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The ecology of anthrax and coinfection trade-offs from an immunological  
perspective: seasonal aspects of host susceptibility

By

Carrie Ann Cizauskas

A dissertation submitted in partial satisfaction of the  
requirements for the degree of

Doctor of Philosophy

in

Environmental Science, Policy, and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Wayne M. Getz, Chair

Professor Justin S. Brashares

Professor Russell E. Vance

Spring 2013

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## Abstract

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Seasonal fluctuations in infectious disease incidence are common, and have been observed for many infectious agents. Immune condition can also change seasonally due to such pathogen fluctuations, as well as to changes in other stress-inducing and immunomodulatory factors such as reproduction and lactation. In addition, most vertebrate hosts are concurrently infected with multiple pathogens, and such coinfections can interact with each other, with the immune system, and with other physiological factors to affect both the individual hosts and population dynamics. While many studies regarding the effects of coinfections and immune trade-offs have been conducted in laboratory settings, similar studies in wildlife are as yet very rare. Fewer studies have been conducted regarding disease and immune seasonality, as these are difficult to model in laboratory settings and require difficult, longitudinal studies in natural systems. In addition, most research regarding disease seasonality in natural systems that has been done has focused on the impacts of abiotic factors on pathogen and vector survival and abundance, or on population-wide dynamics, rather than on physiological mechanisms of host susceptibility. To fully understand the ecology of infectious diseases and the reasons for disease outbreaks, it is necessary to extend laboratory studies into natural hosts in natural systems, as well as to extend wildlife studies to incorporate the complex interactions between environmental, physiological, and coinfection factors. My dissertation research thus focused on the ecological immunology of infectious disease in a natural system, from physiological, seasonal, and coinfection perspectives. I examined the ability of plains zebra (*Equus quagga*), springbok (*Antidorcas marsupialis*), and African elephant (*Loxodonta africana*) to respond immunologically to regular anthrax outbreaks in an endemic anthrax system (Etosha National Park, ENP, Namibia). I also examined how zebra and springbok macroparasite coinfections varied with and affected changes in immune parameters, stress, reproductive hormone levels, and seasonal timing of anthrax outbreaks.

Despite the fact that anthrax is an ancient disease known to affect wildlife, livestock, and humans on nearly every continent, its natural ecology is not well

understood. In particular, little is known about the adaptive immune responses of wild herbivore hosts against *Bacillus anthracis*, the causative agent of anthrax. Thus, I worked to determine the extent to which natural anthrax hosts can fight off sublethal anthrax doses via adaptive immunity. I used enzyme-linked immunosorbent assays, and developed new assay mensuration rules to determine serum antibody titers against the anthrax protective antigen (PA) toxin, an important, potentially protective aspect of adaptive immunity against anthrax. I found that more than 60% of all zebra, up to 15% of springbok, and up to 50% of elephants had measurable anti-PA antibody titers, indicating that these hosts experience and survive sublethal anthrax infections, likely encounter more anthrax in the wet season compared to in the dry, and can partially booster their immunity to *B. anthracis* over time.

Most pathogen-pathogen interactions occur indirectly through the host immune system, and are particularly strong in mixed micro-macroparasite infections because of the strong immunomodulatory effects of helminth parasites. As pathogen transmission changes with season, host immunity may be more strongly influenced by coinfection immunomodulatory effects than by external factors such as changing dietary and demographic patterns. I thus examined the seasonality of immune functionality, pathogen infectivity, and interactions between concurrent infections and immunity in wild zebra in ENP, a system with strongly seasonal patterns of gastrointestinal (GI) helminth infection intensity and concurrent anthrax outbreaks. I found evidence that wet seasons in ENP are characterized by Th2-type immune skewing driven by GI helminth infections, and that these trade-offs make hosts less capable of mounting effective Th1-type immune responses against anthrax infections at this time. I also found evidence that coinfections and immune tradeoffs affect long-term host survival, and that GI parasites likely exert more selection pressure on zebra hosts than do ectoparasites and anthrax, but may actually be a stabilizing force in this host-pathogen system.

Stress and reproductive hormones can modulate each other and the immune system, and can affect disease incidence. Pathogens can also cause host stress, as well as exploit host niches exposed by stress and reproductive hormone-induced immunomodulation. Therefore, I examined seasonal correlations between host stress, reproduction, and GI parasite coinfections in zebra and springbok in ENP. All three macroparasites examined (strongyle helminths, *Strongyloides* helminths, and *Eimeria* coccidia) had strongly seasonal signals, with hosts experiencing the highest parasite infection intensities during times of highest rainfall and highest anthrax outbreaks. Strongyles appeared to be perhaps most potent immunomodulating pathogen in this system, influencing zebra immune responses to anthrax and susceptibility to tick infestations, as well as springbok susceptibility to *Strongyloides* infections. However, helminths were mostly negatively associated with stress hormone concentrations and *Eimeria* had almost no interaction with stress, indicating that most hosts have developed tolerance toward even high macroparasite loads. Stress hormone concentrations were nearly uniformly higher in drier times than in wetter ones, and were positively affected by estrogen

concentrations in females, likely indicating that seasonal nutritional and water stressors, as well as pregnancy stress during the dry season trumped the effects of pathogen infection intensities in causing host stress. In addition, adult animals had higher stress levels than did yearlings, despite yearlings being the largest aggregator of parasites, corroborating the idea that reproductive status is more influential in determining host stress than are pathogens in this system and that mechanisms of tolerance to GI parasites are substantial.

In conclusion, my results indicate that GI parasites play a large role in determining host immune status and susceptibility to micro- and macroparasite coinfections. While ENP herbivores survive anthrax infections with regularity, host immunomodulation by GI parasites likely determines whether a host will mount a successful immune response against this potentially deadly pathogen. GI parasites modulate these coinfection interactions despite causing hosts little direct stress; thus, these coinfection interactions likely take place mostly through direct immunomodulatory effects rather than indirectly through host pathology, nutritional depletion, and other potentially stress-inducing sequelae. ENP zebra and springbok appear to be tolerant of their macroparasite loads, trading off parasite immunomodulatory and pathological effects in favor of balancing resource allocation toward reproductive efforts.

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## Acknowledgements

There are many people who have helped me over the course of my dissertation, with intellectual support, emotional support, fieldwork, and lab work. Firstly, I must thank my dissertation committee: Wayne Getz, Russell Vance, and Justin Brashares. A large thank you to Wayne, my primary advisor, for giving me the opportunity, nay, all but forcing me to build my research program from the ground up and from my own ideas. Wayne, you may be more "hands-off" than many advisors, but you sent me along to the places I needed to go and made me troubleshoot every aspect of my own work, and I am a far better scientist because of it. A very large thanks you as well to Russell Vance, who has acted as my unofficial co-advisor. Thank you, Russell, for your help with all aspects of my immunology work, as well as space and resources in your bustling lab. Another thank you to Justin Brashares, who also chaired my oral exam committee and to whom I still have not returned his physiological ecology notes. Thank you also to Russell Vance again, as well as to Wayne Sousa who rounded out my qualifying exam committee. I must also thank the other faculty, students, and staff of the ESPM department, particularly the other grad students from my cohort.

I thank the Namibian Ministry of Environment and Tourism for giving me permission to conduct my research in Etosha National Park. I thank the following head of Etosha and wardens who were in these positions when I conducted my field research: Michael Sibalatani; park wardens Rehabeam Erckie, Shadrack Kaseba, Immanuel Kapofi, Shayne Kötting, Bonnie Simaata, and Isaskar Uahoo from the Directorate of Parks, Wildlife and Management; and park wardens Nigel Berriman, Seth Guim, Johannes Kapner, Werner Kilian, Birgit Kötting and Wilferd Versfeld from the Directorate of Scientific Services.

To everyone in the Etosha Ecological Institute, I thank you so much for your knowledge, advice, assistance, and patience. I am grateful for the assistance of MET veterinarians Mark Jago and Ortwin Aschenborn, as well for help from veterinarians Nad Brain and Pete Morkel. Thank you so much to my core capture team of Shayne Kötting, Martina Küsters, and Gabriel Shatumbu, as well as to Wendy Turner for a great deal of assistance with many of the captures and other organizational aspects of this project. In addition, I thank Nigel Berriman, Wilferd Versfeld, Johannes Kapner, Marthin Kasaona, Werner Kilian, Piet Nel, and members of Team Anthrax for their field assistance at various stages of this project. I also greatly appreciate the lab help I received from Monika Shikongo. To the tearoom crowd in Etosha, I am so grateful to you all for making me feel like I was part of a family in the field. Werner, thank you for putting up with all of us; we couldn't have done any of this work without you. Shayne, we made a great capture team, and I couldn't have recovered from heat stroke without you putting up with my ramblings as I took over your house. Wilferd, you were fun to learn from and drink tea with, even when you were nukkerig. Nigel, you and Daisy always amused me greatly. Piet, thank you for helping me get into trouble in Afrikaans, yet never really out of it. Gabes, thank you for your unwavering enthusiasm during all of our work together, and just for being



you. Martina, without you, there would be no dissertation. Every project needs a Martina, for her expertise, her work ethic, her pure grit, and her stalwart demeanor that can only be cracked with the persistence of someone as amusingly, endearingly, annoying as me.

I have been fortunate to be a part of an incredible large, collaborative project that has allowed us all to do so much more than we could have done alone. Because of this, I thank Team Anthrax, or at least the portion of Team Anthrax that I worked with during my tenure: Steve Bellan, Holly Ganz, Zepee Havarua, Pauline Kamath, Duduzile Sibanda, Orr Spiegel, Wendy Turner, and Royi Zidon. Thank you, Wendy, for getting this all started, for keeping it all going, and for sharing your samples with me. Anything for data! I have also been fortunate to be part of a large, sprawling lab that should really apply for statehood. During my time at Cal, I have been fortunate to work with: Allison Bidlack, John Eppley, Scott Fortman-Roe, Holly Ganz, Pauline Kamath, Karen Levy, Jamie Lloyd-Smith, Andy Lyons, Niclas Norrström, Matt Plucinski, Sadie Ryan Simonovich, Richard Starfield, Zulima Tablado (honorary Getz lab member), Craig Tambling, Miriam Tsalyuk, Wendy Turner, María Sánchez, Karen Weinbaum, Chris Wilmers, George Wittemyer, and a slew of various post docs. It is only because of my academic siblings that I have ever known how to navigate any part of Cal or this dissertation. I give particular thanks to Sadie Ryan Simonovich for often acting as a co-advisor to me through the final stages of this process.

Thank you as well to the Vance lab, my other home on campus. I am thankful to everyone in the lab for helping me to find space and materials. In particular, I must give a large thank you to Mary Fontana, Dara Burdette, and Kate Monroe for their PCR assistance. And a huge thank you to Katia Sotelo-Troha, the Martina of the Vance lab who helped me with everything, large and small, with unwavering enthusiasm even when I asked the same question for the fifth time.

Outside of Cal, I have worked with so many people during this long research project. Thank you to Peter Turnbull for teaching me specific laboratory methods in Etosha, and to Pauline Lindeque for doing the seminal anthrax work in Etosha upon which all of our work is based. Thank you as well to Hym Ebedes, the first Etosha vet, who provided us with anthrax data dating back to the 1960s. A large thanks you to Neville Pitts for collaborating with me on the hormone research project, and to Bettina Wagner and Heather Freer for collaborating with me on the IgE work. I greatly appreciate the time that Jennifer Johns took to help me refine my blood work protocols. Thank you to Bryan Krantz (actually a Cal professor, but in good company here) for providing me with PA for my many ELISAs, free of charge. Thank you to Dirk Hoffmann for teaching me Afrikaans, and for providing me with several colorful phrases that I was able to use to great effect in the field. I also must thank many of my fellow vets: in addition to the ones already mentioned (Mark Jago, Ortwin Aschenborn, Nad Brain, Pete Morkel, Hym Ebedes, Jennifer Johns, Bettina Wagner), I also am grateful to Melissa Kielty for providing me with negative control horse serum, Stephanie Stevens for providing me with anthrax-vaccinated horse serum, Karen Emanuelson at the Oakland Zoo for providing me with goat serum, and all of the African vets who have taught me so much about wildlife work. I also thank Jeff

Kinzley, the elephant keeper at the Oakland Zoo for helping me get negative control elephant serum, and to Bethyl Labs in Texas for being willing to essentially vaccinate goats against elephants to make me secondary anti-elephant antibodies. Thank you also to the Malilangwe Trust in Zimbabwe (and particularly to Sarah Clegg) for both hosting an amazing pan-Africa anthrax conference, as well as the wildlife capture course I attended and from which I learned all the tricks of the trade.

I could not have completed my research without funding. I have been funded by two Andrew and Mary Thompson Rocca Scholarships, the Julius H. Freitag Memorial Award, the Johannes Joos Memorial Award, two Graduate Division Summer Grants, the Bob Lane Endowed Graduate Student Support Fund, the Carolyn Meek Memorial Scholarship in Environmental Science, a block grant from the department of Environmental Science, Policy, and Management, a Phi Beta Kappa Fellowship, and NIH Grant GM83863 to Wayne Getz. In addition, I have been funded by several teaching opportunities. I am grateful to the Integrative Biology and ESPM departments for allowing me to teach so much (two summers and two semesters in IB, and two semesters in ESPM). I was also fortunate to participate in the NSF-GK12 teaching program for a year, and I am grateful to all of my wonderful seventh graders who are no longer so young but are hopefully still full of questions. On the teaching front, I am also thankful to Robert Beatty in the Molecular and Cell Biology Department for having me back six times thus far to give guest lectures. And I am so fortunate to have been able to teach the Environmental Sciences senior thesis course; thank you, Patina Mendez and Kurt Spreyer, for hiring me, and thank you to all of my amazing undergrads who have done incredible work this year while teaching me a lot about mentoring.

Finally, I of course thank my family and friends. Thank you to my mom and dad and brother (Mary, Robert, and Jonathan, proud Cizauskases all) for always being there; thank you especially to my mom for taking care of Ezra so I could finish my lab work. A big thank you to my mother-in-law, Elizabeth Smith, for her wonderful statistics assistance, and to my father-in-law, Len Smith, for putting up with not receiving enough pictures of Ezra because I was too busy to send them. My friends near and far are also my family. In particular, but in no particular order, Jason Ferrier, Amy Roth, Becky Chaplin-Kramer and Danny Kramer, Ulrika Andersson, Neil Berrett, Andy and Sarah Karlson, Nick Drake, Emily Mills, Chad Stockham, and Joyce Ehrlinger are my extended family, and I could not have done any of this without them. Joyce, thank you so much for also acting as a stellar academic advisor-at-large during so much of this process. A huge thanks you to my pets, who got me through so many years of academic toil and who have always been parts of my soul. Chili and Tomo, I miss you so, and Milo my old man, you have done your best. An enormous thanks you to my son, Ezra, just for being him. And, of course, most especial thanks to my husband, Jeremy, for everything.

# Chapter 1

## Summary of the dissertation

Seasonal fluctuations in infectious disease incidence are common in humans, domestic animals, and wildlife, and have been observed for several diseases (reviewed in Altizer et al. 2006). Most research regarding disease seasonality, however, has focused on the impacts of abiotic factors on pathogen and vector survival and abundance, rather than on host factors. Few studies have been conducted regarding disease and immune seasonality, as these are challenging to model in laboratory settings and require difficult, longitudinal studies in natural systems. In addition, studies that have involved host factors of disease dynamics have largely concentrated on population-wide components, rather than on physiological mechanisms of host susceptibility (but, see Hosseinei et al. 2004; Martin et al. 2008). Fewer studies still (e.g. Jolles et al. 2008; Ezenwa et al. 2010) have examined immunological trade offs in the wild, especially the compromise between the Th1 (anti-microparasite) and Th2 (anti-macroparasite) arms of the immune system.

The field of immunological ecology is relatively new; immunological studies in wildlife have thus far mainly focused on using immunology as a tool to measure the prevalence of disease, rather than as a means toward understanding the immunological mechanisms of disease in wildlife. While immunological studies are common and complex in laboratory settings, few studies have extended this type of research into the wildlife realm (but, see, for example, Cattadori et al. 2005; Beldomenico et al. 2008; Chambert et al. 2012; Pathak et al. 2012). This laboratory research serves to tease apart the complexities of host physiological and immunological responses to pathogens; such research will have limited impact, however, on ecological knowledge and disease management plans, unless these findings are “ground-truthed” in natural, pathogen-endemic systems. As emerging infectious diseases are becoming increasing threats to human, animal, and ecosystem health, as the majority of animal and human populations experience coinfections, and as climate change causes changes in seasonal disease distributions, it is increasingly important to understand infectious disease dynamics in natural systems in concert with seasonality and host immunity.

Wildlife studies have looked at various components of infectious disease dynamics, immune function, disease seasonality, immune seasonality, and coinfections, but rarely, if ever, have they combined all of these interrelated components to examine these complex relationships in natural systems. My research employs a novel approach to disease ecology by examining internal host factors relating to an endemic disease in a natural system, and by relating these factors to the immunological mechanisms of disease in wildlife. In my dissertation work, I examined the seasonal fluctuations in stress, reproductive hormone levels, micro- and macroparasite coinfection status, and several aspects of immune function in plains zebra (*Equus quagga*, formerly *Equus burchelli*), natural hosts of the yearly, seasonal anthrax outbreaks in Etosha National Park (ENP), Namibia. My

zebra research is one of the most comprehensive ecological immunology studies to date, and includes the first longitudinal study simultaneously examining coinfections, seasonality, and complex immunity in wildlife hosts. In these hosts, I measured several immune components (titers of antibodies against *Bacillus anthracis*, and IgG and IgE antibodies; counts of total white blood cells, neutrophils, lymphocytes, monocytes, and eosinophils; and signaling profiles for IFN- $\gamma$  and IL-4 cytokines); general host health factors (hematocrit; age via animal tooth wear patterns); hormonal components (corticosterone, estrogen, progesterone, and testosterone metabolites); and coinfections (anthrax, gastrointestinal helminths, and ectoparasite infections). This is also the first study examining IgE antibody titers in wild equids, and one of the first studies (out of less than five known) to measure this parameter in wildlife.

In my dissertation work, I also examined the seasonal fluctuations in stress, reproductive hormone levels, and macroparasite coinfections in springbok (*Antidorcas marsupialis*), and researched whether zebra, springbok, and elephants (*Loxodonta africana*), all major anthrax hosts in ENP, can mount protective immune responses against *Bacillus anthracis*, the causative agent of anthrax. By examining these factors in concert, one can view a much more detailed picture of immunological changes that may lead to increased host susceptibility to a disease agent (*B. anthracis*) that is likely encountered year-round. This work will not only increase our knowledge of the ecology of a disease that is as yet not well understood in the wild, but will also shed further light on immunological relationships that have thus far been almost entirely confined to laboratory studies.

## **Background and Study System**

Anthrax, a highly virulent, zoonotic, bacterial disease of mammals, is reemerging worldwide, as evidenced by a recent increase in the number and severity of outbreaks in wildlife, livestock, and humans (Clegg et al. 2007; ProMED-mail 2009-2013). It is caused by *Bacillus anthracis*, a gram-positive, rod-shaped, aerobic spore-forming bacterium that can infect most mammalian species but predominantly targets herbivores (Hugh-Jones and de Vos 2002). The most important route of infection for wildlife is thought to be ingestion of the spores (Hugh-Jones and de Vos 2002). Once *B. anthracis* infects an herbivorous host, it reproduces rapidly, releases toxins, and within days to weeks causes acute, fatal septicemia. Vegetative *B. anthracis* cells are released from the host's orifices post-mortem, transforming in the aerobic environment to form hardy, long-lived spores whose length of persistence depends on the external environmental conditions. As anthrax threatens human health, biodiversity, economics, and agricultural security, managing such outbreaks has become a global priority. We must begin, however, by filling in the fundamental gaps in our understanding of anthrax ecology and dynamics. Much of my research aims to shed light on these ecological patterns from the perspective of host susceptibility.

In addition, the majority of animal and human hosts are infected with more than one pathogen at a time, with many host communities experiencing up to 100%

prevalence of coinfections (Petney and Andrews 1998; Lello and Hussell 2008). These infections are often a mix of micro- and macroparasites that can interact with each other to modify pathogen transmission, virulence, and host availability. The majority of these interactions occur indirectly via the immune system (Cox 2001; Page et al. 2006; Pathak et al. 2012), with immunomodulation affecting host susceptibility to other parasites, and enhancing disease intensity, severity, pathology, and/or duration (Chen et al. 2005; Graham et al. 2005; Graham 2008; Lello and Hussell 2008; Pathak et al. 2012). These immunomodulatory effects are particularly strong in micro-macroparasite coinfections, as helminths and other macroparasites are particularly adept at skewing immune responses away from those needed to fight against microparasites such as *B. anthracis* (Abbas et al. 1996; Mosmann and Sad 1996; Graham et al. 2005; Page et al. 2006; Ezenwa et al. 2010). Thus, for many of my studies, I also examined anthrax outbreak patterns in concert with those of macroparasites such as strongyle helminths, *Strongyloides* helminths, and *Eimeria* coccidia, and with those of ectoparasites (Ixodid ticks, previously found on zebra hosts in ENP; Horak et al. 1992). Strongyles (nematodes in the order Rhabditida, suborder Strongylida, primarily within the superfamily Strongyloidea, and family Strongylidae for zebra; and nematodes in the order Rhabditida, superfamily Trichostrongyloidea, and family Trichostrongylidae for springbok), *Strongyloides* (nematodes in the order Rhabditida and family Strongyloididae), and *Eimeria* (class Coccidia, order Eucoccidiorida, and family Eimeriidae) (Jain et al. 2009) are directly-transmitted, orally-ingested parasites that are pathogenic, are known to cause widespread production losses in livestock, and can affect wildlife population dynamics (Dobson and Hudson 1992; Gulland 1992; Murray et al. 1997; Bowman 2003; Newey et al. 2005).

ENP in Namibia is the ideal natural laboratory in which to carry out this research, as *Bacillus anthracis* and the macroparasites mentioned above are endemic to the park. Anthrax in ENP is also unmanaged, providing a model system to evaluate the synergistic roles that the environment, host condition, and coinfections play in shaping the ecology of this disease. Anthrax and macroparasites are the only known pathogens infecting zebra and springbok hosts in ENP; rabies does exist in this system, but there is no evidence that zebra or springbok play a role in its dynamics (Etosha Ecological Institute, EEI, unpublished data; Gasaway et al. 1996). There is also no evidence of the presence of tick-borne pathogens in ENP (EEI, unpublished data).

While ENP zebra have a nearly 100% prevalence year-round with GI strongyles, they experience a significant increase in infection intensity in the wet season compared to in the dry (Turner and Getz 2010). Springbok in ENP also experience significant increases in strongyle, *Strongyloides*, and *Eimeria* prevalence with increased rainfall (Turner and Getz 2010). Anthrax wildlife mortalities occur annually, with plains zebra and springbok as the primary hosts (Lindeque 1991; Lindeque and Turnbull 1994). The Etosha Ecological Institute (EEI) of the ENP has been recording these anthrax mortalities since 1966, with extensive mortality surveillance (recording carcass location by GPS or on a 5x5km grid map) since 1975

(Ebedes 1976; Lindeque 1991). Between 1975-2006, 2,333 carcasses were tested or suspected positive for *B. anthracis*, providing a rich set of data regarding anthrax species and epizootic trends. Although sporadic cases of anthrax occur year-round in ENP, outbreaks there primarily occur in plains ungulates at the end of the wet season, unlike in other systems (Gainer 1987; Gainer and Saunders 1989; de Vos 1990; Lindeque 1991; Clegg et al. 2007; Clegg et al. 2007b). The unique timing of ENP anthrax allows the opportunity to study aspects of the disease otherwise accounted for by dry weather's supposed roles in poor host condition (Hugh-Jones and de Vos 2002). And, unlike in other studied systems, anthrax in ENP affects both sexes equally and does not appear to involve invertebrate mechanical vectors (Gainer and Saunders 1989; Lindeque 1991; Turnbull et al. 1992). Thus, these factors cannot confound hypotheses regarding anthrax outbreaks in ENP.

Given the endemic nature of anthrax in ENP, the prolonged survival times of spores in the environment (Hugh-Jones 1999), and the fact that anthrax deaths do occur throughout the year in this system (Lindeque 1991), it is likely that animals come into contact with anthrax spores year-round. As the traditional spore concentration hypothesis cannot account for the ENP rainy season outbreaks (Hugh-Jones and de Vos 2002), as contracting anthrax from drinking water contaminated with spores is highly unlikely due to dilution effects (Lindeque and Turnbull 1994; Turnbull et al. 1992), and as there is yet no definitive evidence that anthrax reproduces to any extent in the environment (but see Saile and Koehler 2006; Dey et al. 2012) I was led to consider the role of seasonal changes in host condition, immunity, and coinfections in causing anthrax outbreaks in ENP.

## **Questions and Methods**

Beyond this introductory chapter, each of these chapters was written as a stand-alone paper to be submitted for publication in a peer-reviewed journal. As such, some material, particularly methods, is duplicated across chapters. As publications, these papers will all have coauthors; however, for all of these studies, I have acted as lead project designer, main field and lab researcher, and lead author.

In conducting my dissertation research, I asked the following questions:

### *1. Can herbivores survive anthrax infections by mounting an immune response?*

Humoral immunity is believed to play the most important role in immunity to anthrax, and studies have suggested that IgG antibody titers against the anthrax protective antigen (PA) toxin are correlated with level of protection against the disease (Little et al. 1997; Cohen et al. 2000; Pitt et al. 2001; Reuveny et al. 2001; Marcus et al. 2004; Aloni-Grinstein et al. 2005). While previous studies have found that most carnivores can mount a strong, protective antibody response against *B. anthracis* (Turnbull et al. 1992; Turnbull et al. 2008; Hampson et al. 2011; Lembo et al. 2011; Bellan et al. 2012), herbivores have been found to produce very low immune responses in the face of vaccination (Turnbull 1991; Chitlaru et al. 2011) or in the face of natural outbreaks (Turnbull et al. 1992; Lembo et al. 2011). Studies such as these suggest that herbivores, even in endemic anthrax systems, are largely

immunologically naïve, and either don't contact *B. anthracis* at all, or die upon first exposure to the pathogen. Because of the prolonged spore survival time in the environment and the fact that ENP hosts likely do contact anthrax spores year-round, I expected to see both a more constant rate and greater incidence of anthrax deaths in ENP unless exposure to doses exceeding LD<sub>50</sub>s somehow varied with season, host susceptibility changed with season, and/or hosts were able to survive some anthrax infections. If hosts did mount immune responses to sublethal anthrax doses, these responses could then be subject to immunomodulation by coinfection and hormonal factors, or could themselves affect host responses to other pathogens.

I thus used serum samples from 154 zebra captures, 44 elephant captures, and 21 springbok captures to look for the presence of anti-PA antibodies, and to attempt to measure titer changes in recaptured animals over time. These samples were collected between 2008 and 2010, and spanned both wet and dry seasons in ENP. In addition, I pioneered novel, more rigorous methods for determining these antibody titers; while the enzyme-linked immunosorbent assay (ELISA) is the most commonly used method of measuring antibodies in both laboratory settings and wildlife studies, there are no consistent rules governing the determination of endpoint titers and this is often done using very subjective cutoffs. I addressed the common difficulties of adapting this assay to wildlife situations (in which there are usually no titrated positive controls for comparison purposes or no positive controls at all; there are few negative controls to be gotten from zoo animals; and controls and experimental samples are both "moving targets" on assay plates) by producing protocols for both conducting the assay and for applying more rigorous mensuration methods to the determination of final titer cutoffs.

In Chapter 2 of this dissertation, I report on the results of these anti-anthrax titer examinations in zebra, springbok, and elephants, as well as on the results of my different mensuration protocols. I found that between 52 and 87% of unique zebra, between 0 and 15% of unique springbok, and between 3 and 52% of unique elephants sampled had measurable anti-PA antibody titers. In addition, I came to the conclusion that each of the three increasingly conservative endpoint titer rules should be considered together with the data in question; while it was questionable whether springbok and elephants could mount significant anti-anthrax titers, examining these results solely with the most conservative endpoint rules clearly lacked sensitivity for some low responders, while the least conservative rule was clearly less specific. Regardless of the rule used, however, I found that zebras could mount anti-PA responses, and did so frequently. I also found that several animals had increased titers over time, indicating a booster response with subsequent exposures to *B. anthracis*; these titers, however, lasted on average less than six months. While the memory response to anthrax appears to be short-lived, these hosts clearly do allocate immune resources toward fighting this pathogen, and thus this immune response can influence, or be influenced by, other host physiological and pathogenic factors.

## 2. Are there seasonal changes in host immunity?

Previous studies have shown that innate immune factors such as macrophages and neutrophils play a primary role in limiting and clearing *B. anthracis* infections (Cote et al. 2004; Cote et al. 2006). In addition, studies indicate that cell-mediated immunity also plays a role in anthrax prevention (Allen et al. 2006; Parker et al. 2006). Examining the seasonal differences in these cell populations in ENP anthrax hosts was therefore important for gauging immune system functionality against anthrax in different seasons. Seasonality of host immunity can also be caused by other coinfections, or by non-pathogen factors: changing nutrition can bolster or suppress immunity, seasonal pregnancy and lactation can lead to decreased immunity across most adult females at once in a population, and seasonal parturition patterns can lead to a pulse of immunologically naïve hosts into the population (Nelson and Demas 1996; Sheldon and Verhulst 1996; Altizer et al. 2006). Intrinsic aspects of changing host immunity, particularly in concert with parasite dynamics, however, are not yet well studied or understood (Altizer et al. 2006; Beldomenico et al. 2008).

Thus, I examined 154 zebra blood and serum samples from 69 individuals, resampling the same individuals when possible over five seasons (two wet and three dry) between 2008 and 2010. I counted total white blood cells and differential white cell counts, as well as hematocrits for each capture event to compare cellular immune parameters between seasons and in conjunction with other physiological and pathogenic response variables. I also measured IgE antibodies between seasons; IgE is an antibody isotype mediated by the Th2-associated cytokine IL-4 and is important in fighting against helminths (Abbas et al. 1996). I also measured total IgGb antibody titers; IgGb is the most prevalent antibody isotype in equine serum and is able to fix complement via the classical pathway and evoke a respiratory burst from monocytes (Lewis et al. 2008). While IgGb is important in the protective response against intracellular pathogens, suggesting a Th1-associated response, Hooper-McGrevy et al. 2003 found that IgGb is likely a Th2-related (macroparasite-related) antibody in horses. In addition, I measured mRNA signals for the cytokines IL-4 and IFN- $\gamma$  between one wet season and one dry; IFN- $\gamma$  is a key Th1 cytokine that activates macrophages early in infection, while IL-4 is an important Th2 cytokine that induces antibody isotype switching to IgE and augments recruitment of eosinophils to helminths (Mosmann et al. 1986; Abbas et al. 1996).

In Chapters 3 and 4, I report on the seasonal differences of these immune parameters; Chapter 3 discusses these changes mostly in concert with helminth-anthrax immune trade-offs, whereas Chapter 4 discusses them in relation to seasonal changes in stress and reproduction. I found that total white blood cell counts, hematocrits, eosinophil counts, and neutrophil counts were significantly higher in the wet season than in the dry. IgGb titers and lymphocyte counts were significantly higher in the wet season as well, but only for the same individuals compared between different seasons. Monocytes and IgE titers were not significantly different between seasons when compared between simple rainfall



groups; however, more complex models indicated that increased rainfall predicted increased IgE titers. While some of these higher counts in the wet season were likely influenced by better nutritional and water availability in the wet season, I also found evidence that several were driven by host responses to increased GI parasite infection intensities. Thus, while providing some of the first evidence for seasonal immune fluctuations in wildlife, I found that these fluctuations were influenced by coinfection factors that might influence immune responses to other pathogens such as anthrax.

### *3. Do hosts experience immune trade-offs with coinfections?*

Intracellular pathogens such as *B. anthracis* cause the mammalian adaptive immune system to mobilize T-helper-1 (Th1) cells, while extracellular pathogens (most macroparasites) trigger T-helper-2 (Th2) cells. The pathways leading to and from Th1-type responses and Th2-type responses are mutually cross-regulated, with Th1 cytokines inhibiting the activity and development of Th2 factors and vice versa. Hosts therefore have difficulty simultaneously mounting effective Th1 and Th2 responses (Abbas et al. 1996; Mosmann and Sad 1996; Morel and Oriss 1998; Yazdanbakhsh et al. 2002). Many laboratory studies have demonstrated that helminth infections are particularly adept at skewing immune responses toward the Th2 arm, with this polarization down regulating Th1 immunity, even in the face of strong microparasite coinfections (Abbas et al. 1996; Mosmann et al. 1996; Chen et al. 2005; Graham et al. 2005; Page et al. 2006; Diniz et al. 2010; Ezenwa et al. 2010). Chronic helminth infections can also cause host immunosuppression, resulting in maintenance of the immunomodulating worm infection and increased susceptibility to coinfections (Gulland 1992; Graham et al. 2007; van Riet 2007; Diniz et al. 2010).

Zebra in ENP exhibit peak strongyle infection intensities during anthrax outbreak season (Turner and Getz 2010), indicating that parasite loads may be related to anthrax susceptibility through immunomodulation. Thus, I measured GI strongyle infection intensities in fecal samples, along with several Th1-type blood immune parameters (monocyte counts, IFN- $\gamma$  cytokine signaling, anti-PA antibody titers) and Th2-type immune parameters (eosinophil counts, IL-4 cytokine signaling, IgG $\alpha$  and IgE antibody titers) in zebras over three seasons (two wet and three dry) and three years. I collected 154 blood and serum samples and 123 fecal samples from zebras to examine the seasonal, immunomodulatory effects of GI parasites on anthrax outbreak dynamics. In addition, tick infections can also cause host immunomodulation, possibly skewing host immunity toward a Th2 profile (Ferreira and Silva 1999; Ogden et al. 2002; Castagnoli et al. 2008), and some studies have shown that ticks can also be broadly immunosuppressive (Wikel 1999; Slámová et al. 2011). Thus, I also collected ticks from 125 zebras to fully examine the effects of coinfections on zebra immunity.

In Chapter 3, I report on these coinfection immune trade-offs in concert with seasonality of infections in ENP zebras. I found that zebra experienced Th2-type skewing in wet seasons, while Th1-type immunity prevailed in drier seasons. This Th2-type skewing was primarily driven by GI parasite infections, which showed

strong seasonal fluctuations primarily constrained by external environmental effects on free-living parasite stages. As zebra hosts experienced significantly increased GI helminth infection intensities and evidence for Th2-type skewing shortly prior to the population's annual anthrax outbreaks, and as Th2 immunity precludes hosts mounting effective Th1 responses necessary to fight against microparasites like anthrax, this suggests that these anti-parasite Th2 responses made hosts less capable of mounting effective Th1-type immune responses against anthrax infections at this time. I also found that strongyle infections affected tick infestations, and that GI parasites likely exerted more selection pressure on zebra hosts than did ectoparasites or anthrax. I also found evidence that coinfections and immune tradeoffs affected long-term host survival, with older animals possessing less intense GI parasite infections and higher Th2 responses, and few older animals with concurrent intense helminth infections and evidence of anthrax survival.

#### *4. Do seasonal changes in host stress and reproduction affect host immunity to anthrax and macroparasitic coinfections?*

While not strictly seasonal breeders, zebra and springbok in ENP engage in reproductive behavior, birthing, and lactation primarily during the wet season (Gasaway et al. 1996; Turner and Getz 2010). Reproduction and lactation can negatively affect immune function (Olsen and Kovacs 1996; Mallard et al. 1998; Bonizzi et al, 2003; Sugiura et al. 2004; Mougeot et al. 2005). In addition, reproductive hormones can influence stress hormone secretion (Ogilvie and Rivier, 1997; Tilbrook et al, 2000; van Lier et al, 2003; Millspaugh & Washburn, 2004; McCoy and Ditchkoff 2012; Vera et al. 2013). The potential for stress to modulate the immune system and cause increased disease incidence has been demonstrated in numerous studies (Gainer and Saunders, 1989; Besedovsky and Del Rey, 1996; Wilckens & de Rijk, 1997; Khansari et al, 1999; Sapolsky et al, 2000; de Groot et al, 2001; Lafferty and Holt, 2003; Sartorelli et al, 2003; Mahuad et al, 2004). Therefore, measuring stress and stress-inducing factors in conjunction with seasonality and coinfection factors is important for determining host immune condition and the subsequent potential for contracting diseases such as anthrax.

Stress and reproductive hormone status in wildlife is most often determined by measuring excreted products of steroid hormone metabolism. Measuring fecal glucocorticoid metabolites (FGM) provides a noninvasive method of measuring average blood cortisol levels, the principal hormone involved in the adrenal response to a variety of stressors (Wasser et al, 2000; Sapolsky et al, 2000; Mostl and Palme, 2002). Assays for FGM have been established for a large number of domestic and wildlife species (Wasser et al, 2000; Mostl et al, 2002; Millspaugh and Washburn, 2004). Thus, by using fecal samples, I was able to examine seasonal correlations between zebra and springbok stress, reproduction, and GI parasite infections. I used existing fecal samples from a cross-sectional study for which strongyle, *Strongyloides*, and *Eimeria* parasite counts were quantified using a modified McMaster method (Gibbons et al. 2005); I assayed 318 zebra and 272 springbok fecal samples that had been collected over wet and dry seasons between

2005 and 2007 (Turner and Getz 2010; Turner et al. 2012). In addition, I assayed 123 fecal samples from zebra immobilized between 2008 and 2010. For these animals, I was also able to examine hormone concentrations and strongyle infection intensities in conjunction with immune parameters (neutrophil counts to assess inflammatory response status, and lymphocyte counts to assess adaptive immunity and tolerance status), anti-PA antibody titers, and ectoparasite counts.

In Chapter 4, I report on the seasonality of stress and reproductive hormones and parasite infection intensities in zebra and springbok. I also report on the interactions between stress, reproduction, and disease, and interactions between micro-macroparasite coinfections and macro-macroparasite coinfections in these hosts. I found that all GI parasite infection intensities were positively associated with rainfall, as were neutrophil counts in captured zebra, possibly indicating a more intense inflammatory response during the wet season than in the dry. Strongyles appeared to be the most potent immunomodulating pathogens in this system, with increased strongyle loads in zebra potentially increasing host susceptibility to anthrax and ectoparasite infestations, and increased strongyle infection intensities in springbok significantly predicting the presence of higher *Strongyloides* infestations. Estrogen concentrations were highest in both species in the dry season (indicating that most animals were in mid-pregnancy at this time), while progesterone concentrations were highest in the wet season (indicating that most animals were giving birth and entering estrus at this time); thus, zebra and springbok are indeed very seasonal breeders in ENP. Stress hormone concentrations were also nearly uniformly higher in drier seasons than in wetter ones, likely indicating that seasonal nutritional and water stress during the dry season trumped the heightened pathogen stress of the wet season. In fact, while parasite infection intensities were highest in the wet season, these infections did not appreciably affect host stress, nor were higher parasite counts found in more stressed animals. Instead, reproductive hormones were the most significant predictors of increased host stress, and might also have increased susceptibility of mares to strongyles. These results indicated that ENP ungulate hosts are likely very tolerant of their GI parasite loads, trading off parasite immunomodulatory and pathological effects in favor of balancing resource allocation toward reproductive efforts.

## Chapter 2

# The ecological immunology of anthrax in an endemic system: do wild herbivores experience sublethal anthrax infection?

### Abstract

Despite the fact that anthrax is an ancient disease known to affect wildlife, livestock, and humans on nearly every continent, its natural ecology is not well understood. In particular, little is known about the adaptive immune responses of wild herbivore hosts against *Bacillus anthracis*, the causative agent of anthrax. Working in the natural anthrax system of Etosha National Park (ENP), Namibia, I collected 154 serum samples from 69 plains zebra (*Equus quagga*) over 3 years, 21 serum samples from 13 springbok (*Antidorcas marsupialis*) over 2 years, and 45 serum samples from 33 ENP African elephants (*Loxodonta africana*) over 2 years to determine the extent to which these anthrax hosts can fight off sublethal anthrax doses via adaptive immunity. I used enzyme-linked immunosorbent assays to measure serum antibody titers against the anthrax protective antigen (PA) toxin, an important, potentially protective aspect of adaptive immunity as determined by studies in laboratory animals. Comparing wildlife samples to negative control sera and using three increasingly conservative models to analyze our assay results, I found that between 52 and 87% of unique zebra (55-92% of all samples), between 0 and 15% of unique springbok (0-14% of all samples), and between 3 and 52% of unique elephants (2-52% of all samples) had measurable anti-PA antibody titers. While it is still debatable, depending on the model used, whether wild elephants and springbok can mount antibody responses against anthrax, my results indicate that zebra in ENP do experience and survive sublethal anthrax infections, likely encounter more anthrax in the wet season compared to in the dry, and can partially booster their immunity to *B. anthracis* over time. In addition, serological titer assays are often the “bread and butter” of ecological immunology studies, yet the analysis and determination of titers is often based on somewhat subjective criteria. With my three models, I attempt to introduce more mensuration rigor into these assays, even under the often-restrictive conditions that come with adapting laboratory immunology methods to wild systems.

## Introduction

Anthrax, a zoonotic bacterial disease, occurs globally and threatens human health, biodiversity, economics, and agricultural security. While the disease primarily affects herbivores, humans and some carnivores, such as cheetahs, are also susceptible (Sterne 1959; Work et al. 2000; Reuveny et al. 2001; Gaur et al. 2002; Turnbull et al. 2004; Schwanz et al. 2011). The disease has been known since antiquity, but recent evidence suggests that outbreaks are increasing in frequency and severity and are spreading to locations and populations previously free of the disease (Clegg et al. 2007; Hampson et al. 2011). For example, a massive anthrax outbreak in the Malilangwe Wildlife Reserve in 2005 was the first time anthrax had been recorded in Zimbabwean wildlife (Clegg et al. 2007), and an outbreak in Samburu, Kenya in 2005 included the first known deaths of endangered Grevy's zebra from the disease (Muoria et al. 2007). Recent large outbreaks in livestock and some wildlife in the Lower Zambezi Valley in Zimbabwe, in Ghana, in the Northern Cape of South Africa and the Caprivi of Namibia have also resulted in human cases (ProMED-mail 2008-2012). Managing such outbreaks has become a global priority, and must begin by filling in the fundamental gaps in our understanding of anthrax ecology and dynamics. Our research aims to shed light on these ecological patterns from the perspective of host susceptibility.

Etosha National Park (ENP) in Namibia (Figure 1) is the ideal natural laboratory in which to carry out this research, as *Bacillus anthracis* is endemic to the park. The Etosha Ecological Institute (EEI) of the ENP has recorded these anthrax mortalities since 1974 (with previous accounts of the disease in ENP since 1966), providing a rich set of data regarding anthrax host species and epizootic trends. ENP's open habitats are dominated by plains ungulates, three of which, springbok (*Antidorcas marsupialis*), plains zebra (*Equus quagga*), and blue wildebeest (*Connochaetes taurinus*) experience yearly anthrax outbreaks (Lindeque 1991; Lindeque and Turnbull 1994). According to Lindeque and Turnbull (1994), these three species are likely responsible for 97% of anthrax deaths in the park, although they make up only approximately 55% of the park's population of large herbivores. Although sporadic cases of anthrax occur year-round in ENP, anthrax in ENP primarily occurs in plains ungulates at the end of the wet season (Figure 2), unlike in other systems (Gainer 1987; Gainer and Saunders 1989; Braack and de Vos 1990; Lindeque 1991; Clegg et al. 2006; Hampson et al. 2011). Anthrax in ENP affects both sexes equally and does not appear to involve invertebrate mechanical vectors (Lindeque 1991; Turnbull et al. 1992). Elephants (*Loxodonta africana*) also experience anthrax infections in the park, with a slightly increased incidence at the beginning of the wet season (Lindeque 1991) (Figure 2).

Anthrax is caused by *Bacillus anthracis*, a large, gram-positive bacterium that undergoes vegetative reproduction in infected hosts but otherwise exists in a very hardy, long-lived, infectious spore form in the environment. Anthrax can only be transmitted environmentally through the spore form and not via host-to-host contact (Hanna and Ireland 1999). Wildlife hosts are believed to usually contract anthrax via ingestion of a large dose of spores (Watson and Keir 1994; Hugh-Jones

and de Vos 2002), at which point the spores germinate into fast-multiplying vegetative forms. These vegetative bacteria produce three soluble toxic factors: edema factor, an adenylate cyclase which impairs immune cell function (Leppa 1982; Brien et al. 1985; Collier and Young 2003; Comer et al. 2005); lethal factor, a MAP-kinase-kinase that likely suppresses production of several types of cytokines (Duesbery 1998; Vitale et al. 1998; Pellizzari et al. 1999; Erwin et al. 2001; Agrawal et al. 2003; Ribot et al. 2006); and protective antigen (PA), which complexes with the other two factors and allows them to enter host cells through oligomeric PA pores (Little, Leppla, and Cora 1988; Milne et al. 1994; Bradley et al. 2001; Mogridge, Cunningham, and Collier 2002). The collective actions of these toxins results in the peracute-to-acute death of susceptible hosts from edema, vascular collapse, and inflammation, combined with an overwhelming septicemia of up to  $10^9$  bacterial cells per milliliter of blood (Hugh-Jones and de Vos 2002; Collier and Young 2003).

Humoral immunity, particularly against the PA toxin, plays a very important role in a host's fight against anthrax; the presence of anti-PA antibodies appears to be essential for adaptive protection, and several studies have demonstrated that the magnitude of a host's anti-PA IgG antibody titer is correlated with level of protection against the disease (Little et al. 1997; Cohen et al. 2000; Turnbull 2000; Pitt et al. 2001; Reuveny et al. 2001; Little et al. 2004; Marcus et al. 2004; Aloni-Grinstein et al. 2005). Previous studies have found that the majority of carnivores, particularly those living in anthrax-endemic areas with frequent anthrax contact via carcass ingestion, mount strong adaptive immune responses to anthrax (Turnbull et al. 1992; Turnbull et al. 2008; Hampson et al. 2011; Lembo et al. 2011; Bellan et al. 2012). Frequent anthrax contact can act as an immunity booster in these animals, strengthening their anti-anthrax protection over time (Bellan et al. 2012).

Herbivores, however, while capable of forming measurable anti-PA antibodies with anthrax vaccination, often require rigorous vaccination booster schedules to counter their initial low immune responses (Turnbull 1991; Chitlaru et al. 2011). While such vaccination attempts may protect susceptible wildlife species against anthrax infection (Turnbull et al. 2004), the few previous studies of natural immunity against anthrax in wildlife and livestock have indicated that, with the exception of some bovids, many wild herbivores may not develop and maintain measurable anti-anthrax antibody titers (Turnbull et al. 1992; Lembo et al. 2011). As measuring anti-PA titers is the most diagnostically reliable way to determine prior exposure to sublethal anthrax infection (Turnbull et al. 1992), these studies would suggest that most herbivores in these endemic disease systems are anthrax naïve and contract fulminant infection and die on being exposed to a species-specific LD<sub>50</sub> (dose that causes lethality in 50% of hosts) of *B. anthracis* (Glassman 1966; Titball and Manchee 1978; Boyd and Mar 1980; Christie 1987; Bohm et al. 1990; Turnbull et al. 1992; Watson and Keir 1994).

As the traditional spore concentration hypothesis (rains wash spores into depressions; puddles shrink in the dry season, leaving large concentrations of spores leading to dry season outbreaks) (Hugh-Jones 1999) cannot account for the ENP rainy season outbreaks, and there is as yet no definitive evidence that anthrax

can persist over time through its putative ability to undergo vegetative reproduction in the soil (Hanna and Ireland 1999; though see Saile and Koehler 2006 and Dey et al. 2012), it remains unclear why sporadic or cyclic outbreaks of the disease should occur rather than a more constant incidence of anthrax cases. Given the endemic nature of anthrax in ENP, the prolonged survival times of spores in the environment (Hugh-Jones 1999; Nicholson et al. 2000), and the fact that anthrax deaths do occur throughout the year in this system, it is likely that animals can come into contact with anthrax spores in all seasons. This then raises the question of what accounts for the seasonality of death rates from anthrax in ENP; one might expect to see both a more constant rate of anthrax deaths and a greater incidence of herbivore anthrax deaths in endemic systems unless exposure to doses exceeding LD<sub>50</sub>s somehow vary with season, host susceptibility changes with season, and/or hosts are able to survive some anthrax infections.

Given these seeming conundrums in the infection dynamics at the population and individual levels and the relative paucity of anthrax immunity studies in natural systems, I was motivated to more closely examine the immune dynamics of anthrax in the wild. I used anti-PA antibody titers both to gauge anti-anthrax immune responsiveness in plains zebra, African elephants, and springbok in ENP, and as a signature to determine the incidence of sublethal anthrax infections in this system.

In addition, I developed a more rigorous and sensitive method of determining anti-anthrax titers from a common immunology assay protocol (enzyme-linked immunosorbent assay, or ELISA). Serology via ELISA is the “bread and butter” of ecological immunology studies. Using ELISA to determine antibody titers against disease agents can act as both a measure of prevalence of the infectious agent in an ecosystem, as well as a measure of immune function in the hosts. ELISA methods, however, while relatively simple and long-established in laboratory studies, are often not as straightforward in wildlife research: positive controls, quantitatively titrated for standard curve determination or not, are often impossible to come by; usually only a few negative controls can be obtained from zoo collections, and sometimes only from closely- or distantly-related species; relying on commercially-available species-specific secondary antibodies and other reagents may mean accepting varying levels of cross-reactivity with the wildlife species in question; and determination of endpoint titers is usually not quantitative and methods for determining titers vary greatly in their subjectivity, sensitivity, and statistical rigor. While cross-reactivity of reagents should be determined from prior experience and on a case-by-case basis, I have attempted with my current study to address the other concerns listed above.

## **Methods**

### *Study Area*

I conducted this study in Etosha National Park (ENP), a 22,915 km<sup>2</sup> fenced conservation area in northern Namibia, located between 18°30'S-19°30'S and 14°15'E-17°10'E (Figure 1). The vegetation of ENP is largely arid savanna (Huntley, 1982); mopane shrubveld and treeveld covers much of the park, though the central

Okaukuejo plains consist of sweet grassveld and dwarf shrub savanna (le Roux et al. 1988). The park is dominated by a 4,760 km<sup>2</sup> endorheic salt pan, a remnant of a paleolake, which lies north and east of the Okaukuejo plains (Hipondoka et al. 2006).

Rainfall in ENP is highly seasonal: the major rainy season lasts from November through April, with the greatest rainfall occurring during January and February, and 80% of all rain falling between December and March (Gasaway, Gasaway, and Berry 1996; Engert 1997) (Figure 2). The dry season lasts from May through late November, with some local, isolated rains beginning in September (Auer 1997). Animals for this study were largely captured and sampled around the central Okaukuejo area, the location of the Etosha Ecological Institute (EEI) (Figure 1), where the mean annual rainfall is approximately 350mm (Turner et al. 2012). The only perennial water available to the park's wildlife is found in man-made boreholes, or in natural artesian or contact springs (Auer 1997).

### *Study Species*

Plains zebra, springbok, and African elephants were examined for this study. Zebra and springbok are the two most abundant plains ungulates species in ENP, with populations of approximately 13,000 (95% CI rounded to nearest 100: 10,900-15,000) and approximately 15,600 (95% CI rounded to nearest 100: 13,200-17,900) (EEI unpublished aerial survey data 2005). Elephants also exist in this system, with a population of approximately 2,600 (95% CI rounded to nearest 100: 1,900-3,300) (EEI unpublished data 2005).

Zebra, as members of the Family Equidae, are hindgut fermenters that graze almost exclusively on grasses (Codron et al. 2007; Gordon and Prins 2008). They are among the most water-dependent of the plains ungulates found in southern Africa, requiring access to drinking water at least every other day (Skinner and Chimimba 2005; Cain, Owen-Smith, and Macandza 2011). Springbok, members of the family Bovidae, are an arid-adapted species found across southern Africa (Skinner and Chimimba 2005). As intermediate feeders, springbok alternate between grazing and browsing; the majority of their diet (up to 77%) is from browse (Sponheimer et al. 2003), though they increase grazing efforts mainly during rainy periods when grasses are most digestible (Bigalke and van Hensbergen 1990; Hofmann et al. 1995). Springbok will drink when water is available, but are capable of obtaining all of their water needs from nutritional sources when eating succulent shrubs (Nagy and Knight 1994). Elephants are mixed feeders, eating varying amounts of grass and browse depending on location and season (Codron et al. 2006). Elephants in southern Africa have large home ranges, which expand and contract depending on season; home range sizes of elephants in ENP decrease greatly in dry seasons and are dependent on water hole density (de Beer and van Aarde 2008; Young and van Aarde 2010), and daily and hourly movement patterns of elephants in northwestern Namibia cover wider ranges during wet seasons than during dry seasons (Leggett 2009).



### *Animal Capture and Sampling*

Between 2008 and 2010, I obtained serum samples from individuals of all three study species. With a capture team, I fitted animals with VHF (very high frequency) collars (LoxoTrack, Aeroeskoebing, Denmark) or VHF-GPS/GSM (global positioning system/global system for mobile communications) collars (Africa Wildlife Tracking, Pretoria, Republic of South Africa) during the first immobilization to enable resampling of animals over several seasons. We first immobilized and sampled animals on the plains within an approximately 20km radius of Okaukuejo (Figure 1); in subsequent seasons (particularly during dry seasons, when some zebra groups migrate away from Okaukuejo; Stander 1992), we found and resampled several zebra between Okaukuejo and up to approximately 100km to the east, and Okaukuejo and approximately 15km directly to the south, whereas we resampled springbok and elephants again in the original area. We only sampled adult animals for all species. We immobilized, sampled, and released all animals safely under animal handling protocol AUP R217-0509B (University of California, Berkeley).

I collected a total of 154 zebra serum samples, 10 from males and 144 from females. I collected 44 elephant samples, 24 from males and 20 from females. I collected a total of 21 springbok samples, 12 from males and 9 from females. I collected blood from peripheral veins into Vacutainers without anticoagulant (Becton Dickinson, Franklin Lakes, NJ), kept it at approximately 4°C in a mobile refrigerator for up to six hours in the field, left it to clot at room temperature in the laboratory in the Etosha Ecological Institute (EEI) for four hours, placed overnight at 4°C, and then centrifuged it to allow for serum to be removed and aliquoted. I kept serum initially at -20°C for up to six months, and thereafter stored samples at -80°C until analyzed.

I sampled zebra over five seasons total, two wet and three dry (Table 1). I made an effort to resample as many animals as possible to reduce variation at the individual level and to be able to assess temporal immune changes. Several animals were resampled between two and five times over the seasons, for a total of 69 unique animals sampled and 154 samples total (Table 1). I sampled springbok over two seasons, one wet and one dry, and I sampled elephants over four seasons, one wet and three dry. There were eight springbok and 12 elephants resampled twice (Table 1).

### *Anti-PA ELISAs*

I adapted the ELISA procedure used to measure anti-PA antibody titers in serum from Pitt et al. 2001, Little et al. 2004, Marcus et al. 2004, and Turnbull et al. 2008. I used wild type PA provided in a concentration of 8.5mg PA/ml of phosphate buffered saline (PBS). I coated each well of a 96 well ELISA plate with PA at a concentration of 0.375µl per well in ELISA coating buffer (Bethyl Laboratories, Inc., Montgomery, TX), covered the plate to prevent drying, and incubated it at 20°C for at least one hour (up to 48 hours). I washed the plate with a solution of PBS containing 10% (v/v) Tween-20 (PBST), and then blocked wells for 30 minutes with

200ml per well of a mixture of PBS, 0.5ml/l Tween-20, and 10% (w/v) skim milk powder (Oxoid Laboratory Preparations; Basingstoke, Hampshire, England) (PBSTM).

After washing with PBST, I made serial, two-fold dilutions to the ends of rows in duplicate for all samples and negative controls in PBSTM, starting at a dilution of 1:4 and ending at 1:8192. I ran a duplicate negative control full titration series on each ELISA plate, even when we ran multiple plates simultaneously. I incubated the plate at room temperature for an hour before washing with PBST. I used commercially-available goat-anti-horse IgG-heavy and light chain horseradish peroxidase (HRP) conjugate (for zebra) and rabbit-anti-goat IgG-heavy and light chain HRP conjugate (for springbok) (Bethyl Laboratories, Montgomery, TX) as the secondary antibody, at the suggested dilution of 1:60,000. Previous studies have shown that the use of secondary antibodies against domestic and laboratory species related to the wildlife in question has good cross-reactivity against several wildlife species (e.g. Turnbull et al. 2004; Turnbull et al. 2008). As there are no domestic species closely related to elephants, Bethyl Laboratories manufactured goat-anti-elephant IgG heavy-and-light chain secondary antibodies for this study, using elephant blood from several of our captured elephants. As these antibodies were not conjugated, I added rabbit-anti-goat HRP-conjugated antibodies to the elephant plates after an additional incubation and washing step.

After further incubation and washing, I added TMB substrate (Kirkegaard & Perry Laboratories; Gaithersburg, MD) and stopped the reaction after 30 minutes with 2N sulfuric acid. I read well absorbance as optical density (OD) at 450nm on a SpectraMax M2 Microplate Reader using SoftMax Pro software v5.3 (Molecular Devices; Sunnyvale, CA).

For our negative control samples, I obtained 6 serum samples from plains zebra from the Woodland Park Zoo (Seattle, Washington), and 4 serum samples from African elephants and 7 serum samples from domestic goats (as proxies for springbok) from the Oakland Zoo (Oakland, California). Using the preceding ELISA procedure, I first analyzed negative controls individually in duplicate, two-fold serial dilution titration series (1:4-1:8192). I randomly chose one zebra sample as the negative control sample for all subsequent zebra sample ELISAs, whereas I combined all of the elephant negative controls and four of the lowest goat negative control responders in equal parts into separate, mixed samples to be used as the negative controls for all subsequent elephant and springbok ELISAs, respectively.

#### *ELISA Endpoint Titer Determination*

Previous ecological immunology and anthrax serology studies have used a variety of methods for determining ELISA endpoint titers, with varying degrees of subjectivity. While some studies benefit from the availability of quantitative, commercially-available kits for the antibodies in question (e.g. Abolins et al. 2011; Chambert et al. 2012), most involve the development of novel, more qualitative assays using available materials. Some studies have had access to positive controls, and have used these to determine sample endpoint titers in varying ways, for

example as percentages of positive control values (*e.g.* Zysling, Garst, and Demas 2009), by using positive control titration curves to establish arbitrary units against which to measure experimental samples (*e.g.* Svensson, Sinervo, and Comendant 2001), or as a function of negative and positive control differences (*e.g.* Cabezas, Calvete, and Moreno 2011; Murphy et al. 2011). As I was unable to obtain positive controls (samples from anthrax-vaccinated animals) for our assays, a common limitation in wildlife disease immunology studies, I developed the following endpoint titer determination protocols based solely on the more readily available negative control samples (samples from zoo animals).

Endpoint titer determination using only negative controls as comparisons has previously been fraught with subjectivity and has largely been devoid of a unifying method for titer determination. Studies have determined positive titers as the reciprocal of those sample dilutions showing absorbance above a pre-determined OD cutoff (*e.g.* Pezard et al. 1995; Little et al. 1997; Chabot et al. 2004); those that measure at a two- or three-fold increase above negative control or background ODs (*e.g.* Altboum et al. 2002; Aloni-Grinstein et al. 2005); those with a colorimetric change greater than mean negative control OD plus two or three standard deviations (SDs determined using various methods) (*e.g.* Work et al. 2000; Reuveny et al. 2001; Gaur et al. 2002; Schwanz et al. 2011); or those samples showing a greater than 20% difference over several titrations compared to competitively inhibited titrations of the same samples, with or without comparisons to negative controls (*e.g.* Turnbull et al. 1992; Turnbull et al. 2008). Frey, Di Canzio, and Zurakowski (1998) addressed some of these problems by establishing a statistically defined method for determining ELISA endpoint titers, based on the *t*-distribution of negative control readings. Their method increased sensitivity for detecting weak immune responses, and established good statistical power even with relatively low numbers (5-30) of negative controls. I expand on these methods here to make them even more relevant to ecological immunology studies, in which there are often larger amounts of variation, even amongst negative control samples.

Specifically, I addressed the following issues:

- 1) Even when different ELISA plates are run at the same time on the same day, there is often great enough inter-plate variability that real differences between low responders and negative controls may be swamped out by background variation. I addressed this by running a negative control sample on every plate to try to account for the “moving target” of plate variability and build it into the endpoint titer calculation
- 2) Very high and low dilutions of sera can have very variable degrees of binding in ELISA, compared to middle-range dilutions.
- 3) There can be high background noise particularly when crude antigens are used to coat ELISA plates (Frey, Di Canzio, and Zurakowski 1998). I addressed issues 2 and 3 by running the negative control, in duplicate on each plate, in the same serial dilution titration series as is used for the experimental samples, and calculating a cutoff for each dilution; while Frey et al. suggested this step might be tried in a pilot experiment, I believe that the

background noise and variability in ecological immunology samples (controls or otherwise, especially owing to the not-uncommon use of reagents made for species other than the ones being examined) makes it imperative that this step be included at all times, on all plates.

- 4) Negative control sera may only be available in very small amounts (*i.e.* depending on species and source).
- 5) Plate “real estate” is limited, and reagents may be as well. I addressed issues 4 and 5 by examining the titrated response curves of all negative controls (in duplicate) prior to the experimental assays, and then either combining the low-responding negative controls into a 1:1 pooled negative control mixture or by choosing one, low-responding abundant sample at random for use as the negative control for all subsequent plates. This allowed for the negative control to be analyzed in full titration and in duplicate on each plate, only required two rows of space out of eight on a 96 well plate, and still provided data with which to calculate inter-negative control (and inter-plate) variability for one method of endpoint titer determination.

Thus, to determine endpoint titers using our equations derived below, I suggest the following protocol:

- 1) Analyze all negative controls, in duplicate and in full titration series, prior to analyzing experimental samples.
- 2) Either pool equal amounts of each negative control into one master negative control sample, or choose at random one, low-responding (*i.e.* non-outlier), abundant negative control to be used for all subsequent plates.
- 3) Run the negative control on every plate with experimental samples.
- 4) Run the negative control, in duplicate, for the same titration series as is used for the experimental samples.

From the results of this rubric, I established three rules for endpoint titer determination:

**Rule 1:** Liberal cutoff. The titer is the last (highest) dilution in the titration series for which the mean sample OD for that dilution is greater than the mean negative control OD titration curve. This is similar to the subjective cutoff rules used in previous studies, though is potentially more sensitive (and potentially less specific) than rules using mean negative control OD  $\pm 2$  or 3SD.

**Rule 2:** Medium-conservative cutoff. The within-plate inter-duplicate standard deviation is estimated as:

$$\hat{\sigma}_{dup,d} = \sqrt{\frac{\sum_i (r_{d,i,1} - r_{d,i,2})^2}{n - 1}}$$

where  $d = 1:4, 1:8, \dots, 1:8192$ ;  $r_{d,i,1}$  and  $r_{d,i,2}$  are the two (duplicate) optical densities of the  $i$ -th sample at dilution  $d$ ; and  $n$  is the number of samples (indexed by  $i$ ) that were run in duplicate. For this rule, then, the endpoint titer is the last dilution at which

$$\overline{OD}_{s,d} > \overline{OD}_{nc,d} + 1.96 * \hat{\sigma}_{dup,d}$$

where  $\overline{OD}_{s,d}$  is the mean sample OD at a dilution, and  $\overline{OD}_{nc,d}$  is the mean negative control OD at a dilution buffered by the following 95% confidence interval determined by the inter-duplicate error at that dilution, across all samples. I recommend doing these calculations after all experimental samples have been analyzed, and re-calculating all titers for a project if additional samples are run later but are to be compared to the previous batch.

**Rule 3:** Ultra-conservative cutoff. The inter-negative control standard deviation is estimated as:

$$\hat{\sigma}_{ncont,d} = \sqrt{\frac{\sum_i (r_{d,i,1} - r_{d,i,2})^2}{n-1}}$$

where  $d = 1:4, 1:8, \dots, 1:8192$ ;  $r_{d,i,1}$  and  $r_{d,i,2}$  are the two (duplicate) optical densities of the  $i$ -th negative control at dilution  $d$ ; each  $i$  represents each of the  $n$  negative controls run on the negative control-only plates; and  $n$  is the number of negative controls (indexed by  $i$ ) that were run in duplicate. For this rule, the endpoint titer is the last dilution at which

$$\overline{OD}_{s,d} > \overline{OD}_{nc,d} + 1.96 * \hat{\sigma}_{ncont,d}$$

where  $\overline{OD}_{s,d}$  is the mean sample OD at a dilution, and  $\overline{OD}_{nc,d}$  is the mean negative control OD at a dilution buffered by the following 95% confidence interval determined by the inter-negative control (and therefore also inter-plate) error at that dilution, across all samples. This rule is usually more conservative than rule 2 due to the fact that variation between ODs of negative control samples run on different plates is greater than variation between ODs of the same sample run in duplicate on one plate.

#### *Examining Titer Patterns With Age and Season*

I developed generalized estimating equation (GEE) models using R v2.15.2 and the 'geepack' package (Højsgaard et al. 2006) to examine the influences of age, rainfall, and capture area on anti-PA titers in zebra. I chose GEEs because they can deal with data that violate assumptions of normality and independence and can account for different autocorrelation patterns in repeated measures data (Liang and Zeger 1986; Hardin and Hilbe 2003). These models are particularly useful for longitudinal data with many individuals (zebras) but relatively few longitudinal observations (sampling seasons) per individual (Zuur et al. 2009). For all models, I used a working correlation matrix with a first-order autoregressive relationship (AR-1) because, while individual disease and immune factors are likely correlated

through time, these correlations should decrease between later time points and earlier samplings (Liang and Zeger 1986; Zuur et al. 2009). I used zebra identification as the grouping structure, and used the waves argument in geepack with the capture number to account for missing sampling times for individuals.

In the first model, I included presence or absence of an anti-PA titer via rule 2 as the dependent variable, and capture area (Okaukuejo or Halali to the east), animal age in days, and season type (wet or dry) as independent variables. I determined age to half a year by combining tooth eruption observations, caliper measurements of upper incisors, and patterns of wear (Smuts 1974; Penzhorn 1982). I converted year age to age in days, and then "aged" each animal at recapture by the number of days between that event and the previous sampling for that individual. I fitted this GEE with a binomial distribution linked to a logit function.

In the second model, I included the  $\log_2$  of anti-PA titer via rule 2 as the dependent variable, and capture area, animal age in days, and cumulative rainfall one and two months prior to sampling as independent variables. Previous studies in this system determined that gastrointestinal helminth infection intensity in zebras is significantly related to rainfall one and two months prior, with both strongly correlated with helminth eggs shed per gram of feces (Turner 2009). As these parasite infections occur only a few weeks prior to peak anthrax outbreaks in these hosts (Turner 2009), I used these measures of rainfall both as more fine-scaled representations of season, and as a potential proxy for other seasonal effects on anthrax exposure. I determined cumulative rainfall over the one and two months prior to sampling by adding up daily rainfall amounts (in mm) over the 30 or 60 days prior to each individual capture event. I used rainfall gauge data from Okaukuejo for those capture events that took place surrounding that area, and rainfall gauge data from Halali for those capture events that took place on the Halali plains. I fitted this GEE model with a Poisson distribution linked with a log function, as  $\log_2$  titers were essentially discrete counts.

I developed the GEE models by using a backwards stepwise refinement method based on comparing the quasi-likelihood under the independence model criterion (QIC) values between maximal models and models with variables removed (Pan 2001). The QIC is equivalent to Akaike's information criterion (AIC) for repeated measures; a smaller QIC indicates a better fitting model. I used the 'yags' package in R to determine QIC values (Carey 2004). After this first refinement, I added biologically-sensible interaction terms between the remaining explanatory variables and further refined the models by comparing QIC's. I validated the models by plotting Pearson's residuals against fitted values to look for residual patterns, examined residual histograms to assess the normality of error distributions, and plotted residuals against each explanatory variable to test for homogeneity of error variances. For the final, best-fit models, I used Wald chi-square tests to determine the significance of each parameter estimate.

## Results

### *Prevalence of Anti-PA Antibodies*

Prevalence of anti-PA antibodies in the three study species varied with the ELISA endpoint titer rule used (Table 2; Figure 3). While few springbok and elephants showed positive titers for the more rigorous rules 2 and 3, more than half of the zebra samples (and unique individuals) tested had measurable anti-anthrax titers for all rules.

Antibody prevalences for unique zebras ranged from 52-87%, depending on the endpoint titer rule used (Table 2). Rules 1 and 3 zebra titers had the largest ranges, with animals having antibody titers between 4 and 4096 ( $\log_2$  titer range=2-12); the highest zebra titer for rule 2 was 256 ( $\log_2$ =8) (Figure 3). As rule 3 showed decreased sensitivity for the zebra assays, and as I did not run any sample duplicates on different ELISA plates for zebra (and thus did not need to account for inter-plate variability using rule 3), I decided to use the titer results from the other rules in our further analyses and discussions. For similar reasons, I also decided to not give further consideration to rule 3 results for springbok and elephants. Because rule 2 appeared to be sensitive enough to capture a large number of positive zebra titers in our assay while employing a rigorous assessment rubric, I decided to examine the zebra titers generated with the rule 2 calculations only for further analysis and discussion.

Prevalences of anti-PA antibodies in unique springbok ranged from 0-15%, depending on the rule used (Table 2). While rule 1 appeared to be 1.5 times as sensitive for detecting positive titers as was rule 2 for this assay, it is difficult to determine from the lower springbok sample size and low positive sample size whether the more rigorous rule 2 significantly affected these prevalence rates. Because anthrax titer prevalence in springbok was so much lower than that for zebra while the two species experience anthrax outbreaks in the same season, I did independent-samples, two-tailed *t*-tests comparing monthly totals for zebra and springbok deaths over both the historical anthrax sampling period of 1974-2010 (mean, SD: zebra: 80.6, 93.8; springbok: 23.5, 15.8) ( $t=2.18$ ,  $p=0.049$ ) and over the study period of 2008-2010 (mean, SD: zebra: 16.2, 18.1; springbok: 4.1, 2.5) ( $t=2.39$ ,  $p=0.030$ ). Thus, there are potentially significant differences in anthrax deaths between these two species.

Positive prevalences of anti-PA antibodies in unique elephants ranged from 3-52%, depending on the rule used (Table 2). As there was only one positive animal detected with rule 2, but 17 unique positives detected with rule 1, I suspect that rule 2 may not have been sensitive enough for this particular assay to detect true positive titers in elephants. As the positive animal via rules 2 and 3 indicate that elephants can, in fact, form measurable anti-PA antibody titers, I am confident that at least some of the positive animals via rule 1 were true positives. While understanding that rule 1 may also give a relatively high number of false negative titers, I decided to focus my further examinations and discussions on the elephant titer results calculated via rule 1. Positive animals via rule 1 had titers ranging from

4-8192 ( $\log_2=2-13$ ), with an even distribution of samples across this range (Figure 3).

#### *Changes in Anti-PA Titers Over Time*

Eleven resampled zebra positively seroconverted (changed from a negative anti-PA titer to a positive, measurable titer) at some point during the course of my study. In addition, 13 resampled zebra converted from a positive titer in one season to a negative titer in a subsequent season (Figure 4; some negative seroconverting animals not shown in this figure). As my sampling period was somewhat coarse (between three and eight months for individual recaptures), I was not able to determine exactly when a negatively seroconverting animal lost its measurable titer; I was, however, able to determine that this animals did not maintain a titer for longer than that sampling period. In this manner, examining just those zebra that measured positive in one season and negative in the subsequent sampling season, I found that the mean time to negative seroconversion was less than  $191 \pm 39$  days ( $6.3 \pm 1.3$  months; mean  $\pm$  SD;  $N=9$ ), with a range of less than 107-225d (3.5-7.4mo). In addition, by looking at zebra sampled four or five times over the two-year study period, I was able to track changes in titers over time. I found that, while some zebra titers changed somewhat erratically by repeatedly rising and falling, some animals maintained relatively stable titers over several seasons while others decreased or increased steadily over time (Figure 4).

Only one of the springbok with a positive titer by rule 2 was sampled twice; this animal positively seroconverted between seasons from a negative titer to one of 128 ( $\log_2=7$ ). This same animal showed a positive seroconversion to 1024 ( $\log_2=10$ ) via rule 1 calculations. Of the three seropositive springbok via rule 1, only one was resampled while also negatively seroconverting from one season to the next; this animal changed from a very low titer (4) to a negative titer sometime over the course of 238 days (7.8 months). Given the low sample size of resampled springbok and the low number of sampling times, this is obviously a coarse estimate of seroconversion time for this group.

Four elephants positively seroconverted during the course of my study. Three elephants negatively seroconverted over time; due to the long intermittent times between elephant sampling, the mean time to negative seroconversion of  $533 \pm 140$ d ( $17.5 \pm 4.6$ mo; mean  $\pm$  SD), and range of 383-661d (12.6-21.7mo), are rather coarse estimates of the time required for elephants to lose anti-PA seropositive status.

#### *Relationships Between Anti-PA Titers, Season, and Age*

The best fitting first model contained only season type, while age and area were both insignificant and not included in the final model. Season type was significantly associated with having an anti-PA titer ( $B=0.86 \pm 0.32$  SE, Wald statistic=7.03,  $p=0.008$ ), with animals in the wet season being more likely to have a titer than those in the dry season. This model had a 1.8% improvement in QIC over the initial, maximal model. As titers dropped in nearly all animals over time, and as



several animals were found to vacillate between positive and negative titers at different capture events, I then performed a Pearson Chi-square test for independence between seasons using each titer as a separate event without taking into account individual identification. I found that there was a significant effect of season on being titer-positive ( $\chi^2=4.97$ ,  $p=0.026$ ), with a positive titer prevalence of 76% in the wet season and 58% in the dry (Figure 6).

The best fitting second GEE model contained both cumulative rainfall one month prior to capture and cumulative rainfall two months prior to capture (hereafter simply "Rain1" and "Rain2"). It did not contain age or area, nor were any interaction terms significant or improve the model fit. Rain1 was significantly, negatively associated with anti-PA titer ( $B=-0.01 \pm 0.01$  SE, Wald statistic=6.66,  $p=0.009$ ), while Rain2 was significantly, positively associated with anti-PA titer ( $B=0.01 \pm 0.01$  SE, Wald statistic=5.82,  $p=0.016$ ). This model had a 5.5% improvement in QIC over the initial, maximal model.

## Discussion

### *Hosts in ENP Experience Sublethal Anthrax Infections*

My data show that, regardless of which of my rules are used for ELISA endpoint antibody titer calculations, zebra in ENP experience sublethal anthrax infections and survive, bearing the immune signatures of these infections. Using anti-PA titers as a proxy for sublethal anthrax infection occurrence, and given the fact that anthrax either results in subacute-to-acute death or survival after the same short time period (*i.e.* it is not a chronic disease) (Collier and Young 2003; Hugh-Jones and de Vos 2002), the cumulative incidence of sublethal anthrax in the ENP zebra population over the three years of this study can be roughly estimated at approximately 50% (Table 2). Given the fact that the mean time to titer loss in resampled zebras was less than six months, with the shortest loss of titer occurring over the course of 3.5 months, my survey rate of roughly six months likely underestimates the number of sublethal anthrax infections in these hosts.

In addition, I found that zebras are more likely to have measurable anti-PA antibody titers in wet seasons compared to in dry seasons, perhaps indicating that anthrax encounter rates differ between seasons (see discussion below). However, when actual amounts of rainfall are taken into account, a more complex picture emerges. While more cumulative rainfall in the two months prior to sampling predicts higher anti-PA antibody titers, more rainfall in the one month prior to sampling predicts lower anti-PA antibody titers. Given the strong correlation between rainfall and gastrointestinal parasite infection intensities in this system, the time lag between GI parasite peaks and anthrax deaths in the wet season (Turner, 2009), and the fact that GI parasites can cause strong immunomodulatory effects on hosts resulting in the inability to fight off bacterial coinfections (Mosmann et al. 1996; Brady et al. 1999; Chen et al. 2005; Graham et al. 2005; Ezenwa et al. 2010), it is likely that there are coinfection and immunomodulatory effects here not being accounted for.

My antibody titer results in elephants and springbok are somewhat equivocal, as only one elephant and two springbok samples were positive under the tighter assessment protocols of rule 2. However, the fact that I did see at least one animal of each of these anthrax host species with a measurable titer indicates that both elephants and springbok are also capable of mounting measurable adaptive immune responses against anthrax, and that they experience and survive anthrax infection upon occasion. As greater than 50% of elephant samples (both of unique animals, and including all resamples) showed measurable anti-PA titers under rule 1 (Table 2), with some titers being quite high (Figure 3), it is likely that rule 2 was simply not sensitive enough in this case to detect all of the true positive titers. As such, it is likely that the cumulative incidence of sublethal anthrax in elephants over the course of this study was somewhere in between that estimated by rule 2 (3%) and that estimated by rule 1 (about 50%).

### *Comparison to Previous Studies*

The results of my study differ from those of the few other previous wildlife anthrax serology studies. Out of 72 herbivore sera from ENP tested previously, only four (three springbok and one giraffe) had positive, measurable anti-PA titers, and no zebras ( $N=24$ ) and no elephants ( $N=12$ , including 4 that had been vaccinated 12-18 months prior) tested with positive titers (Turnbull et al. 1992). That study, however, used a competitive inhibition ELISA to measure anti-PA antibodies. While this kind of assay is able to detect obvious positive samples with high titers (*e.g.* Turnbull et al. 1992 found that 47/48 ENP carnivores had positive titers, with a range of 128-32,768), it may not be sensitive enough to detect lower responders; this assay requires a high enough antibody concentration for there to be at least two consecutive dilutions at which the sample and its counterpart row (competitively inhibited with soluble antigen) show at least 0.2OD units difference from each other. As such, this previously-used assay behaves more sensitively when there is enough antibody present that there will, at some point in the dilution curves, exist a bound antibody excess compared to soluble antigen.

While Lembo et al. (2011) found some measurable anti-PA titers in Serengeti herbivores, particularly in buffalo and wildebeest, they found no positive titers in zebras ( $N=85$ ). Lack of assay sensitivity for low responders may have been an issue; this study used a new, commercially available anti-PA antibody kit (QuickELISA anthrax PA kit immunoassay, Immunetics, Inc., Boston, MA), which, while promising, has yet to be validated for many species (Lembo et al. 2011). The real difference in titers, however, likely lies in the fact that anthrax appears to be less ubiquitous in the Serengeti ecosystem than it is in ENP. The Serengeti study only detected 283 suspected (non-culture-confirmed) anthrax positive cases (and only 34 of them zebra) over 14 years, whereas we found 254 culture-confirmed anthrax carcasses in our three study species (270 including other host species) over the three-year study period of 2008-2010 alone, 194 of which were zebras (Figure 2). Thus, the environmental level of anthrax in the Serengeti ecosystem may be significantly

lower than that in ENP, resulting in their herbivores encountering and ingesting significantly fewer anthrax spores than do herbivores in ENP.

### *Sublethal Sources of Anthrax*

How might anthrax hosts in ENP might encounter sublethal doses of anthrax? There is as yet no definitive evidence that van Ness' incubator area theory of anthrax multiplication within soil is true (van Ness 1971); however, while a very low portion of vegetative anthrax bacteria survive from a carcass to sporulate (Lindeque and Turnbull 1994), a carcass with up to  $1 \times 10^9$  bacteria per milliliter at the time of death (Collier and Young 2003) can still seed the environment with potentially high enough concentrations of *B. anthracis* to cause sublethal infection. Lindeque and Turnbull (1994) found that *B. anthracis* spores were not uncommonly found in ENP at low levels, with anthrax in 3.3% of water and 3% of soil samples that had no known historical association with anthrax carcasses. Scavengers may play a role in spreading anthrax over the environment in a place like ENP; Lindeque and Turnbull (1994) found that between 50% and 72% of fecal samples from scavengers seen feeding on anthrax-positive carcasses contained viable *B. anthracis* spores. They also found that 26% of water samples collected from western ENP during an anthrax outbreak in elephants contained viable spores; while spore levels in water declined rapidly with dilution and time, final concentrations in water sources may indeed still be enough to result in sublethal anthrax infection and immune response. In fact, herbivores that ingest even a large dose of anthrax spores from a locally infectious zone may not suffer from fulminant anthrax and death; doses of *B. anthracis* required to kill even very susceptible hosts are remarkably high (Watson and Keir 1994), and a significant portion of anthrax spores may pass through host guts post-ingestion without taking hold and invading, potentially converting high ingested doses into low enough numbers of invading spores to result in sublethal infection (Aloni-Grinstein et al. 2005).

### *Surviving Anthrax Exposure*

My evidence shows that so-called "susceptible" anthrax hosts can not only survive anthrax exposure, but that hosts in endemic systems can often become infected with, and survive, anthrax. How might they survive such infections? Innate immune responses likely play a large role in preventing at least lower doses of anthrax from becoming established. While macrophages act as incubators for the initial germination of anthrax, they also play an important role in controlling early anthrax (Cote et al. 2004; Kang et al. 2005). Neutrophils may also help to control and clear anthrax early in infection (Mayer-scholl et al. 2005; Cote, Rooijen, and Welkos 2006). Evidence from imaging studies indicates that anthrax spores germinate and establish infections at the initial site of inoculation, such as the Peyer's Patches in the gut, and do not spread systemically until nearly 1.5-2 days after infection (Glomski et al. 2007; Dumetz et al. 2011). This delay may give the immune system enough time to prevent dissemination and fulminant infection. A recent study by Weiner et al. (2012) supports the idea that, at a low enough spore

dose, immune mechanisms locally and in the draining lymph nodes can overwhelm anthrax infection and prevent septicemia.

Adaptive immune mechanisms, including anti-PA titers, may also be protective early in anthrax infection. At first glance, this appears counterintuitive. A full plasma cell/antibody memory response requires at least three to six days to reach appreciable concentrations and increased antibody avidity (maturation) (Zinkernagel et al. 1996; Marcus et al. 2004) which is usually too slow to be effective against a fulminant anthrax infection that can kill the host within that time period. In addition, exotoxin production reaches its peak during the exponential growth phase of the bacteria and, while toxins may be produced to a lower degree earlier in infection, they are produced at low enough concentrations within the first one to two days of infection that they do not yet affect immune cell functioning at this time (Cote et al. 2005; Weiner et al. 2012). In this case, having even high concentrations of circulating anti-PA antibodies would seem irrelevant to preventing establishment of infection early on. However, PA has been detected on the surfaces of *B. anthracis* spores as early as one hour into the germination process, and anti-PA antibodies have been found to bind more strongly to the surface of ungerminated spores than to germinated spores (Welkos et al. 2001; Cote et al. 2005). This early antibody-spore binding has direct consequences for the fate of the spore: it increases the rate of macrophage phagocytosis of spores, and enhances the rate of intracellular spore germination, which is essential as macrophages can only kill germinating spores (Welkos et al. 2001).

#### *Maintaining Immunity to Anthrax*

While all three study species mounted measurable adaptive immune responses against anthrax toxin, these responses could be rather short-lived. The average time to loss of titer in zebra of six months is not that surprising, given what is known from anthrax vaccine and livestock studies; human anthrax vaccines require several initial doses and annual boosters to maintain effective anti-PA titers (Turnbull 1991), and most cattle surviving anthrax outbreaks and anthrax-vaccinated horses fail to maintain significant anti-PA titers for more than ten months to a year (Turnbull et al. 1992). My sampling time course was unfortunately not often enough to detect negative seroconversion more finely than at an 8-12 month scale for springbok and elephants. While I cannot, due to this issue, as well as to the low sample size of positive springbok, make strong conclusions regarding duration of immunological memory in elephants and springbok, the evidence suggests that anti-anthrax adaptive immunity in these hosts can also be short-lived.

As I did not find any evidence of a higher anti-anthrax immune responses as animals age, it appears likely that most animals, while experiencing repeated exposures to *B. anthracis*, do not encounter the pathogen on a regular enough basis to consistently maintain a positive titer trajectory over the very long-term. However, I did find evidence for at least short-term maturation of the anti-anthrax immune response in several animals (Figures 4 and 5). Several zebras and one elephant showed steadily increasing titers over the course of two or more seasons, indicating

a kind of natural booster effect with succeeding infections. Evidence from laboratory studies indicates that a maturing memory response can, in fact, occur even with extremely low initial doses of *B. anthracis*. Marcus et al. (2004) vaccinated guinea pigs with a recombinant PA vaccine at low enough first doses that, in some cases, a post-vaccine anti-PA titer could not be measured. Those animals, after being boosted two weeks later with the same low dose, seroconverted, and their antibodies showed a partial maturation with increased avidity toward PA. While the memory response was stronger (both in terms of titer and avidity) in animals given higher initial and booster doses of the vaccine, even animals given an intermediate vaccine dose showed increased rates of full protection in the face of an extreme *B. anthracis* dose challenge. Some intermediately vaccinated animals also showed full survival after a large *B. anthracis* dose, even though their titers had decreased to non-detectable levels in the months prior to challenge. These findings may have important implications for wild herbivores becoming infected with low doses of *B. anthracis*; by doing so, they may be effectively repeatedly “vaccinating” themselves against anthrax, and even low anti-PA titers may be more protective against subsequent infection than has been previously thought. Given the fact that very low infections with *B. anthracis* might be defeated by a host without creating an immune signature (*e.g.* Aloni-Grinstein et al. 2005 had to booster guinea pigs three times with an oral anthrax vaccine to obtain measurable titers), the first anti-PA titer in my study animals may represent two or three previous, undetected infections.

#### *Species Differences in Anthrax Immunity*

While 62% of unique zebras showed measurable anti-PA titers as signatures of sublethal anthrax infection, the results from springbok and elephants were more equivocal, with 8-15% of unique springbok and 3-52% unique elephants showing positive anti-anthrax titers, depending on which rule is used (*i.e.* rule 2 and rule 1, respectively) (Table 2). These species variances may, in part, be due to differences in sampling efforts: springbok and elephant individual animals were, at most, resampled once, and then only at a timescale of eight to twelve months later. My sample sizes for springbok, especially, were also small compared to those for zebras.

However, there may be other, ecological and life history-related reasons for the observed species differences. Springbok in ENP do experience anthrax outbreaks in the wet season, but at much lower observable numbers than do zebra (Figure 2). This could be due, in part, to the fact that springbok are much smaller than zebra, and therefore springbok carcasses are more difficult to find due to size and quicker full predation after death; however, *t*-tests did show significant differences in anthrax deaths between the two species and so there may be real differences in anthrax infection rates between these two species, regardless of detection bias. Though springbok do graze more during wet seasons when grasses are more digestible (Bigalke and van Hensbergen 1990), they are always predominantly browsers (Sponheimer et al. 2003) while zebra are almost exclusively grazers (Codron et al. 2007). Interestingly, a recent study found that, while soil ingestion increased significantly during the wet season compared to in the

dry for both zebra and springbok, springbok ingested more than seven times less soil per day than did zebras (Turner et al. 2013). Thus, springbok very likely have less overall contact with anthrax spores than do zebras. In addition, I found that zebras are significantly more likely to have a positive anti-PA titer in the wet season compared to in the dry season (Figure 6), indicating that they are likely encountering more anthrax at this time. Zebras, as hindgut fermenters, also eat significantly more per body weight than do ruminants such as springbok, which may also increase the amount of spores ingested and contact rate for zebras (Gordon and Prins 2008; Hampson et al. 2011). In addition, zebra are significantly more water-dependent than are the more arid-adapted springbok; zebra in ENP drink from water sources nearly every day (R. Zidon, unpublished data), whereas springbok drink only once out of every four or five days (Auer 1997). While waterborne spread is unlikely to cause fulminant anthrax infection (Durrheim et al. 2009), the low concentrations of anthrax spores found in some ENP watering sources may be enough to “booster” zebras more often than springbok with sublethal anthrax infections (Lindeque and Turnbull 1994).

Elephant anthrax outbreaks occur with different timing in ENP than those in the other plains herbivores, with most anthrax deaths in elephants at the end of the dry season/beginning of the wet season (Figure 2). Recent work by Beyer et al. (2012) revealed that zebras, springbok, and elephants in ENP are infected with and die from the same strains of anthrax, so outbreak timing differences are not microbiological in nature. Elephant movement patterns in ENP, however, are quite different from those of the other plains herbivores; when the majority of zebras and springbok are congregating in the wet season in the area surrounding Okaukuejo, elephants are ranging widely (especially north and west of Okaukuejo) throughout the park, and are very scarce on the Okaukuejo plains (J. W. Kilian, unpublished data). Elephants return to these plains, where the majority of anthrax outbreaks have occurred throughout known ENP history, in the dry season. During this season, elephants are prone to taking dust baths (Rees 2002; EEI, unpublished data); thus, as has been hypothesized for bison anthrax outbreaks in the Northwest Territories in Canada, perhaps elephants contract inhalational anthrax while dust-wallowing (Gates, Elkin, and Dragon 1995; Dragon et al. 1999). Recent zebra deaths in this area during the previous season may create potent locally infectious zones (LIZs) of concentrated anthrax; even if these spore sinks become dispersed over the landscape with time, it is likely that these LIZs remain for at least the next several months for elephants to contact (W. Turner, unpublished data). As elephants are mostly browsers (Codron et al. 2006), they, like springbok, may not ingest anthrax as much and as often as do zebras, thereby resulting in lower anti-PA titers compared to those in zebras and potentially less protection against subsequent doses. The LD<sub>50</sub> (lethal dose 50%) for inhalational anthrax is also nearly always significantly lower than that for intestinal anthrax (Watson and Keir 1994). These combined factors may help account for the differences in elephant anti-anthrax titer prevalence, as well as the differences in outbreak timing in this species compared to in other ENP hosts.

## Conclusions

In summary, zebra in ENP often become infected with, and survive, *B. anthracis* infection. In the process, zebra can build up their immunity to *B. anthracis* over time. Zebra appear to have a higher exposure to *B. anthracis* in the wet season, though factors other than season also clearly affect the complex immune dynamics of anthrax in these hosts. Elephants and springbok in ENP also appear to experience sublethal anthrax infections, though at lower rates than do zebras. Though adaptive immunity to anthrax wanes rapidly in all three of our study species, there is evidence that subsequent sublethal *B. anthracis* infections occurring at a constant enough rate (*i.e.* approximately every three to six months) act as immune boosters that result in maturation of the anti-PA response in these hosts. This antibody maturation response also serves as an immune signature proving that even so-called susceptible hosts in an anthrax-endemic system survive multiple infections with anthrax over their lifetimes.

These results show that, rather than being an all-or-nothing disease, anthrax often occurs as a sublethal infection in susceptible herbivore hosts in an endemic anthrax system. Plains herbivore populations in ENP exist well below their food resource ceiling, and it has been hypothesized that, alongside predation, anthrax is a main population-limiting factor in this system (Ebedes 1976; Berry 1981; Gasaway, Gasaway, and Berry 1996). However, others have argued that anthrax deaths occur at too low a level compared to overall population sizes to cause population declines in plains ungulates (Lindeque 1991). While some have hypothesized that anthrax outbreaks may occur when hosts are immunocompromised due to co-infections and other stressors (Gainer and Saunders 1989; Gates, Elkin, and Dragon 1995; Dragon et al. 1999), my results indicate that sublethal anthrax infections may themselves act as immunomodulators, as hosts use immune system resources to successfully clear them. Thus, although anthrax death rates may be too low in ENP to limit host population growth, sublethal anthrax infections could potentially be population modulators through indirect immune system and co-infection effects.

## Acknowledgments

I thank the Namibian Ministry of Environment and Tourism for permission to do this research, the Directorate of Parks, Wildlife and Management for permission to work throughout Etosha, and the staff in the Directorate of Scientific Services at the Etosha Ecological Institute for logistical support and assistance. I would like to give special thanks to veterinarians Mark Jago, Conrad Brain, Peter Morkel, and Ortwin Aschenborn for their assistance with animal captures, as well as to Martina Küsters, Shayne Kötting, Gabriel Shatumbu, Wendy Turner, Wilferd Versfeld, Marthin Kasaona, Royi Zidon, and Werner Kilian, among others, for their tremendous help in the field. I especially thank Russell Vance for allowing me to work in his immunology lab and for giving me assistance and guidance. I also greatly thank Steve Bellan for assistance with the ELISA endpoint titer determinations. Thank you to Wendy Turner for her comments on this manuscript. I thank Bryan Krantz for

providing PA for the ELISAs, Bethyl Laboratories for being willing to manufacture anti-elephant antibodies, keepers at the Woodland Park Zoo for providing zebra control serum, keeper Jeff Kinzley at the Oakland Zoo for providing elephant control serum, and veterinarian Karen Emanuelson at the Oakland Zoo for providing goat control serum. This research was supported by Andrew and Mary Thompson Rocca Scholarships, a James S. McDonnell grant, and NIH grant GM83863.



## Tables

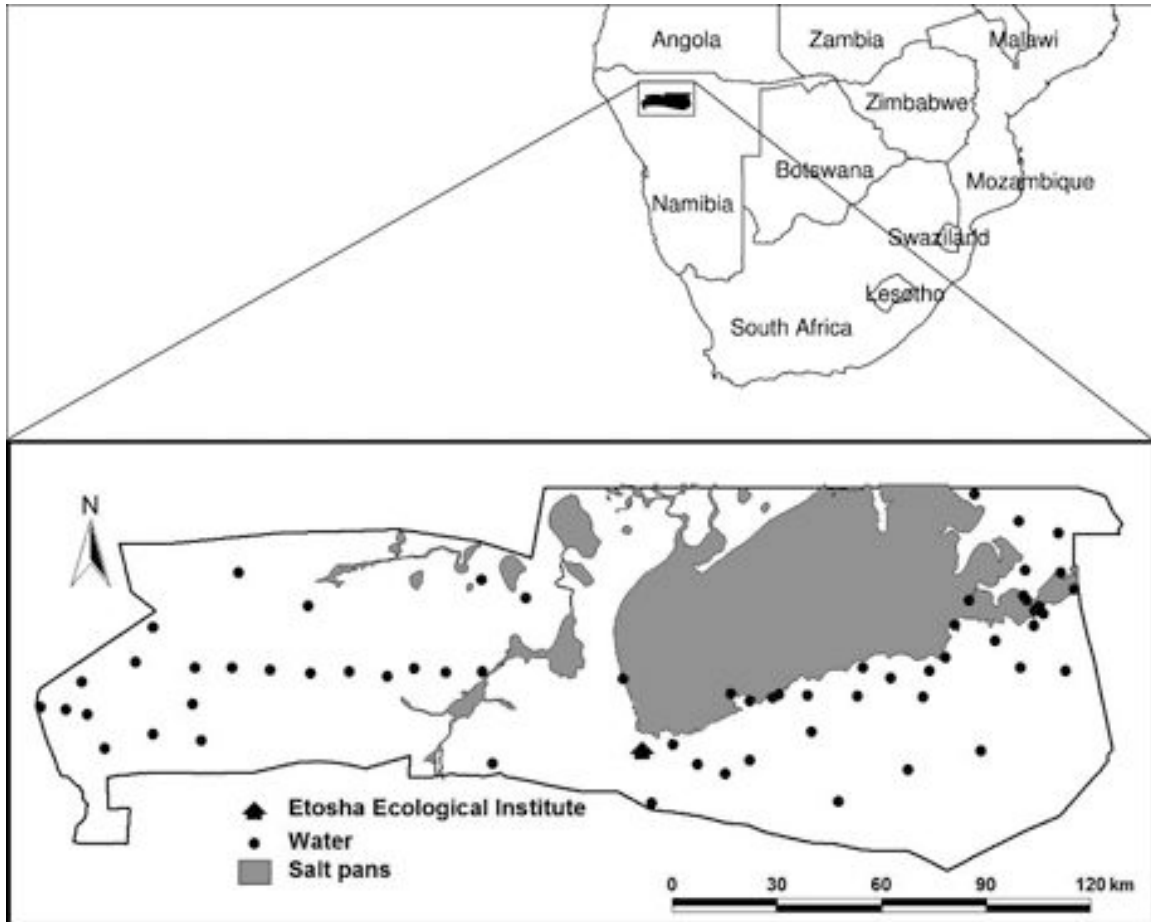
**Table 1.** Capture seasons and timing for plains zebra (*Equus quagga*), springbok (*Antidorcas marsupialis*), and African elephant (*Loxodonta africana*) in Etosha National Park (ENP), Namibia. "#New" refers to new individuals and their samples; "#Re-S" refer to animals resampled at least once in that season. #Re-S x2, 3, 4, or 5 are the total numbers of animals for each species resampled 2, 3, 4, or 5 times, respectively.

Species	Capture Season	Nominal Season	Date (Mo/Yr)	#New	#Re-S	#Re-S x2	#Re-S x3	#Re-S x4	#Re-S x5
Zebra	S1	Wet	3-4/08	45	0				
	S2	Dry	10-11/08	14	22				
	S3	Wet	4-5/09	6	29				
	S4	Dry	9-11/09	4	9				
	S5	Dry	8/10	0	25				
	Totals	5		69	85	20	11	12	2
Springbok	S1	Dry	8-9/09	13	0				
	S2	Wet	4-5/10	0	8				
	Totals	2		13	8	8	0	0	0
Elephant	S1	Dry	10/08	10	0				
	S2	Dry	7-10/08	18	0				
	S3	Wet	3/10	1	1				
	S4	Dry	8/10	5	11				
	Totals	4		34	12	12	0	0	0

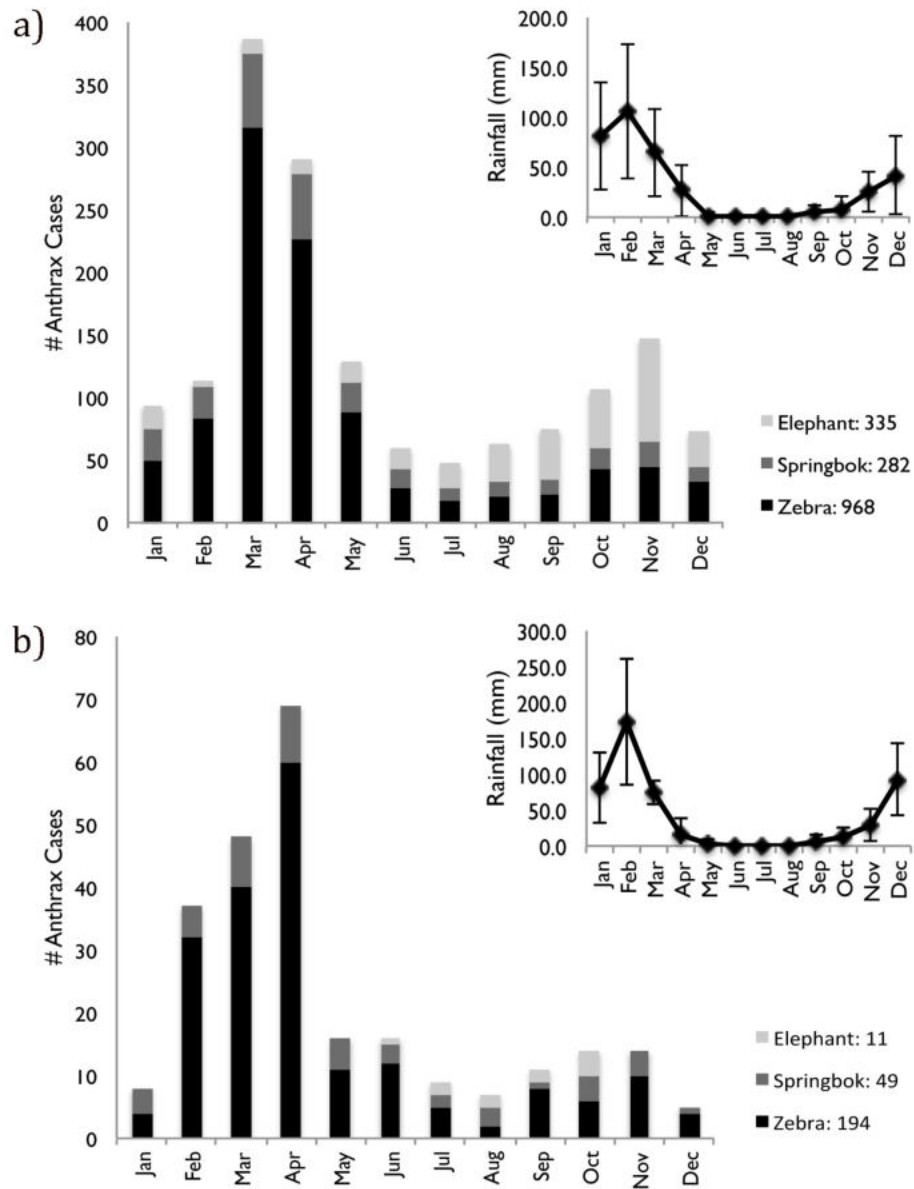
**Table 2.** Prevalence (%) of antibodies against *Bacillus anthracis* PA toxin in zebra, springbok, and elephant in ENP. Positive titers were determined by indirect anti-PA ELISA and three different endpoint titer determination rules. “All” refers to all samples tested from that species, including resampled individuals. “Unique” refers to unique individuals: animals that were resampled in multiple seasons but were only counted once per positive titer. Numbers in parentheses are sample sizes (same for each species across rules).

Species	Rule 1		Rule 2		Rule 3	
	All	Unique	All	Unique	All	Unique
Zebra	91.6 (154)	86.9 (69)	67.5	62.3	54.5	52.2
Springbok	14.3 (21)	15.4 (13)	9.5	7.7	0.0	0.0
Elephant	52.3 (44)	51.5 (33)	2.3	3.0	2.3	3.0

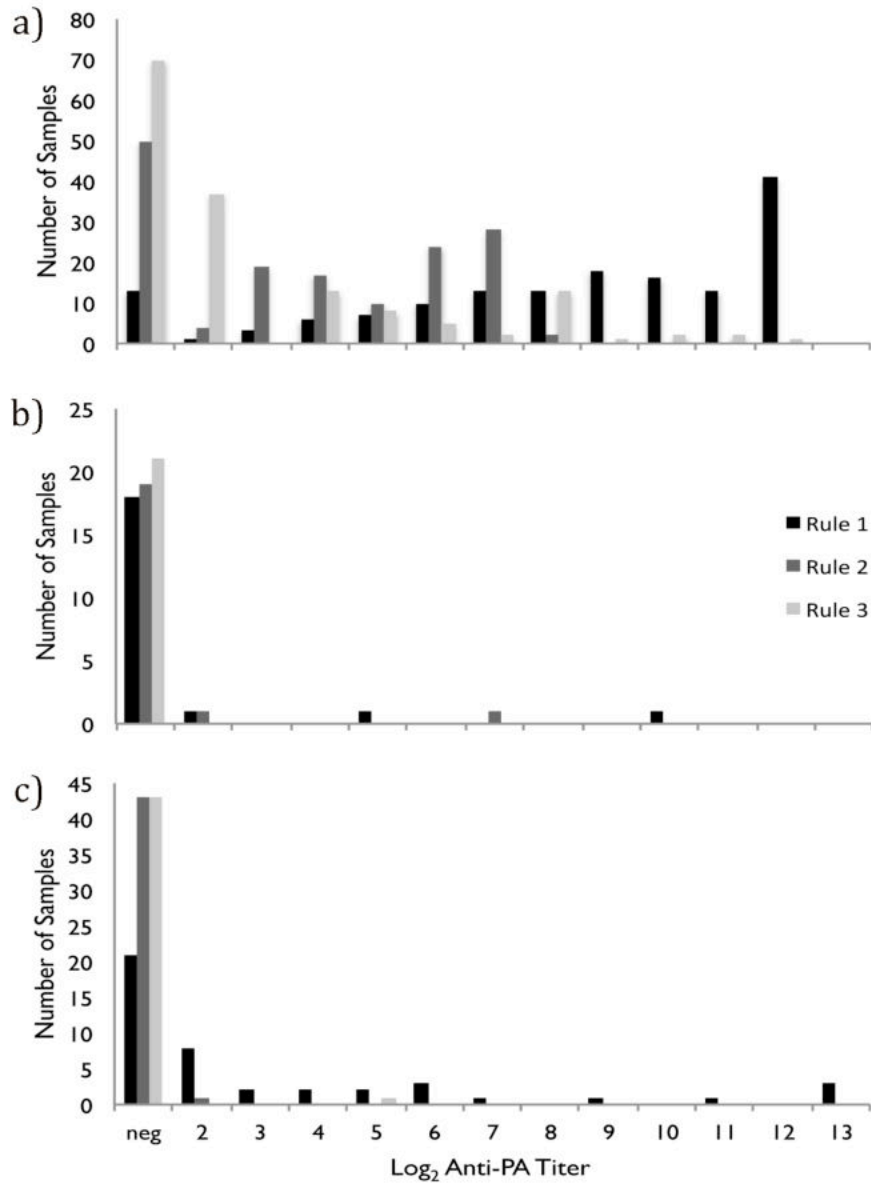
## Figures



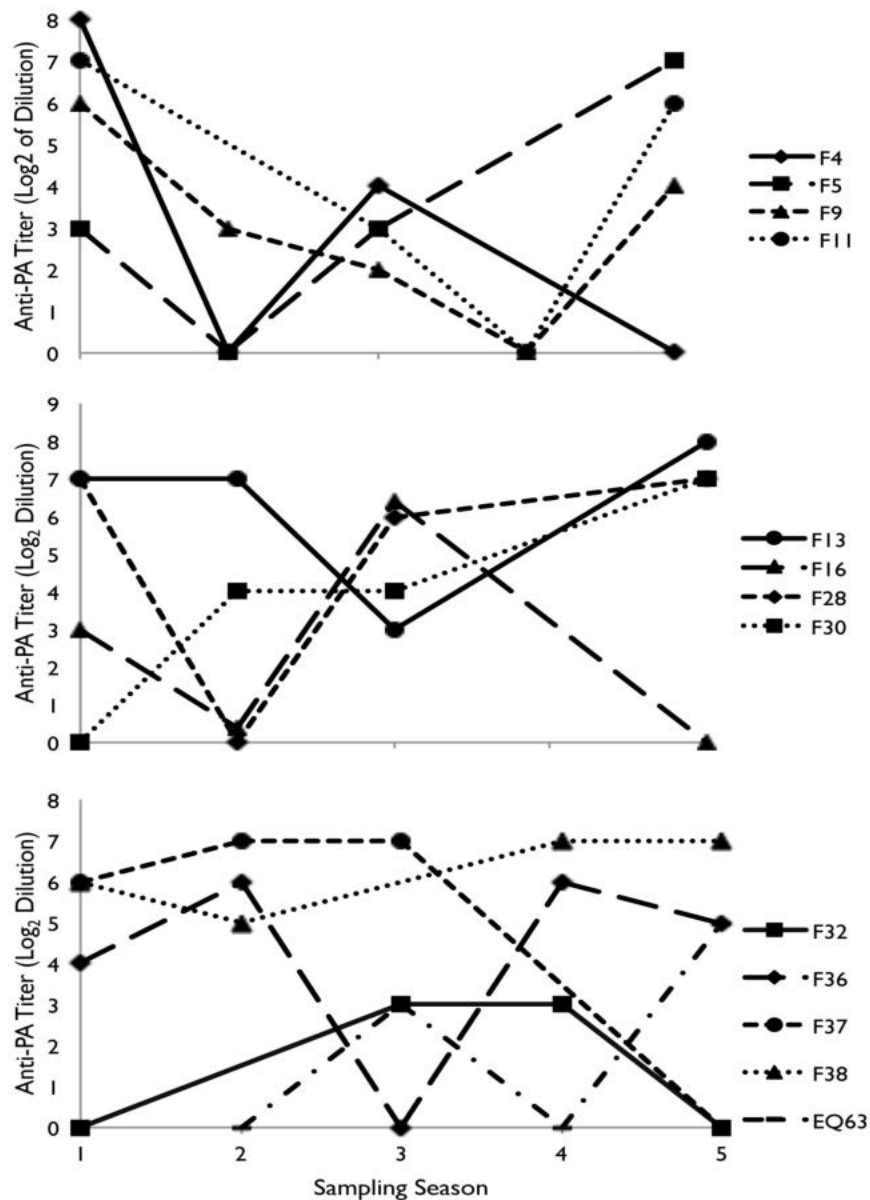
**Figure 1.** Etosha National Park in northern Namibia. The Etosha Ecological Institute is located in Okaukuejo in the center of the park; the majority of animal sampling for this study occurred in the nearby surrounding area, within a radius of approximately 20km (in the plains outside of the salt pans). Perennial watering points (springs or boreholes) are marked with black circles.



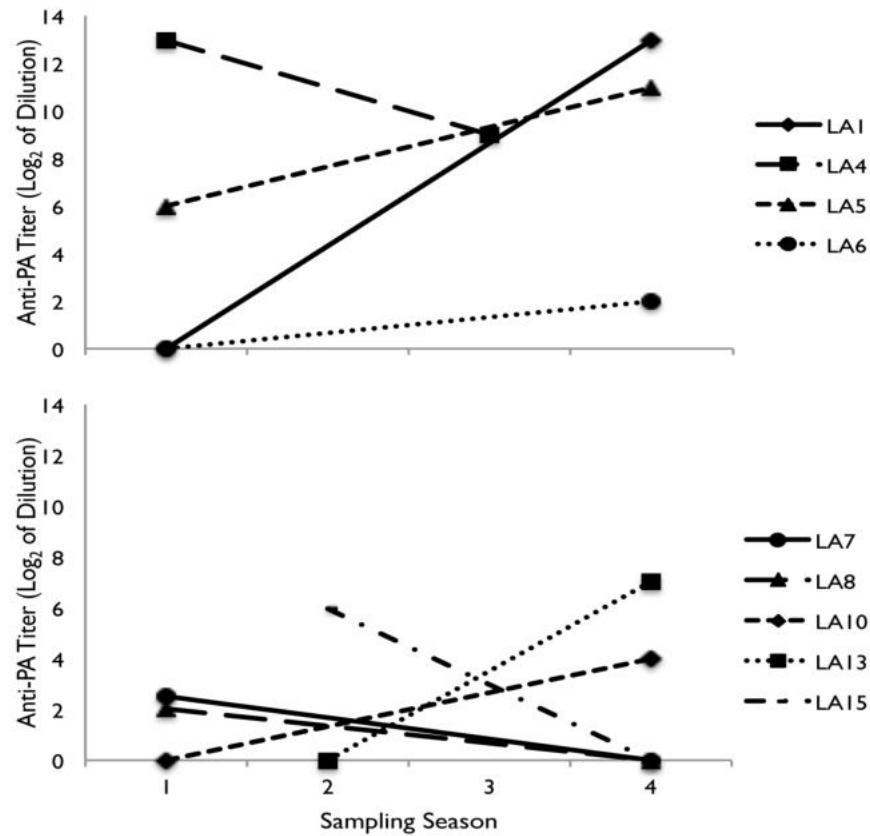
**Figure 2.** Culture-confirmed anthrax cases for elephant, springbok, and plains zebra by month in ENP for a) 1974-2010 (the entire time of reliable anthrax sampling in the park) and b) 2008-2010 (the years of this study). Elephant cases in (a) are driven by a few large outbreaks in the 1980s (Lindeque 1991). Numbers listed in the legends are the total anthrax cases for each species for the years referred to in each graph. The graph inserts indicate the mean monthly rainfall  $\pm$  standard deviation in the Okaukuejo region for these same time periods, respectively.



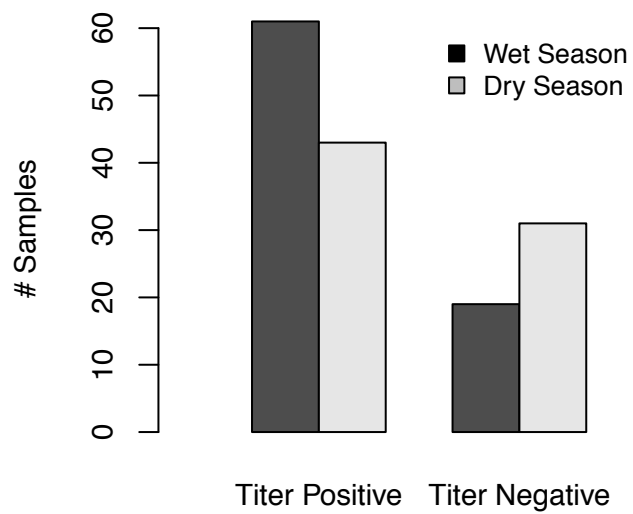
**Figure 3.** Antibody endpoint titers to *Bacillus anthracis* protective antigen (PA) toxin as determined by indirect ELISA in a) plains zebra, b) springbok, and c) elephant in ENP. Samples were all assayed in serial two-fold dilutions; endpoint titers are the reciprocal of the highest dilution above the cutoff determined by one of three rules. The figure shows numbers of individuals possessing each titer (shown as log<sub>2</sub> of the reciprocal of the dilution, for clarity) for each endpoint titer rule calculation.



**Figure 4.** Change over time of zebra antibody titers to *Bacillus anthracis* protective antigen toxin, as determined by indirect ELISA. Titers are shown as the  $\log_2$  of the reciprocal of the dilution endpoint as determined by rule 2 (based on the mean of the negative control plus a 95% confidence buffer determined by the inter-duplicate error for each dilution). Only zebra sampled 4 or 5 times are shown. Sampling seasons 1, 2, 3, and 4 are roughly 6 months apart from each other, whereas S5 is approximately 12 months after S4. Overlapping portions of some plots have been jittered slightly for clarity.



**Figure 5.** Change over time of elephant antibody titers to *Bacillus anthracis* protective antigen toxin, as determined by indirect ELISA. Titers are shown as the  $\log_2$  of the reciprocal of the dilution endpoint as determined by rule 1 (the highest mean sample dilution above the mean negative control curve). Only elephants sampled twice are shown. Sampling seasons 1 and 2 are 12 months apart, 2 and 3 are roughly 6 months apart, and 3 and 4 are 5 months apart. Overlapping portions of some plots have been jittered slightly for clarity.



**Figure 6.** Number of zebra samples with positive antibody titers against *Bacillus anthracis* protective antigen toxin and number of samples with no measurable titers compared across wet and dry seasons.



## Chapter 3

# Seasonal immune trade-offs driven by microparasite-macroparasite coinfections: evidence that gastrointestinal helminths affect seasonal susceptibility to anthrax?

### Abstract

The majority of vertebrates are concurrently infected with multiple pathogens. Most pathogen-pathogen interactions occur indirectly through the host immune system, and are particularly strong in mixed micro-macroparasite infections because of the strong immunomodulatory effects of helminth parasites. While these interactions and immune tradeoffs have been examined extensively in laboratory animals, few studies have extended such research into natural systems. In addition, many wildlife pathogens exhibit seasonal fluctuations, likely due, at least in part, to seasonal host immune fluctuations. As pathogen transmission changes with season, host immunity may be more strongly influenced by coinfection immunomodulatory effects than by external factors such as changing dietary and demographic patterns. I examined the seasonality of immune functionality, pathogen infectivity, and interactions between concurrent infections and immunity in wild plains zebra (*Equus quagga*) in Etosha National Park (ENP), Namibia, a system with strongly seasonal patterns of gastrointestinal (GI) helminth infection intensity and concurrent anthrax outbreaks. I found evidence that wet seasons in ENP are characterized by Th2-type immune skewing driven by GI helminth infections, and that these trade-offs make hosts less capable of mounting effective Th1-type immune responses against anthrax infections at this time. I found that immunity to GI parasite infections also helps to mitigate ectoparasite infestations, in contrast to previous studies showing that ectoparasites succumb to Th1-type immune responses. I also found evidence that coinfections and immune tradeoffs affect long-term host survival, with older animals possessing less intense GI parasite infections and higher Th2 responses, and few older animals with concurrent intense helminth infections and evidence of anthrax survival. GI parasites likely exert more selection pressure on zebra hosts than do ectoparasites and anthrax, but may actually be a stabilizing force in this host-pathogen system. This study is the one of the first to examine seasonal changes in multiple immune parameters in concert with macroparasite-microparasite coinfections in wildlife hosts in a natural system, one of the first to follow the same hosts longitudinally while examining these interactions, one of the first wildlife ecology studies to make use of multiple imputation methods for missing data, and the first to measure IgE antibody titers in wild equids.

## Introduction

Microparasites (bacteria, viruses, fungi, protozoa) and macroparasites (helminths, arthropods) are important components of both external and within-host ecosystems (Pedersen and Fenton 2007). The majority of animal and human hosts are infected with more than one pathogen at a time, with many host communities experiencing up to 100% prevalence of coinfections (Petney and Andrews 1998; Lello and Hussell 2008). These infections are often a mix of micro- and macroparasites that can interact with each other to modify pathogen transmission, virulence, and host availability. Pathogens can compete with each other for within-host niches (Lello et al. 2004), and fatal pathogens cause ecological interference by killing hosts and removing them from the susceptible pool for another parasite (Rohani et al. 2003; Jolles et al. 2008). The majority of pathogen-pathogen interactions, however, appear to occur indirectly via the immune system (Cox et al. 2001; Page et al. 2006; Pathak et al. 2012). Through immunomodulation, infections can increase host susceptibility to other parasites (Chen et al. 2005; Abu-Raddad et al. 2006), enhance the intensity of other pathogen infections (Borkow et al. 2001; Graham et al. 2008; Pathak et al. 2012), increase disease severity and pathology (Chen et al. 2005; Graham et al. 2005; Hughes and Shafran 2006; Lello and Hussell 2008), and increase disease duration (Pathak et al. 2012; Walzl et al. 2000).

Immunomodulation and immune tradeoffs are particularly strong in mixed microparasite-macroparasite infections. Intracellular pathogens (most microparasites) cause the mammalian adaptive immune system to mobilize T-helper-1 (Th1) cells, while extracellular pathogens (macroparasites) trigger T-helper-2 (Th2) cells. The most important Th1 cytokines include gamma interferon (IFN- $\gamma$ ), interleukin-2 (IL-2), tumor necrosis factor alpha (TNF- $\alpha$ ), and many subtypes of immunoglobulin G (IgG antibodies), while the Th2 pathway involves the cytokines IL-4, IL-5, IL-6, and IL-13, white blood cells such as eosinophils, and immunoglobulin E (IgE) antibodies (Mosmann et al. 1986; Abbas et al. 1996; van Riet 2007; Friberg et al. 2010). The pathways leading to and from Th1-type responses and Th2-type responses are mutually cross-regulated. Th1 cytokines inhibit the activity and development of Th2 factors and vice versa. Furthermore, both Th1 and Th2 cells are derived from the same T cell lineage within the hematopoietic stem cell line, and hence are ultimately limited by the maximum rate at which a host can produce undifferentiated T cells. These factors help explain why hosts have difficulty simultaneously mounting effective Th1 and Th2 responses (Abbas et al. 1996; Mosmann et al. 1996; Morel and Oriss 1998; Yazdanbakhsh et al. 2002). While Th1-Th2 cross-regulation may represent more of a continuum than a strict dichotomy, there is overwhelming evidence at a population level that the shift toward a distinct Th1 or Th2-type response is distinguishable, polarized, and non-random (Kelso 1995; Mosmann et al. 1996).

Many studies have demonstrated that helminth infections (trematodes and cestodes in the phylum Platyhelminthes, and roundworms in the phylum Nematoda) are particularly adept at skewing immune responses toward the Th2 arm, with this polarization becoming stronger with infection chronicity (Abbas et al. 1996;

Mosmann et al. 1996; Page et al. 2006). These Th2 responses have been found to downregulate Th1 immunity, even in the face of strong microparasite coinfections (Brady et al. 1999; Borkow et al. 2001; O'Neill et al. 2001; Chen et al. 2005; Graham et al. 2005; Page et al. 2006; Diniz et al. 2010; Ezenwa et al. 2010). Chronic helminth infections may also cause host immunosuppression, resulting in maintenance of the immunomodulating worm infection and increased susceptibility to coinfections (Gulland 1992; Graham et al. 2007; van Riet 2007; Diniz et al. 2010). Other macroparasites, such as arthropod ectoparasites, also elicit immune responses when feeding on hosts, with primarily Th1 responses being potentially more effective against factors in tick saliva (Wikel et al. 1982; Wikel et al. 1989; Wikel 1999; Ogden et al. 2002; Prelezov et al. 2002). Tick infections can also cause host immunomodulation, possibly skewing host immunity toward a Th2 profile (Ferreira et al. 1999; Ogden et al. 2002; Castagnolli et al. 2008), and some studies have shown that ticks can also be broadly immunosuppressive (Wikel 1999; Slámová et al. 2011). In addition to these immune effects, helminths contribute to host malnutrition through protein loss (Gulland 1992; Bowman 2003; Pullan and Brooker 2008), decrease host appetite and grazing behavior (Arneberg et al. 1996; Forbes et al. 2000; Colditz 2007), decrease host growth and weight gain (Gulland 1992; Lello et al. 2005; Pullan and Brooker 2008), increase probability of predation (Hudson et al. 1992; Murray et al. 1997) decrease host fecundity and survival (Hudson 1986; Fuller et al. 1996; Newey et al. 2004; Lello et al. 2005) and, through these effects, exert population-wide pressure and even cause population destabilization (Anderson and May 1979; Dobson and Hudson 1992; Gulland 1992; Hudson et al. 1998; Coltman et al. 1999; Tompkins 1999; Newey et al. 2005). Some ectoparasites can also affect host body mass and fecundity (Neuhaus 2003; Hawlena et al. 2006; Ballestreros et al. 2012). Thus, understanding macroparasite infections are important for understanding within-host to population level dynamics.

Many infectious diseases also experience seasonal fluctuations, patterns that cannot be ignored when examining effects of infections on each other, on hosts, and on populations (Grassly and Fraser 2006). Seasonality can influence pathogen survival outside of hosts, parasite survival within hosts, host behavior leading to differences in disease transmission, host immune function, and the abundance of disease vectors and reservoir hosts (Klein and Nelson 1999; Altizer et al. 2006; Grassly and Fraser 2006). Seasonality is especially prominent in directly transmitted helminth infections, as free-living stages require temperature and moisture thresholds to develop and survive outside of hosts (Waller et al. 2004; Cattadori et al. 2005); given the seasonality of these infections and their propensity to immunomodulate hosts, it is likely that helminths can help drive seasonal patterns of coinfections. Seasonality can also be affected by changes in host immunity due to non-pathogen factors: changing nutrition can bolster or suppress immunity, seasonal pregnancy and lactation can lead to decreased immunity across most adult females at once in a population, and seasonal parturition patterns can lead to a pulse of immunologically naïve hosts into the population (Nelson and Demas 1996; Sheldon and Verhulst 1996; Altizer et al. 2006). Intrinsic aspects of

changing host immunity, particularly in concert with parasite dynamics, however, are not yet well studied or understood (Koelle and Pascual 2004; Altizer et al. 2006; Beldomenico et al. 2008).

Many studies regarding the effects of coinfections and immune trade-offs have been conducted in laboratory settings, yielding a wealth of knowledge regarding these interactions. Similar studies in wildlife, however, are rare. Fewer studies have been conducted regarding disease and immune seasonality, as these are challenging to model in laboratory settings and require difficult, longitudinal studies in natural systems. Wildlife studies have looked at infectious disease seasonality without immune measures (Altizer et al. 2004), coinfections without seasonal or immune measures (or with only one simple measure of immunity) (Lello et al. 2005), single diseases with some measures of immunity (Gulland 1992; Cornell et al. 2008; Beechler et al. 2009; Chambert et al. 2012), immune seasonality without disease measures (Cattadori et al. 2005; Owen and Moore 2006; Beldomenico et al. 2008), coinfections and seasonality with only one simple measure of immunity (Telfer et al. 2010), and coinfections and immune trade-offs without seasonality (Jolles et al. 2008 and Ezenwa et al. 2010; Pathak et al. 2012; Bertó-Moran et al. 2013). Few studies examining infectious diseases and immunity link these measures in the same individuals and follow them through time (Telfer et al. 2010; Chambert et al. 2012). As emerging infectious diseases are becoming increasing threats to human, animal, and ecosystem health (Daszak et al. 2000; Corvalan et al. 2005), as the majority of animal and human populations experience coinfections (Petney and Andrews 1998), and as climate change causes changes in parasite distributions (Patz et al. 2000), it is increasingly important to understand infectious disease dynamics in natural systems in concert with seasonality and host immunity. Here, I present what I believe to be the first longitudinal study simultaneously examining coinfections, seasonality, and complex immunity in wildlife hosts.

I examined a natural population of plains zebra (*Equus quagga*, formerly *Equus burchelli*) in Etosha National Park (ENP), Namibia, over three years, encompassing five seasons, resampling many individuals over several seasons. I examined coinfections (or immune signatures of infection) with ectoparasites (ticks), gastrointestinal (GI) helminths, and anthrax in concert with several measures of immune function in these hosts. These three pathogens are the only known pathogens infecting zebra hosts in ENP; rabies does exist in this system, but there is no evidence that zebra play a role in its dynamics (Etosha Ecological Institute, EEI, unpublished data; Gasaway et al. 1996). Previous studies in ENP have found several tick species on zebra, all within the family Ixodidae (Horak et al. 1992). There is no evidence of the presence of tick-borne pathogens in ENP, and thus tick burdens can be assessed solely for their impact as ectoparasites on their hosts (EEI, unpublished data). Seasonal patterns of tick infection intensity are largely unknown for this system (but see Horak et al. 1992). While ENP zebra have a nearly 100% prevalence year-round with GI helminths, they experience a significant increase in infection intensity in the wet season compared to in the dry (Turner and

Getz 2010). The wet season is also when ENP plains ungulates experience large, annual outbreaks of anthrax, an environmentally-transmitted microparasitic infection; zebra account for 52% of all anthrax cases in ENP, and 57% of all anthrax cases occur in March and April at the middle and end of the rainy season (Turner et al. 2013). The reasons for anthrax outbreaks are unclear; the traditional spore concentration hypothesis (Hugh-Jones 1999) cannot account for the ENP rainy season outbreaks, and there is as yet no definitive evidence that anthrax can multiply in the soil (Hanna and Ireland 1999; though see Saile and Koehler 2006 and Dey et al. 2012). Given the endemic nature of anthrax in ENP, the prolonged survival times of spores in the environment (Hugh-Jones 1999; Nicholson et al. 2000), the fact that anthrax deaths and sublethal infections do occur throughout the year in this system, it is likely that animals come into contact with anthrax spores in all seasons. Thus, here, I examine seasonal changes in host coinfection and immune factors that may influence host susceptibility to this environmental pathogen. The goals of this study are: (1) to determine how host immune function changes seasonally in system with strong seasonal disease patterns; (2) to further examine the correlation with seasonal rainfall of the three pathogens in question; (3) to examine the host immune responses and immunomodulatory relationships that correlate with each of the three pathogens being examined; (4) to specifically determine if GI parasites skew host immune responses toward Th2-type immunity that correlates with timing of anthrax outbreaks and/or influences immunity to anthrax; and (6) to examine how patterns of disease and immune changes might affect long-term host survival.

## **Methods**

### *Study Area*

Etosha National Park (ENP) is a 22,915 km<sup>2</sup> fenced conservation area in northern Namibia, located between 18°30'S-19°30'S and 14°15'E-17°10'E (Figure 1). The park is dominated by arid savanna and mopane shrubveld and treeveld, though the central Okaukuejo plains consist of sweet grassveld and dwarf shrub savanna (Huntley, 1982; le Roux et al. 1988). A 4,760 km<sup>2</sup> endorheic salt pan, the remnant of a paleolake, dominates the northeastern quadrant of the park (Hipondoka et al. 2006).

Rainfall in ENP is highly seasonal: the major rainy season lasts from November through April, with the greatest rainfall occurring during January and February, and 80% of all rain falling between December and March (Gasaway et al. 1996; Engert 1997) (Figure 2). The dry season lasts from May through January (Auer 1997). Zebra for this study were largely captured and sampled around the central Okaukuejo area, the location of the Etosha Ecological Institute (EEI) (Figure 1), where the mean annual rainfall is approximately 350mm (Turner et al. 2012). During dry seasons, several re-captures took place in the Halali plains, an area in the near east of the park that receives a mean annual rainfall of approximately 420mm (EEI, unpublished data). The only perennial water available to the park's wildlife is found in man-made boreholes, or in natural artesian contact springs (Auer 1997).

### *Study Species*

Plains zebra (*Equus quagga*, formerly *E. burchelli*) are one of the most abundant plains ungulates species in ENP, with a population of 13,000 (95% CI rounded to nearest 100: 10,900-15,000) (EEI unpublished aerial survey data 2005). Zebra, as members of the Family Equidae, are hindgut fermenters that graze almost exclusively on grasses (Codron et al. 2007; Gordon and Prins 2008). They are among the most water-dependent of the plains ungulates found in southern Africa, requiring access to drinking water at least every other day (Skinner and Chimimba 2005; Cain et al. 2011). Zebra live in relatively stable harems of one stallion (>5 years old) and up to six females plus offspring, whereas younger males usually live in similarly-sized bachelor herds. In ENP, plains ungulates typically congregate in large, mixed groups around water or moving toward water, making group composition and size difficult to determine; thus, I did not take group size into account in this study (Turner and Getz 2010). Though not strictly seasonal breeders, zebra mating usually peaks at the start of a rainy season (Estes, 1991), with peak birthing in ENP zebras in December and April (Turner & Getz 2010).

### *Animal Capture and Sampling*

I obtained whole blood, serum, fecal, and ectoparasite samples from zebra over five seasons between 2008 and 2010 (Table 1). With veterinarians employed by the Namibian Ministry of the Environment and Tourism, and with assistance from the Etosha Ecology Institute and other researchers, I fitted animals with VHF (very high frequency) collars (LoxoTrack, Aeroeskoebing, Denmark) or VHF-GPS/GSM (global positioning system/global system for mobile communications) collars (Africa Wildlife Tracking, Pretoria, Republic of South Africa) during the first immobilization to enable resampling of animals over several seasons. We first immobilized and sampled all animals on the plains in the immediate area (within an approximately 20km radius) surrounding the EEI in Okaukuejo (Figure 1); in subsequent seasons (particularly during dry seasons, when some zebra groups travel away from Okaukuejo; Stander 1992), we found and resampled several zebra between Okaukuejo and up to approximately 100km to the east in the Halali plains, and Okaukuejo and approximately 15km directly to the south near Ombika gate. We only sampled adult animals, nearly all of which were females to control for sex differences. We immobilized, sampled, and released all animals safely under animal handling protocol AUP R217-0509B (University of California, Berkeley).

We conducted a total of 154 zebra capture events with 10 males and 144 females. We sampled 69 individuals overall, with 20 resampled twice, 11 resampled three times, 12 resampled four times, and two resampled five times. I collected whole blood and serum samples from each individual-capture event, but was only able to collect fecal and ectoparasite samples from a subset of the captures (Table 1).

I collected blood from peripheral veins for protocols using both whole blood and serum. Whole blood for eosinophil counts was collected into Vacutainers (Becton Dickinson, Franklin Lakes, NJ) containing EDTA anticoagulant, whereas that for cytokine stimulation studies was collected using sodium heparin Vacutainers.

For serum needed for antibody assays, I collected blood into Vacutainers without anticoagulant. I kept all tubes at approximately 4°C in a mobile refrigerator for up to six hours in the field. I stored whole blood samples at 4°C for up to 24 hours prior to use, while I left blood for white blood cell counts to clot at room temperature in the laboratory in the Etosha Ecological Institute (EEI) for four hours, placed it overnight at 4°C, and then centrifuged it to allow for serum to be removed and aliquoted. I kept serum initially at -20°C for up to six months, and thereafter stored samples at -80°C until analyzed.

I collected feces by observing an individual prior to capture and collecting a homogenized sub-sample within ten minutes of defecation. For animals that were not observed defecating, I collected fecal samples when possible by inserting a gloved hand into the rectum. As capture events took place between 9:00 and 14:00, fecal samples were collected within this same time window, thereby controlling for potential differences in timing of fecal egg shedding (Villanúa et al. 2006). I stored feces in sealed plastic bags at 4°C for up to 48 hours prior to analysis.

### *Animal Aging*

We sampled only adult animals, the vast majority of them female to control for sex differences. I determined zebra sex and rough age based on relative size, pelage, and genitalia (Smuts 1975). I further determined age to half a year by combining tooth eruption observations, caliper measurements of upper incisors, and patterns of wear (Smuts 1974; Penzhorn 1982). I converted year age to age in days, and then "aged" each animal at recapture by the number of days between that event and the previous sampling for that individual.

### *Rainfall Quantification*

Previous studies in this system determined that helminth infection intensity in zebras is significantly related to rainfall one and two months prior, with both strongly correlated with helminth eggs shed per gram of feces (Turner, 2009). I determined cumulative rainfall over the one month prior to sampling, and cumulative rainfall over the two months prior to sampling by adding up daily rainfall amounts (in mm) over the 30 or 60 days prior to each individual capture event. I used rainfall gauge data from Okaukuejo for those capture events that took place surrounding that area, and rainfall gauge data from Halali for those capture events that took place on the Halali plains.

I used cumulative rainfall two months prior to sampling as my primary indicator of seasonal wetness in our models, as this measurement was better than the one month cumulative measurement for taking into account one or two days of rainfall followed by no precipitation for weeks. Histograms of cumulative rainfall prior to each individual sampling event revealed a strongly bimodal distribution in the individually-linked rainfall, regardless of qualitatively named seasons (Figure 3). I thus chose to, when carrying out "seasonal" comparisons across and between individuals, group by quantitative rainfall groups rather than by qualitative seasons. This made both ecological and biological sense: the S3 sampling began more than

one month later than did sampling in the S1 "wet season," (Table 1) and those S3 animals sampled late in April and the beginning of May (the typical start of the cold dry season) accordingly fell into the lower rainfall group; and external environmental changes have been shown to influence the further development and egg-producing activity of gastrointestinal parasites living within hosts, thus potentially biasing an important measure in my study if I did not take actual rainfall into account (Horak 1981; Shaw 1988). The rain groups were thus: RG1, the high rainfall group, containing individuals that had experienced  $\geq 200\text{mm}$  rainfall two months prior to sampling; and RG2, the low rainfall group, containing individual samplings connected with  $\leq 100\text{mm}$  rainfall in the two months prior.

#### *Gastrointestinal Parasite Species and Quantification*

The gastrointestinal nematodes examined in this host species were in the order Rhabditida, suborder Strongylida, primarily within the superfamily Strongyloidea, and family Strongylidae; this group contains both the "large strongyles" (spp. in the subfamily Strongylinae) and the "small strongyles" (spp. in the subfamily Cyathostominae) (Jain et al. 2009). While Strongylids in the superfamily Trichostrongyloidea, family Trichostrongylidae are most often parasites of ruminants, some species have been known to use plains zebras as hosts and most are generalists for hosts (Krecek et al. 1987b; Matthee et al. 2004). A previous study in plains zebra in ENP found only Strongyloidea species and no Trichostrongyloidea species in zebra guts (Krecek et al. 1987); however, as only nine zebras were examined in this previous study, and as the eggs for Trichostrongylids and Strongylids are indistinguishable to the naked eye (Bowman 2003), I conservatively refer to the Strongylida eggs that I sampled as "strongyles" without assigning these eggs to species within one of the two superfamilies mentioned above.

These parasites are oviparous and exhibit a direct life cycle with three, free-living larval stages. The first two moults to the infectious L3 stage occur over one to two weeks, after which L3's are ingested by herbivore hosts (Durette-Desset et al. 1994). The first two free-living stages are highly susceptible to desiccation, and develop more quickly and survive for longer in relatively warm temperatures and humid conditions (Nielson et al. 2007). L3 larvae are less susceptible to desiccation, but require a film of moisture to move (O'Connor et al. 2006); thus, it is unsurprising that previous studies in ENP found a strongly seasonal pattern in zebra strongyle infection intensities, with hosts exhibiting greater new infections during the wet season than in the dry (Turner and Getz 2010). Once ingested, L3 larvae encyst in the gut mucosa and develop into L4 larvae, causing damage to the intestinal mucosa in the process. L4 stages of some strongyle species travel throughout host tissues before returning to the gut, causing tissue damage and inflammation along the way, while others remain in the gut lumen and mucosal epithelium to develop into adult stages that then colonize the intestines (Anderson 2000; Bowman 2003).



I evaluated fecal samples for strongyle eggs using a modified McMaster technique for fecal egg counts (Gibbons et al. 2005), a commonly used non-invasive method for quantifying parasitism (Bowman 2003). Briefly, I combined 4g of homogenized fecal matter with 56ml of a saturated NaCl solution (specific gravity 1.2), removed any large debris with a strainer, and obtained a homogenized filtrate. I placed an aliquot of filtrate into each chamber of a McMaster slide and counted the number of eggs observed in each chamber using a compound microscope at 10x magnification. I obtained a measure of eggs per gram of feces by adding the number of eggs for both chambers and multiplying by 50.

Fecal egg counts (FECs) provide an accurate estimate of how the input of parasite eggs into the environment varies with other factors of interest (Turner and Getz 2010). While the actual relationship between fecal egg count and total nematode burden within a host is of unknown specificity and sensitivity, these counts provide a nonlethal and often noninvasive method for estimating these infection burdens (Stear et al. 1995; Wilson et al 2001; Seivwright et al. 2004). In addition, W. Turner and I previously assessed the influence of fecal water content on measurement of fecal egg counts, as fecal water content can vary widely between seasons in ENP. We found that fecal water content had no effect on seasonal and age-related patterns in strongyle egg counts, thus increasing our confidence regarding the overall accuracy of this measurement (Turner et al. 2009).

#### *Ectoparasite Collection*

I collected and counted all visible ectoparasites, regardless of life stage, on zebras during capture events; as these parasites congregate almost entirely around the ventrum, perineum, and ears, I was confident that I was able to collect the vast majority of ticks present even while a zebra lay on its side. I used forceps to detach ectoparasites, and placed them together into a separate glass jar for each host. I identified five tick species from the family Ixodidae and genera *Hyalomma* and *Rhipicephalus*. The majority of ticks observed were *R. evertsi mimeticus*, a tick species found throughout Namibia in wild equids and greater kudu (Horak 1984; Horak et al, 1984; Horak et al. 1992). These ticks parasitize hosts year-round, with more adults present from November to May and immature stages peaking from February to March and May to September (Biggs and Langenhoven 1984).

#### *Eosinophil and Monocyte Counts*

Eosinophils are often important for eliminating helminths, and have been shown to be a good measure of Th2 responsiveness (Abbas et al. 1996; Scott and Koski 2000; Claerebout et al. 2005; Jolles et al. 2008). Monocytes are recruited and activated by IFN- $\gamma$  in Th1 immune reactions, and were used as a measurement of potential Th1 immune activity (Abbas et al. 1996).

I created thin blood smears on glass slides, fixed them with methanol, and stained them with Diff-Quik (Dade Behring, Deerfield, IL). I performed manual total white blood cell (WBC) counts using a compound microscope; I counted cells in ten fields at 40x magnification and multiplied mean cell count per field by 1600

(magnification<sup>2</sup>) to obtain total WBCs per  $\mu\text{l}$  of blood. I did differential counts by determining the percent of each of the most common WBC types (neutrophils, monocytes, lymphocytes, eosinophils) in 200 WBCs counted at 40x and multiplying this by total WBC concentration to obtain numbers of eosinophils or monocytes per  $\mu\text{l}$  of blood. All counts were done in duplicate and averaged.

### *IgE ELISA*

IgE is an antibody isotype mediated by the Th2-associated cytokine IL-4 and important in fighting against helminths (Abbas et al. 1996). I used an enzyme-linked immunosorbent assay (ELISA) method developed to detect total serum immunoglobulin isotype E (IgE) in domestic horses (Wagner et al. 2003). Briefly, I used monoclonal antibody anti-IgE 176 for the coating antibody and biotinylated monoclonal anti-IgE 134 as the secondary antibody; these two antibodies recognize different epitopes in the IgE heavy chain constant region. Coating antibody was used at a concentration of  $10\mu\text{g}/\text{ml}$ . Beginning with a dilution of 1:200, I titrated samples in serial, two-fold dilutions to 1:25,000. After incubation with the secondary antibody, I added peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) and substrate solution. I determined IgE concentration in  $\text{mg}/\text{ml}$  of serum by comparing to a titrated, purified IgE standard of known concentration.

### *Anti-PA ELISA*

Anthrax is caused by *Bacillus anthracis*, a large, gram-positive bacterium that exists in a hardy, long-lived spore form in the environment. Anthrax can only be transmitted environmentally through these spores, and is usually orally ingested by susceptible herbivore hosts (Hanna and Ireland 1999; Watson and Keir 1994). Anthrax can cause death within hours to days (Hugh-Jones and de Vos 2002), though there is evidence that even very susceptible host species can experience a sublethal dose of anthrax and survive, in part, due to a humoral immune response against the anthrax protective antigen (PA) toxin (Lembo et al. 2011; and see dissertation Chapter 2). These anti-PA antibodies have been shown to be essential for adaptive protection against anthrax (Little et al. 1997; Turnbull 2000; Little et al. 2004; Marcus et al. 2004). As a bacterial infection, anthrax provokes primarily a Th1 type immune response (Hanna 1999; Pickering et al. 2004).

I used an ELISA procedure previously described (see dissertation Chapter 2). Briefly, I used wildtype *Bacillus anthracis* protective antigen (PA) as coating antigen at a concentration of  $0.375\mu\text{l}$  per well. I made serial, two-fold dilutions to the ends of rows in duplicate for all samples and negative controls, starting at a dilution of 1:4 and ending at 1:8192. I ran a duplicate negative control full titration series on each ELISA plate. I used goat-anti-horse IgG-heavy and light chain horseradish peroxidase (HRP) conjugate (Bethyl Laboratories, Montgomery, TX) as the secondary antibody, at the suggested dilution of 1:60,000. I then added TMB substrate (Kirkegaard & Perry Laboratories; Gaithersburg, MD) and stopped the reaction with 2N sulfuric acid. I read well absorbance as optical density (OD) at

450nm on a SpectraMax M2 Microplate Reader using SoftMax Pro software v5.3 (Molecular Devices; Sunnyvale, CA). As we had no known, titrated standards to establish a standard curve, I determined the endpoint titers as the  $\log_2$  of the last sample dilution at which the mean OD for that sample at that dilution was greater than the mean OD for all negative controls at that dilution, buffered by a 95% confidence interval determined by the inter-duplicate error at that dilution, across all samples analyzed.

### *IgGb ELISA*

IgGb is the most prevalent antibody isotype in equine serum and is able to fix complement via the classical pathway and evoke a respiratory burst from monocytes (Lewis et al. 2008). While IgGb is important in the protective response against intracellular pathogens, suggesting a Th1-associated response, Hooper-McGrevy et al. 2003 found that IgGb is likely a Th2-related antibody in horses.

I used a commercially-available horse IgGb ELISA kit (Bethyl Laboratories) to quantify immunoglobulin isotype G subclass b (IgGb) in serum. I used sheep-anti-horse IgGb as the capture antibody, at a concentration of 10 $\mu$ g/ml. I added sample serum at a dilution of 1:128,000 and a titrated standard to determine a standard curve. All samples were run in triplicate and standards were run in duplicate. I used goat-anti-horse HRP-conjugated secondary antibody. I added TMB substrate, stopped the reaction, and read well absorbance as per the anti-PA ELISA protocol above. I determined concentration of IgGb in serum in mg/ml by comparing to the standard curve and adjusting for dilution amount.

### *Ex Vivo Cytokine Stimulation*

I performed whole blood, ex vivo stimulation to investigate interleukin-4 (IL-4) and interferon-gamma (IFN- $\gamma$ ) cytokine production by T cells, using an adapted protocol (Thurm and Halsey 2005). IFN- $\gamma$  is a key Th1 cytokine that activates macrophages early in infection. IL-4 is an important Th2 cytokine that induces antibody isotype switching to IgE and augments recruitment of eosinophils to helminths (Mosmann et al. 1986; Abbas et al. 1996).

Ex vivo stimulation by antigens has been shown to produce cytokine patterns reflective of those occurring in vivo (Heinzel et al., 1991). Briefly, I added phytohemagglutinin (PHA) diluted in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2mM L-glutamine to 0.5ml whole blood to achieve a final concentration of 10 $\mu$ g/ml PHA. I incubated these stimulated samples in an incubator held at 37°C, 5% CO<sub>2</sub>, and constant humidity for between 20 and 24 hours. I then added 0.8-1.3ml RNeasy RNA Stabilization Reagent (Qiagen, Valencia, CA) to each sample. I kept these samples between 0°C and 4°C during shipping, and thereafter at -80°C prior to qPCR analysis for amounts of IFN- $\gamma$  and IL-4 mRNA.

### *Quantitative RT-PCR for IL-4 and IFN- $\gamma$*

I thawed on ice our PHA-stimulated whole blood samples in RNeasy and transferred the entire contents of each sample to a polypropylene tube. I extracted

RNA with TRIzol Reagent using the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, I added 3:1 TRIzol per sample volume, vortexed, added 0.2ml of chloroform per 1ml of TRIzol used, centrifuged at high speed, and transferred the aqueous phase containing total RNA to a new tube. I added 0.5ml isopropyl alcohol per 1ml TRIzol originally added to precipitate RNA and washed the pellet with 75% ethanol. I dissolved RNA in 100 $\mu$ l RNase-free water and analyzed the RNA content with a NanoDrop spectrophotometer and ND-1000 software, v3.3.0 (Thermo Scientific, Wilmington, DE).

I treated 1000ng of RNA for each sample with RQ1 DNase (Promega, Madison, WI). For samples that contained less than 1000ng of RNA in 8 $\mu$ l, we used as much RNA in 100ng increments that were contained in  $\leq$ 8 $\mu$ l of sample (e.g. 300, 400, 500, 800, or 900ng, depending on the sample) and adjusted for this lower concentration in our final analysis. For each sample, I prepared cDNA using SuperScript III reverse transcriptase (RT) (Invitrogen) and a negative control sample lacking RT. I performed amplification reactions in a thermal cycler (iCycler Thermalcycler, BioRAD, Hercules, CA). cDNA reactions were primed with poly(dT).

I performed quantitative PCR using a Step One Plus RT-PCR system (Applied Biosystems, Foster City, CA) with Platinum *Taq* DNA polymerase (Invitrogen) and EvaGreen (Biotium, Hayward, CA). The target genes of interest were equine IFN- $\gamma$  and interleukin-4, and I used GAPDH as our housekeeping gene. Primer sequences are from domestic equines and are listed in Table 2 (Ainsworth et al. 2003). Primers were provided by Elim Biotech (Hayward, CA). Reaction mixtures for qPCR had a final volume of 25 $\mu$ l, consisting of 5 $\mu$ l cDNA and 20 $\mu$ l of master mix. I analyzed each sample in triplicate for target gene and included negative controls (water) on each plate.

For one sample on each plate, I analyzed serial ten-fold dilutions for each primer pair. I ran each dilution in duplicate. The endpoint in qPCR is  $C_T$ , the cycle number at which product is reliably detected. Using  $C_T$ s for these dilutions and the  $\log_{10}$ cDNA (calculated from the amount of RNA added to the RT reaction), I constructed a relative standard curve for each target gene. Out of our several dilution series, I chose reference samples with standard curves with  $R^2$  values of  $>0.9$  for GAPDH and IL-4, and  $>0.65$  for IFN- $\gamma$ . I used these standard curve equations and the  $C_T$  value for each sample to calculate the  $\log_{10}$ cDNA amount as a proxy for ng of target RNA. For samples for which RNA amount added to the RT reaction was different from that of the reference sample, I adjusted our calculations by the appropriate fold factors. I normalized these transcript levels to those of GAPDH for each sample by calculating the cytokine:GAPDH ratio. Samples for which the RT ratio:noRT ratio was less than one were discarded from further analysis.

### *Statistical Analyses*

#### *Multiple Imputation of Missing Data*

Imputation is a method of replacing missing observations with plausible estimates based on available data. Multiple imputation (MI) methods are particularly useful for imputing multivariate missing data (Little and Rubin 2000;

van Buuren and Groothuis-Oudshoorn 2011). Multiple imputation uses available data from multiple predictors and covariates to create a set of datasets for each missing value, each containing different sets of those missing values drawn from their predictive distributions. This method, unlike single imputation methods, provides a variance of an estimate and also estimates the contribution of uncertainty due to the fact that the value in question was imputed rather than observed (Rubin 1996; Little and Rubin 2000; van der Heijden et al. 2006).

Multiple imputation is most often used in human public health studies in which some data are missing for individuals sampled repeatedly over time (Little and Rubin 2000; van der Heijden et al. 2006; Johansen et al. 2012). Comparisons of analyses using multiply imputed datasets versus complete case analysis (CCA), in which cases with any missing data are eliminated from the analysis, have found that MI produces much less biased results. This is true when both small and large amounts of data points are missing (van der Heijden et al. 2006; Vergouw et al. 2012). In addition, CCA has been found to be appropriate only when data are known to be absolutely missing completely at random (MCAR); because there are often underlying, potentially unobserved causes for missing data, using CCA is often suboptimal (Rubin 1996; Little and Rubin 2000; Vergouw et al. 2012).

For my data, I imputed missing values for an individual capture event but did not impute completely missing capture events for an individual animal (i.e. if a zebra was sampled in S1 and S3 but not S2, S4, or S5, I did not impute all data for that animal for S2, S4, and S5; if that zebra was, however, missing a GI parasite count for S1, I imputed that missing value). I imputed five missing values for IgGb titer (3.2% of total IgGb data), 15 (9.7%) for ectoparasite count, and 35 (22.7%) for GI parasite count. Few of these missing variables were overlapping for the same individual-capture; thus, discarding these cases in favor of CCA would have resulted in disregarding upwards of 79% of the capture events in my analyses. While I used the IFN- $\gamma$  and IL-4 PCR data in my predictor matrix, I did not impute missing values for these variables given the large number of missing data for each (102 for each of IFN- $\gamma$  and IL-4). Four individual-captures were missing all hematological parameters; I discarded these four from the imputation and analyses as their data were clearly missing due to related, non-random reasons. I also did not include in my imputations and analyses the few obviously biased data points identified in my preliminary data analyses.

For imputation, I used the Multiple Imputation by Chained Equations (MICE) method with the 'mice' package (van Buuren and Groothuis-Oudshoorn 2011) in R v2.15.2 (R Core Development Team, Vienna, Austria). This method specifies the imputation model for each variable by building, and iterating over, a set of conditional densities for each variable. I built my predictor matrix by first using all variables in this study (Rubin 1996), and then refined the predictor matrix for each variable to avoid collinearity. I preserved all data transformations by passively imputing each transformed variable linked to its original variable (van Buuren and Groothuis-Oudshoorn 2011). I validated my imputations by confirming convergence, examining density plots and strip plots to ensure that imputed values overlapped

existing data, and comparing distributions of observed versus imputed data based on propensity scores (van Buuren and Groothuis-Oudshoorn 2011). I performed 50 imputation cycles and generated 20 imputations. I averaged the 20 estimates for each data point to produce single mean estimates for use in  $t$  tests, while I used the multiply imputed datasets directly in my generalized estimating equations and then determined mean estimates for the model parameters (Vergouw et al. 2012). I adjusted standard errors, Wald statistics, and  $p$  values according to Rubin's rules (Rubin 1987).

### *Comparing First Captures*

I first examined the effects of season for my various pathogen and immune factors, as very few studies have examined simple seasonal differences for most of these variables. I started by compared data from only the first sampling events for animals across the two rain groups. This ensured that I was only comparing unique individuals across rain groups, without the possibility of autocorrelation and the issue of repeated measures (Table 3A).

I transformed variables for normality when necessary and possible (Table 4). I compared  $\log_{10}$ -transformed eosinophil counts, monocyte counts, and IgE titers between rain groups using Welch's two-tailed  $t$  tests. I compared square root-transformed IgGb titers and GI parasite counts between rain groups using Welch's two-tailed  $t$  tests. I compared ectoparasite counts and  $\log_2$  anti-PA titers between rain groups using two-sided Wilcoxon rank sum tests. I adjusted  $p$  values to control for the familywise error rate by using the Holm-Bonferroni method (Holm 1979).

I also compared IFN- $\gamma$  PCR results and IL-4 PCR results between seasons using two-sided Wilcoxon rank sum tests. Because these tests were only conducted for S2 and S3 samples, grouping them into our regular rain groups resulted in a very small sample size for RG1. I thus modified rain groups for these variables only, so that RG1 included individuals linked with  $\geq 75$ mm rainfall and RG2 included individuals linked with  $< 75$ mm rainfall in the two months prior to sampling. This rubric still separated the animals sampled in the last week of April and the beginning of May (the start of the nominal dry season) from those sampled slightly earlier in April (the end of the nominal wet season). I compared only first captures for animals in each rain group; due to the small sample size for RG1 animals in this analysis ( $N=6$ ), I also compared between rain groups for all samples regardless of potential autocorrelation issues.

### *Comparing Recaptures*

I then did pairwise comparisons across the same animals, between rain groups, for first and second captures to control for potential individual variation (Table 3B). I compared the same transformed variables using two-tailed paired  $t$  tests and two-sided paired Wilcoxon signed rank tests, and then corrected for familywise error rate as per above. I also compared resampled animals for IFN- $\gamma$  and IL-4 in their modified rain groups using two-sided paired Wilcoxon signed rank tests.

### *Generalized Estimating Equations*

I developed generalized estimating equations (GEE) using R v2.15.2 and the 'geepack' package (Højsgaard et al. 2006) to examine the correlations between pathogen types and immune parameters. I chose GEEs because they can incorporate both random and fixed effects, can model counts and binary data as well as continuous data, can deal with data that violate assumptions of normality and independence, and can account for different autocorrelation patterns in repeated measures data (Liang and Zeger 1986; Hardin and Hilbe 2003). These models are particularly useful for longitudinal data with many individuals (zebras) but relatively few longitudinal observations (sampling seasons) per individual (Zuur et al. 2009). In all models I used a working correlation matrix with a first-order autoregressive relationship (AR-1) because, while individual immune and disease factors are likely correlated through time, these correlations should decrease between later time points and earlier samplings (Liang and Zeger 1986; Zuur et al. 2009). I used zebra identification as the grouping structure, and used the waves argument in geepack with the capture number to account for missing sampling times for individuals.

To avoid multicollinearity between explanatory variables, I first assessed pairwise scatterplots, correlation coefficients, and variance inflation factors, and excluded one of each pair of variables with correlations of  $r > 0.8$  or  $VIF > 3$  (Farrar and Glauber 1967; Zuur et al. 2009). I developed each GEE by using a backwards, stepwise refinement method based on comparing the quasi-likelihood under the independence model criterion (QIC) values between maximal models and models with variables removed (Pan 2001). The QIC is equivalent to Akaike's information criterion (AIC) for repeated measures; a smaller QIC indicates a better fitting model. I used the 'yags' package in R to determine QIC values (Carey 2004). After this first refinement, I added biologically-sensible interaction terms between the remaining explanatory variables and further refined the models by comparing QIC's. I validated the models by plotting Pearson's residuals against fitted values to look for residual patterns, examined residual histograms to assess the normality of error distributions, and plotted residuals against each explanatory variable to test for homogeneity of error variances. I used transformed variables when necessary for eliminating residual patterns. For the final, best-fit models, I used Wald chi-square tests to determine the significance of each parameter estimate.

I first used GEE models to address the relationship between my study's three pathogen types, seasonal factors, and host parameters (Table 4). While assigning directionality with anthrax as the response variable in all cases may seem a straightforward choice, I decided to allow each pathogen (GI helminths as determined by FEC; ectoparasites as determined by tick counts; and sublethal anthrax exposure, as determined by anti-PA titers or the presence or absence of a measureable titer) to play the role of response variable in separate models for several reasons. While spikes in GI parasite infection intensity do occur in this system a few weeks prior to anthrax outbreaks, there is a 100% prevalence of GI

helminth infections year-round in ENP zebras (Turner 2009; Turner and Getz 2010). Because of this, and because cases of anthrax do occur throughout the year, assigning one pathogen as the dependent variable in this case would have been potentially biased. The same reasons apply for examining host ectoparasite loads as a potential outcome of other infections. In addition, as immunity is not unidirectional and the immune system involves a very complicated interplay between different immune factors and the influences of co-infections, I wanted to allow each pathogen "outcome" to be examined from the standpoint of potential predisposing factors toward that infection. I fitted the GEE models for GI helminths and ectoparasite counts with a Poisson distribution linked with a log function, while I fitted the GEE model for presence or absence of a PA titer with a binomial distribution linked to a logit function (Table 5).

For these same reasons, I then used each of my immune parameters as response variables in separate models to allow me to more directly examine the cross-relationships between them and the other immune parameters and pathogens. I fitted the GEE models for eosinophil counts and monocyte counts with a Poisson distribution linked to a log function. I fitted the GEE models for normally-transformed IgE titers and IgGb titers with a Gaussian distribution for continuous variables (Table 5).

Finally, I examined a model using animal age as the dependent outcome; could disease status and immune function explain individual age? I fitted this GEE model with a Poisson distribution linked to a log function (Table 5). As little is known about how disease status and immune function influence animal aging and survival, this model allowed me to examine more basic ecological immunological questions.

## **Results**

### *Rain Group Comparisons for First Captures*

I found highly statistically significant differences between rain groups for GI parasites and eosinophil counts for unique animals (no autocorrelation, no paired samplings) (Table 6 and Figure 4). While both variables were transformed for normality prior to using *t* tests, I also observed large differences between rain groups for the non-transformed versions of these variables (GI parasites mean difference of 1771 epg, with a range of  $3801 \pm 1665$  epg — mean  $\pm$  SD — for RG1, to  $2110 \pm 750$  epg for RG2; eosinophil mean difference of 240 cells/ $\mu$ l blood with a range of  $397 \pm 278$  cells/ $\mu$ l for RG1, to  $157 \pm 79.4$  cells/ $\mu$ l for RG2). Prior to the Holm's Bonferroni *p*-value correction, IgGb titer was also significantly different in the two rain groups (Table 6), with a mean difference of 1.44mg/ml for the non-transformed variables (range of  $7.15 \pm 2.64$  mg/ml in RG1 to  $5.71 \pm 2.10$  mg/ml in RG2). I found no significant differences in anti-PA antibody titers, ectoparasite counts, monocyte counts, or IgE antibody titers compared between rain groups for these animals (Table 6). Thus, my conclusions for my unique animals are that GI parasite infection intensities and eosinophil counts (and possibly IgGb antibody titers) were all greater during wetter times than during dry ones.



As an internal check on the validity of my data imputations, I did the same transformations and tests on my non-imputed data for each variable, and found that the presence/absence of significant differences between rain groups and the directionality of any differences were the same for non-imputed and imputed variables, both before and after correcting for familywise error rates (data not shown).

#### *Pairwise Rain Group Comparisons for Recaptures*

I found highly statistically significant differences for paired individuals between rain groups for GI parasite counts and eosinophil counts (Table 7 and Figure 5) (mean difference for non-transformed GI parasites of 1878 epg, with a range of  $3963 \pm 1629$  epg — mean  $\pm$  SD — in RG1, to  $2086 \pm 1184$  epg in RG2; mean difference for non-transformed eosinophil counts of 216 cells/ $\mu$ l blood, with a range of  $414 \pm 268$  cells/ $\mu$ l in RG1, to  $198 \pm 223$  cells/ $\mu$ l in RG2). I also found statistically significant differences between rain groups for ectoparasite counts and IgGb antibody titers (Table 7) (mean difference for ectoparasite counts of 2.41 ticks, with a range of  $3.72 \pm 2.59$  ticks in RG1, to  $6.13 \pm 5.55$  ticks in RG2; mean difference for non-transformed IgGb titers of 1.91 mg/ml, with a range of  $7.28 \pm 2.95$  mg/ml in RG1, to  $5.36 \pm 2.47$  mg/ml in RG2). I found no significant differences in anti-PA titers, monocyte counts, or IgE antibody titers compared between rain groups for these animals (Table 7).

Thus, my conclusions for paired animals are that GI parasite infection intensities, eosinophil counts, and IgGb antibody titers were all greater during times of higher rains than during those of low to no rains, whereas ectoparasite infection intensities were greater during drier times than during wet ones (Figure 6). These results also indicate that controlling for individual variation allowed the subtler, yet present rain group differences in ectoparasite counts and IgGb titers to be revealed.

#### *Cytokine Comparisons*

I found no statistically significant differences when comparing IFN- $\gamma$  transcript ratios between rain groups for all animals (Wilcoxon  $U=245$ ,  $N_1=16$ ,  $N_2=37$ ,  $p=0.317$ ), for first captures only (Wilcoxon  $U=78.0$ ,  $N_1=6$ ,  $N_2=37$ ,  $p=0.249$ ), or for paired recaptures (Wilcoxon  $T=9.50$ ,  $N_1=N_2=6$ ,  $p=0.916$ ). I also compared fold differences for transcript levels between rain groups; these indicated that, despite the non-significance in Wilcoxon tests, there may still be rain group differences worth examining, especially in the light of small sample sizes with high variances. Comparing mean ratios in the different rain groups, I found a 2.35-fold increase in mean IFN- $\gamma$  transcript in RG2 compared to in RG1 for all samples, and a 2.98-fold increase in transcript in RG2 compared to in RG1 for first captures only (unique animals). The level of transcript was nearly the same in both rain groups (only a 1.13-fold increase, this time in RG2 compared to in RG1) for resampled animals; this group, however, had the lowest sample size of all comparisons ( $N=6$ ). Thus, I conclude that there is some evidence showing an increase in IFN- $\gamma$  production during drier times compared to in wetter seasons, but more data is needed.

I found no statistically significant differences when comparing IL-4 transcript ratios between rain groups for all animals (Wilcoxon  $U=232$ ,  $N_1=17$ ,  $N_2=36$ ,  $p=0.145$ ), for first captures only (Wilcoxon  $U=101$ ,  $N_1=5$ ,  $N_2=37$ ,  $p=0.686$ ), or for paired recaptures (Wilcoxon  $T=8.50$ ,  $N_1=N_2=9$ ,  $p=0.608$ ). Comparing fold differences, however, I found a mean 4.56-fold increase in IL-4 transcript in RG1 over RG2 for all samples, a 15.2-fold increase in transcript in RG1 compared to in RG2 for unique animals, and a 1.58-fold increase in RG1 over RG2 for resampled animals. Thus, I conclude that there is some evidence showing an increase in IL-4 production during wetter times compared to in drier seasons, but more data is needed.

### *Influences on Pathogen Markers*

I square root transformed GI parasite and ectoparasite response variables for my GEE models to deal with overdispersion. In addition, I used a square root-transformed GI parasite explanatory variable in my ectoparasite GEE model, and used presence/absence of an anti-PA titer as my response variable for that model. These transformations did not affect model selection or significance, but improved residual patterns and normality. As these transformations do affect the direct interpretation of coefficient estimates, interpretations here are based solely on sign and significance of coefficients to determine predictors of the response variables and the directions of these effects.

The best fitting GI parasite model included cumulative rainfall two months prior to sampling (hereafter simply "rainfall"),  $\log_2$  of anti-PA titer, eosinophil count, and IgE titer. Rainfall and eosinophil count significantly predicted GI parasite infection intensity; while increased rainfall predicted higher GI parasite loads, as expected from rain group analyses (Figures 4 and 5), higher eosinophil counts predicted decreased GI parasite infection intensity (Table 8). IgE titer was nearly significant in the model ( $p=0.085$ ), and higher IgE titer predicted decreased GI parasite loads. While anti-PA titer was not statistically significant in the model ( $p=0.236$ ), its negative coefficient suggests that higher anti-PA titers may be associated with lower GI parasite counts (Table 8).

The best fitting PA model indicated that presence or absence of an anti-PA antibody titer was significantly influenced by rainfall, eosinophil count, and an interaction between the two. The correlation between rainfall and presence of a titer was negative, while the influence of eosinophil count was positive on having a titer (Table 8): as rainfall increases, titer prevalence decreases, and as eosinophil count increases, titer prevalence increases. The interaction term of rainfall with eosinophil count was highly statistically significant and negative; this directionality may be due to rainfall being more influential in the model than eosinophil count. While age and GI parasite count were necessary in the model but not significant, both were potentially negatively associated with having an anti-PA titer (Table 8).

The best fitting ectoparasite model included rainfall, age, GI parasite count (square root-transformed), and the interaction between transformed GI parasite count and age. All of these terms significantly influenced the response variable (Table 8). Decreased rainfall predicted higher ectoparasite counts in this model,

consistent with the rain group comparisons of paired samples (Figure 5). Age and GI parasite counts were both positively associated with ectoparasite counts: being older predicted higher ectoparasite loads, and having more GI parasites predicted higher ectoparasite loads, at least independently. The interaction term between GI parasites and age, however, negatively influenced ectoparasite count, indicating that perhaps animals that are both older and have high GI parasite infection intensities (or both younger and with fewer GI parasites) have fewer ectoparasites (Table 8). A simple linear model explaining square root transformed ectoparasite counts by age also indicated that ectoparasites were likely positively, significantly associated with increasing age ( $N=154$ ,  $F_{1,152}=4.83$ ,  $R^2=0.03$ ,  $p=0.029$ , coefficient= $1.47\text{e-}04$ ). Relationships for all three pathogen models are illustrated in Figure 6.

### *Influences on Immune Factors*

I log<sub>10</sub> transformed eosinophil counts, monocyte counts, and IgE titer response variables for their respective models, and square root transformed the IgGb titer response variable for its model to deal with overdispersion. Again, interpretations here are therefore based solely on sign and significance of coefficients to determine significant predictors of the response variables and the directions of these effects.

The best fitting eosinophil GEE model was significantly influenced by rainfall, GI parasite counts, and monocyte counts (Table 9). Higher rainfall predicted higher eosinophil counts here, corroborating results from unique animal and pairwise rain group analyses (Figures 4 and 5). Higher monocyte counts were also associated with higher eosinophil counts. GI parasite counts were negatively associated with eosinophil counts, as was observed in the GI parasite GEE model (Tables 8 and 9). Ectoparasite counts were necessary for model fitting but were not statistically significant; however, the model showed that there might be a weakly negative association between ectoparasites and eosinophils. This relationship was also suggested by a linear regression model of ectoparasites on log-transformed eosinophil counts ( $N=154$ ,  $F_{1,152}=6.99$ ,  $R^2=0.03$ ,  $p=0.009$ , coefficient= $-0.02$ ).

The final monocyte GEE model contained rainfall, eosinophil counts, IgE titer, IgGb titer, and an interaction term between rainfall and eosinophils. Rainfall and eosinophil counts both significantly positively influenced monocyte counts: increased rainfall predicted increased monocytes, and increased eosinophil counts predicted increased monocyte counts as well (Table 9). The interaction term between rainfall and eosinophils, however, was nearly significant and negatively associated with monocyte counts, indicating a complex interplay between these three variables. A simple linear regression of rainfall on log-transformed monocyte counts indicated that there was a significant, positive relationship between the two variables ( $N=154$ ,  $F_{1,152}=8.42$ ,  $R^2=0.05$ ,  $p=0.004$ , coefficient= $7.51\text{e-}04$ ), though this was not observed in rain group comparisons (Figures 4 and 5). IgE and IgGb antibody titers both significantly negatively predicted monocyte counts in the GEE model: increased IgE titers and IgGb titers were associated with decreased monocyte counts (Table 9).

The best fitting IgE antibody titer GEE model was significantly influenced by rainfall, and also contained GI parasite counts and ectoparasite counts. Higher rainfall was predictive of higher IgE titers in this model (Table 9), though this relationship was not observed in unique animal or pairwise comparisons (Figures 4 and 5) or in a linear regression exploration of rainfall on log-transformed IgE titers ( $N=154$ ,  $F_{1,152}=2.36$ ,  $R^2=0.02$ ,  $p=0.127$ , coefficient= $3.12e-04$ ). While GI parasite counts and ectoparasite counts were not statistically significant in the model ( $p=0.312$  and  $p=0.204$ , respectively), they both showed a potentially negative association with IgE titer.

The final IgGb antibody titer GEE model was significantly influenced by rainfall, age, and monocyte counts, and also contained  $\log_2$  of anti-PA titers, ectoparasite counts, and eosinophil counts (Table 9). Rainfall was positively associated with IgGb titers: higher rainfall predicted higher IgGb titers, corroborating the similar, significant trend seen in pairwise comparisons (Figure 5) and the near-significant relationship between rainfall and IgGb titer observed for unique animals (Table 6). Age was also positively predictive of IgGb titers: older animals were associated with higher titers in this model. Monocyte counts were negatively associated with titers, with higher monocyte counts predicting lower IgGb titers. While I did not observe this relationship in the monocyte GEE model (Table 9), a linear regression exploration of monocyte count on square root transformed IgGb titers supported this trend ( $N=154$ ,  $F_{1,152}=7.42$ ,  $R^2=0.05$ ,  $p=0.007$ , coefficient= $4.94e-04$ ). Ectoparasite count and  $\log_2$  of anti-PA titer were nearly significant in the model ( $p=0.098$  and  $p=0.127$ , respectively); anti-PA titer showed a possible positive relationship with IgGb titer, while ectoparasite count showed a potentially negative relationship with titer. Eosinophil counts were also not significant in the model, though showed a potentially negative relationship with IgGb antibodies. Relationships for all four immunity models are illustrated in Figure 7.

#### *Influences of Pathogens and Immune Factors on Age*

I square root-transformed age as a response variable to deal with residual patterns. The best fitting age GEE model contained GI parasite counts, ectoparasite counts, eosinophil counts, and IgGb titer (Table 9). GI parasite infection intensities were significantly, negatively predictive of age: fewer GI parasites predicted older animals. A linear regression of square root transformed GI parasite counts on age supported this trend ( $N=154$ ,  $F_{1,152}=4.07$ ,  $R^2=0.02$ ,  $p=0.045$ , coefficient= $-9.22$ ). Ectoparasite counts were significantly, positively associated with age: more ectoparasites predicted older animals, similar to the relationships between these variables seen in the ectoparasite GEE model (Table 8). IgGb titer was significantly, positively predictive of age as well: higher IgGb titers were associated with older animals, suggesting that IgGb titers were both maintained and boosted over time. These results corroborated those observed in the IgGb titer GEE model (Table 9). Eosinophil counts were nearly significantly ( $p=0.058$ ) associated with age, also in a positive manner: higher eosinophil counts were associated with older animals.

While age was not a factor included in the eosinophil GEE model, the opposite relationships seen here between GI parasite loads and age (negative), and eosinophil counts and age (positive) support similar relationships between GI parasites and eosinophils observed in several models thus far (GI parasite and PA GEEs, Table 8: eosinophil GEE, Table 9). These relationships are illustrated in Figure 7.

## **Discussion**

This study evaluated seasonality of immune functionality, seasonality of infectious diseases, and the seasonal interactions between concurrent infections and immunity. I aimed to determine how host immunity changes in a strongly seasonal disease system, the applicability of mostly lab-derived immune trade-off patterns to mixed parasite infections in natural systems, and if such immune-parasite interactions help describe anthrax seasonality in Etosha National Park, a primarily wet-season anthrax system. This study is one of the first to examine seasonal changes in multiple immune parameters in concert with macroparasite and microparasite coinfections in wildlife hosts in a natural system. In addition, it is one of the first studies to follow the same wildlife hosts longitudinally while examining these interactions, one of the first studies examining IgE antibody titers in wildlife (and the first in wild equids), and one of the first wildlife ecology studies to make use of multiple imputation methods for missing data.

### *Seasonal Changes in Immunity and Pathogen Intensity*

I found strong differences in several pathogen and immune parameters based on rainfall groups (hereafter referred to as seasons). As I expected from previous work (Turner and Getz 2010), GI parasite infection intensities were significantly higher in wetter seasons than in dry ones (Figure 5). The reasons for this are likely to be at least partly environmental; free-living stages of GI nematode larvae develop more consistently and survive for longer in wetter, cooler conditions than in hotter, drier ones (Gordon 1948; Banks et al. 1990). In addition, studies have suggested that helminths in strongly seasonal environments have adapted their life stages to correspond with the most favorable external environmental conditions; parasite infections are often maintained at least to some extent in hosts year-round because it is too immunologically costly for hosts to completely eliminate all parasites (Colditz 2008). Adult worms often delay egg production until rainy seasons, and L4 stage larvae often arrest in a state of hypobiosis within hosts, essentially lying dormant and delaying becoming adults until it is advantageous to be able to mate and produce eggs (Horak 1981; Krecek et al. 1987; Krecek et al. 1987b; Jacquiet et al. 1995). Internal host conditions such as changes in diet, hormones, and immunity can also influence these seasonal GI parasite patterns (discussed below) (Horak 1981; Cornell et al. 2008).

While strong seasonal patterns in anthrax mortality incidence have been noted in ENP since the disease was first recorded there in 1966, I did not find significant seasonal differences in anti-PA antibody titers (Figure 5; however, see

discussion below). The reasons for this are likely because my previous work with zebra anti-PA titers showed that mean time for negative seroconversion in titer-positive animals was six months (see dissertation Chapter 2), long enough to obscure any seasonal differences in sublethal anthrax exposure at the scale I was examining.

Ectoparasite counts were significantly higher in the drier seasons than in the wetter ones in paired samples but not in unique animal comparisons (Figures 4 and 5); this provides evidence that individual susceptibility, likely mediated by immune responses and/or immunomodulation, was at least partly responsible for seasonal differences in ectoparasite infection intensities. Previous studies have found that hard-bodied ticks in most systems prefer higher humidity and intermediate temperatures and are at their lowest numbers on hosts in very hot or very cold times and in very dry seasons (Papazahariadou et al. 1995; Mushi et al. 1996; Yakchali et al. 2011). As ticks were actually lower on ENP zebras during what should have been their preferred season, this lends more evidence that immunomodulatory conditions influenced tick infestation of hosts. I did not, however, distinguish between tick life stages when determining tick burdens. While previous studies in southern Africa zebras have found both immature and adult ticks on zebra hosts (Horak 1984; Horak et al. 1984), it is possible that different tick life stages were more concentrated on non-zebra hosts at certain times of year, potentially confounding my seasonal zebra-tick patterns (R. Lane, pers. comm.).

Eosinophil counts were also significantly higher in the wetter seasons than in the drier ones, consistent with hosts fighting against new GI parasite infections during wetter conditions (Figure 5). In a parasite immunity model, Fenton et al. 2008 found that increasing the mortality rate of macroparasite free-living stages resulted in decreased macroparasite infection pressure and decreased host investment in Th2 responses, while increased macroparasite transmission selected for increased host Th2 investment; thus, increased survival of L3 larvae in ENP in the wetter seasons likely led to increased transmission and to increased host investment in eosinophil responses during this time. Other work in ENP has found that zebra shed significantly more strongyle larvae during wetter seasons than in drier ones, lending further evidence of an active immune response against new infections during the wet season (W. Turner, unpublished data). Eosinophil counts in ENP zebras ranged from  $198 \pm 223$  cells/ $\mu$ l (mean  $\pm$  SD) in the dry season to  $414 \pm 268$  cells/ $\mu$ l in the wet season, a 2.3-4.7-fold increase over the average eosinophils in plains zebras in zoos ( $88 \pm 86$  cells/ $\mu$ l) (ISIS 2008); clearly, GI parasite infections greatly affect these wild hosts immunologically compared to their consistently dewormed captive counterparts.

Unlike for eosinophils, there were no significant changes in monocyte counts between seasons (Figure 5). Monocytes, however, are more broadly responsive innate immune responders than are the Th2-specific eosinophils, and thus consistent monocyte counts may reflect constant host responses to a wide variety of pathogens (also, see discussion below). Monocyte counts for ENP zebras averaged  $456 \pm 273$  cells/ $\mu$ l (mean  $\pm$  SD), however, a two-fold increase over typical counts

measured in zoo zebras ( $226 \pm 171$  cells/ $\mu$ l) (ISIS, 2008). This indicates that wild zebras are allocating significantly greater resources to immune functions than are captive zebras.

Interestingly, IgE antibody titers did not differ significantly between seasons (Figure 5). However, while eosinophil counts drop within a few months of dropping strongyle worm infections, high IgE titers can last for years even after complete elimination of GI helminths (Poirrez 2001; Mitre and Nutman 2006). Thus, it is likely that IgE levels persist from season to season, obscuring any seasonal differences in zebra hosts. IgE titers in recaptured zebra ranged from  $8.50 \pm 4.90$   $\mu$ g/ml (mean  $\pm$  SD) in the dry season to  $10.4 \pm 4.90$   $\mu$ g/ml in the wetter seasons. While these were at least ten times the mean IgE level in non-atopic humans (Ledin et al. 2008), they were quite a bit lower than IgE titers found in non-atopic domestic horses treated with anthelmintics ( $84 \pm 90$   $\mu$ g/ml) (Wagner et al. 2003). However, while Ledin et al. (2008) found that wild wolves had increased IgE titers compared to domestic dogs, and while Devalapalli et al. (2006) found that wild rats and mice had a 4-to-12-fold increase in IgE titers compared to their domestic conspecifics, Ledin et al. also found that their captive wolf controls had higher IgE titers than did their free-ranging counterparts; laboratory animals kept in sterile cages are unlikely to become re-infected with parasites after deworming, while those living in small, outdoor pastures may experience rapid helminth re-infection soon after anthelmintics wear off, leading to the disparity in these IgE titer comparisons. There is also a chance that domestic or captive animals can mount stronger IgE responses against parasite threats because they lack seasonal nutritional and coinfection stressors of their wild counterparts. This study is the first examination of IgE titers in wild equids and one of the first measures of IgE in wildlife, and thus while IgE titer interpretations remain complex, this study provides valuable baseline information regarding this immune component in natural systems.

IgGb antibody titers were significantly greater in wetter seasons than in drier ones, but only for recaptured animals, indicating that there are individual differences in immune allocation, immunomodulation, or both (Figures 4 and 5). While vaccine studies have found that IgG antibodies and IgG memory B cells can last between months and years, the clear differences at an approximate six-month scale here indicate that zebra IgGb antibody titers can rise and fall rather quickly, especially compared to IgE titers (Kohler et al. 2000; Harrod et al. 2001; Wing et al. 2011; Grabenstein and Manoff 2012). The few studies examining IgG titers in wildlife have found conflicting evidence regarding seasonal changes; while some have found that IgG titers differ seasonally in helminth-infected hosts (Pathak et al. 2012), others have found no seasonal differences in IgG titers (Owen and Moore 2006). However, Gonzales et al. (1999) found that house sparrows fed a protein-restricted diet exhibited reduced immunoglobulin concentration, and Beldomenico et al. (2008) found that immune investment was lowest in field voles in winter likely due to nutritional constraints on immune investment. In addition, while IgGb is likely a Th2-type antibody subtype in equids (Hooper-McGrevy et al. 2003), IgGb is also the most prevalent antibody isotype in equine serum and is potentially involved

in protection against other types of pathogens as well (Lewis et al. 2008). Thus, while changes in IgGb titers may in part reflect seasonal changes in GI parasite infection intensity in ENP zebras, they may also reflect immune resource allocation fluctuations during nutritionally depleted dry seasons.

While IL-4 levels were not significantly different between seasons, I found evidence that there may be increases in IL-4 signaling in wetter times compared to in drier ones. As this cytokine has been shown to be instrumental in protecting against or clearing many helminth infections, measuring a 15-fold increase in IL-4 signal in recaptured animals while these animals were experiencing their highest GI parasite infection intensities of the year makes immunological sense (Abbas et al. 1996; Mosmann and Sad 1996; Diniz et al. 2010). IL-4 levels have been found to peak soon following new parasite infections and to fall a couple of months after removal or attenuation of infection (Pathak et al. 2012); thus, drops in IL-4 seen in the dry season likely also correlate with drops in new GI parasite infections at this time.

While IFN- $\gamma$  levels were also not significantly different between seasons, I also found evidence that there may be increases in IFN- $\gamma$  signaling in drier times than in wetter ones. As IFN- $\gamma$  has been found to significantly decrease in the face of increased GI helminth infection intensity and to increase after anthelmintic treatment, it is logical that I found an approximately 2-fold decrease in IFN- $\gamma$  signal in wet seasons when hosts were experiencing peak new GI helminth infections compared to in the relatively parasite-depleted dry season (Chen et al. 2005; Graham et al. 2008; Ezenwa et al. 2010).

#### *Correlates of Immunity and Coinfection with Immunomodulatory Effects*

Several of the seasonally changing pathogen and immune factors provide evidence for seasonal Th1-Th2 tradeoffs in zebra hosts. GI parasite infections clearly greatly affect zebra during the wet season, as evidenced by the significant increase in infection intensity during this time, coupled with peak eosinophil responses and host shedding of at least some of the newly infecting larvae. This eosinophil peak, combined with significantly higher IgGb antibody titers and evidence for increased IL-4 and decreased IFN-g signaling at the same time in the same hosts provides strong evidence that host immune responses are skewed toward a Th2-type response in the wet season due to GI helminth infections. The significant drop in eosinophil counts and IgGb titers in the dry season, coupled with evidence of increased IFN-g signaling at that time supports the hypothesis that immunomodulation toward a Th2 response by GI parasites is attenuated when free-living GI parasite stages are less capable of survival. Interestingly, there is evidence that pregnancy in many mammals suppresses Th1 responses in favor of Th2-type immunity (Mosmann and Sad 1996; Spellberg and Edwards 2001). Zebra in ENP are largely seasonal breeders, with most females giving birth between December and April (Turner and Getz 2010). As the gestation period for plains zebra is 12 months (Estes 1991) and with animals able to become pregnant even while lactating, many adult female zebras in the population are pregnant year-round. In addition, while



Turner et al. (2012) found evidence for a periparturient rise in GI parasite intensity in springbok (indicating a relative loss in Th2 immunity against parasites following parturition), this pattern was not observed in zebras. Thus, it appears that any Th2-type skewing in zebra hosts is driven primarily by GI parasite infections and that this immunomodulation is strong enough to overcome strong hormonal fluctuations.

This Th2-type skewing by helminths has been found to have significant consequences for hosts fighting against concurrent microparasitic infections. Chen et al. (2005) found that increased intestinal helminth infection correlated with increased susceptibility to a GI bacterial infection in mice, and also correlated with significant downregulation of IFN- $\gamma$  protective against these bacteria. In a meta-analysis, Graham (2008) found that when helminths reduced production of microparasite-specific IFN- $\gamma$ , this predicted greater microparasite density in hosts. Thus, the wet season Th2-type skewing observed in this system, in correlation with the timing of peak anthrax incidence, suggests that hosts may experience increased anthrax susceptibility shortly following periods of peak GI helminth infection intensity due, at least in part, to immunomodulatory effects and immune tradeoffs.

Why, then, did the GEE models not show this relationship more clearly? There is evidence, though not statistically significant, that decreased anti-PA titers (or absence of titers) were correlated with increased GI parasite infection intensities, and vice versa (Figure 6). Thus, as parasite numbers increase, hosts may be less likely to mount a humoral immune response against anthrax. This relationship, however, is likely complicated by the fact that the anti-PA memory response appears to last, at least in part, for a few months across seasons. In addition, *B. anthracis* may provoke both Th1 and Th2-type responses at different stages of infection, patterns that are not uncommon in pathogens that involve both intra- and extracellular stages of infection (Boyaka et al. 2003; Maizels et al. 2004; Pickering et al. 2004b; Aloni-Grinstein et al. 2005; Allen et al. 2006). Such mixed responses can potentially complicate analyses of tradeoffs. It is possible that animals experiencing low levels of anthrax might ultimately mount at least a minor Th1-type immune response, even in the face of strong Th2-skewing; Potian et al. (2011) found that mice coinfecting with GI helminthes and lung tuberculosis bacteria were able to mount both Th2 and Th1 responses, respectively, to these pathogens, but only once the parasites colonized separate organs. Interestingly, low antigen concentrations and low-dose infections are more likely to induce Th1 responses, whereas high doses of antigens induce Th2-type responses (Abbas et al. 1996). As animals ingesting very high doses of anthrax spores are more likely to develop fulminant infection and die subacutely (Watson and Keir 1994), animals that have measurable anti-anthrax titers are even more likely to have experienced low infectious doses and a potential for fighting with a measureable Th1 response against an established Th2-type skewing. Animals that experience high GI parasite infections and large anthrax doses are also likely at much greater risk for mortality, thus effectively removing very helminth-susceptible and coinfecting animals from this study but still benefiting anthrax transmission (Hugh-Jones and de Vos 2002; Jolles et al. 2008). As I was not able to sample anthrax carcasses for GI parasite and

immune parameters, my results may be biased against accurately measuring those animals that succumbed to anthrax due to macroparasite Th2-type skewing potentially aided by *B. anthracis* itself. In addition, though anthrax outbreaks are quite apparent in ENP, the number of deaths recorded per outbreak season are very low compared to the large zebra population; the highest number of anthrax deaths predicted from empirical evidence and a hierarchical distance sampling model is approximately 600 for all ENP anthrax hosts out of roughly 13,000 zebra (Bellan et al. 2013). While anthrax likely acts with predation in ENP to limit ENP population growth (Gasaway et al. 1996), it is a relatively minor source of total mortality in this system (Lindeque 1991). Thus, lack of statistical significance in the relationships between GI parasites and anthrax in my models may in part be due to the probable low sample size of animals that had survived sublethal anthrax infection and developed measurable infection markers (only about 60% of my measured population; see dissertation Chapter 2).

The relationship of anti-PA titers with rainfall and eosinophils is perhaps more informative. To understand these correlations, we must first examine the relationships between eosinophils and GI parasite infection intensities. While higher eosinophils and higher GI parasite infections were both observed in the wet season and while both were significantly, positively related to rainfall in GEE models (Figures 6 and 7), eosinophil counts and GI parasite load in the same animals show a more complex relationship. Lower eosinophils significantly predicted higher GI parasite counts, and lower GI parasite counts were associated with higher eosinophil counts (Figures 6 and 7). Previous studies in wildlife have found a similar pattern, with a strongly negative association between GI helminth FECs and eosinophil counts, even when taking into account other host factors (Stear et al. 1995; Kanobana et al. 2002; Jolles et al. 2008; Ezenwa et al. 2010). These findings suggest that individuals that mount stronger Th2 responses (higher eosinophil counts) are more resistant to parasite infections, and thus exhibit lower FECs. Other studies have demonstrated that eosinophils are important for killing newly ingested and migrating helminth larvae and for decreasing adult worm body size and fecundity, supporting the hypothesis that these higher eosinophil counts, lower FECs, and the increased numbers of shed larvae we observed in the wet season are directly related (Stear et al. 1995; Winter et al. 1997; Balic et al. 2000; Kanobana et al. 2002; Rowe et al. 2008).

Although I did not see any direct differences in anti-PA titer when comparing animals across rain groups, having an anti-PA titer was significantly, negatively related to rainfall in the PA GEE (Figure 6). While eosinophil count alone was positively correlated with presence of a PA titer in this model, the interaction between rainfall and eosinophil count was much more strongly significant in predicting the absence of a PA titer, suggesting the effects of parameters not included in the model. The strongly negative association of rainfall:eosinophils with PA titer, the non-significant but essential inclusion of a negative GI parasite term in this model, and the strongly associated matrix of rainfall, eosinophil counts, and GI parasite infection intensity in other models here suggest that GI helminths affect

hosts' ability to mount an immune response to anthrax, through immunomodulatory routes. Similar directional tradeoffs were observed in studies of concurrent GI helminth and tuberculosis infections in buffalo (Jolles et al. 2008; Ezenwa et al. 2010). In addition, GI helminth infections could influence anthrax transmission in more physical ways. Turner et al. (2012) found a significant relationship between strongyle infection intensity and lower body condition in springbok, and Lello et al. (2005) found that rabbits with more helminths had less body fat and were in poorer body condition; Lello et al. thus suggested that parasite-weakened animals may become less selective in their feeding and also need to graze more. Turner et al. (2013) found that ENP zebra ingested significantly more soil in the wet season compared to in the dry, likely in part due to grazing new grass shoots close to the ground and to pulling up entire plants from the dampened, loosened soil. If hosts with more GI parasites also need to ingest more food and are less selective, these animals may eat more soil still during the wet season, exposing them to potentially much higher doses of soil-borne anthrax at this time.

While IgE titers were not significantly different comparing across seasons, lower GI parasite intensities significantly predicted higher IgE titers (Figure 7), and lower IgE titers were nearly significant in predicting higher GI parasite counts in GEE models (Figures 6 and 7). It is therefore likely that, as fellow components of the Th2-type arm of immunity and as important anti-helminth factors, higher eosinophil counts and IgE titers both render hosts less susceptible to GI parasites in similar ways.

IgGb titers were also significantly, positively associated with increased rainfall in GEE models (Figure 7). Their suggested, but not significant, positive relationship with anti-PA titer and negative relationship with eosinophils, and their inclusion as a significant negative predictor of monocyte counts paints a complex picture. The fact that IgGb antibodies are present in large concentrations in equine serum and potentially play mixed functional roles may make straightforward interpretation of these IgGb titers impossible.

While monocyte counts were not significantly different when compared across rain groups, increasing rainfall was significantly correlated with increased monocytes in the monocytes GEE model (Figure 7). Interestingly, while monocytes were significantly, positively predicted by increased eosinophils, they were non-significantly, negatively predicted by a rainfall-eosinophil interaction and significantly, negatively predicted by both IgE and IgGb titers. Monocytes and anthrax have a complex relationship; while classically activated (Th1-type) macrophages (CAM) play an important role in limiting and clearing anthrax infection (Welkos et al. 2002; Kang et al. 2005; Cote et al. 2006), anthrax spores germinate and survive within macrophages and anthrax can cause macrophage apoptosis through the effects of toxins (Hanna et al. 1993; Guidi-Rontani et al. 1999; Park et al. 2002; Ribot et al. 2006). Chronic helminth infections also may have differing relationships with macrophages; alternatively activated macrophages (AAM) are macrophages activated by Th2 cytokines such as IL-4 and IL-13 in the face of chronic helminth infections (Noël et al. 2004; Anthony et al. 2007; Potian et

al. 2011). These AAM also express chemokines that preferentially attract Th2 cells to sites of helminth infections, and thus can play a critical role in host immune responses to macroparasites (Loke et al. 2000; Rodriguez-Sosa et al. 2002). Alternative activation downregulates the Th1-driven killing of intracellular parasites through a nitric oxide (NO) burst, compromising host ability to restrict bacterial infections such as tuberculosis (Gordon 2003; Potian et al. 2011). As classically activated macrophages kill *B. anthracis* via NO synthesis, AAM triggered by helminth infections could greatly compromise early host immune defenses against anthrax spores (Raines et al. 2006). It is therefore conceivable in my current study that the monocytes associated with increased rainfall and increased eosinophil counts represent pools mobilized to become AAM in the face of high GI helminth infections, whereas those significantly associated with decreased IgE and IgGb titers represent CAM active during drier seasons and lower GI helminth infection intensities (Figure 7). AAM can respond to Th1 cytokines after removal or attenuation of Th2 cytokines, and macrophages can live in tissues for months to years; thus, the same cellular pool can switch protective roles in the face of changing pathogen pressures (Parihar et al. 2010; Potian et al. 2011). However, the half-life of monocytes in the circulation prior to either apoptosis or mobilization into tissues to become macrophages is only approximately 3 days (Whitelaw and Bell 1966). Thus, it is unlikely that I measured the same cells in different activation states in different seasons even if these cells were mobilized to replace the same macrophages already laboring in affected tissues.

While studies suggest that ectoparasites are more active in parasitizing hosts during milder and more humid times, ectoparasite infection intensities in my study were both higher on hosts in the dry season in rain group comparisons and were significantly negatively associated with rainfall in GEE models (Figure 6; though see seasonal tick discussion above). Interestingly, higher GI parasite infection intensities were significantly associated with higher ectoparasite counts, despite the opposite interaction of ectoparasites with rainfall. While not significant, ectoparasite counts were included in eosinophil and IgE GEE models and suggested similar relationships to these variables as for GI parasite infection intensity (Figure 7). Several studies suggest that a Th1-type immune response is most effective against tick infestations and that ticks can suppress this response in favor of a less effective Th2-type skewing (Ferreira et al. 1999; Wikel 1999; Ogden et al. 2002; Castagnolli et al. 2008). However, my study suggests that a Th2-type immune response is actually more effective in fighting against tick infestations, the more so because otherwise the combination of preferred environmental conditions coupled with a strong Th2 skewing would be expected to result in very high tick burdens in the wet season. As hard-bodied ticks are more capable of survival during dry seasons than are desiccation-prone GI helminth free-living stages (Biggs and Langenhoven 1984; Horak 1994), the increase in tick infestations in the dry season may represent ticks taking advantage of a decrease in GI parasite-driven Th2 immune pressure at this time. The directionality of these interactions, with GI parasites driving a Th2-type immune response and indirectly affecting ectoparasites, is supported by the fact

that rare major histocompatibility complex (MHC) alleles were found to be associated with increased GI parasite infection intensities, and common alleles with increased ectoparasites in ENP zebras (Kamath 2011). This suggests that GI parasites exert stronger selective pressure at the gene locus in question than do ectoparasites, with GI parasites having higher fitness costs to hosts and likely driving host immune allocation.

### *Predictors of Host Age*

I found that, while ectoparasites and IgGb titer significantly increased with age and also significantly predicted older animals, lower GI parasite infection intensities significantly correlated with older animals (Figures 6 and 7). Increased eosinophils were also nearly significantly predicted older animals, in concert with the relationships between eosinophils and GI parasite infections already discussed. Thus, it appears that GI parasites exert a stronger selection pressure than do ectoparasites, as discussed above: higher GI parasite counts predict that animals are less likely to survive to older ages, while animals appear to accumulate higher tick burdens with age without overtly negative consequences to survival time. The negative effects of macroparasite infections on wildlife survival have been well-documented (Anderson and May 1979; Keith et al. 1985; Murray et al. 1997; Hudson et al. 1998).

The fact that increasing eosinophil counts predict older animals is likely due to less susceptible animals carrying lower GI helminth burdens and surviving for longer rather than eosinophils accumulating over time with increased parasite exposure. However, there is also evidence from a few previous studies that adaptive immunity to GI parasites may increase over time with cumulative exposure (Gulland 1992; Cattadori et al. 2005; Cattadori et al. 2007; Cornell et al. 2008; Pathak et al. 2012). This may help account for some of the rise in IgGb titers with age. The lack of association between higher IgE titers and older animals is puzzling, though trends in this parameter may again be obscured by the long-term persistence of IgE antibodies. Ledin et al. (2008) found that IgE titers in wild wolves actually tended to decrease with age, and hypothesized that this may be due to a positively reinforcing cycle of successful immunity against parasites and a related decrease in new infections. Other work in ENP has found that older zebra were significantly more likely to shed L4 strongyle than were younger ones, indicating accumulating immunity against parasites with age (W. Turner, unpublished data). In addition, while macroparasites tend to be overdispersed within populations, with a few hosts harboring the majority of the parasite numbers, strongyles are not aggregated in ENP zebras (Turner and Getz 2010). This pattern is consistent with high GI parasite loads causing higher mortality and cleansing the population of these highly susceptible, highly infected animals (Gulland 1992; Gulland and Fox 1992; Jolles et al. 2008).

Anti-PA antibody titers did not show a significant correlation with age. I previously found that successive sublethal anthrax exposures can cause animals to booster their anti-PA titers over time; however, even with multiple infections,

immunity is short lived ( $\leq 6$  months on average) (see dissertation Chapter 2). Thus, it is unlikely that animals would repeatedly experience enough anthrax dosing at the proper intervals to boost their adaptive immune response, but not high enough doses to cause fulminant infection and death.

### *Conclusions*

In summary, I found evidence that GI helminth infection intensities, eosinophil counts, monocyte counts, IgE antibody titers, IgGb antibody titers, and IL-4 cytokine signaling were increased in wetter seasons than in drier ones, and that ectoparasite infestations, IFN- $\gamma$  cytokine signaling, and anti-PA antibody titers were increased in drier seasons than in wetter ones. Taken together, this supports the hypothesis that wet seasons in ENP are characterized by Th2-type immune skewing in zebra, while Th1-type immunity prevails in drier seasons. This Th2-type skewing is primarily driven by GI parasite infections, which show strong seasonal fluctuations primarily constrained by external environmental effects on free-living parasite stages.

As zebra hosts experience significantly increased GI helminth infection intensities and evidence for Th2-type skewing shortly prior to the population's annual anthrax outbreaks, this suggests that anti-parasite Th2 responses make hosts less capable of mounting effective Th1-type immune responses against anthrax infections at this time. Increased eosinophil counts during wet seasons represent an active, and partly successful, immune response against primarily new GI parasite infections. As the interaction between rainfall and eosinophil counts is significantly, negatively correlated with anti-anthrax adaptive immune responses, this also provides more direct evidence for the immunomodulatory effects of GI parasite infections on anthrax susceptibility. GI parasites also appear to negatively affect ectoparasites, with evidence that Th2-type immune responses are more effective than Th1-type responses in preventing and controlling tick infestations.

I also found evidence that these coinfections and immune tradeoffs affect long-term host survival. Animals with stronger Th2-type immune responses were less susceptible to new GI parasite infections and were more likely to be older. Ectoparasite infestations increased with host age, corroborating previous evidence that GI parasites exert more selection pressure on ENP zebra hosts than do ectoparasites (Kamath 2011), and strengthening the hypothesis that GI parasites are the primary drivers of immunomodulation in this system. In addition, the fact that anthrax incidence is factors of magnitude lower than that of GI helminth and ectoparasite infections, and that immunity to anthrax does not increase with age suggest that anthrax exerts less selection pressure on zebra than do these other pathogens. However, I also found support for previous evidence showing that anthrax is likely a population-controlling factor in ENP plains ungulates, in concert with other factors (Gasaway et al. 1996); the lack of a strong relationship between high GI parasite counts and lower anti-PA titers, and the lack of parasite aggregation in this population suggest that coinfections with intense GI parasite infestations and anthrax result in increased host mortality (similar to Jolles et al. 2008).

Ezenwa and Jolles (2011) suggested that effects of helminth coinfection on disease dynamics were most evident for microparasites that cause mild, acute infections rather than those that cause chronic or severe acute disease. As a microparasite, anthrax is an interesting case to test this scenario; while it can cause severe, acute disease, there is also evidence that it can cause acute, sublethal infection in its herbivore hosts (see dissertation Chapter 2). *Bacillus anthracis*, unlike perhaps any other vertebrate pathogen, also directly relies on host death for disease spread. Thus, the GI parasite-anthrax coinfection system in ENP may perpetuate because GI parasites are ubiquitous enough to persist even in the face of cleansing of highly parasitized hosts through mortality, and increased deaths due to anthrax have the opposite of a cleansing effect. In addition, Ezenwa et al. (2010) found that South African buffalo harbored a relatively common allele that was associated with a 60% increase in GI helminth FEC, but that also gave these hosts the ability to mount at least a partial Th1-type immune response against tuberculosis; tradeoffs such as this may exist in ENP zebras as well, accounting for the near 100% year-round persistence of strongyles in these hosts. The concurrent presence of these pathogens in ENP may even have a stabilizing effect on the population. Fenton (2008) found that, unlike the strong oscillations between epidemics observed in a disease model with only microparasite infections, adding a macroparasite to the system caused the model to settle into a stable equilibrium with hosts, microparasites, and macroparasites coexisting in an endemic community. This scenario appears to describe ENP well, in which plains ungulates exist at quite stable population levels with relatively small annual anthrax epidemics.

Finally, in concert with the ideas of Pedersen and Fenton (2007), I used the wealth of data from this study to develop a proposed interaction network of within-host pathogen connectivity for this system (Figure 8). Like community ecology trophic cascades, such a network involves a basal level (host resources), an intermediate level (pathogens), and a top trophic level (immune system, corresponding to predators). Unlike an external ecosystem, however, the basal and top levels of a within-host interaction network are inextricably, and directly, linked. Such networks reveal the high connectivity of within-host parasite communities; as coinfection interactions may explain more variation in infection risk than do more external factors relating to host condition and transmission risk (Telfer et al. 2010), it is essential that we not shy away from the internal host complexity involved in studying multiple disease systems, but embrace it.

## **Acknowledgments**

I thank the Namibian Ministry of Environment and Tourism for permission to do this research, the Directorate of Parks, Wildlife and Management for permission to work throughout Etosha, and the staff in the Directorate of Scientific Services at the Etosha Ecological Institute for logistical support and assistance. I would like to give special thanks to veterinarians Mark Jago, Conrad Brain, Peter Morkel, and Ortwin Aschenborn for their assistance with animal captures, as well as to Martina Küsters, Shayne Kötting, Gabriel Shatumbu, Wendy Turner, Wilferd Versfeld, Marthin

Kasaona, Royi Zidon, and Werner Kilian, among others, for their tremendous help in the field. Among those in the field capture team, I especially thank Wendy Turner and Martina Küsters for their extra assistance with sample collection and GI parasite quantification. I especially thank Russell Vance for allowing me to work in his immunology lab and for giving me assistance and guidance. I also greatly thank Bettina Wagner and Heather Freer for assisting with the IgE ELISAs. I thank Steve Bellan for assistance with the anti-PA ELISA endpoint titer determinations, Jennifer Johns for consulting with me regarding the manual blood cell counts, and Mary Fontana and Dara Burdette for assisting me with the PCR protocols and interpretations. I thank Bryan Krantz for providing PA for the ELISAs and keepers at the Woodland Park Zoo for providing zebra control serum. This research was supported by Andrew and Mary Thompson Rocca Scholarships and NIH grant GM83863.



## Tables

**Table 1.** Zebra capture seasons, timing, animals involved, and samples taken. "CS" is Capture Season and "NS" is Nominal Season. Data refer to Total#(#New, #Resampled), where and "New" refers to new individuals and their samples and "Resampled" refers to animals resampled at least once in that season and their corresponding samples collected. "Ticks" refers to the number of zebras sampled for total tick burden.

CS	NS	Date (Mo/Yr)	Blood	Feces	Ticks
S1	Wet	3-4/08	45(45,0)	38(38,0)	45(45,0)
S2	Dry	10-11/08	36(14,22)	29(17,12)	18(0,18)
S3	Wet	4-5/09	35(6, 29)	32(4,28)	30(5,25)
S4	Dry	9-11/09	13(4,9)	10(3,7)	13(4,9)
S5	Dry	8/10	25(0,25)	14(0,14)	19(0,19)
<b>Totals</b>			154(69,85)	123(62,61)	125(54,71)

**Table 2.** Primer sequences used in quantitative PCR assays. Primer sequences are taken from Ainsworth et al. 2003.

<b>Gene</b>	<b>Forward primer 5' → 3'</b>	<b>Reverse primer 5' → 3'</b>
Eq IL-4	tcg tgc atg gag ctg act gta	gcc ctg cag att tcc ttt cc
Eq IFN-g	tct tta aca gca gca cca gca a	gcg ctg gac ctt cag atc at
Eq GAPDH	aag tgg ata ttg tcg cca tca at	aac ttg cca tgg gtg gaa tc

**Table 3.** A. Number of zebra captured in each season for first captures only, grouped by rain groups. B. Number of zebra captured in each season for paired recaptured only, grouped by rain groups. There were no resampled animals that fell into Cap1RG2 or Cap2RG1 groups. Cap1 is capture 1; Cap2 is capture 2 for the same individual; RG1 is rain group 1 (experience of cumulative rainfall  $\geq 200$ mm over the two months prior to capture); RG2 is rain group 2 (experience of cumulative rainfall  $\leq 100$ mm over the two month prior to sampling).

A.	Capture Season	Cap1RG1	Cap1RG2	B.	Capture Season	Cap1RG1	Cap2RG2
	S1	45	0		S1	32	0
	S2	0	14		S2	0	23
	S3	0	6		S3	0	8
	S4	0	4		S4	0	1
	S5	0	0		S5	0	0

**Table 4.** List of variables used in models, with their abbreviations and descriptions.

<b>Variable</b>	<b>Abbreviation</b>	<b>Description</b>
Cumulative rain 2 months prior	Rain	Rain (mm) experienced by an individual in 60 days prior to a capture event
Individual age	Age	Age (days) at a sampling event, determined first by dental wear
GI parasite burden	GIP, or GIsqrt when square root transformed	GI parasite infection intensity (nematode eggs/gram of feces)
Sublethal anthrax exposure	log2PA or PA	Anti-PA antibody titer as measured in log <sub>2</sub> of final dilution (log2PA) or as presence or absence of a titer (PA)
Ectoparasite burden	Ecto, or Ectosqrt when square root transformed	Total number of ticks
Eosinophil count	Eos, or logEos when log <sub>10</sub> transformed	Number of eosinophils/ $\mu$ l of blood
Monocyte count	Monos, or logMonos when log <sub>10</sub> transformed	Number of monocytes/ $\mu$ l of blood
IgE Titer	IgE, or logIgE when log <sub>10</sub> transformed	Serum concentration of IgE antibodies ( $\mu$ g/ml)
IgGb Titer	IgG, or IgGsqr when square root transformed	Serum concentration of IgGb antibodies (mg/ml)

**Table 5.** Maximal generalized estimating equation models evaluated.

<b>Pathogen Models</b>	
Glsqrt	~ Rain2 + Age + log2PA + Ecto + Eos + Monos + IgE + IgG
PA	~ Rain2 + Age + GIP + Ecto + Eos + Monos + IgE + IgG
Ectosqrt	~ Rain2 + Age + Glsqrt + log2PA + Eos + Monos + IgE + IgG
<b>Immune Models</b>	
logEos	~ Rain2 + Age + GIP + log2PA + Ecto + Monos + IgE + IgG
logMonos	~ Rain2 + Age + GIP + log2PA + Ecto + Eos + IgE + IgG
logIgE	~ Rain2 + Age + GIP + log2PA + Ecto + Eos + Monos + IgG
IgGsqrt	~ Rain2 + Age + GIP + log2PA + Ecto + Eos + Monos + IgE
<b>Age Model</b>	
Agesqrt	~ GIP + log2PA + Ecto + Eos + Monos + IgE + IgG

**Table 6.** Results of two-tailed Welch's *t* tests and Wilcoxon rank sum tests comparing variables between rain groups for first animal samplings (unique animals) only. Significant tests ( $p \leq 0.05$ ) are in bold.

Variable	N (RG1, RG2)	df	<i>t</i> or <i>U</i>	<i>p</i> -value before correction	Holm's corrected <i>p</i> -value	Mean Difference <sup>#</sup>	Higher Group
<b>Glsqrt</b>	45, 24	66.9	5.36	<b>0.000***</b>	<b>0.000***</b>	1771	<b>RG1</b>
log2PA	45, 24		643 <sup>+</sup>	0.095.	0.379	4.34	RG1
Ecto	45, 24		493 <sup>+</sup>	0.553	1.000	0.15	RG2
<b>logEos</b>	45, 24	55.3	5.07	<b>0.000***</b>	<b>0.000***</b>	240	<b>RG1</b>
logMonos	45, 24	43.1	0.45	0.653	1.000	34.1	RG2
logIgE	45, 24	32.6	0.40	0.690	1.000	1.01	RG2
IgGsqrt	45, 24	59.1	2.36	<b>0.022*</b>	0.109	1.44	RG1

<sup>+</sup> are Wilcoxon rank sum test results, using the test statistic *U*.

<sup>#</sup> Mean differences are differences between non-transformed means in the two rain groups for all variables. Units for mean differences are ep<sub>g</sub> for GLP; log<sub>2</sub> titer for log<sub>2</sub>PA; number of ticks for Ecto; cells/μl of blood for Eos and Monos; μg/ml for IgE; and mg/ml for IgG.

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 7.** Results of paired two-tailed *t* tests and Wilcoxon signed rank tests comparing variables between rain groups for first and second samplings of the same individuals. Significant tests ( $p \leq 0.05$ ) are in bold.

Variable	N (RG1, RG2)	df	<i>t</i> or <i>T</i>	<i>p</i> -value before correction	Holm's corrected <i>p</i> - value	Mean Difference <sup>#</sup>	Higher Group
<b>Glsqrt</b>	32, 32	31	5.35	<b>0.000***</b>	<b>0.000***</b>	1877	<b>RG1</b>
log2PA	32, 32		273 <sup>+</sup>	0.663	1.000	0.19	RG2
<b>Ecto</b>	32, 32		78.0 <sup>+</sup>	<b>0.005**</b>	<b>0.022*</b>	2.41	RG2
<b>logEos</b>	32, 32	31	5.11	<b>0.000</b>	<b>0.000***</b>	214	<b>RG1</b>
logMonos	32, 32	31	0.19	0.850	1.000	17.3	RG2
logIgE	32, 32	31	1.99	0.056.	0.167	1.82	RG1
<b>IgGsqrt</b>	32, 32	31	2.70	<b>0.011*</b>	<b>0.044*</b>	1.85	<b>RG1</b>

<sup>+</sup> are Wilcoxon signed rank test results using the test statistic *T*.

<sup>#</sup> Mean differences are differences between non-transformed means in the two rain groups for all variables. Units for mean differences are epg for GLP; log<sub>2</sub> titer for log<sub>2</sub>PA; number of ticks for Ecto; cells/μl of blood for Eos and Monos; μg/ml for IgE; and mg/ml for IgG.

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 8.** Maximum likelihood estimates for the best fit generalized estimating equation pathogen models. Significant coefficients ( $p \leq 0.05$ ) are in bold.

Response	Coefficients	Estimate $\pm$ SE	Wald statistic	$p$ -value	% QIC reduction from full model
Glsqrt	<b>Intercept</b>	3.83 $\pm$ 0.06	4614	<b>0.000***</b>	0.05
	<b>Rain2</b>	2.00e-03 $\pm$ 1.00e-03	14.7	<b>0.000***</b>	
	log2PA	-8.00e-03 $\pm$ 8.00e-03	1.02	0.236	
	<b>Eos</b>	-1.00e-03 $\pm$ 1.00e-03	3.43	<b>0.039*</b>	
	IgE	-5.00e-03 $\pm$ 3.10e-03	2.28	0.085.	
PA	Intercept	0.47 $\pm$ 0.68	0.48	0.455	8.42
	<b>Rain2</b>	-1.03e-04 $\pm$ 4.40e-3	9.66	<b>0.001**</b>	
	Age	-1.92e-04 $\pm$ 2.00e-4	0.90	0.268	
	GIP	-1.03e-04 $\pm$ 2.00e-4	0.45	0.478	
	<b>Eos</b>	4.00e-03 $\pm$ 2.10e-03	3.64	<b>0.034*</b>	
	<b>Rain*Eos</b>	-3.57e-05 $\pm$ 1.19e-05	9.01	<b>0.002**</b>	
Ectosqrt	Intercept	-0.06 $\pm$ 0.29	0.05	1.000	1.31
	<b>Rain2</b>	-8.56e-04 $\pm$ 4.00e-04	5.70	<b>0.010**</b>	
	<b>Age</b>	2.82e-04 $\pm$ 9.64e-05	8.57	<b>0.002**</b>	
	<b>Glsqrt</b>	0.01 $\pm$ 5.5e-03	6.42	<b>0.006**</b>	
	<b>Glsqrt*Age</b>	-4.58e-06 $\pm$ 1.88e-06	5.93	<b>0.009**</b>	

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

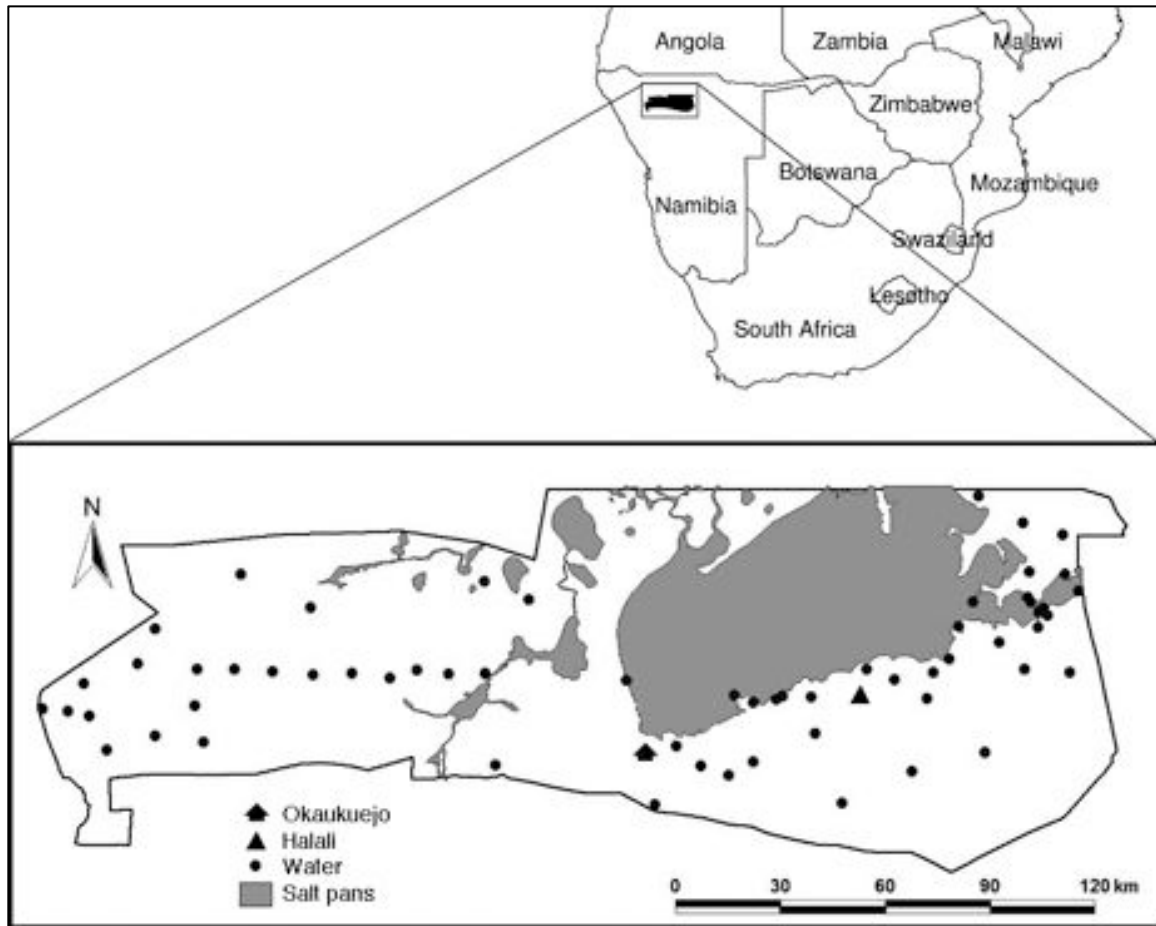


**Table 9.** Maximum likelihood estimates for the best fit generalized estimating equation immunity and age models. Significant coefficients ( $p \leq 0.05$ ) are in bold.

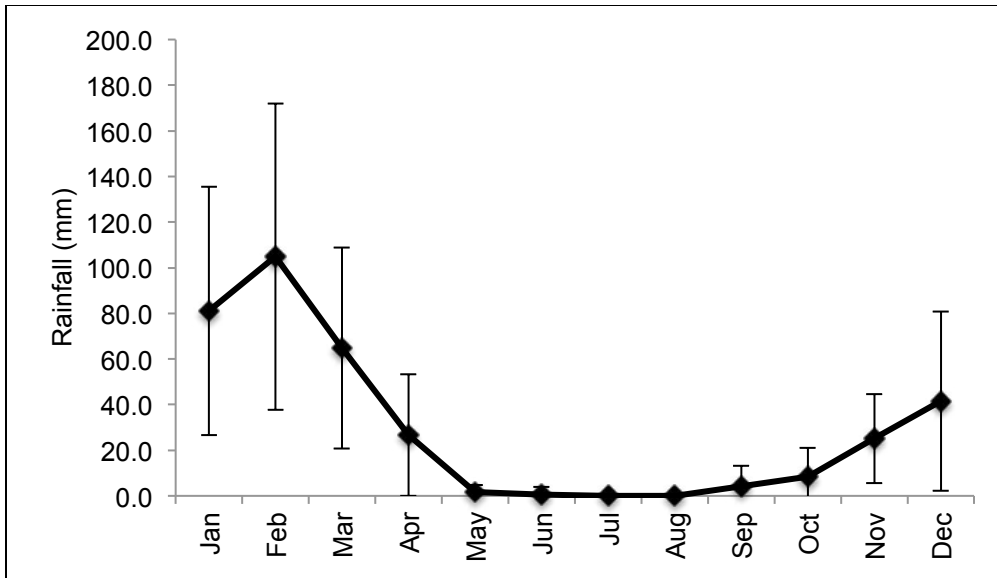
Response	Coefficients	Estimate $\pm$ SE	Wald statistic	p-value	% QIC reduction from full model
logEos	<b>Intercept</b>	0.75 $\pm$ 0.03	549	<b>0.000***</b>	0.06
	<b>Rain2</b>	8.21e-04 $\pm$ 1.00e-04	41.0	<b>0.000***</b>	
	<b>GIP</b>	-1.57e-05 $\pm$ 8.09e-06	3.77	<b>0.031*</b>	
	Ecto	-3.10e-03 $\pm$ 3.20e-3	0.99	0.245	
	<b>Monos</b>	9.39e-05 $\pm$ 3.67e-05	6.54	<b>0.006**</b>	
logMonos	<b>Intercept</b>	0.91 $\pm$ 0.03	699	<b>0.000***</b>	0.29
	<b>Rain2</b>	4.41e-04 $\pm$ 2.00e-04	7.84	<b>0.003**</b>	
	<b>Eos</b>	2.19e-04 $\pm$ 8.93e-05	5.99	<b>0.008**</b>	
	<b>IgE</b>	-2.60e-03 $\pm$ 1.10e-03	5.56	<b>0.012*</b>	
	<b>IgG</b>	-9.80e-03 $\pm$ 3.90e-03	6.27	<b>0.007**</b>	
	Rain*Eos	-7.65e-07 $\pm$ 4.40e-07	3.03	0.050.	
logIgE	<b>Intercept</b>	3.97 $\pm$ 0.05	6381	<b>0.000***</b>	0.65
	<b>Rain2</b>	3.93e-04 $\pm$ 2.00e-04	3.57	<b>0.036*</b>	
	GIP	-1.32e-05 $\pm$ 1.51e-05	0.76	0.3126	
	Ecto	-6.76e-03 $\pm$ 6.2-e-03	1.18	0.2046	
IgGsqr	<b>Intercept</b>	2.44 $\pm$ 0.181	184	<b>0.000***</b>	0.41
	<b>Rain2</b>	8.55e-04 $\pm$ 5.00e-04	3.60	<b>0.035*</b>	
	<b>Age</b>	8.66e-05 $\pm$ 4.65e-05	3.47	<b>0.038*</b>	
	log2PA	0.02 $\pm$ 0.02	2.12	0.095.	
	Ecto	-0.02 $\pm$ 0.01	2.08	0.098.	
	Eos	-2.82e-04 $\pm$ 2.00e-04	1.73	0.127	
	<b>Monos</b>	-4.57e-04 $\pm$ 2.00e-04	7.58	<b>0.003**</b>	
Agesqr	<b>Intercept</b>	3.79 $\pm$ 0.06	4512	<b>0.000***</b>	0.01
	<b>GIP</b>	-2.48e-05 $\pm$ 9.84e-06	6.33	<b>0.007**</b>	
	<b>Ecto</b>	0.012 $\pm$ 4.20e-03	8.39	<b>0.002**</b>	
	Eos	1.16e-04 $\pm$ 6.93e-05	2.82	0.058.	
	<b>IgG</b>	0.01 $\pm$ 5.60e-03	5.00	<b>0.015*</b>	

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

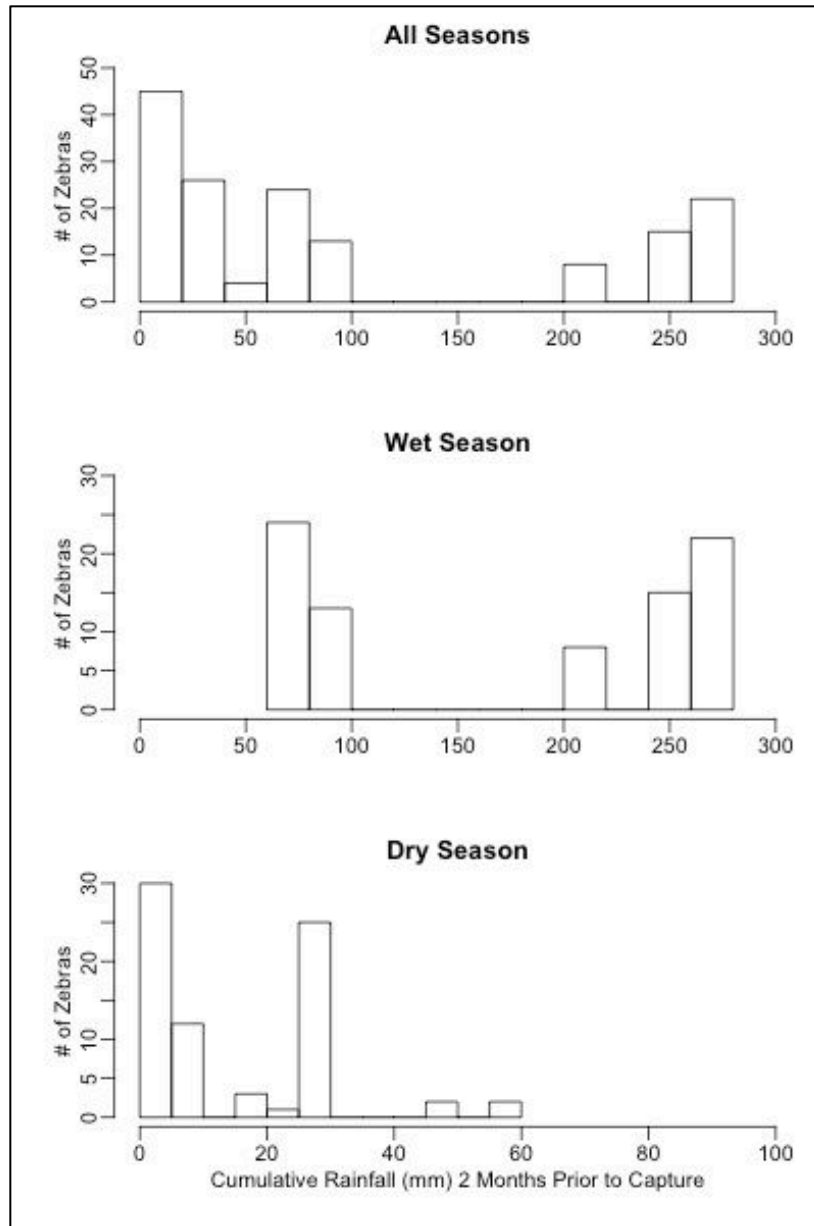
## Figures



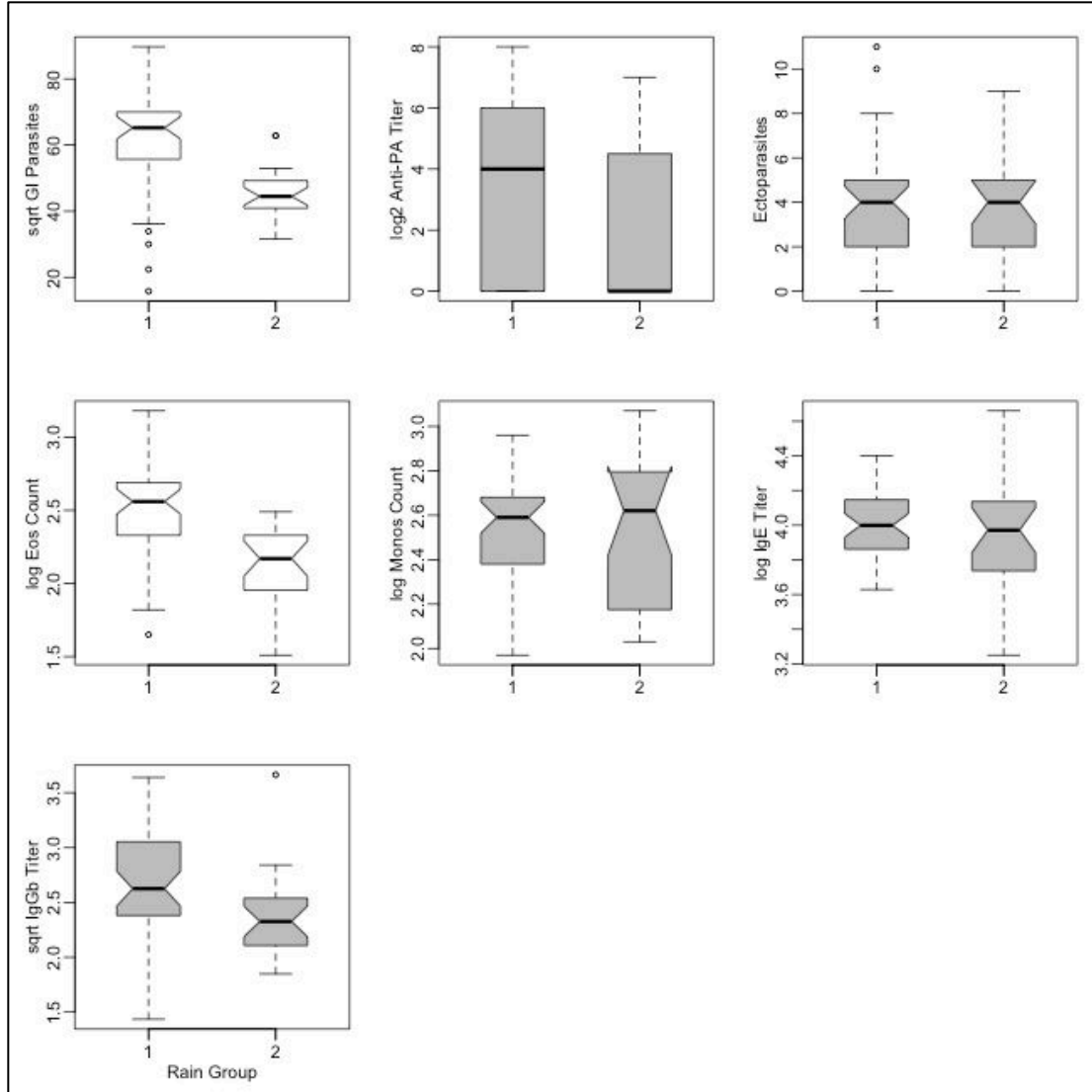
**Figure 1.** Etosha National Park in northern Namibia. The Etosha Ecological Institute is located in Okaukuejo in the center of the park; the majority of animal sampling for this study occurred in the nearby surrounding area, within a radius of approximately 20km (in the plains outside of the salt pans). During drier seasons, some sampling took place up to 100km to the east of Okaukuejo, around the Halali plains, and 15km south of Okaukuejo.



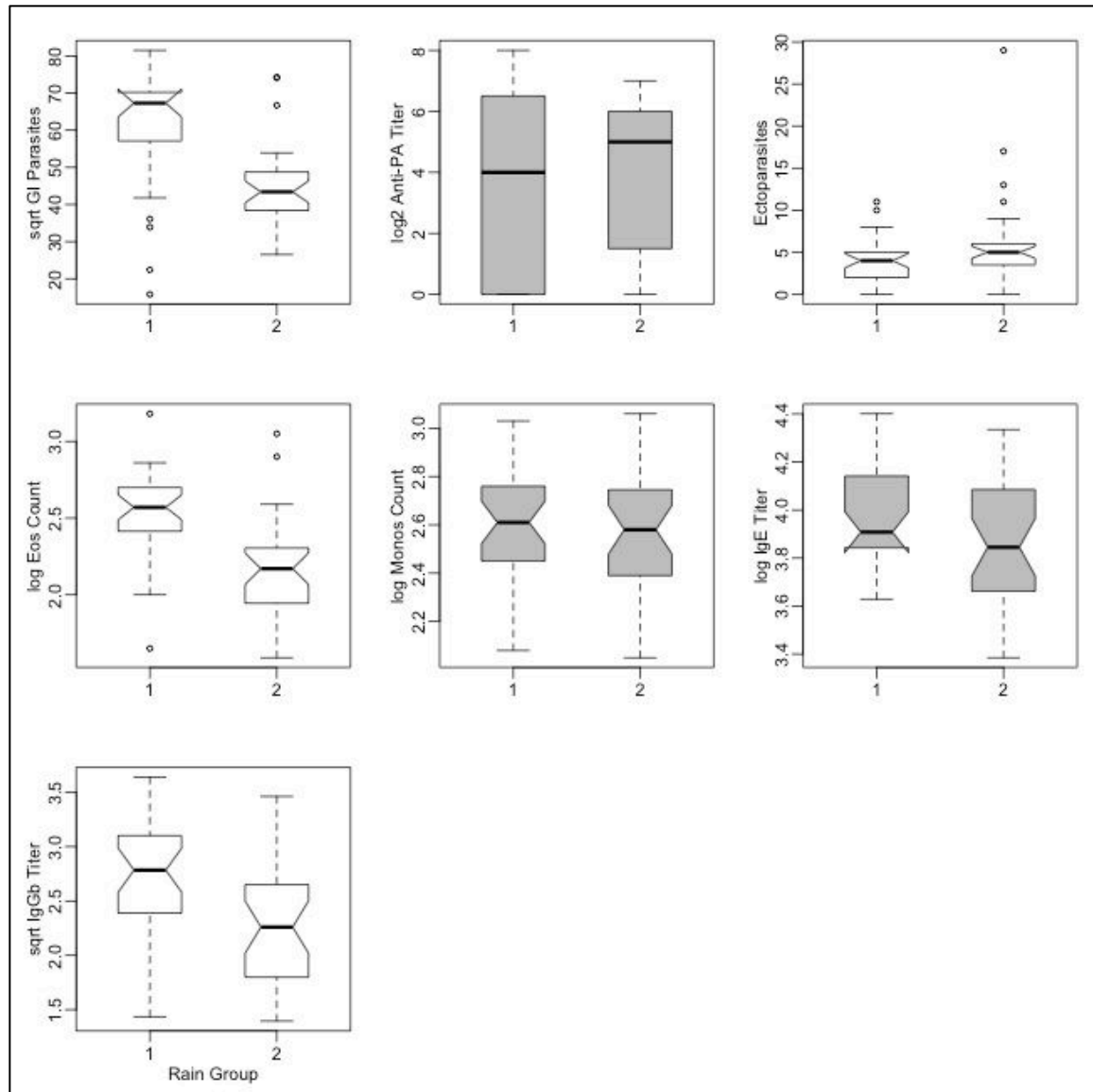
**Figure 2.** Mean ( $\pm$ SE) monthly Okaukuejo rainfall from 1974-2010. This encompasses the start of the most reliable anthrax sampling in ENP through the years of the current project (2008-2010).



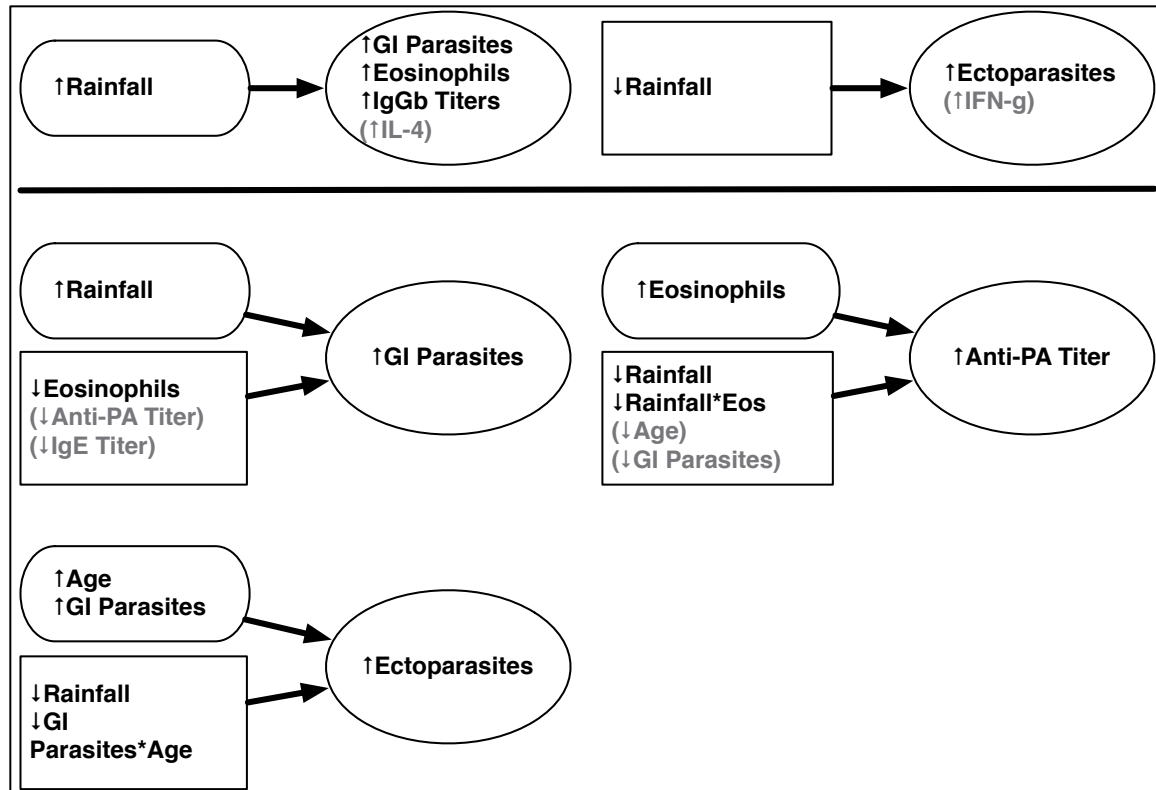
**Figure 3.** Cumulative rainfall 2 months prior to each zebra capture. The total rainfall in the 60 days prior to capture was determined for each individual zebra capture event, and that number was assigned to that individual-capture as its associated rainfall amount. While we sampled animals in nominally "wet" or "dry" seasons, we saw a clear bimodal pattern in rainfall amounts that did not necessarily align with seasons (particularly see the Wet Season data). We therefore used rainfall amounts to assign each individual-capture to a rain group: RG1, the high rainfall group, containing individuals that had experienced  $\geq 200\text{mm}$  rainfall two months prior to sampling; and RG2, the low rainfall group, containing individual samplings connected with  $\leq 100\text{mm}$  rainfall in the two months prior.



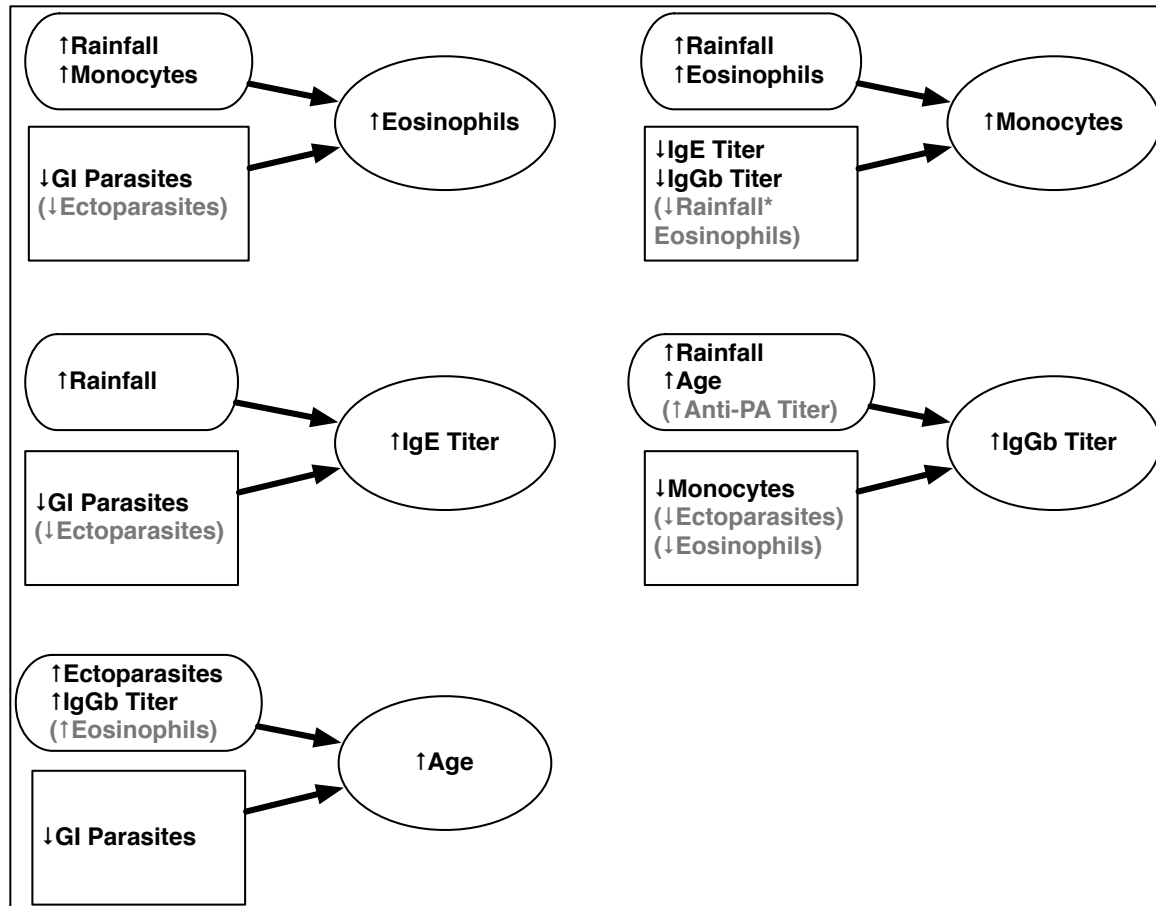
**Figure 4.** Comparisons of pathogens and immune factors between rain groups for different individuals. Only first captures are represented; thus, there is no autocorrelation between rain groups here. Center lines in boxplots represent medians, with notches extending to  $\pm 1.58 \text{ IQR} / \sqrt{N}$  where IQR is the interquartile range and  $N$  is the length of  $x$ . Notches that do not overlap provide strong evidence that the medians differ (Chambers et al. 1983) (not shown for log2PA because notches grossly overlapped the ends of the boxes). The box hinges represent nearly the first and third quartiles, with whiskers showing the largest and smallest observations  $1.5x$  the box size from the nearest hinge. Points outside this range are shown as open circles. Boxplots in white are for variables that are significantly different from each other by  $t$  tests or Wilcoxon rank sum tests, whereas grey boxplots are not significantly different.



**Figure 5.** Pairwise comparisons of pathogens and immune factors between rain groups for the same individuals resampled twice. Boxplot statistics are interpreted as in Figure 4. Boxplots in white are for variables that are significantly different from each other by *t* tests or Wilcoxon rank sum tests, whereas grey boxplots are not significantly different.

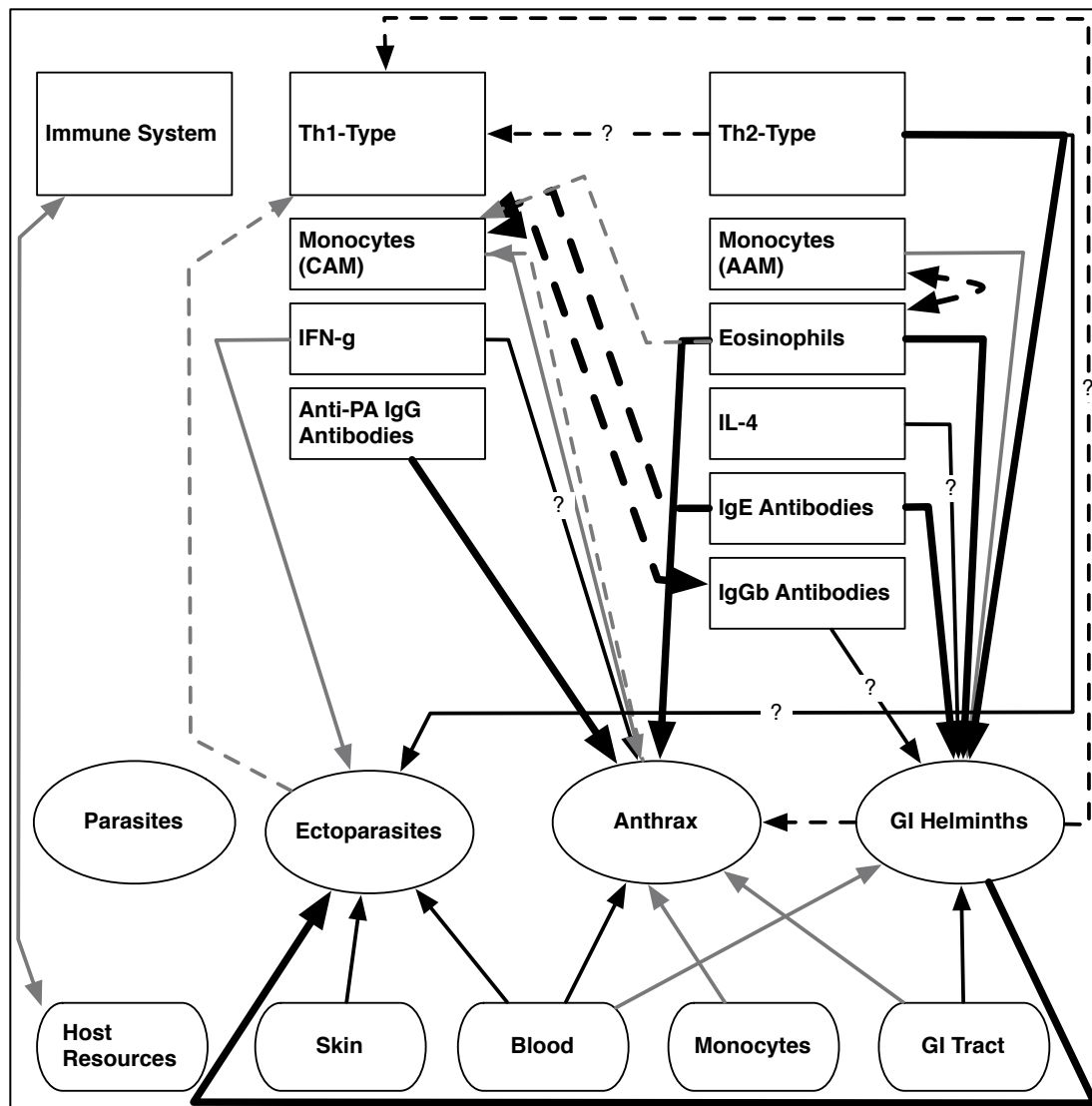


**Figure 6.** Illustrated relationships between rainfall and other variables for pairwise rain group comparisons (above line in figure), and for pathogen GEE models (below line). Response variables are contained in ovals. Explanatory variables showing a positive relationship to response variables are in pill-shaped boxes, while those showing a negative relationship to response variables are in rectangular boxes. Significant explanatory or response variables are in black print, while those variables that were included in the models but were statistically not significant are in gray.



**Figure 7.** Illustrated relationships for immunity and age GEE models. Response variables are contained in ovals. Explanatory variables showing a positive relationship to response variables are in pill-shaped boxes, while those showing a negative relationship to response variables are in rectangular boxes. Significant explanatory variables are in black print, while those variables that were included in the models but were statistically not significant are in gray.





**Figure 8.** The known community of parasites in plains zebra (*Equus quagga*) in ENP and their within-host interaction networks, based on a community ecology trophic structure (Pedersen and Fenton 2007). The basal level, analogous to primary producers, includes the known host resources involved in maintaining the three pathogens. The intermediate level includes the parasites known to this host. The top trophic level, analogous to predators, includes the immune system components that interact with these pathogens. In contrast to an external trophic structure, within-host basal and top levels of interaction networks are directly linked to each other as shown here. Black, solid lines show direct links demonstrated in this study; thicker lines represent statistically significant interactions, and "?" represent probable interactions determined indirectly through our analyses. Black dashed lines show immunomodulatory links demonstrated (or probable, "?") through this study, with thicker lines corresponding to statistically significant results. Gray solid and dashed lines indicate direct and immunomodulatory interactions, respectively, known from other sources that were not directly investigated or observed in this study.

## Chapter 4

# Seasonal patterns of hormones, macroparasites, and microparasites in wild African ungulates: the interplay between stress, reproduction, and disease

### Abstract

Stress can modulate the immune system and cause increased disease incidence. Sex hormones and seasonal reproductive status can also influence immune competence, as well as stress hormone secretion. Pathogens can also cause host stress, as well as exploit host niches exposed by stress and reproductive hormone-induced immunomodulation. Therefore, measuring stress and stress-inducing factors concurrently with coinfection issues is important for determining host immune condition and the subsequent potential for contracting other diseases. Using steroid hormone metabolites from fecal samples, I examined seasonal correlations between host stress, reproduction, and gastrointestinal (GI) parasite coinfections in zebra and springbok in Etosha National Park (ENP), Namibia. I found strong seasonality of all factors, with infection intensities of all three GI macroparasites examined (strongyle helminths, *Strongyloides* helminths, and *Eimeria* coccidia) highest in the wet season, concurrent with the timing of anthrax outbreaks. Parasites also declined with age and with increased acquired immune responses. I found hormonal evidence that both mares and ewes are strongly seasonal breeders in ENP, and that reproductive hormones are correlated with immunosuppression and higher susceptibility to GI parasite and ectoparasite infections. While stress hormones exhibit seasonality in both zebra and springbok, stress hormones largely peak in the dry season when parasite infection intensities are lowest. In fact, peak stress hormone levels are mostly driven by dry season environmental challenges and host reproductive status, rather than by pathogen prevalence or infection intensity in this system. Given the evidence that GI parasites are capable of causing host pathology, causing immunomodulation and immunosuppression, and controlling population age structures, the fact that these parasites persist in ENP hosts without causing chronic stress responses supports the hypothesis that hosts are tolerant of their parasites. Such tolerance would help to explain the ubiquity of these pathogens in ENP herbivores, even in the face of their immunomodulatory trade-offs with anti-anthrax immunity. This study is one of the first to examine seasonal changes in stress responses in concert with reproductive changes, macroparasite and microparasite coinfections, and immune parameters in wildlife hosts in a natural system. In addition, it is one of the first studies to follow wildlife hosts longitudinally while examining these interactions.

## Introduction

Vertebrates experience regular stress. Stressors can take the form of acute, unpleasant, and usually unpredictable stimuli (*e.g.* predator threat, aggression) that precipitate a stress response; these hormonal (increase in glucocorticoids), physiological (mobilization of energy reserves and enhancement of immune function), and behavioral (*e.g.* running, fighting) changes help animals cope with the stressor and enhance survival (Dhabhar and McEwen 1997; Sapolsky et al. 2000; Romero 2004; Martin 2009). Acute stress responses take place within seconds to minutes, during which glucocorticoids (GCs), adrenal hormones of the hypothalamic-pituitary-adrenal (HPA) axis and the primary mediators of the stress response, are secreted and immediately begin increasing metabolic energy use, enhancing the cardiovascular effects of catecholamines of the sympathetic nervous system, and stimulating immune function. Over the course of minutes to hours, GCs decrease reproductive physiology and behavior to save resources for more pressing concerns (Dhabhar and McEwen 1997; Sapolsky et al. 2000). Once the acute stressor is gone, GCs also help to suppress the stress response, promote recovery from the stressor, and prepare the animal to respond to future, similar stressors, in part by enhancing innate and adaptive immunity to pathogens and promoting immunological memory responses (Romero 2004; Dhabhar and McEwen 2007).

While the stress response is finely tuned to help animals positively respond to and recover from acute noxious stimuli, problems arise when this response is triggered for several hours a day over weeks to months (Dhabhar and McEwen 1997). This chronic increase in GCs has several negative physiological effects, including metabolic disruption, reproductive suppression, immunosuppression and immunomodulation, and increased susceptibility to disease. While increased GC levels can help animals by shifting to a catabolic metabolism and promoting gluconeogenesis to ensure enough fuel for body maintenance in the absence of sufficient food resources, chronically elevated GC levels result in severe muscle wasting and fat redistribution (Foster and McGarry 1988; Smith et al. 1990; Dallman et al. 1993). The effects of chronic stress in the suppression of reproductive performance are also well documented, and can result in both decreased release of reproductive hormones from the hypothalamus and pituitary, and decreased gonadal responsiveness to these hormones (Liptrap 1993; Dobson and Smith 1995; Ferin 1999; Sapolsky et al. 2000; Lafferty and Holt 2003; Wingfield and Sapolsky 2003). While acute stress results in the rapid and significant decrease in numbers of T cells, B cells, NK cells, and monocytes in blood, these changes represent a useful redistribution of effector cells to vulnerable organs such as skin, and are reversible (Dhabhar et al. 1995; Dhabhar and McEwen 1996). Chronic stress, however, causes widespread immunosuppression by reducing total numbers of circulating white blood cells, inhibiting synthesis, release, and actions of many cytokines, decreasing activation of T and B cells, suppressing effector cell functions, inhibiting antigen presentation, and even causing thymus atrophy and triggering apoptosis of immature T and B cells and mature T cells (Black et al. 1994; Dhabhar and McEwen 1997; Morale et al. 2003; Sapolsky 2003; Glaser and Kiecolt-Glaser 2005; Martin 2009). While it has been suggested that such immunosuppression may represent a positive sacrifice under resource limitation to provide for other essential metabolic

functions (Sheldon and Verhulst 1996), immunity down-regulation takes weeks and thus does not yield any short-term energy benefits, apoptosis of leukocytes is actually an energy-intensive process, and immune responsiveness is beneficial against many stressors (Dhabhar and McEwen 2007; Martin 2009). Thus, all immunosuppression under stress conditions has been argued to be maladaptive (Dhabhar and McEwen 2007). There is also evidence that chronically elevated levels of GCs may shift the balance of an ongoing immune response in favor of Th2-type immunity, with suppression of Th1-type immunity (Daynes and Araneo 1989; Miyaura and Iwata 2002; Elenkov 2004). While Th2 immune responses are often protective against macroparasite infections (ectoparasites, endoparasitic worms), Th1 responses are most effective against many microparasites (bacteria, viruses), and both are mutually inhibitory; thus, stress-induced Th2-skewing can result in increased susceptibility to a host of pathogens (Abbas et al. 1996; Mosmann and Sad 1996; Yazdanbakhsh et al. 2002; Graham et al. 2007; van Riet 2007; Diniz et al. 2010). Through such immunosuppression and immunomodulation, chronic stress responses have been found to increase host susceptibility to viruses (Cohen et al. 1991), to bacteria (Edwards and Dean 1977; Chao et al. 1990), to delayed wound healing (Marucha et al. 1998), and to several other types of pathogens (Khansari et al. 1999 and Sapolsky et al. 2000). Through the combination of these mechanisms, chronic stress can affect entire host populations and even infectious disease dynamics by reducing host fecundity and survivorship. Thus, while stress at an individual level usually decreases overall host health, stress at a population level can make disease transmission less efficient by decreasing host density through reducing host carrying capacity (Scott 1988; Holmes 1996; Lafferty and Holt 2003).

Pathogens themselves, however, can also cause chronic stress in their hosts. While evidence for the ability of macroparasites to cause host stress is equivocal, several studies have found significant, positive relationships between parasite infection intensity or prevalence and GC concentration (Belden and Kiesecker 2005; Muehlenbein 2006; Arriero et al. 2008; Pedersen and Greives 2008). Zebra in ENP experience infections with strongyle helminths, anthrax, and ectoparasites. Springbok in ENP experience infections with strongyles, *Strongyloides* helminths, *Eimeria* coccidia parasites, anthrax, and ectoparasites (Lindeque 1991; Turner and Getz 2010; Kamath 2011; see dissertation Chapters 2 and 3). Strongyle helminths have been shown to have significant negative fitness impacts on hosts, and to contribute to growth impairment, weight loss, undernutrition, and reduced fecundity (Hudson 1986; Gulland 1992; Newey and Thirgood 2004; Lello et al. 2005; Pullan and Brooker 2008). These parasites can also cause significant pathology in several host tissues during migration, and to intestinal mucosa (Bowman 2003). *Strongyloides* species usually cause little pathology in normal, healthy individuals, but can compromise host nutritional status. However, hosts can experience disseminated strongyloidiasis if immunocompromised (Viney and Lok 2007). Coccidia can also negatively affect total body mass, impair growth and food utilization, cause epithelial cell damage and inflammation, and result in diarrhea, weight loss, and even death (Yun et al. 2000; Lello et al. 2005). Even in the absence of clinical disease, coccidia can affect host digestive processes and host homeostasis (Daugochies and Najdrowski 2005). Ectoparasites such as ticks can be very

energetically expensive, and therefore have negative impacts on host body mass and effect fitness costs (Allan 2001; Neuhaus 2003; Ballesteros et al. 2012). Plains ungulates in ENP experience yearly outbreaks with anthrax, caused by the bacterium *Bacillus anthracis* (Lindeque 1991; Lindeque and Turnbull 1994). The majority of anthrax cases in ENP occur in March and April, with just over half of all cases occurring in zebra (Turner et al. 2013). Sublethal infections are also common in these hosts, with approximately 60% of zebra and up to 15% of springbok demonstrating measurable anti-anthrax antibody titers (see dissertation Chapter 2); thus, even infections that animals survive have fitness costs in terms of resources devoted to anti-anthrax immune responses. Mixed macro-macroparasite and micro-macroparasite infections can also influence each other, either by enhancing or suppressing coinfections; the majority of these interactions occur through the immune system, and thus mixed infections may influence host fitness more than singular infections (Behnke 2008; Lello and Hussell 2008; Pathak et al. 2012).

Other, more normal, life history tasks such as breeding can push animals into allostatic overload; allostatic load is the energy requirement needed to accomplish a life history task, and thus allostatic overload is experienced when more energy is required for that task than an animal can obtain from environmental resources (McEwen and Wingfield 2003). While stress itself (for example, from acute stressors or from pathogens) can lead an animal into allostatic overload, energy-heavy tasks in the face of a spare environment or concurrent stressors can also push an animal into overload. Although energy demand may provoke helpful GC spikes that mobilize stored energy, animals in chronic allostatic overload are likely to experience chronic GC elevations, and therefore to suffer the negative consequences of chronic stress. Reproductive tasks are particularly energy demanding, and most last for at least several hours, if not days, weeks, or months. For example, the energy requirements for gestating females are 17-32% higher than for non-gestating females, and lactating females require two-to-three times more energy than do non-lactating animals (Robbins 1993). In fact, lactation is considered to be the most energetically costly reproductive investment for mammals, more demanding than gestation, estrus, or ovulation (Pond 1977; Pluháček et al. 2010; Olléová et al. 2012). Male reproductive efforts can also be energetically costly; male springbok in rut have been measured with metabolic rates 155% of those expected for similar sized animals, and rutting males typically lose weight because of the combined effects of high metabolic rate and spending less time eating and drinking and more time engaging in reproductive behaviors (Nagy and Knight 1994). Rutting behavior has thus also been correlated with increased GC concentration (Feher et al. 1994).

External environmental factors can also affect stress levels. Seasonally reduced food availability often invokes increased GC secretion in mammals (Saltz and White 1991; DelGiudice et al. 1992; Murphy and Wideman 1992; Tsuma et al. 1996), and this stress can have measurably negative effects on susceptibility to disease (Beck and Levander 2000). While elevated GC production might, in part, help animals adapt to harsh environmental conditions (Huber et al. 2003), there is evidence that this preparative response can have negative consequences as harsh seasons continue, resulting in significant immunosuppression (Demas and Nelson 1998; Eraud et al. 2008). Lack of available water in dry seasons can also likely cause

stress responses in animals dependent upon free drinking water. Zebra in the dry season have been found to spend 12-17% fewer hours per month resting, 10-15% fewer hours foraging, and 45-100% more hours traveling than they spend in the wet season, all to get to water every one to two days (Cain et al. 2011). Such energy expenditures may therefore push animals into allostatic overload and into chronic stress responses.

Etosha National Park (ENP), Namibia, is a highly seasonal environment; it experiences one rainy season for approximately three to four months per year, followed by a cold dry and then a hot dry season during which daytime temperatures can reach a high of 35°C (Gasaway et al. 1996; Turner et al. 2012). With these seasonal changes in rainfall and temperature come changes in food and free water resources. Two of the predominant plains ungulates in ENP, plains zebra (*Equus quagga*) and springbok (*Antidorcas marsupialis*) are affected by these seasonal changes. Grass production is positively, linearly related to rainfall once grass growth begins; while leaf production in woody plants is influenced by rainfall during the previous rainy season, leaf retention is influenced by current seasonal rainfall (Gasaway et al. 1996). While increased rainfall is correlated with declining nutrition due to increased crude fiber in plants, overall nutritional quality of foodstuffs for these herbivores is significantly higher in the wet season than in the dry (Turner et al. 2013). The rainy season, however, also brings increases in gastrointestinal (GI) parasite infection intensities in both of these hosts. While there is nearly 100% prevalence of strongyle nematodes in zebra in this system year-round, zebra experience a significant increase in strongyle infection intensity in the wet season compared to in the dry (Turner and Getz 2010). Springbok in ENP also experience both greater prevalence and infection intensity in three gastrointestinal parasites during the wet season, strongyle nematodes, *Strongyloides* nematodes, and *Eimeria* coccidia (Turner and Getz 2010). These three parasites have direct lifecycles (or mostly direct, in the case of *Strongyloides*; see Methods), with transmission of infectious stages through the environment. Development of shed eggs and oocysts in feces is significantly improved in warm but not extreme temperatures and with increased humidity; development to infectious stages takes up to a week, and intermediate stages are very susceptible to desiccation (Banks et al. 1990; O'Connor et al. 2006; Nielsen et al. 2007). Thus, the infection dynamics of these parasites are primarily driven by environmental factors (Turner and Getz 2010; see dissertation Chapter 3). Zebra and springbok also experience annual anthrax outbreaks in the wet season, shortly after parasite infection intensities peak (Turner 2009), and GI parasites likely contribute to increased zebra susceptibility to this pathogen at this time due to immunomodulatory effects (see dissertation Chapter 3). In addition, while springbok and zebra are not strictly seasonal breeders, the springbok population in ENP experiences a birth peak in the early wet season (January through March, with juveniles weaned at the end of May and mating occurring in July (Gasaway et al. 1996; Turner et al. 2012), and zebra birth mainly from January through March and mate and lactate during this same season (Gasaway et al. 1996).

While many studies have examined stress responses and the impact of chronic stress in wildlife (e.g. Wasser et al. 1997; Creel et al. 2002; Dehnhard et al.

2003; Mashburn et al. 2004; Archie et al. 2012), several have examined the seasonality of stress responses (*e.g.* Saltz et al. 1991; Monfort et al. 1993; Boswell et al. 1994; Schiml et al. 1996; Ingram et al. 1999; Romero et al. 2002; Huber et al. 2003; Rangel-Negrin et al. 2009), several have examined stress in conjunction with wildlife reproduction efforts (*e.g.* Bubenik et al. 1983; Nilssen et al. 1985; Ingram et al. 1999; Monello et al. 2010; Vera et al. 2013), and some have examined stress due to pathogens in wildlife (*e.g.* Goldstein et al. 2005; Muehlenbein 2006; Raouf et al. 2006; Arriero et al. 2008; Pedersen and Greives 2008; Monello et al. 2010), few, if any, have examined the seasonality of stress responses simultaneously in conjunction with environmental changes, reproductive efforts, pathogen pressures, pathogen interactions, and immune function. As all of these factors are closely interlinked, it is important that studies begin examining this complex array of factors in concert with each other, despite the challenge of teasing apart the important relationships from a convoluted web. This study examines the fluctuations in stress hormones, in concert environmental parameters, reproductive and gestational status, and pathogen interactions in zebra and springbok in ENP between the wet and the hot dry seasons. As springbok are colonized by several macroparasite species (strongyles, *Strongyloides*, and *Eimeria*), I also examine the macroparasite interactions in concert with these factors for these hosts. In addition, because a subset of zebra were immobilized, sampled, and collared, I will also examine immune status, macro-macroparasite interactions (between strongyles and ticks), and macro-microparasite interactions (between strongyles and anti-anthrax immune responses) longitudinally in conjunction with reproductive changes and stress status for these hosts.

Because there is such strong seasonality in this system for most parameters examined, I hypothesize that stress responses, as measured by fecal GC metabolites, will be significantly different between the hot dry and the wet seasons for both zebra and springbok. I expect stress to be highest in both ungulates in the wet season. While zebra gestate for just over one year (Wackernagel 1965; Barnier et al. 2012) and thus females should mostly be in mid-gestation in the dry season in ENP, parturition itself is a potent stimulus for GC secretion (Sapolsky et al. 2000); thus, parturition followed by highly energy demanding lactation may lead to chronic GC elevation in the face of other trade-offs such as high pathogen loads. In fact, in dairy cattle, the periparturient period (three weeks before and after parturition) is the most critical for health and production, and is the time when cattle are most susceptible to infectious diseases due to stress and stress- and reproduction-related immunosuppression (Bonizzi et al. 2003). Thus, I hypothesize that the wet season combination of parturition, most intense lactation, and estrus behavior in females, and corresponding energy-demanding rutting behavior in males is likely more stressful in zebras than are mid-gestational energy demands and hormonal fluctuations. Although springbok predominantly come into estrus and rut during the cold dry season and thus are also in mid-gestation during the hot dry season (Estes 1991; Skinner et al. 2001), females predominantly birth and lactate during the wet season, and mating rams can remain territorial throughout the year (Skinner et al. 2002); thus, I also expect that the wet season is more reproductively stressful for springbok as well. While nutritional quality is better in the wet season, zebra and

springbok in ENP are below their food resource ceiling, and as such may not be significantly nutritionally deprived during the dry season (Gasaway et al. 1996). While springbok body condition was significantly affected by a severe drought in ENP in 1992, springbok fertility rate was still high during this time; thus, even in the face of severe drought there were at least enough nutrients available to sustain energetically demanding pregnancies. Zebra, on the other hand, maintained excellent body condition during this same drought, and there was no added mortality of either species during this time (Gasaway et al. 1996). Both zebra and springbok experience the highest infection intensity with all of their macro-endoparasites during the wet season. We have previously found that zebra GI parasites likely exert more selection pressure than do the other pathogens in this system, and that they strongly influence host immunity during peak infection intensities (Kamath 2011; see dissertation Chapter 3). Thus, I expect GI parasites to also influence stress more than do environmental factors, increasing stress during the wet season. Because these parasites are likely so influential in many ways in these hosts, I hypothesize that they will also be the primary drivers of host chronic stress responses, and that this effect, combined with the added reproductive trade-offs at this time, will significantly increase host stress levels in the wet season compared to in the parasite-depauperate dry season. Understanding the primary drivers of host stress is important for determining the full extent of pathogen effects, as well as for determining potential avenues for intervention in conservation programs.

## **Methods**

### *Study Area*

Etosha National Park (ENP) is a 22,915 km<sup>2</sup> fenced conservation area in northern Namibia, located between 18°30'S-19°30'S and 14°15'E-17°10'E (Figure 1). The park is dominated by arid savanna and mopane shrubveld and treeveld, though the central Okaukuejo plains consist of sweet grassveld and dwarf shrub savanna (Huntley, 1982; le Roux et al. 1988). A 4,760 km<sup>2</sup> endorheic salt pan, the remnant of a paleolake, dominates the northeastern quadrant of the park (Hipondoka et al. 2006).

Rainfall in ENP is highly seasonal: the major rainy season lasts from November through April, with the greatest rainfall occurring during January and February, and 80% of all rain falling between December and March (Gasaway et al. 1996; Engert 1997) (Figure 2). The dry season lasts from May through January (Auer 1997), with daytime temperatures reaching highs of 35°C between October and December (Turner et al. 2011). The only perennial water available to the park's wildlife is found in man-made boreholes, or in natural artesian contact springs (Auer 1997).

Captured zebra for this study were largely immobilized and sampled around the central Okaukuejo area, the location of the Etosha Ecological Institute (EEI) (Figure 1), where the mean annual rainfall is approximately 350mm (Turner et al. 2012). During dry seasons, several re-captures took place in the Halali plains, an area in the near east of the park that receives a mean annual rainfall of approximately 420mm (EEI, unpublished data). Fecal samples from non-captured



zebra (NC zebra) and springbok were chosen from animals sampled only in the Okaukuejo area.

### *Study Species*

Plains zebra (*Equus quagga*, formerly *E. burchelli*) and springbok (*Antidorcas marsupialis*) are the two most abundant plains ungulates species in ENP, with populations of approximately 13,000 (95% CI rounded to nearest 100: 10,900-15,000) and 15,600 (13,200-17,900) (EEI unpublished aerial survey data 2005). Zebra, as members of the Family Equidae, are hindgut fermenters that graze almost exclusively on grasses (Codron et al. 2007; Gordon and Prins 2008). They are among the most water-dependent of the plains ungulates found in southern Africa, requiring access to drinking water at least every other day (Skinner and Chimimba 2005; Cain et al. 2011). Zebra live in relatively stable harems of one stallion (>5 years old) and up to six females plus offspring, whereas younger males usually live in similarly-sized bachelor herds. In ENP, plains ungulates typically congregate in large, mixed groups around water or moving toward water, making group composition and size difficult to determine; thus, I did not take group size into account in this study (Turner and Getz 2010). Though not strictly seasonal breeders, zebra mating usually peaks at the start of a rainy season (Estes, 1991), with peak birthing in ENP zebras in March and April (Turner & Getz 2010). Zebra females can foal when they are as young as three years old, and gestation lasts a mean of 371.2 days (Wackernagel 1965; Barnier et al. 2012). Plains zebra can enter estrus as soon as eight days after parturition, with at least half of plains zebras conceiving while still lactating with a mean inter-birth interval of 13.9 months (King 1965; Klingel 1969; Smuts 1976; Estes 1991). Given that wild plains zebra reproduction rates have been found to be as high as 79% and 88% and that zebra can conceive so soon after giving birth, it is conceivable that the majority of adult zebra in ENP are pregnant at any given time (Smuts 1976; Grange et al. 2004).

Springbok, members of the family Bovidae, are an arid-adapted species found across southern Africa (Skinner and Chimimba 2005). As intermediate feeders, springbok alternate between grazing and browsing; the majority of their diet (up to 77%) is from browse (Sponheimer et al. 2003), though they increase grazing efforts mainly during rainy periods when grasses are most digestible (Bigalke and van Hensbergen 1990; Hofmann et al. 1995). Springbok will drink when water is available, but are capable of obtaining all of their water needs from nutritional sources when eating succulent shrubs (Nagy and Knight 1994). Springbok live in dynamic groups of up to several thousand animals and often within mixed assemblages; thus, I also did not take springbok group size into account in this study (Estes 1991; Turner and Getz 2010). While not strictly seasonal breeders, springbok typically mate while in top condition in the wet season and early dry season, gestate for 6.5-7 months, and calve at the beginning of the rainy season (Estes 1991; Skinner et al. 2001). Springbok in ENP follow this pattern, with a strongly seasonal birth peak in February and March (Gasaway et al. 1996; Turner and Getz 2010). Female springbok can conceive as early as 6-7 months old and calve around one year of age, while males mature at two years of age (Estes 1991). The male rut lasts for five to 23 days, during which males forage less and engage in intense

territoriality, vocalization, boundary marking, and aggression toward other males (Skinner et al. 2001); this rutting of rams can also bring ewes into synchronous estrous within days (Skinner et al. 2002).

### *Zebra Capture and Sampling*

I obtained whole blood, serum, fecal, and ectoparasite samples from zebra over five seasons between 2008 and 2010 (Table 1). With veterinarians employed by the Namibian Ministry of the Environment and Tourism, and with assistance from the Etosha Ecology Institute and other researchers, I fitted animals with VHF (very high frequency) collars (LoxoTrack, Aeroeskoebing, Denmark) or VHF-GPS/GSM (global positioning system/global system for mobile communications) collars (Africa Wildlife Tracking, Pretoria, Republic of South Africa) during the first immobilization to enable resampling of animals over several seasons. We first immobilized and sampled all animals on the plains in the immediate area (within an approximately 20km radius) surrounding the EEI in Okaukuejo (Figure 1); in subsequent seasons (particularly during dry seasons, when some zebra groups travel away from Okaukuejo; Stander 1992), we found and resampled several zebra between Okaukuejo and up to approximately 100km to the east in the Halali plains, and Okaukuejo and approximately 15km directly to the south near Ombika gate. We only sampled adult animals, nearly all of which were females to control for sex differences. We immobilized, sampled, and released all animals safely under animal handling protocol AUP R217-0509B (University of California, Berkeley).

We conducted a total of 144 zebra capture events; all captured animals for this study were adult females to control for sex differences. We sampled 69 individuals overall, with 20 resampled twice, 11 resampled three times, 12 resampled four times, and two resampled five times. I collected whole blood and serum samples from each individual-capture event, but was only able to collect fecal and ectoparasite samples from a subset of the captures (Table 1).

I determined zebra sex and rough age based on relative size, pelage, and genitalia (Smuts 1975). I further determined age to half a year by combining tooth eruption observations, caliper measurements of upper incisors, and patterns of wear (Smuts 1974; Penzhorn 1982). I converted year age to age in days, and then "aged" each animal at recapture by the number of days between that event and the previous sampling for that individual. I recorded, when possible, whether a mare was actively caring for a foal (0 for no foal; 1 for foal present); whether a mare was pregnant (0 for not pregnant; 1 for possibly pregnant based on palpation and visual determination; and 2 for definitely pregnant); and whether a mare was lactating (0 for no lactation; 1 for watery, straw-colored liquid able to be expressed from teats; 2 for milk able to be expressed from teats).

I collected blood from peripheral veins for protocols using both whole blood and serum. Whole blood for hematocrit (HCT) and neutrophil and lymphocyte counts was collected into Vacutainers (Becton Dickinson, Franklin Lakes, NJ) containing EDTA anticoagulant. For serum needed for antibody assays, I collected blood into Vacutainers without anticoagulant. I kept all tubes at approximately 4°C in a mobile refrigerator for up to six hours in the field. I stored whole blood samples at 4°C for up to 24 hours prior to use, while I left blood for white blood cell counts to

clot at room temperature in the laboratory in the Etosha Ecological Institute (EEI) for four hours, placed it overnight at 4°C, and then centrifuged it to allow for serum to be removed and aliquoted. I kept serum initially at -20°C for up to six months, and thereafter stored samples at -80°C until analyzed. I measured HCT, a measure of percent of red blood cells per volume of blood, within five hours of whole blood collection using heparinized capillary tubes and a micro-hematocrit card style reader (StatSpin, Westwood, MA).

I collected feces by observing an individual prior to capture and collecting a homogenized sub-sample within ten minutes of defecation. For animals that were not observed defecating, I collected fecal samples when possible by inserting a gloved hand into the rectum. As capture events took place between 9:00 and 14:00, fecal samples were collected within this same time window, thereby controlling for potential differences in timing of fecal egg shedding (Villanúa et al. 2006). I stored feces in sealed plastic bags at 4°C for up to 48 hours prior to parasite analysis and then at -20°C for up to one year prior to hormone metabolite extraction.

#### *Non-Captured Zebra and Springbok Sampling*

Fecal samples were collected noninvasively from zebra and springbok in the Okaukuejo area monthly between August 2005 and May 2007 (Turner and Getz 2010; Turner et al. 2012). Fecal collection took place in the first week of each month, with a mean of 37 and 35 samples collected per month for zebra and springbok, respectively. Samples were collected within 100m of roads and between 7:00 and 13:00 to control for potential differences in timing of fecal egg shedding (Villanúa et al. 2006). Animals were observed defecating and a homogenized subsample of feces was collected within 10 minutes of deposition. For each fecal sample, date, time, species, sex, and age category were recorded. Age and sex were determined from horn growth and morphology and genitalia for springbok (Rautenbach 1971), while age and sex for zebra were assessed based on body size, pelage, and genitalia (Smuts 1975; Smuts 1976). Age was determined within one of three age classes: juveniles for those < 1 year old; yearlings for those 1-2 years of age; and adults for those 2+ years old. Fecal samples were collected into sealed plastic bags and stored at 4°C for up to 48 hours prior to GI parasite analysis. After using a small subsample (4g) for parasite analysis, all fecal samples were frozen at -20°C for up to one year prior to hormone metabolite extraction.

I subsampled this large sample set for hormone analyses. I selected only yearlings and adults to control for effects of sexual maturity or near sexual maturity on hormone profiles; as the sample set represented mostly adults, I selected yearling and adult samples approximately in proportion to their prevalence in the larger set. I selected male and female samples, and zebra and springbok samples in approximately equal numbers. I chose samples representing similar seasons to those of captured zebra samplings: February-May for the "wet" season, and August-October for the "dry" season. Ultimately, 318 zebra (22 male yearlings, 11 female yearlings, 136 male adults, and 149 female adults) and 272 springbok extracted samples (22 male yearlings, 35 female yearlings, 106 male adults, and 107 female adults) were deemed in acceptable shape for hormonal analysis.

### *Rainfall Quantification*

Previous studies in this system determined that helminth infection intensity in zebras and springbok is significantly related to rainfall one and two months prior, with both strongly correlated with helminth eggs and coccidia oocysts shed per gram of feces (Turner, 2009). I determined cumulative rainfall over the one month prior to sampling, and cumulative rainfall over the two months prior to sampling by adding up daily rainfall amounts (in mm) over the 30 or 60 days prior to each individual sampling event. I used rainfall gauge data from Okaukuejo for those capture and sampling events that took place surrounding that area, and rainfall gauge data from Halali for those capture events that took place on the Halali plains.

I used cumulative rainfall two months prior to sampling as my primary indicator of seasonal wetness in our models, as this measurement was better than the one month cumulative measurement for taking into account one or two days of rainfall followed by no precipitation for weeks. Histograms of cumulative rainfall prior to each individual sampling event for captured zebras revealed a strongly bimodal distribution in the individually-linked rainfall, regardless of qualitatively named seasons (Figure 3). I thus chose to, when carrying out "seasonal" comparisons across and between individuals, group by quantitative rainfall groups rather than by qualitative seasons. This made both ecological and biological sense: the S3 sampling began more than one month later than did sampling in the S1 "wet season," (Table 1) and those S3 animals sampled late in April and the beginning of May (the typical start of the cold dry season) accordingly fell into the lower rainfall group; and external environmental changes have been shown to influence the further development and egg-producing activity of gastrointestinal parasites living within hosts, thus potentially biasing an important measure in my study if I did not take actual rainfall into account (Horak 1981; Shaw 1988). The rain groups were thus for captured zebra: RG1, the high rainfall group, containing individuals that had experienced  $\geq 200\text{mm}$  rainfall two months prior to sampling; and RG2, the low rainfall group, containing individual samplings connected with  $\leq 100\text{mm}$  rainfall in the two months prior.

I divided NC zebra and springbok into similar rain group categories. While individually-experienced rainfall was not as strongly bimodal for these samples as it was for captured zebra, all "wet season" rainfall values below 105mm of rain were experienced by those animals sampled after 1 April. The rainfall groups were thus for NC zebra and springbok: RG1 for those experiencing  $>120\text{mm}$  rainfall in the two months prior to sampling; and RG2 for those experiencing  $\leq 120\text{mm}$  rainfall in the two months prior (there were no rainfall values between 105 and 122mm).

### *Gastrointestinal Parasite Species and Quantification - Zebras*

The gastrointestinal nematodes examined in this host species were in the order Rhabditida, suborder Strongylida, primarily within the superfamily Strongyloidea, and family Strongylidae; this group contains both the "large strongyles" (spp. in the subfamily Strongylinae) and the "small strongyles" (spp. in the subfamily Cyathostominae) (Jain et al. 2009). While Strongylids in the superfamily Trichostrongyloidea, family Trichostrongylidae are most often parasites of ruminants, some species have been known to use plains zebras as hosts.

Most Strongylida are generalists for equid hosts, and most wild zebras in southern Africa simultaneously harbor multiple strongylid species (Krecek et al. 1987b; Matthee et al. 2004). A previous study in plains zebra in ENP found only Strongyloidea species and no Trichostrongyloidea species in zebra guts (Krecek et al. 1987); however, as only nine zebras were examined in this previous study, and as the eggs for Trichostrongylids and Strongylids in zebra are indistinguishable to the naked eye (Bowman 2003), I conservatively refer to the Strongylida eggs that I sampled as “strongyles” without assigning these eggs to species within one of the two superfamilies mentioned above.

These parasites are oviparous and exhibit a direct life cycle with three, free-living larval stages. The first two moults to the infectious L3 stage occur over one to two weeks, after which L3's are ingested by herbivore hosts (Durette-Desset et al. 1994). The first two free-living stages are highly susceptible to desiccation, and develop more quickly and survive for longer in relatively warm temperatures and humid conditions (Nielson et al. 2007). L3 larvae are less susceptible to desiccation, but require a film of moisture to move (O'Connor et al. 2006); thus, it is unsurprising that previous studies in ENP found a strongly seasonal pattern in zebra strongyle infection intensities, with hosts exhibiting greater new infections during the wet season than in the dry (Turner and Getz 2010). Once ingested, L3 larvae encyst in the gut mucosa and develop into L4 larvae, causing damage to the intestinal mucosa in the process. L4 stages of some strongyle species travel throughout host tissues before returning to the gut, causing tissue damage and inflammation along the way, while others remain in the gut lumen and mucosal epithelium to develop into adult stages that then colonize the intestines (Anderson 2000; Bowman 2003).

I evaluated all captured zebra fecal samples and the NC zebra fecal sample subset described above for strongyle eggs using a modified McMaster technique for fecal egg counts (Gibbons et al. 2005), a commonly used non-invasive method for quantifying parasitism (Bowman 2003). Briefly, I combined 4g of homogenized fecal matter with 56ml of a saturated NaCl solution (specific gravity 1.2), removed any large debris with a strainer, and obtained a homogenized filtrate. I placed an aliquot of filtrate into each chamber of a McMaster slide and counted the number of eggs observed in each chamber using a compound microscope at 10x magnification. I obtained a measure of eggs per gram of feces by adding the number of eggs for both chambers and multiplying by 50.

Fecal egg counts (FECs) provide an accurate estimate of how the input of parasite eggs into the environment varies with other factors of interest (Turner and Getz 2010). While the actual relationship between fecal egg count and total nematode burden within a host is of unknown specificity and sensitivity, these counts provide a nonlethal and often noninvasive method for estimating these infection burdens (Stear et al. 1995; Wilson et al 2001; Seivwright et al. 2004). In addition, W. Turner and I previously assessed the influence of fecal water content on measurement of fecal egg counts, as fecal water content can vary widely between seasons in ENP. We found that fecal water content had no effect on seasonal and age-related patterns in strongyle egg counts, thus increasing our confidence regarding the overall accuracy of this measurement (Turner et al. 2009).

### *Gastrointestinal Parasite Species and Quantification - Springbok*

Springbok typically experience infections with strongyles in the superfamily Trichostrongyloidea (Bowman 2003). While previous studies of these parasites in ENP have not distinguished these parasites beyond the family level, evidence from springbok studies in South Africa suggest that *Trichostrongylus* and *Paracooperia* species (family Trichostrongylidae, subfamily Trichostrongylidae) and *Cooperia* species (family Cooperiidae) are the dominant intestinal strongyles in springbok in this region (Horak et al. 1982; De Villiers et al. 1985; Jain et al. 2009). Springbok in ENP also experience intestinal infections with *Strongyloides* species, nematodes in the order Rhabditida and family Strongyloididae (Jain et al. 2009; Turner and Getz 2010). Only parasitic adult females reside within host guts; these worms reproduce parthenogenetically, shedding eggs in host feces (Viney and Lok 2007). In the external environment, eggs hatch into L1 larvae, develop to the infective L3 stage, and penetrate host skin. L3 larvae in the environment can also continue developing into L4 larvae and then into free-living male or female adult worms; the adults mate, the females lay eggs, larvae develop into infectious L3 stages, and then either infect hosts or again develop into free-living adult worms (Viney and Lok, 2007). Once in a host, L3 larvae migrate rostrally, developing into L4 larvae along the way. After being swallowed by the host, L4s make their way to the small intestine and develop into adult female worms. These parasites can also undergo several series of autoinfection within hosts, in which they repeatedly complete their life cycle all within the same host. Worms can also arrest their migration and development in various tissues; larvae commonly undergo hypobiosis within mammary glands, and only reactivate development at lactation, thus allowing for effective transmammary transmission (Viney and Lok 2007).

Springbok in ENP also experience infections with coccidian parasites in the genus *Eimeria* (class Coccidia; order Eucoccidiorida; family Eimeriida) (Jain et al 2009). *Eimeria* are typically very host-specific, and three species of this parasite have been identified in ENP springbok (Turner 2009). *Eimeria* are transmitted fecal-orally between hosts, via an environmental stage. Oocysts are shed in host feces, undergo development (sporulation), and become infectious to new hosts within approximately one week (Dauguschies and Najdrowski 2005). While oocysts are fairly hardy, they require moisture and lower temperatures for long-term environmental survival (Dauguschies and Najdrowski 2005). When ingested, oocysts travel to host intestines and excyst sporozoites within the intestinal lumen that then penetrate intestinal epithelial cells. These undergo asexual reproduction within cells, burst the host cells, and then infect neighboring cells in which they undergo asexual reproduction to form sexual stages capable of producing new oocysts to be shed in the feces (Dauguschies and Najdrowski 2005; McDonald and Shirley 2009).

The springbok fecal samples used in this study were previously evaluated for GI parasite egg and oocyst counts using the same modified McMaster technique for fecal egg counts as described above (Gibbons et al. 2005). In rare cases in which the number of oocysts on the slide was too high to count accurately, the sample was diluted and counts were adjusted accordingly.

### *Ectoparasite Collection – Captured Zebras*

I collected and counted all visible ectoparasites, regardless of life stage, on zebras during capture events; as these parasites congregate almost entirely around the ventrum, perineum, and ears, I was confident that I was able to collect the vast majority of ticks present even while a zebra lay on its side. I used forceps to detach ectoparasites, and placed them together into a separate glass jar for each host. I identified five tick species from the family Ixodidae and genera *Hyalomma* and *Rhipicephalus*. The majority of ticks observed were *R. evertsi mimeticus*, a tick species found throughout Namibia in wild equids and greater kudu (Horak 1984; Horak et al, 1984; Horak et al. 1992). These ticks parasitize hosts year-round, with more adults present from November to May and immature stages peaking from February to March and May to September (Biggs and Langenhoven 1984).

### *Total White Blood Cell, Neutrophil and Lymphocyte Counts – Captured Zebras*

Total white blood cell (WBC) count is often used to represent an individual's total investment in immunity (Nunn et al. 2003, 2009; Nunn 2002; Semple et al. 2002). Neutrophils are white blood cells instrumental in the innate immune and inflammatory responses against primarily extracellular pathogens and possibly against certain intracellular bacteria (Mantovani et al. 2011). While the roles of neutrophils in the immune response are being revealed as increasingly complex, I am using neutrophil counts in this study primarily as markers of early infection, nonspecific responses, and inflammation. Lymphocytes include the different types of T and B cells that are activated in an antigen-specific manner as components of the adaptive immune system (Viney et al. 2005). Again, while the diverse roles of different lymphocytes are exceedingly complex, I am using lymphocyte counts in this study as simple proxies of specific anti-pathogen responses, chronic infection, and immunological memory.

I created thin blood smears on glass slides, fixed them with methanol, and stained them with Diff-Quik (Dade Behring, Deerfield, IL). I performed manual total white blood cell (WBC) counts using a compound microscope; I counted cells in ten fields at 40x magnification and multiplied mean cell count per field by 1600 (magnification<sup>2</sup>) to obtain total WBCs per  $\mu$ l of blood. I did differential counts by determining the percent of each of the most common WBC types (neutrophils, monocytes, lymphocytes, eosinophils) in 200 WBCs counted at 40x and multiplying this by total WBC concentration to obtain numbers of neutrophils or lymphocytes per  $\mu$ l of blood. All counts were done in duplicate and averaged.

### *Anti-PA ELISA – Captured Zebras*

Anthrax is caused by *Bacillus anthracis*, a large, gram-positive bacterium that exists in a hardy, long-lived spore form in the environment. Anthrax can only be transmitted environmentally through these spores, and is usually orally ingested by susceptible herbivore hosts (Hanna and Ireland 1999; Watson and Keir 1994). Anthrax can cause death within hours to days (Hugh-Jones and de Vos 2002), though there is evidence that even very susceptible host species can experience a sublethal dose of anthrax and survive, in part, due to a humoral immune response

against the anthrax protective antigen (PA) toxin (Lembo et al. 2011; and see dissertation Chapter 2). These anti-PA antibodies have been shown to be essential for adaptive protection against anthrax (Little et al. 1997; Turnbull 2000; Little et al. 2004; Marcus et al. 2004). As a bacterial infection, anthrax provokes primarily a Th1 type immune response (Hanna 1999; Pickering et al. 2004).

I used an ELISA procedure previously described (see dissertation Chapter 2). Briefly, I used wildtype *Bacillus anthracis* protective antigen (PA) as coating antigen at a concentration of 0.375 $\mu$ l per well. I made serial, two-fold dilutions to the ends of rows in duplicate for all samples and negative controls, starting at a dilution of 1:4 and ending at 1:8192. I ran a duplicate negative control full titration series on each ELISA plate. I used goat-anti-horse IgG-heavy and light chain horseradish peroxidase (HRP) conjugate (Bethyl Laboratories, Montgomery, TX) as the secondary antibody, at the suggested dilution of 1:60,000. I then added TMB substrate (Kirkegaard & Perry Laboratories; Gaithersburg, MD) and stopped the reaction with 2N sulfuric acid. I read well absorbance as optical density (OD) at 450nm on a SpectraMax M2 Microplate Reader using SoftMax Pro software v5.3 (Molecular Devices; Sunnyvale, CA). As we had no known, titrated standards to establish a standard curve, I determined the endpoint titers as the log<sub>2</sub> of the last sample dilution at which the mean OD for that sample at that dilution was greater than the mean OD for all negative controls at that dilution, buffered by a 95% confidence interval determined by the inter-duplicate error at that dilution, across all samples analyzed.

#### *Hormone Metabolite Extraction and Measurement*

Steroid hormones can be detected in blood and their metabolites can be detected in feces; however, measurements of hormone metabolites in feces are often best in wildlife because fecal collection can often be done noninvasively and is free from stress hormone feedback mechanisms, and fecal samples reflect longer term patterns of hormone trends, dampening potential acute stressor responses or diurnal variations in hormone levels (Wasser et al. 2000; Millspaugh et al. 2002; Möstl and Palme 2002; Keay et al. 2006; Millspaugh and Washburn 2004; Chinnadurai et al. 2008). Fecal hormone metabolite concentrations reflect the state of circulating hormones an individual experienced approximately 10-12 hours prior (for most ruminants; Möstl and Palme 2002) or approximately one day prior (for equids; Asa et al. 2001). Fecal assays for concentrations of stress and reproductive hormone metabolites have been developed and used extensively in wildlife (*e.g.* Chapeau et al. 1993; Monfort et al. 1997; Goymann et al. 1999; Asa et al. 2001; Dehnhard et al. 2001; Franceschini et al. 2008; Black et al. 2011; Ncube et al. 2011).

Prior to extraction, I thawed frozen fecal samples for 24 hours at 4°C. I extracted all captured zebra samples, and the subset of NC zebra and springbok fecal samples described above. Using methods modified from a previous similar study (Palme and Möstl 1997), I manually mixed samples within bags prior to selecting a subsample for powdering with an electric coffee grinder. After removing large particulate matter, I selected roughly half of each powdered sample for determining dry weight; samples were dried in foil packets in a drying oven at 80-100°C for 24-48 hours. For extractions, I selected 1g (weighed to the nearest



0.001g) of non-dried, powdered sample, added 5ml methanol, and vortexed for 30-60 minutes. I then centrifuged samples at 3000rpm for 15 minutes and stored supernatants at -20°C prior to analysis.

Radioimmunoassays (RIA) for fecal glucocorticoid, fecal progesterin, fecal estrogen, and fecal testosterone metabolites were conducted by Neville Pitts (School of Physiology, University of the Witwatersrand). Hereafter, these will be referred to simply as stress hormones, progesterone, estrogen, and testosterone.

Concentrations of cortisol metabolite immunoreactivity in fecal extracts were quantified using a double antibody  $^{125}\text{I}$ -corticosterone radioimmunoassay (RIA) (MP Biomedicals, Santa Ana, CA) validated for zebra and other antelope species (Chinnadurai et al. 2008) and used to assess translocation stress in zebra (Franceschini et al. 2008). Fifty microliters of fecal extract was added to 50 $\mu\text{l}$  of steroid diluent (MP Biomedicals) and then assayed according to the manufacturer's instructions. Parallelism between serial dilutions of fecal extracts for both zebra and springbok and the standard curve were obtained, validating use of the assay in each species. Assay sensitivity was 12.5 ng/ml, with the minimum and maximum detection limits of 25 ng/ml and 1000 ng/ml respectively. Intra-assay and inter-assay coefficients of variation were <4% and <9 % respectively. No corticoid immunoreactivity was detected in zebra or springbok fecal extracts using a  $^{125}\text{I}$ -cortisol RIA (Coat-a-Count, Diagnostic Products, CA, USA). All extract concentrations were used to express the detected fecal glucocorticoid metabolites (FGM) as ng/g dry feces.

Concentrations of progesterin metabolite immunoreactivity in fecal extracts were quantified using an  $^{125}\text{I}$ - antibody coated tube RIA (Diagnostic Systems Laboratories, Webster, TX) (Sanders et al., 1994). Assays such as this one with cross-reactivity to a wide range of progesterone metabolites have been used to successfully quantify progesterone in a wide range of species (Schwarzenberger 2007), and previous studies have found strong correlations between serum progesterone concentration and fecal progesterone metabolites (Capezzuto et al. 2008). Between 5 and 20 microliters of fecal extract was made up to 25  $\mu\text{l}$  with steroid diluent (Cat. No. 07-166197 MP Biomedicals) and then assayed according to the manufacturer's instructions. Parallelism between serial dilutions of fecal extracts for both zebra and springbok and the standard curve were obtained, validating use of the assay in each species. Assay sensitivity was 0.2 ng/ml, with the minimum and maximum detection limits of 0.3 ng/ml and 90 ng/ml respectively. Intra-assay and inter-assay coefficients of variation were <5% and <10 % respectively. All extract concentrations were used to express the detected fecal progesterone metabolites (FPM) as ng/g dry feces.

Concentrations of estrogen metabolite immunoreactivity in fecal extracts were quantified using an  $^{125}\text{I}$ - double antibody RIA (Diagnostic Systems Laboratories). Previous studies have found significant correlations between serum estrogen concentrations and fecal estrogen metabolite levels (Capezzuto et al. 2008). Between 25 and 50 microliters of fecal extract was made up to 100  $\mu\text{l}$  with steroid diluent (MP Biomedicals) and then assayed according to the manufacturer's instructions. Parallelism between serial dilutions of fecal extracts for both zebra and springbok and the standard curve were obtained, validating use of the assay in each

species. Assay sensitivity was 0.6 pg/ml, with the minimum and maximum detection limits of 5 pg/ml and 750 pg/ml respectively. Intra-assay and inter-assay coefficients of variation were <5% and <10 % respectively. All extract concentrations were used to express the detected fecal estrogen metabolites (FEM) as pg/g dry feces.

Concentrations of testosterone metabolite immunoreactivity in fecal extracts were quantified using an  $^{125}\text{I}$ - antibody coated tube RIA (Immunotech, Marseille, France). Previous studies have found significant positive correlations between fecal testosterone metabolites and serum testosterone concentration (Chang et al. 2009). Fifty microliters of fecal extract was assayed according to the manufacturer's instructions. Parallelism between serial dilutions of fecal extracts for both zebra and springbok and the standard curve were obtained, validating use of the assay in each species. Assay sensitivity was 0.05 ng/ml, with the minimum and maximum detection limits of 0.1 ng/ml and 20 ng/ml respectively. Intra-assay and inter-assay coefficients of variation were <8% and <12% respectively. All extract concentrations were used to express the detected fecal testosterone metabolites (FTM) as ng/g dry feces.

### *Statistical Analyses*

#### *Multiple Imputation of Missing Data - Captured Zebras*

Imputation is a method of replacing missing observations with plausible estimates based on available data. Multiple imputation (MI) methods are particularly useful for imputing multivariate missing data (Little and Rubin 2000; van Buuren and Groothuis-Oudshoorn 2011). Multiple imputation uses available data from multiple predictors and covariates to create a set of datasets for each missing value, each containing different sets of those missing values drawn from their predictive distributions. This method, unlike single imputation methods, provides a variance of an estimate and also estimates the contribution of uncertainty due to the fact that the value in question was imputed rather than observed (Rubin 1996; Little and Rubin 2000; van der Heijden et al. 2006). Comparisons of analyses using multiply imputed datasets versus complete case analysis (CCA), in which cases with any missing data are eliminated from the analysis, have found that MI produces much less biased results. This is true when both small and large amounts of data points are missing (van der Heijden et al. 2006; Vergouw et al. 2012).

For my data, I imputed missing values for an individual capture event but did not impute completely missing capture events for an individual animal (i.e. if a zebra was sampled in S1 and S3 but not S2, S4, or S5, I did not impute all data for that animal for S2, S4, and S5; if that zebra was, however, missing a GI parasite count for S1, I imputed that missing value). I imputed two missing values for HCT (1.4% of total HCT data), 15 (9.7%) for ectoparasite count, 33 (22.9%) for GI parasite count, 47 (32.6%) for FGM, 59 (40.9%) for FPM, 53 (36.8%) for FEM, 16 (11.1%) for presence or absence of a foal, 21 (14.6%) for pregnancy status, and 16 (11.1%) for lactation status. Few of these missing variables were overlapping for the same individual-capture; thus, discarding these cases in favor of CCA would have resulted in disregarding upwards of 90% of the capture events in my analyses. Four

individual-captures were missing all hematological parameters; I discarded these four from the imputation and analyses as their data were clearly missing due to related, non-random reasons. I also did not include in my imputations and analyses the few obviously biased data points identified in my preliminary data analyses.

For imputation, I used the Multiple Imputation by Chained Equations (MICE) method with the 'mice' package (van Buuren and Groothuis-Oudshoorn 2011) in R v2.15.2 (R Core Development Team, Vienna, Austria). This method specifies the imputation model for each variable by building, and iterating over, a set of conditional densities for each variable. I built my predictor matrix by first using all variables in this study (Rubin 1996), and then refined the predictor matrix for each variable to avoid collinearity. I preserved all data transformations by passively imputing each transformed variable linked to its original variable (van Buuren and Groothuis-Oudshoorn 2011). I validated my imputations by confirming convergence, examining density plots and strip plots to ensure that imputed values overlapped existing data, and comparing distributions of observed versus imputed data based on propensity scores (van Buuren and Groothuis-Oudshoorn 2011). I performed 100 imputation cycles and generated five imputations. I averaged the five estimates for each data point to produce single mean estimates for use in  $t$  tests, while I used the multiply imputed datasets directly in my generalized estimating equations and then determined mean estimates for the model parameters (Vergouw et al. 2012). I adjusted standard errors, Wald statistics, and  $p$  values according to Rubin's rules (Rubin 1987).

I did not impute missing data for NC zebra and springbok analyses, due to both the large number of missing hormone measurements and the fact that most missing variables were missing for the same individuals and/or same seasons. For NC zebra, there were 5 missing FGM, 92 missing FPM, 99 missing FEM, and 55 missing FTM measurements; for springbok, there were 1 missing FGM, 78 missing FPM, 102 missing FEM, and 51 missing FTM measurements. Instead of imputing missing variables, I partitioned these data in subsets based on the parameters of interest and performed several nested models of decreasing overall sample size (see below).

### *Comparing Captured Zebra Between Rain Groups*

I first examined the effects of season for my various pathogen and immune factors, as very few studies have examined simple seasonal differences for most of these variables. I started by compared data from only the first sampling events for animals across the two rain groups. This ensured that I was only comparing unique individuals across rain groups, without the possibility of autocorrelation and the issue of repeated measures (Table 2a).

I transformed variables to better approximate assumptions of normality when necessary and possible (Table 3); normality was determined through a combination of visual assessment with histograms and statistical testing using Shapiro-Wilk tests. I compared square root-transformed GI parasite counts, WBC counts, lymphocyte counts, HCT, and FPM concentrations between rain groups using Welch's two-tailed  $t$  tests. I compared  $\log_{10}$ -transformed neutrophil counts, FGM concentrations, and FEM concentrations between rain groups using Welch's two-

tailed  $t$  tests. I compared ectoparasite counts and  $\log_2$  anti-PA titers between rain groups using two-sided Wilcoxon rank sum tests. I adjusted  $p$  values to control for the familywise error rate by using the Holm-Bonferroni method (Holm 1979).

I then did pairwise comparisons across the same animals, between rain groups, for first and second captures to control for potential individual variation (Table 2b). I compared the same transformed variables using two-tailed paired  $t$  tests and two-sided paired Wilcoxon signed rank tests, and then corrected for familywise error rate as per above.

#### *Generalized Estimating Equations – Captured Zebra*

I developed generalized estimating equations (GEE) using R v2.15.2 and the 'geepack' (Højsgaard et al. 2006) to examine the correlations between pathogen types, immune parameters, and hormone concentrations. I chose GEEs because they can incorporate both random and fixed effects, can model counts and binary data as well as continuous data, can deal with data that violate assumptions of normality and independence, and can account for different autocorrelation patterns in repeated measures data (Liang and Zeger 1986; Hardin and Hilbe 2003). These models are particularly useful for longitudinal data with many individuals (zebras) but relatively few longitudinal observations (sampling seasons) per individual (Zuur et al. 2009). In all models I used a working correlation matrix with a first-order autoregressive relationship (AR-1) because, while individual immune, disease, and hormonal factors are likely correlated through time, these correlations should decrease between later time points and earlier samplings (Liang and Zeger 1986; Zuur et al. 2009). I used zebra identification as the grouping structure, and used the waves argument in geepack with the capture number to account for missing sampling times for individuals.

To avoid multicollinearity between explanatory variables, I first assessed pairwise scatterplots, correlation coefficients, and variance inflation factors, and excluded one of each pair of variables with correlations of  $r > 0.8$  or  $VIF > 3$  (Farrar and Glauber 1967; Zuur et al. 2009). I developed each GEE by using a backwards stepwise refinement method based on comparing the quasi-likelihood under the independence model criterion (QIC) values between maximal models and models with variables removed (Pan 2001). The QIC is equivalent to Akaike's information criterion (AIC) for repeated measures; a smaller QIC indicates a better fitting model. I used the 'yags' package in R to determine QIC values (Carey 2004). After this first refinement, I added biologically-sensible interaction terms between the remaining explanatory variables and further refined the models by comparing QIC's. I validated the models by plotting Pearson's residuals against fitted values to look for residual patterns, examined residual histograms to assess the normality of error distributions, and plotted residuals against each explanatory variable to test for homogeneity of error variances. I used transformed variables when necessary for eliminating residual patterns. For the final, best-fit models, I used Wald chi-square tests to determine the significance of each parameter estimate.

I first used GEE models to address the relationship between my study's three pathogen types, seasonal factors, and host parameters (Table 4). While assigning directionality with GI parasites or anthrax as the response variable in all cases may

seem a straightforward choice, I decided to allow each pathogen (GI helminths as determined by FEC; ectoparasites as determined by tick counts; and sublethal anthrax exposure, as determined by anti-PA titers or the presence or absence of a measureable titer) to play the role of response variable in separate models for several reasons. While spikes in GI parasite infection intensity do occur in this system a few weeks prior to anthrax outbreaks, there is a 100% prevalence of GI helminth infections year-round in ENP zebras (Turner 2009; Turner and Getz 2010). Because of this, and because cases of anthrax do occur throughout the year, assigning one pathogen as the dependent variable in this case would have been potentially biased. The same reasons apply for examining host ectoparasite loads as a potential outcome of other infections. In addition, as immunity is not unidirectional and the immune system involves a very complicated interplay between different immune factors and the influences of co-infections, I wanted to allow each pathogen "outcome" to be examined from the standpoint of potential predisposing factors toward that infection. I fitted the GEE models for transformed GI helminth and ectoparasite counts with a Gaussian distribution; response variables were transformed to eliminate residual patterns, which effectively changed counts into continuous variables. I fitted the GEE model for presence or absence of a PA titer with a binomial distribution linked to a logit function (Table 4).

For these same reasons, I then used each of my immune parameters as response variables in separate models to allow me to more directly examine the cross-relationships between them and the other immune and hormonal parameters and pathogens. I did the same with FGM as the response variable for similar reasons. I did not fit individual models with FPM or FEM as response variables as these hormones are more clearly directionally determined by maturity, mating, and pregnancy status; these hormones were measured as means of controlling for underlying seasonal changes in mating and pregnancy rather than as individual response variables of interest. I fitted the GEE models for transformed neutrophil counts, lymphocyte counts, and FGM concentration with a Gaussian distribution for continuous variables (Table 4).

Finally, I examined a model using animal age as the dependent outcome; could disease status, immune function, and hormone status explain individual age? I fitted transformed age with a GEE model with a Gaussian distribution (Table 4). As little is known about how disease status, immune function, and stress influence animal aging and survival, this model allowed me to examine more basic ecological immunological questions.

#### *Comparing NZ Zebra and Springbok Between Rain Groups, Sex, and Age*

Similar to the rain group comparisons for captured zebra, I performed bulk group comparisons for NC zebra and springbok between rain groups, sexes and age classes. For those response variables that could be successfully transformed to normality (GIP and FTM for NC zebra; FPM, FEM, and FTM for springbok), I performed Type III ANOVAs for unbalanced data to explore seasonal differences and potential interaction effects. I used R v2.15.2 and the 'car' package for these analyses (Fox and Weisberg 2011). I used Tukey pairwise post hoc tests to determine the significance of multiple comparisons. For other response variables that were

incapable of being transformed to complete normality, I used Type II ANOVAs and Tukey post hoc tests to explore potentially significant interaction effects. I then used Wilcoxon rank sum tests to examine rain group, sex, and age differences, and performed these same comparison tests for any interactions significant in the ANOVAs at a  $p \leq 0.1$  level. I adjusted  $p$  values from Wilcoxon rank sum tests to control for the familywise error rate by using the Holm-Bonferroni method discussed above.

For all GI parasite comparisons, I included interactions between sex and age but not between rainfall and sex or rainfall and age; given previous studies showing that GI parasite counts in ENP ungulates are very statistically significantly higher in wetter seasons than in drier ones, these interaction terms were nonsensical (Turner and Getz 2010; see dissertation Chapter 3). For FGM, I included interactions between sex and age, rainfall and sex, and rainfall and age. For FPM, FEM, and FTM, I did not include interactions between sex and age; numbers of yearlings were quite low for this dataset for NC zebras ( $N=9$ ), and these reproductive hormones were expected to be higher in adults of reproductive age in both species than in yearlings. I did, however, explore interactions between rainfall and sex for FPM and FEM. For FTM, only males were examined. The yearling age group in the NC zebra dataset was also quite low ( $N=15$ ), but I explored interactions between rainfall and age with caution. The yearling age group in the springbok dataset only included 10 individuals, and thus I did not explore rainfall-age interactions for springbok testosterone.

#### *Generalized Linear Models – NC Zebra and Springbok*

I developed generalized linear models (GLM) using R v2.15.2 and the 'MASS' package (Venables and Ripley 2002) to examine the correlations between parasite types and stress hormone metabolite (FGM) concentrations. I used negative binomial (NB) GLMs with a log link function for NC zebra GIP models and springbok GIP and GIC models to deal with large amounts of overdispersion in these counts variables (Zuur et al. 2009) (Tables 5 and 6). I used a gamma distribution with an inverse link function for FGM GLMs for both NC zebra and springbok (Tables 5 and 6). For *Strongyloides* parasites (GIS) models, zero inflated negative binomial (ZINB) mixture models were used to deal with the large numbers of zero GIS counts (80 samples out of 273, 29.3%) and the large overdispersion present in these data (Zuur et al. 2009) (Table 6); I used the 'pscl' package in R to develop these models (Zeileis et al. 2008). The ZINB method applies two models to the data simultaneously: a counts model using a negative binomial distribution with a log link that includes "true zeros" and non-zero counts versus all other data; and a binomial model with a logit link that accounts for excess "false zeros" versus all other data (Zuur et al. 2009; Pilosof et al. 2012). True zeros are the true absence of parasites because the host is, for example, unsuitable for that parasite or has a strong immune reaction that clears the parasite. False zeros are generated by, for example, observer or measurement errors. I verified that ZINB models were more useful for modeling the GIS data than were regular negative binomial GLMs by applying Vuong tests to compare both types of models; a high positive Vuong statistic favors the ZINB over the NB GLM (Vuong 1989; Shankar et al. 1997).

To avoid multicollinearity between explanatory variables, I first assessed pairwise scatterplots, correlation coefficients, and variance inflation factors, and excluded one of each pair of variables with correlations of  $r > 0.8$  or  $VIF > 3$  (Farrar and Glauber 1967; Zuur et al. 2009). I developed each GLM or ZINB model by using a backwards, stepwise refinement method based on comparing the Akaike's information criterion (AIC) values between maximal models and models with variables removed. After this first refinement, I added biologically-sensible interaction terms between the remaining explanatory variables and further refined the models by comparing AIC's. I validated the models by plotting deviance residuals for NB GLM models and Pearson's residuals for ZINB models (Zuur et al. 2009) against fitted values to look for residual patterns, and plotted residuals against each explanatory variable to test for homogeneity of error variances. I used transformed variables when necessary for eliminating residual patterns. For the final, best-fit ZINB models, I determined the fitted values for the logistic regression model components and determined the odds ratios of observing excess false zeros in conjunction with each predictive parameter (Pilosof et al. 2012).

Similar to the GEE models built for captured zebra, I used each parasite type and FGM as response variables in their own models (Tables 5 and 6). This allowed me to examine the effects of other parasites, stress, reproductive hormones, and basic host and environmental variables on each parasite outcome, and to examine the effects of parasites, reproductive status, and basic host and environmental factors on the development of chronic stress. As zebras in ENP are only host to GI strongyles and not *Strongyloides* or *Eimeria* in any measurable amounts (Turner and Getz 2010), NC zebra GLM models included only GIP and FGM as response variables (Table 5). Conversely, as ENP springbok are host to strongyles, *Strongyloides*, and *Eimeria*, each of these (and FGM) in turn were used as response variables for springbok models (Table 6). In addition, as, for various logistical reasons, there were several missing variables for many NC zebra and springbok samples (see discussion above), I built a series of nested models for each response parameter. This allowed me to take advantage of the largest possible dataset for each iteration. I began with models of the response variable in question explained solely by simple host and environmental factors (rainfall, age, sex) and FGM (for parasite models) or by those factors and the GI parasite counts (for FGM models); this allowed me to use a sample size of 302 for NC zebra and greater than 260 for springbok (Tables 13 and 14). I then added the FPM and FEM reproductive hormones to each model ( $N=163$  complete cases for NC zebra;  $N=113-114$  for springbok). Finally, I built males-only models to account for the effects of FTM on parasite counts and FGM concentrations ( $N=103$  complete cases for NC zebra;  $N=77$  for springbok) (Tables 13 and 14).

## Results

### *Rain Group Comparisons for Captured Zebra*

For both unique animals and paired recaptures, I found statistically significantly higher GI strongyle counts, total white blood cell counts and neutrophil counts in the wet season, and significantly higher estrogen concentrations in the dry season (Tables 7 and 8; Figure 4) While these variables were transformed for normality prior to using  $t$  tests, I also observed large differences between rain

groups for the non-transformed versions of these variables (Tables 7 and 8). I also found statistically significant differences between rain groups for ectoparasite counts and lymphocyte counts, but only for paired individuals (Tables 7 and 8; Figure 4); this indicates that controlling for individual variation allowed these subtler, yet present rain group differences to be revealed. Animals experienced higher tick burdens in the dry season, and higher lymphocyte counts in the wet season. HCT was significantly and nearly significantly higher in the wet season for unique and paired animals, respectively; this was also significant for paired animals prior to the Holm's  $p$  value correction ( $p=0.020$ ). Stress hormone concentrations were also significantly higher in drier seasons compared to in the wetter ones for paired animals, though only prior to the Holm's  $p$  correction ( $p=0.049$ ); this corroborates similar near-significantly higher stress in the dry season observed for unique animals ( $p=0.098$  prior to Holm's correction). I found no significant differences in anti-PA titers or progesterone concentrations compared between rain groups (Tables 7 and 8). Zebra were no more likely to be pregnant, have a nearby foal, or be lactating in the wet season versus the dry season ( $t=0.380$ ,  $p=0.706$ ;  $t=-1.379$ ,  $p=0.347$ ;  $t=-2.045$ ,  $p=0.137$ , respectively). However, slightly more mares were observed with foals and as obviously pregnant in the dry season, and were visibly lactating in the wet season.

Thus, my conclusions for captured zebra are that GI parasite infection intensities, total white blood cell counts, neutrophil counts, and lymphocyte counts (and possibly HCT) were all greater during times of higher rains than during those of low to no rains, whereas ectoparasite infection intensities and estrogen concentrations (and possibly stress hormone concentrations) were greater during drier times than during wet ones. These relationships are incorporated into and illustrated in Figure 9.

#### *Rain Group Comparisons for Non-Captured Zebras*

Similar to the results for comparisons in captured zebra, I found that GI strongyle counts were significantly higher in wetter seasons than in drier ones, whereas estrogen concentrations were significantly higher during the dry seasons (Table 11; Figure 5). Mean differences between these groups, however, were much smaller than they were in captured zebras (mean difference of 479epg in NC zebras versus 1807epg and 1926epg for unique and paired captured zebras, respectively; mean difference of 1961pg/g in NC zebras versus 6592pg/g and 5194pg/g for unique and paired captured zebras, respectively) (Tables 8 and 11). Stress hormone concentrations were highly statistically significantly different between rain groups; however, unlike in captured zebra, FGM was significantly greater in wetter seasons in NC zebra than it was in drier ones. The magnitude of the mean seasonal difference for stress hormones in NC zebras (693ng/g) was, interestingly, nearly identical to that measured in paired, captured animals (649ng/g) (Tables 8 and 11; Figure 5). Progesterone was also significantly higher in wetter seasons than in drier ones for NC zebra (Table 11; Figure 6). Testosterone concentration was not different between seasons (Table 11). Thus, overall, higher rainfall was associated with higher strongyle counts and higher stress and progesterone hormone



concentrations, while lower rainfall was associated with higher estrogen concentrations.

Males had significantly more intense GI strongyle infections than did females, with nearly the same mean difference as was measured for GIP compared between rain groups (Table 11; Figure 5). Male yearlings harbored significantly more parasites than other groups. The highest overall GIP signatures in NC zebra, therefore, were in the wet season, males, and yearlings.

Females had significantly higher stress hormone concentrations than did males, overall and for several age group comparisons (Table 11; Figure 5). Adults in general had higher stress hormone levels, both in the simple comparison, and when compared between seasons (Table 11). The general trends, therefore, were for stress hormones to be highest in the wet season, in females, and in adults; season may have played the largest role in determining stress, particularly because, while females had higher stress levels than males in nearly every instance, males in the wet season had significantly higher FGM than did females (Table 11).

Progesterone was significantly higher in females and adults than in males and yearlings (Table 11; Figure 6). In corroboration with the general rain group trends discussed above, females in RG1 had significantly higher progesterone concentrations than did males in RG2; however, there were no differences between females alone in the two seasons or males alone in the two seasons, indicating that the rain group differences must have been largely driven by the gulf between FPM in wet season females and FPM in dry season males. Overall, then, progesterone was higher in the wet season, in females, and in adults, but was predominantly driven by levels in females in the wet season. Estrogen concentrations were not significantly different between males and females, or between adults and yearlings when examined alone (Table 11; Figure 6). Females and adults did, however, have measurably higher concentrations of this hormone than did their comparative counterparts (1574pg/g and 1886pg/g higher, respectively). In corroboration with the higher FEM levels measured in RG2 as discussed above, females in RG2 had significantly higher estrogen concentrations than did males or females in RG1 (Table 11). Overall, then, estrogen tended to be highest in the dry season, in females, and possibly in adults. Testosterone concentrations were not significantly different between seasons, though trended somewhat toward being higher in RG2 (Table 11; Figure 6). Adults had significantly higher FTM than did yearlings. All of these relationships are incorporated into and illustrated in Figure 9.

#### *Rain Group Comparisons for Springbok*

Similar to the results for comparisons in captured and NC zebra, I found that GI strongyle counts were significantly higher in wetter seasons than in drier ones, whereas estrogen concentrations were significantly higher during the dry seasons (Table 12; Figures 7 and 8). In addition, *Strongyloides* and *Eimeria* counts were also both significantly higher in springbok in the wet season compared to in the dry (Table 12; Figure 7). Stress hormone concentrations were not statistically significantly different between rain groups; however, similar to captured zebra, FGM was measurably higher in the drier seasons than in the wetter ones, with a greater difference between seasons for springbok than was measured for zebra

(971ng/g for springbok, versus 357ng/g and 649ng/g for unique and paired captured zebra, respectively, and versus 693ng/g for NC zebra). Similar to the trends measured in NC zebra, springbok progesterone concentrations were significantly higher in the wet season compared to in the dry (Table 12; Figure 8). Testosterone concentration was not different between seasons (Table 12). Overall, higher rainfall was associated with higher strongyle, *Strongyloides*, and *Eimeria* counts and progesterone concentrations. Lower rainfall was associated with higher estrogen and stress hormone concentrations.

I found no statistically significant differences between sexes for strongyle, *Strongyloides*, or *Eimeria* counts when sex was examined alone (Table 12; Figure 7). Age was not a significant factor across both sexes in determining strongyle counts, though yearlings harbored significantly more *Strongyloides* and *Eimeria* parasites. Male yearlings had significantly higher strongyle counts than did male and female adults, and possibly more than did female yearlings ( $p=0.084$ ). Male yearlings also harbored significantly more *Eimeria* than did female adults (Table 12). The highest overall parasite infection intensities, therefore, were in wet seasons and in yearlings, with male yearling springbok being the highest parasite aggregators. These patterns are essentially the same as those observed for NC zebra.

There was no significant difference in stress hormone concentration between sexes when examined across all age groups and seasons, though males had a marginally higher mean FGM concentration than did females (459ng/ml) (Table 12; Figure 8). I also found no significance of age alone, though adults had measurably higher stress hormone levels than did yearlings (1128ng/g mean difference). Comparing interaction terms, I found further evidence for higher FGM in males, counter to my findings in NC zebra in which females had higher FGM (Table 12). While females compared between seasons did not have significantly different stress hormone levels, there were measurably higher FGM in dry season female springbok (1604ng/g mean difference). The general trends, therefore, were for stress hormones to be somewhat higher in the dry season, in males, and in adults. This is counter to the group comparison season trends, and the sex trends I observed for NC zebra, but similar to the seasonal trends seen for captured zebra.

I found no significant differences in progesterone concentrations between sexes and ages. In addition, measured differences between sex and age groups were very small (21.5ng/g and 36.6ng/g mean differences, respectively) (Table 12; Figure 8). By these measurements, FPM was marginally higher in males and adults than in females and yearlings, though overall, I can only determine that progesterone was higher in the wet season than in the dry. Similarly, I found no significant differences in estrogen concentrations between sexes and ages alone (Table 12; Figure 8). Adults had only marginally higher FEM (197pg/g) than yearlings. Females in the dry season had significantly higher estrogen levels than did females in the wet season, corroborating the higher FEM in RG2 seen in both springbok and all zebra. Overall, then, springbok estrogen tended to be highest in the dry season, and perhaps in adults. Similar to my findings for NC zebra, I found no significant differences in testosterone concentrations between seasons. However, like zebra, springbok had a slight trend of increased FTM in the dry season compared to in the wet (Table 12;

Figure 8). All of these relationships are incorporated into and illustrated in Figure 11.

### *Zebra GEE Models*

I square root transformed GI parasite, ectoparasite, lymphocyte, and age response variables, and log transformed neutrophil and FGM response variables for my GEE models to deal with overdispersion. In addition, I used log-transformed FGM in my PA model and square root-transformed GIP in my lymphocyte model (Table 4). These transformations did not affect model selection or significance, but improved residual patterns and normality. As these transformations do affect the direct interpretation of coefficient estimates, interpretations here are based solely on sign and significance of coefficients to determine predictors of the response variables and the directions of these effects.

Higher cumulative rainfall two months prior to sampling (hereafter simply "rainfall") predicted higher GI parasite loads, as expected from rain group analyses (Table 9; Figure 4). Mares in full milk were more likely to harbor more strongyles than were those lactating water or not lactating at all. While higher lymphocyte counts predicted more strongyles, higher neutrophil counts predicted fewer GI parasites (Table 9). Anti-PA titer was also negatively correlated with strongyle counts, as I have previously demonstrated (see dissertation Chapter 3). Overall, rainfall, lactation, and lymphocytes were positively associated with strongyle counts, while neutrophils and anti-PA titer were negatively associated with GIP.

Increased lymphocyte counts and increased stress hormone concentrations both predicted the presence of an anti-PA titer (Table 9). While strongyle counts and rainfall were not statistically significant, they were necessary for best PA model fit and maintained previously demonstrated inverse relationships with PA titer presence (see dissertation Chapter 3). As FEM increased, PA titer prevalence decreased. However, I found that animals experiencing more rainfall interacting with higher estrogen levels were more likely to mount a measurable anti-anthrax immune response. Overall, lymphocyte counts, FGM, and rainfall in conjunction with estrogen positively predicted the presence of an anti-PA titer, whereas strongyles, rainfall alone, and estrogen alone predicted the absence of an anti-PA titer.

Rainfall alone predicted higher ectoparasite counts in this model, contrary to the patterns I found in my group comparisons (Table 8). This term was not significant, however, and the rainfall interaction terms were both inversely associated with tick counts (Table 9). Pregnancy status and estrogen level alone positively predicted higher ectoparasite loads; however, given the year-long gestation period and several estrogen fluctuations over the course of zebra gestation, the opposite trends seen with the rainfall interaction terms may actually suggest similar effects but at different stages of pregnancy. Overall, pregnancy and estrogen alone predicted higher ectoparasite infection intensities, but rainfall in conjunction with pregnancy and rainfall in conjunction with estrogen were negatively associated with tick counts, indicating different effects of different gestational stages on infection status.

Higher rainfall, lymphocyte counts, HCT, and progesterone concentrations significantly predicted higher neutrophil counts (Table 10). The relationship with

rainfall corroborates similar trends I observed with my group comparisons (Tables 7 and 8). Lactating mares were more likely to have higher neutrophil counts, though those with higher estrogen levels were not (Table 10). Strongyle counts negatively predicted neutrophil counts, corroborating the similar trends I observed in my GIP GEE model (Table 9). Higher ectoparasite infection intensity also negatively predicted neutrophil counts. While anti-PA titer was not included in the final model, a linear regression of log2 PA titer on log-transformed neutrophil count suggested that higher PA titers correlated with higher neutrophil counts ( $N=144$ ,  $F_{1,142}=4.59$ ,  $R^2=0.03$ ,  $p=0.034$ , coefficient=0.01). Overall, rainfall, lymphocyte counts, hematocrit, progesterone, and lactation were positively associated with neutrophil counts, whereas estrogen, strongyle counts, and ectoparasite counts were negatively predictive of neutrophil counts.

Contrary to what I observed in my group comparisons, increased rainfall in the GEE model predicted that hosts would carry fewer lymphocytes (Table 10). Neutrophils and strongyles positively predicted lymphocyte counts, similar to the relationships I observed in my neutrophil and GIP models. Pregnant mares had significantly fewer lymphocytes, as did older individuals. Overall, neutrophil and strongyle counts positively predicted lymphocyte counts, while rainfall, pregnancy, and older age were negatively associated with lymphocytes.

While differences in FGM between rain groups was not significant in group comparisons, they suggested that stress hormones were higher during the dry season than during the wet (Table 7 and 8). Corroborating these trends, rainfall negatively predicted stress hormone levels in my GEE model (Table 10). Both higher rainfall and higher progesterone levels together, however, predicted higher FGM; similar to the trends in my ectoparasite GEE model, these opposite effects between rainfall and its interaction term may reflect gestational timing effects. In accordance with this idea, increasing FEM and FPM alone both positively predicted FGM levels. Similar to the trends in my PA GEE model, anti-PA titer was positively associated with stress hormone concentration in this model. Overall, estrogen and progesterone concentrations, rainfall in conjunction with progesterone, and anti-PA titer positively predicted higher stress hormone levels, whereas rainfall alone was negatively associated with stress.

Lymphocyte counts were significantly, negatively predictive of age (Table 10). As WBC was highly collinear with both lymphocyte and neutrophil counts, I could not include it in the overall age model. However, a simple GEE model of WBC on transformed age revealed that fewer WBC also nearly significantly predicted older animals (coefficient $\pm$ SE=-5.79e-04 $\pm$ 2.95e-04, Wald statistic=3.70,  $p=0.054$ ). Milk lactation alone significantly predicted older mares, though both lactating and having a foal predicted younger animals. As all captured zebra were adults, these trends are perhaps simply capturing slightly different individual life stages. No pathogens or hormones were included in the final age model. However, a linear model of FGM on square root-transformed age was strongly significant, with higher FGM predicting older animals ( $N=144$ ,  $F_{1,142}=7.43$ ,  $R^2=0.05$ ,  $p=0.007$ , coefficient=0.01). A linear model of strongyle count on transformed age was also nearly significant, with fewer parasites predicting older animals ( $N=144$ ,  $F_{1,142}=3.17$ ,  $R^2=0.02$ ,  $p=0.08$ , coefficient=-0.01). This corroborates previous evidence that older

zebra are less likely to harbor high strongyle loads (see dissertation Chapter 3). Overall, lactation, the presence of a foal, and higher stress hormone levels predicted older animals, while higher lymphocyte counts, WBC, and strongyle counts, and having a visible foal while lactating milk predicted younger animals. All of these relationships are incorporated into and illustrated in Figure 10.

#### *NC Zebra GLM Models*

I log transformed FEM and fourth root transformed FPM in all models to improve model residual patterns. In accordance with all other zebra strongyle models and comparisons, more rainfall predicted higher GI parasite loads in all GIP models (Table 13). However, higher rainfall in conjunction with higher stress hormone concentrations predicted less intense strongyle infections in the GIP-hormones model. This relationship was perhaps driven primarily by the stress effects; higher stress levels also significantly, negatively predicted strongyle counts in the simplest GIP-FGM model, corroborating my simple linear model exploration between these two variables (see above), as well as the interaction effects in the more complex GIP-hormones model. This strongly significant, negative relationship between FGM and strongyle count also held for the GIP-males only model (Table 13). Males were more likely to have higher strongyle counts in the simplest GIP-FGM model, similar to what I observed in my group comparisons (Table 11). Thus, overall, these models indicate that strongyles in zebras were predominantly positively associated with rainfall and maleness, and were negatively associated with stress hormone concentrations.

More rainfall predicted lower stress hormone concentrations in all models, counter to the FGM rain group comparison results for NC zebra but in concordance with the patterns seen in captured zebra (Tables 10 and 11). Stress hormone concentration was significantly, positively associated with strongyle counts in all three FGM GLM models, contrary to the patterns observed in GIP GLM models and my simple linear model explorations. While all estimates comparing these two variables were small, FGM inversely predicted strongyle counts in GIP models with greater magnitude than GIP counts positively predicted FGM levels in FGM models (Table 13). In addition, rainfall-GIP interaction terms were nearly significant and highly significant in inversely predicting stress hormone levels in both the FGM-GIP and the FGM-males only models. Sex terms alone in these models also provided conflicting information. Older animals also predicted lower FGM, the opposite of the trend observed in the simple linear model discussed above. However, older females significantly predicted higher stress hormone concentrations in the FGM-GIP model, and males with higher testosterone levels were associated with lower FGM in the FGM-males only model. These results, taken together with the fact that females had significantly higher FGM in group comparisons (Table 11) suggests that female zebras likely do experience higher stress hormone levels than do males. Overall, then, it appears that increased rainfall was associated with lower stress, females likely had higher stress hormone concentrations than did males, and stress may decrease with age. All of these relationships are incorporated into and illustrated in Figure 10.

### Springbok GLM Models

As for the NC zebra GLM models, I transformed certain explanatory variables (GIP, GIS, and GIC) in all springbok models to improve model residual patterns (Table 6). In all GIP models, higher rainfall predicted more strongyles, as I have observed in all strongyle models thus far (Table 14). Higher *Strongyloides* counts also predicted higher strongyle counts in all models. Interestingly, however, in all three models, higher rainfall interacting with higher *Strongyloides* counts predicted fewer strongyles in springbok hosts. Higher stress hormones nearly significantly ( $p=0.052$ ) predicted higher strongyle counts in the GIP-FGM model, counter to the trends observed in zebra. Overall, increased rainfall, increased *Strongyloides* counts, and increased FGM were positively predictive of strongyle counts in springbok, while *Eimeria* counts did not affect strongyle loads. Animals experiencing both high rainfall and high *Strongyloides* loads were prone to having less intense strongyle infections, indicating a complex relationship between season and coinfections.

*Strongyloides* counts were negatively predicted by rainfall in the final GIS-FGM model; however, increased rainfall also predicted fewer false zeros in *Strongyloides* counts, indicating a complex relationship between these parasites and rainfall (Table 14). A simple linear model of rainfall on transformed GIS was highly significant and indicated a positive relationship between rainfall and *Strongyloides* infection intensities ( $N=266$ ,  $F_{1,264}=45.9$ ,  $R^2=0.15$ ,  $p=0.000$ , coefficient=0.06). Increased host loads of *Coccidia* parasites significantly predicted that a host would also carry a measurable *Strongyloides* infection (odds ratio=0.74: it was 34% less likely that animals experiencing high *Eimeria* counts would also experience no *Strongyloides* infections). While strongyle counts had no direct influence on magnitude of *Strongyloides* infections, GIP was highly significantly, negatively predictive of false zeros (odds ratios=0.92 and 0.86 for GIS-FGM and GIS-hormones models, respectively: it was 8.8-16.6% less likely that an animal with high strongyle loads would also harbor no *Strongyloides*). Males alone, however, experienced the opposite relationships: both increased strongyles and increased *Eimeria* significantly predicted more zero *Strongyloides* counts (odds ratios=1.11 and 1.34, respectively). Increased stress hormone concentrations significantly predicted fewer *Strongyloides* in the GIS-FGM model, while more estrogen significantly predicted fewer *Strongyloides* and more GIS zeros in the GIS-hormones model (Tables 14 and 15). Overall, higher *Strongyloides* infection intensities were associated with higher *Eimeria* and strongyle counts in females, with males experiencing the opposite trends. In addition, higher stress and estrogen hormone concentrations predicted lower *Strongyloides* loads. Rainfall associations with *Strongyloides* parasites were complex.

*Eimeria* counts were significantly, positively associated with rainfall in all three GIC models (Table 15), corroborating trends observed in the group comparisons (Table 12). While *Strongyloides* counts significantly, positively predicted *Eimeria* counts in all three models, rainfall-GIS interactions were significant and negative for both GIC-FGM and GIC-males only models. *Eimeria* counts were also significantly, negatively associated with age, corroborating trends observed in group comparisons (Table 12). In general, high *Eimeria* counts were associated with high rainfall and younger animals, and had a complex relationship

with *Strongyloides* parasites but not with strongyles. Stress and other hormones did not play a role in predicting *Eimeria* counts.

Higher rainfall significantly, positively predicted higher stress hormones in the FGM-parasites model (Table 15). Higher *Strongyloides* infection intensities also significantly, positively predicted FGM, the opposite of the trend observed in the GIS models. However, higher rainfall in conjunction with higher GIS significantly, negatively predicted FGM, corroborating trends observed in GIS models and rain group comparisons (Table 12). Strongyle loads significantly, negatively predicted FGM, similar to the inverse relationship between GIP and FGM in most NC zebra GIP models, but counter to the relationships observed in springbok GIP models and NC zebra FGM models (Table 13). *Eimeria* counts did not significantly influence FGM in any models, though they were necessary for the best fit of the males-only FGM model (Table 15), and were slightly negatively associated with stress levels. While higher testosterone concentrations in males predicted lower stress, higher *Eimeria* counts in conjunction with higher testosterone levels predicted higher stress hormone concentrations, corroborating evidence from group comparisons indicating that male springbok in general have higher FGM (Tables 14 and 12). However, females with high estrogen levels also significantly predicted higher stress hormone concentrations in the FGM-hormones model, indicating that reproductive status cannot be discounted. Finally, older animals predicted lower stress levels, contrary to the patterns observed in the group comparisons (Table 12). Overall, higher stress hormone concentrations were associated with lower rainfall and higher *Strongyloides* infection intensities. Higher strongyle counts predicted lower FGM, counter to the strongyle-FGM trends observed in most previous springbok and zebra models and comparisons. Maleness was associated with higher FGM, particularly in conjunction with *Eimeria* infections and higher testosterone levels, though females with high estrogen levels also experienced high levels of stress. Age effects in these models ran counter to previous FGM age effects, with older animals having lower stress hormone concentrations. All of these relationships are incorporated into and illustrated in Figure 12.

## Discussion

This study evaluated the seasonality of stress responses in conjunction with environmental, reproductive, pathogenic, and immunologic factors. I aimed to determine how the strong seasonality of environmentally transmitted pathogens such as helminths and coccidia parasites correlated with changes in host reproduction, immune function, and measures of chronic stress, as well as with the occurrence of coinfections. As I have previously found that wet season-driven GI strongyles are strongly immunomodulatory in zebra hosts and therefore may influence susceptibility to anthrax in Etosha National Park, I wanted to determine if these pathogens also influence host susceptibility by invoking stress responses and stress-related immunosuppression. In addition, such correlations would provide evidence that these GI parasites likely affect host fecundity and survival through mechanisms other than direct pathology and resource use. Instead, I found that, while stress responses do exhibit seasonality in zebra and springbok, stress hormone levels largely peak in the dry season when parasite infection intensities

are lowest. Rather than by pathogen prevalence or infection intensity, peak stress hormone levels are mostly driven by dry season environmental challenges and host reproductive status. This study is one of the first to examine seasonal changes in stress responses in concert with reproductive changes, macroparasite and microparasite coinfections, and immune parameters in wildlife hosts in a natural system. In addition, it is one of the first studies to follow wildlife hosts longitudinally while examining these interactions.

### *Seasonal Patterns*

As expected, I found strong seasonality in nearly all parameters examined. Previous studies in this system have found that GI parasite infection intensities in zebra and springbok peak in the wet season, and that prevalence of strongyles, *Strongyloides*, and *Eimeria* in springbok reaches nearly 90% during times of highest rainfall (Turner and Getz 2010; see dissertation Chapter 3). Thus, these trends held in this study, even when using subsets of previously examined samples.

Corroborating previous findings, ectoparasite infestation was related to decreased rainfall and decreased GI parasite infection intensities, and anti-anthrax antibody titers were most prevalent when GI parasite infection intensities were lowest (see dissertation Chapter 3). Pathogen seasonality is common in many systems, particularly for those like the GI parasites in this study that have free-living stages whose survival depends on permissive environmental conditions (Cattadori et al. 2005; Altizer et al. 2006; Grassly and Fraser 2006). Thus, the timing of new GI parasite infections is relatively fixed in ENP, as these species require moderate temperatures and high environmental moisture for survival prior to transmission (Gordon 1948; Horak 1981; Banks et al. 1990).

While the seasonality of GI macroparasites in ENP is largely determined by environmental conditions, transmission rates can change seasonally due to changes in host behaviors and the birth of large numbers of immune naïve hosts, and host susceptibility can change due to physiological factors such as reproductive hormonal fluxes and changing nutrition (Altizer et al. 2006; Grassly and Fraser 2006; Cornell et al. 2008). Though technically aseasonal breeders (Estes 2001), both zebra and springbok in ENP have been observed to breed on a largely seasonal basis, with birth peaks in the wet season (Gasaway et al. 1996; Turner and Getz 2010). This study provides definitive hormonal evidence of strong reproductive seasonality for both of these species in this system. Previous studies in zebra have indicated that estrogen peaks in mid-gestation; while estrogen concentration is comparable to non-pregnant values until the beginning of the third month of gestation, it can increase up to 60 times baseline value in mid-pregnancy before declining sharply in the last trimester (Chapeau et al. 1993; Ncube et al. 2011; Barnier et al. 2012). Progesterone peaks in the last 50 days prior to foaling, and declines sharply in the short periparturient period (Asa et al. 2001; Ncube et al. 2011). Estrogen and progesterone also play roles in preparing mares for ovulation and estrus behavior, and thus can exhibit shorter and smaller rises around times of mating (Asa et al. 2001). Springbok progesterone levels have also been found to increase significantly with introduction of a ram, followed by peaks in estrogen and estrus. Estrogen also reaches a sustained peak in mid-pregnancy in this species, with progesterone peaks



just prior to parturition (Skinner et al. 2001 and 2002). Thus, my findings that FEM levels were significantly, negatively associated with rainfall for both species indicates that the majority of the zebra and springbok examined were in mid-gestation in the dry season, whereas the significantly increased FPM in the wet season indicates that the majority of females of both species were giving birth and/or coming into estrus at this time. Given the high reproductive rates of plains zebra in other systems (79% in Kruger, 88% in Serengeti, 74% in Hwange; Barnier et al. 2012) and the fact that springbok can raise up to three lambs in two years (Skinner et al. 1987), it is conceivable that pregnancy rates in my study animals were indeed very high. Though Gasaway et al. 1996 found that springbok fertility rates in ENP were high even in the face of severe drought, and that zebra body condition did not fluctuate greatly even under the same drought conditions, the strong seasonality of breeding in this system still makes sense as it capitalizes on the significantly better nutritional resources of the wet season during the intensely energetically demanding times of parturition and lactation (Olléová et al. 2012; Turner et al. 2013). Interestingly, I also found seasonal signals for these hormones in males as well. Both male zebra and springbok in the dry season had significantly higher FEM than did females in the wet season, indicating, unlike in other similar studies (Ncube et al. 2011), that there is likely an environmental, seasonal component to reproductive hormone secretion as well.

I also found seasonal patterns in WBC counts, HCT, lymphocytes, and neutrophils, in corroboration with the seasonality of several other immune factors previously measured in these zebra (see dissertation Chapter 3). Higher HCT in the wet season likely reflects a higher nutritional state, especially in light of the fact that dehydration often results in an elevated HCT (Beechler et al. 2009; Voyles et al. 2012); the fact that HCT in the wet season was still significantly higher than that of the more dehydrated animals in the dry season makes it likely that overall red blood cell numbers were even higher during periods of more rainfall. Higher WBC in the wet season may also reflect this higher plane of nutrition, as higher WBCs in general often represent a higher investment in immunity (Demas and Nelson 1998; Nunn 2002; Nunn et al. 2003). The higher total numbers of neutrophils and lymphocytes in zebras in the wet season may also at least partly reflect this nutritional trend. However, Neutrophils and lymphocytes had opposite relationships with rainfall, and thus simple seasonality and nutrition effects cannot completely account for these patterns (discussed below).

Higher stress hormones were overall correlated with decreased rainfall, particularly for zebras; while springbok had a complex relationship with stress hormone levels, there was some indication that females in the dry season had higher FGM than did females in the wet season (Table 12). Some of this seasonality was likely driven by environmental factors such as decreased nutrition and water availability in the dry season. Elevations of GCs in the face of altered nutrition could be adaptive to help mobilize energy stores, rather than indications of distress; there is evidence that predictable aversive conditions such as seasonal changes may cause animals to preemptively raise GC levels and to prepare for the expected GC-related immunosuppression by elevating immune function (Demas and Nelson 1998; Martin 2009). However, the interaction between FGM and other factors during the

dry season indicates that animals also likely do experience chronic stress responses at this time (discussed below).

### *Stress and Reproduction*

Stress hormone levels were not only higher at the same time most females were in mid-gestation, but were also significantly correlated with reproductive hormone concentrations. Increased FEM significantly predicted higher FGM for zebra, and increased FEM in females predicted higher FGM for springbok (Figures 11 and 12). The lack of significance of FEM in the NC zebra FGM-hormone model is likely due to the fact that this model incorporated FEM measurements for both males and females; the strongly significant relationships between FEM and FGM for only female zebra (captured zebra) and for female springbok indicate that this correlation was not simply due to estrogen fluctuations, but to those changes in concert with pregnancy; thus, it appears that pregnancy and its attendant hormone increases causes a significant stress response in these animals. The lack of strong correlations between researcher-determined pregnancy status and FGM in this study are likely due to the subjective nature of pregnancy assessment by visual inspection and light palpation. While there are many studies illustrating the effects of stress on reproductive hormone concentrations and reproductive success, this is one of the first studies (also see Foerster et al. 2012) examining how pregnancy itself may cause chronic stress in wildlife. Interestingly, FPM in zebra was significantly negatively correlated with stress hormone levels (Table 13). However, in models examining only female zebra (captured animals), FPM was positively associated with FGM, and high rainfall interacting with high FPM significantly predicted increased stress hormone levels (Table 10); this likely reflects females in the periparturient period and just prior to estrus, and the distinct possibility that these reproductive efforts cause stress in mares. Progesterone in springbok was not related to stress, perhaps because these animals tend to give birth earlier in the wet season and mate later in the wet season than samples were examined in this study.

In accordance with these findings, female zebras overall had higher stress hormone levels than did males; though males predicted significantly higher FGM levels in the NC zebra GIP-FGM model, older females in this model were also significantly predictive of high FGM levels, and femaleness was nearly significantly correlated with increased FGM levels in the more complex FGM-hormones model (Table 13). Other wildlife studies have found similar sex-stress patterns and have suggested that, in addition to the stress of reproduction, higher GC secretion in females may be adaptive to promote longer-term survival and the energy demands of reproduction (Vera et al. 2013).

While stress in male zebras was highest in the wet season, stress hormone levels in male zebras were negatively related to testosterone. This indicates either that the wet season, testosterone-driven mating events in male zebras are not stressful, or that subordinate males experience more stress at this time than do dominant stallions. Previous studies have found that dominant males of social species (which also tend to have the highest testosterone levels) can experience less stress than do subordinate males due to higher mating success and fewer altercations (Archie et al. 2012), though the opposite can also be the case (Creel et al.

1996; Roberts et al. 2009). Male springbok, on the other hand, experienced overall more stress than did their female counterparts, and did so mostly in the wet season. While this timing may have been capturing the beginning of the energetically and socially stressful rut, testosterone itself was negatively predictive of FGM levels in springbok (Table 15). There were, however, other male-related factors likely at play here (see below).

### *Reproduction, Pathogens, and Immunity*

Synchronized reproduction as seen in zebra and springbok in ENP can result in a pulse of new, immunologically naïve hosts at the same time each year, and parasites can synchronize their life cycles with those of hosts to ensure that pastures are contaminated with infective stages when transmission is most likely (Lloyd 1983; Altizer et al. 2006). In accordance with these ideas, GI macroparasites in this study were overwhelmingly aggregated in younger animals of both species. This also provides evidence for acquired immunity against GI parasites in this system. Cell-mediated immunity has been found to play a large role in protective immunity against coccidiosis, and is often long-lasting (Yun et al. 2000; Hong et al. 2006). No absolute protection is achieved against coccidia, however, which accounts for the prevalence of *Eimeria* even in older springbok (Daugeschies and Najdrowski 2005). Hosts also mount immune responses against both adult and larval *Strongyloides*, and can even clear infections over time (Viney and Lok 2007).

Acquired immunity appeared to also be important in fighting against zebra strongyles in this study. Although neutrophil counts were highest in the wet season, increased strongyle counts negatively predicted neutrophil counts. While decreased neutrophils predicted more strongyles, indicating that an inflammatory response may be at least partially helpful in decreasing new worm infections (Hayes et al. 2004), strongyles can strongly suppress the Th1 responses in which neutrophils play a large role (Allen and Maizels 2011). In contrast, more strongyles predicted higher lymphocyte counts, despite the fact that lymphocytes were also negatively correlated with rainfall (Figure 10). Reduction in numbers of larvae establishing themselves in host tissues is largely due to acquired immunity to GI nematodes and memory responses, and is driven primarily by Th2-type lymphocytes (Balic et al. 2000; Allen and Maizels 2011). While lymphocytes do not usually result in expulsion of adult worms, immune responses to adult worms have been found to be enhanced by ongoing larval challenge and can still result in reductions in adult worm size and fecundity (Rowe et al. 2008).

Interestingly, increased lymphocyte numbers also predicted higher strongyle fecal egg counts, perhaps reflecting the arms race between helminths and the immune system; while acquired immunity can be effective in controlling helminth infections, helminths can also cause widespread immunosuppression (Claerebout and Vercruysse 2000; van Riet et al. 2007). In addition, zebra in this system are host to strongyles year-round. While immune resistance to parasites protects hosts at the expense of the parasite, sterilizing immunity is energetically expensive, and risks immunopathology with the required rigorous immune responses (Balic et al. 2000; Colditz 2008; Råberg et al. 2009). Tolerance of parasites, in contrast, saves the host from harm with lowered negative effects on the parasite; this represents a trade-off

for hosts between dealing with parasites or saving resources, but may result in a fitter animal overall (Råberg et al. 2009). Thus, the complex interplay between lymphocytes and strongyles in this study likely reflects the hosts' attempts to control parasites, parasites' attempts to control host immunity, and increased numbers of regulatory T cells, the primary immune agents of host tolerance.

Reproductive hormones also influenced immunity in this study. Non-pregnant zebras had higher lymphocyte counts and lower FEM was significantly correlated with higher neutrophil counts (Figure 10). Breeding and immunity are both expensive and somewhat incompatible, as elevations of one often compromise the other (Bonneaud et al. 2003; Hanssen et al. 2004). Despite the fact that lactation and estrus behaviors are also very energetically costly (Pluháček et al. 2010; Olléová et al. 2012), both lactation and FPM significantly predicted higher neutrophil counts in zebras. This may reflect an inflammatory response to the potentially injurious activities against mares of suckling young and mating stallions.

Reproductive status and sex were also important factors in predicting pathogen infection intensity. Pregnant and lactating mares and mares with higher FEM in the dry season had higher tick infestations, despite the fact that ticks are significantly higher in the dry season and mares lactate most intensively in the wet. The fact that increased rainfall interacting with pregnancy, and increased rainfall interacting with FEM predicted fewer ectoparasites was most likely driven by immunomodulation by wet season GI parasites preventing tick infestation of hosts (see dissertation Chapter 3). Animals with higher FEM also had lower anti-PA titers, indicating reproductive hormone immunomodulatory effects. The relationship between increased rainfall in conjunction high FEM and higher anti-PA titers is complex; perhaps this reflects the generally higher rate of immunity in females compared to males, and a heightened response at a time when these hosts may be encountering more anthrax spores (Rolff 2002; Nunn et al. 2009; Turner et al. 2013). Lactating zebra and those with higher FEM levels in the wet season had more intense strongyle infections, likely indicating a periparturient rise in parasitism. Such increases in parasite infections are common around the time of parturition due to energy trade-offs between reproduction and immunity, and the immunosuppressive actions of fluctuating reproductive hormones (Coop and Kyriazakis 1999; Beasley et al. 2010). In addition, behavior could have played a role here, as lactating zebra in ENP were found to eat significantly more and take more bites per minute than did males and non-lactating females; this increased and less discriminate grass intake could have resulted in lactating mares ingesting more strongyle larvae (Neuhaus and Ruckstuhl 2002). Springbok, however, showed no obvious periparturient rise in any of their GI parasite species. Turner et al. 2012, however, did find evidence that springbok females had both a higher strongyle infection intensity and lower body condition than did adult males, and that these trends were coincident with the time of peak parturition and first two months of lactation. While the female springbok subset in my study had marginally higher strongyle infection intensities than did males, this difference was quite low (115epg) and was not at all significantly different ( $p=0.626$ ). The reasons for these discrepancies may be partly due to the fact that I subsampled between February and May, whereas Turner et al., using the entire springbok sample set, found the largest

strongyle infection intensity differences in January (with female GIP being significantly higher) and in late May/June (with male GIP being significantly higher); strongyle intensities between February and May were largely similar between the sexes. However, it may also be the case that, when reproductive hormones and stress hormones were taken into account, periparturient ewes did not actually have more intense strongyle infections than did non-pregnant ewes and males. Reproductive status did not actually play a large role in predicting intensities of any of the three species of springbok parasites, though FEM was actually significantly, negatively correlated with *Strongyloides* counts (Table 14).

Sex, however, was important in determining parasite infection intensities for both zebra and springbok. Male zebra had significantly higher strongyle counts, and strongyles were most aggregated in yearlings (Tables 11 and 13). While springbok group comparisons for males and females were not significantly different for any parasite, male yearlings again shed significantly more strongyle eggs and *Eimeria* oocysts than did other sex-age groups (Table 12). These sex aggregations, however, were not related to testosterone levels, as FTM was not included in any final male parasites models. Male-biased parasitism is relatively common in wildlife (Zuk and McKean 1996; Grear et al. 2009), which has often been attributed to the immunosuppressive effects of testosterone; the immunocompetence handicap hypothesis (ICHH) suggests that males trade off testosterone-mediated sexual selection traits for immunocompetence, and thus "brighter" males must tolerate more parasites (Folstad and Karter 1992). However, insects also often have male-biased parasitism, though do not have testosterone (Rolff 2002). Bateman's principle suggests that males gain fitness by increasing mating success, whereas females increase fitness through longevity due to higher reproductive efforts, and to promote this longevity females must invest more in immunity than do males (Bateman 1948). Rolff (2002) suggests that Bateman's rule can be applied to parasite susceptibility, with females being overall more immunocompetent and resistant to infections and males trading off immunity for quicker reproductive fitness. He suggests that immune trade-offs in males and females are due to life history trade-offs and different life history trajectories that can manifest as immune differences even before the onset of reproduction. While several studies have found support for the ICHH (e.g. Norris et al. 1994; Muehlenbein et al. 2005; Muehlenbein and Bribiescas 2005), the results of my study go counter to this and instead support Rolff's hypothesis. Behavioral differences in young males may also play a role here in parasite intensity differences (Ezenwa 2004); young male zebras and springbok segregate into bachelor herds, whereas rutting male springbok are lone and territorial and stallions preside over harems and mixed young (Estes 2001; Skinner and Chimimba 2005).

#### *Stress, Pathogens, and Immunity*

While stress hormone levels were significantly, positively correlated with several reproductive parameters, increased FGM largely did not correlate with increased pathogen infection intensity. In fact, decreased FGM significantly predicted increased strongyles in zebra and increased *Strongyloides* in springbok, and higher strongyle counts in springbok significantly predicted lower FGM levels

(NB there is no evidence that parasites can directly or indirectly downregulate FGM production; Monello et al. 2010). While strongyle infection intensity in zebras and *Strongyloides* infection intensity in springbok did predict higher FGM levels, the overall magnitude of the negative relationships between FGM and these parasites was much higher. Thus, while there is some indication here that these two parasites may cause host stress, there are clearly more complex interactions between parasites and the HPA axis taking place. The negative relationship between FGM and some parasites could indicate that being able to mount a small stress response is actually beneficial for animals fighting against these pathogens, by helping mobilize energy resources or by holding an overzealous immune reaction to parasites in check. Mounting a stronger stress response against these parasites would be costly, resulting in further immunosuppression and likely rendering parasite-infected hosts even more susceptible to anthrax than do parasites already. In addition, acclimation to a stressor can occur when animals experience the same stressor repeatedly. Over time, such a stressor can be perceived to be less noxious, decreasing the GC response (Dhabhar et al. 1997; Romero 2004). This could particularly be the case for predictable stressors such as seasonally increased GI parasite larval transmission. Parasites have been found to interact with seasonally poor nutrition to predict higher GC levels in hosts (Chapman et al. 2007). However, parasite transmission in ENP is strongly tied to the wet season when animals experience better nutrition and free water availability, and thus ENP hosts may be able to tolerate parasites without being pushed into distress. Alternatively, chronically stress can actually cause GCs to decrease with intrinsic control of adrenal function (Linklater et al. 2010). However, this is likely not the case here, as lowered GCs often represent a highly distressed situation, and zebra and springbok were still capable of secreting significant GCs in response to reproductive and environmental stressors in both seasons.

Thus, overall, macroparasites in this system do not appear to affect host stress levels. This is even more apparent as male yearlings of both species harbored the most parasites of any group, but females of nearly all age-sex comparisons had higher stress levels in zebras. While male springbok had higher stress hormone concentrations than did females, male adults had nearly significantly higher FGM than did male yearlings, despite yearlings harboring significantly higher parasite intensities. In addition, Turner et al. 2012 found that body condition of adult female springbok was significantly, negatively related to strongyle intensity, whereas that of yearlings and adult males was significantly, negatively related to rainfall; thus, the springbok age-sex class with the highest condition impact from parasites did not have the highest FGM levels, even in the face of concurrent parturition, lactation, and estrus. In fact, the only relationship between stress, reproduction, and parasites was seen in male springbok experiencing both high coccidia intensities and high testosterone concentrations; this parasite-FGM combination significantly predicted high FGM levels. Thus, only through interaction with significant amounts of parasites did testosterone affect male stress, and only through the interaction with significant amounts of testosterone (and also likely behavioral stress, as these were more likely to be adult, rutting males) were parasites important in producing a stress response.

Increased stress hormone levels predicted the presence of an anti-PA titer, potentially indicating the beneficial aspects of GC responses. However, increased PA titers also significantly predicted increased FGM, and thus mounting a resource-intensive immune response to anthrax may cause an animal some stress. No relationship was seen between tick loads and stress hormones. Though ticks can be energetically demanding, other studies have found that eliminating ticks can have no effect on FGM levels, even when ticks cause host tissue inflammation, hemorrhage, and irritation (Monello et al. 2010). There were also no direct relationships between the immune parameters measured and stress hormone levels. Stress is known to decrease circulating lymphocytes and increase circulating neutrophils, the pattern in zebras that was correlated with increased rainfall. However, the fact that this pattern was observed when stress hormones were not at their highest suggests that this did not indicate a stress leukogram, and that the wet season neutrophilia was instead more likely due to inflammatory responses against mating and lactation injuries.

While there were significant macroparasite coinfection interactions, none of these interactions were significantly correlated with host stress levels. As increasing GI parasite species richness has been associated with poorer host body condition, the lack of association between coinfection richness and stress hormones supports the idea that parasites do not cause these hosts significant stress (Lello et al. 2005). While the intensities of strongyles and *Strongyloides* significantly predicted the presence of each other, higher rainfall in conjunction with higher *Strongyloides* intensities predicted lower strongyles, perhaps indicating competition between the two parasites. Interestingly, there were no interactions between strongyles and *Eimeria*, even though *Eimeria* is usually controlled by a Th1-type immune response potentially down-regulated by the strongyle-invoked Th2-type response (Hong et al. 2006). *Eimeria* and *Strongyloides* predicted increases in each other's intensities, though *Eimeria* appeared to be more permissive toward *Strongyloides* establishment; it was 34% less likely for females with high *Eimeria* counts to have no *Strongyloides*. Interestingly, while coinfections overall predicted the presence of *Strongyloides*, both strongyles and *Eimeria* significantly predicted more zero *Strongyloides* counts in males; thus, male springbok appeared more likely than females to have singular parasite infections. This finding supports the hypothesis that male behavior plays a part in parasite transmission differences.

Finally, age affected stress and pathogens. Older zebras were more likely to have higher stress hormone levels, lower strongyle intensities, and lower lymphocyte counts, whereas younger springbok were more likely to have higher FGM and higher intensities of all three GI macroparasites. The declining parasite intensities with age suggest that anti-parasite acquired immune responses with cumulative parasite exposure may be at least somewhat effective in controlling parasite loads (Cattadori et al. 2005; Cornell et al. 2008). In addition, older zebra and springbok in ENP shed significantly more L4 larvae than did younger animals, indicating more active immunity against parasites with age (W. C. Turner, unpublished data). This also supports the hypothesis that parasites are age limiting in ENP, and that animals that survive for longer are those that are less genetically susceptible to higher parasite loads (Keith et al. 1985; Gulland 1992; Coltman et al.

1999; Kamath 2011; see dissertation Chapter 3). The lower lymphocyte counts in older zebra also supports this idea, as animals less genetically susceptible, and more tolerant, to parasites would have to allocate fewer immune resources toward maintaining immunity to these pathogens. The increased stress in younger springbok was somewhat equivocal, as age and stress had different relationships in different models. The increased stress levels with age in zebra may reflect aging stress alone (Archie et al. 2012), or could partially reflect the increased reproductive stress in adults compared to in pre-breeding yearlings.

### *Conclusions*

In summary, I found evidence that stress, reproduction, immunity, and pathogens exhibited strong seasonality in ENP. Most zebra and springbok in ENP are indeed seasonal breeders, with both species experiencing parturition and lactation primarily in the wet season, zebra experiencing most estrus and breeding behaviors in the wet season, and both species experiencing mid-gestation in the hot dry season. Overall immune condition is best during the wet season, likely driven by nutritional factors, though wet season neutrophilia also may reflect increased inflammatory responses likely driven by breeding behaviors. Stress hormone levels are highest overall in the dry season, though these seasonal signals are stronger in zebras. While peaks in reproductive hormone levels are correlated with immunosuppression and increased parasite infection intensity, stress hormone levels are largely unaffected by pathogen prevalence or infection intensity. Instead, stress hormone levels are driven primarily by environmental and reproductive stressors. Given the evidence that GI parasites are capable of causing host pathology, causing immunomodulation and immunosuppression, and controlling population age structures, the fact that these parasites persist in ENP hosts without causing chronic stress responses supports the hypothesis that hosts are tolerant of their parasites.

Hosts that are good at reducing or clearing parasite burdens are not necessarily the healthiest; eliminating parasites is immunologically costly, and hosts risk immunopathology with vigorous immune responses (Allen and Maizels 2011). In addition, as immunity and reproductive efforts fight for the same resources, increased immune responses, particularly in the face of external resource limitations, can reduce host fecundity. Thus, hosts that can tolerate parasites may have advantages over those that cannot. Tolerance reflects the rate of decline in fitness as parasite burden increases; less fitness decline with increased parasite infection intensity indicates higher parasite tolerance (Råberg et al. 2009). Mechanisms of tolerance can involve immune responses against parasite-derived substances rather than against the parasites themselves, or can involve pathways that decrease immunopathology such as increased regulatory T cells (Råberg et al. 2011; Allen and Maizels 2011). Such tolerance mechanisms are selected for when there are high rates of infection but relatively low virulence (Restif and Koella 2004); while GI parasites in ENP do affect host condition, immune function, and susceptibility to other pathogens, the fact that they are nearly ubiquitous in herbivore hosts suggests that their overall virulence is low, particularly compared to other pathogens in the system such as *B. anthracis*. In addition, while the evolution



of resistance should reduce parasite prevalence in host populations, the evolution of tolerance to parasites should have a neutral or positive effect on parasite prevalence, as is observed in herbivores in ENP (Råberg et al. 2011). Thus, it appears that, while GI parasites may influence susceptibility to anthrax and ticks in ENP hosts through immunomodulation (see dissertation Chapter 3), these parasites do not increase host disease susceptibility through stress. Understanding such mechanisms of pathogen-hormone-immune interaction are essential for determining how and why pathogens persist in ecosystems, and what factors may influence the establishment of emerging infectious diseases in sensitive populations.

### **Acknowledgments**

I thank the Namibian Ministry of Environment and Tourism for permission to do this research, the Directorate of Parks, Wildlife and Management for permission to work throughout Etosha, and the staff in the Directorate of Scientific Services at the Etosha Ecological Institute for logistical support and assistance. I would like to give special thanks to veterinarians Mark Jago, Conrad Brain, Peter Morkel, and Ortwin Aschenborn for their assistance with animal captures, as well as to Martina Küsters, Shayne Kötting, Gabriel Shatumbu, Wendy Turner, Wilferd Versfeld, Marthin Kasaona, Royi Zidon, and Werner Kilian, among others, for their tremendous help in the field. Among those in the field capture team, I especially thank Wendy Turner and Martina Küsters for their extra assistance with sample collection and GI parasite quantification, and Wendy for the use of her stockpiled samples and data. I greatly thank Neville Pitts for doing all of the steroid hormone metabolite assays on my extracted samples. I thank Russell Vance for allowing me to work in his immunology lab and for giving me assistance and guidance, and Steve Bellan for assistance with the anti-PA ELISA endpoint titer determinations. This research was supported by Andrew and Mary Thompson Rocca Scholarships and NIH grant GM83863.

## Tables

**Table 1.** Zebra capture seasons, timing, animals involved, and samples taken. "CS" is Capture Season and "NS" is Nominal Season. Data refer to Total#(#New, #Resampled), where and "New" refers to new individuals and their samples and "Resampled" refers to animals resampled at least once in that season and their corresponding samples collected. "Ticks" refers to the number of zebras sampled for total tick burden.

CS	NS	Date (Mo/Yr)	Blood	Feces	Ticks
S1	Wet	3-4/08	45(45,0)	38(38,0)	45(45,0)
S2	Dry	10-11/08	36(14,22)	29(17,12)	18(0,18)
S3	Wet	4-5/09	35(6, 29)	32(4,28)	30(5,25)
S4	Dry	9-11/09	13(4,9)	10(3,7)	13(4,9)
S5	Dry	8/10	25(0,25)	14(0,14)	19(0,19)
<b>Totals</b>			154(69,85)	123(62,61)	125(54,71)

**Table 2.** A. Number of zebra captured in each season for first captures only, grouped by rain groups. B. Number of zebra captured in each season for paired recaptured only, grouped by rain groups. There were no resampled animals that fell into Cap1RG2 or Cap2RG1 groups. Cap1 is capture 1; Cap2 is capture 2 for the same individual; RG1 is rain group 1 (experience of cumulative rainfall  $\geq 200$ mm over the two months prior to capture); RG2 is rain group 2 (experience of cumulative rainfall  $\leq 100$ mm over the two month prior to sampling).

A.	Capture Season	Cap1RG1	Cap1RG2	B.	Capture Season	Cap1RG1	Cap2RG2
	S1	45	0		S1	32	0
	S2	0	14		S2	0	23
	S3	0	6		S3	0	8
	S4	0	4		S4	0	1
	S5	0	0		S5	0	0

**Table 3.** List of variables used in models, with their abbreviations and descriptions.

Variable	Abbreviation	Subjects	Description
Cumulative rain 2 months prior	Rain2	All	Rain (mm) experienced by an individual in 60 days prior to a capture event
Sex	Sex; M or F	All	Male (M) or female (F)
Individual age	Age	Captured (C) Zebras	Age (days) at a sampling event, determined first by dental wear
Age Class	AgeC; A or Y	NC zebras; springbok	Adults (>2 years old; A) or yearlings (<2 years old; Y)
GI Strongyle parasite burden	GIP, or GIPsqrt when square root transformed	All	GI strongyle helminth infection intensity (nematode eggs/g of feces)
GI coccidia burden	GIC, GICsqrt, or GIC4 when 4 <sup>th</sup> root transformed	Springbok	GI coccidian infection intensity ( <i>Eimeria</i> oocysts/g of feces)
GI <i>Strongyloides</i> burden	GIS, GISsqrt, or GIS4	Springbok	GI <i>Strongyloides</i> helminth infection intensity (nematode eggs/g of feces)
Sublethal anthrax exposure	log2PA or PA	C zebras	Anti-PA antibody titer as measured in log <sub>2</sub> of final dilution (log2PA) or as presence or absence of a titer (PA)
Ectoparasite burden	Ecto, or Ectosqrt	C zebras	Total number of ticks
Hematocrit	HCT or HCTsqrt	C zebras	% of blood volume comprised of red blood cells
White blood cell count	WBC, or WBCsqrt	C zebras	Number of total white blood cells / $\mu$ l of blood
Neutrophil count	Neut, or logNeut when log <sub>10</sub> transformed	C zebras	Number of neutrophils/ $\mu$ l of blood
Lymphocyte count	Lymph, or Lymphsqrt	C zebras	Number of lymphocytes/ $\mu$ l of blood
Fecal glucocorticoid metabolites	FGM, logFGM, or FGM4	All	Glucocorticoid metabolite concentration in feces (ng/g of dry fecal weight)
Fecal progesterone metabolites	FPM, FPMsqrt, or FPM4	All (females)	Progesterone metabolite concentration in feces (ng/g of dry fecal weight)
Fecal estrogen metabolites	FEM, logFEM, or FEM4	All (females)	Estrogen metabolite concentration in feces (pg/g of dry fecal weight)
Fecal testosterone metabolites	FTM	NC zebra, springbok (males)	Testosterone metabolite concentration in feces (ng/g dry fecal weight)
Foal presence	Foal	C zebras	Presence or absence of a foal with a mother
Pregnancy	Preg	C zebras	Obvious presence (2), obvious absence (0), and likely but unconfirmed presence (1) of pregnancy
Lactating	Lact	C zebras	Lactating milk (2), watery discharge (1), or no lactation (0)

**Table 4.** Maximal generalized estimating equation models evaluated for captured zebras.

<b>Pathogen Models</b>	
GIPsqr	~ Rain2 + Neut + Lymph + HCT + FGM + FPM + FEM + log2PA + Ecto + Age + Foal + Preg + Lact
PA	~ Rain2 + Neut + Lymph + HCT + logFGM + FPM + FEM + GIP + Ecto + Age + Foal + Preg + Lact
Ectosqr	~ Rain2 + Neut + Lymph + HCT + FGM + FPM + FEM + log2PA + GIP + Age + Foal + Preg + Lact
<b>Immune Models</b>	
logNeut	~ Rain2 + Lymph + HCT + FGM + FPM + FEM + log2PA + GIP + Ecto + Age + Foal + Preg + Lact
sqrLymph	~ Rain2 + logNeut + HCT + FGM + FPM + FEM + log2PA + GIPsqr + Ecto + Age + Foal + Preg + Lact
<b>Hormone Model</b>	
logFGM	~ Rain2 + Neut + Lymph + HCT + FPM + FEM + log2PA + GIP + Ecto + Age + Foal + Preg + Lact
<b>Age Model</b>	
Agesqr	~ Neut + Lymph + HCT + FGM + FPM + FEM + log2PA + GIP + Ecto + Foal + Preg + Lact

**Table 5.** Maximal generalized linear models evaluated for **non-captured zebras**.

<b>Strongyle Helminth Models</b>	
GIP	~ Rain2 + AgeC + Sex + FGM
GIP	~ Rain2 + AgeC + Sex + FGM + FPM4 + logFEM
GIP (males only)	~ Rain2 + FGM + FTM
<b>Hormone Models</b>	
FGM	~ Rain2 + AgeC + Sex + GIP
FGM	~ Rain2 + AgeC + Sex + GIP + FPM4 + logFEM
FGM (males only)	~ Rain2 + GIP + FTM

All GIP models use the negative binomial distribution with a log link, while all FGM models use the gamma distribution with an inverse link.

**Table 6.** Maximal generalized linear models and zero-inflated models evaluated for **springbok**.

<b>Strongyle Helminth Models</b>	
GIP	~ Rain2 + AgeC + Sex + GISqrt + GIC4 + FGM
GIP	~ Rain2 + AgeC + Sex + GISqrt + GIC4 + FGM + FPM4 + logFEM
GIP (males only)	~ Rain2 + GISqrt + GIC4 + FGM + FTM
<b>Strongyloides Helminth Models</b>	
GIS	~ Rain2 + AgeC + Sex + GIPsqrt + GIC4 + FGM
GIS	~ Rain2 + AgeC + Sex + GIPsqrt + GIC4 + FGM + FPM + FEM
GIS (males only)	~ Rain2 + GIPsqrt + GIC4 + FGM + FTM
<b>Eimeria Models</b>	
GIC	~ Rain2 + AgeC + Sex + GIPsqrt + GISqrt + FGM
GIC	~ Rain2 + AgeC + Sex + GIPsqrt + GISqrt + FGM + FPM + FEM
GIC (males only)	~ Rain2 + GIPsqrt + GISqrt + FGM + FTM
<b>Hormone Models</b>	
FGM	~ Rain2 + AgeC + Sex + GIPsqrt + GISqrt + GIC4
FGM	~ Rain2 + AgeC + Sex + GIPsqrt + GISqrt + GIC4 + FPM + FEM
FGM (males only)	~ Rain2 + GIPsqrt + GISqrt + GIC4 + FTM

All GIP and GIC generalized linear models use the negative binomial distribution with a log link, while all FGM generalized linear models use the gamma distribution with an inverse link. All GIS models are zero inflated negative binomial models; counts portions of the models use the negative binomial distribution with a log link, while zero-inflated portions of the models use a binomial distribution with a logit link.

**Table 7.** Results of two-tailed Welch's *t* tests and Wilcoxon rank sum tests comparing variables between rain groups for **first captured zebra** samplings (unique animals) only. Significant tests ( $p \leq 0.05$ ) are in bold.  $N=38$  for all RG1 variables and 24 for all RG2.

Variable	df	<i>t</i> or <i>U</i>	<i>p</i> -value before correction	Holm's corrected <i>p</i> - value	Mean Difference <sup>#</sup>	Higher Group
<b>GIPsqr</b>	54.5	5.07	<b>0.000***</b>	<b>0.000***</b>	1807	<b>RG1</b>
log2PA		561 <sup>+</sup>	0.117	0.490	1.18	RG1
Ecto		420 <sup>+</sup>	0.604	1.000	0.23	RG2
<b>WBCsqr</b>	57.7	2.80	<b>0.007**</b>	<b>0.042*</b>	1213	<b>RG1</b>
<b>logNeut</b>	54.9	3.41	<b>0.001***</b>	<b>0.010**</b>	870	<b>RG1</b>
Lymphsqr	49.1	0.70	0.488	1.000	148	RG1
<b>HCTsqr</b>	46.1	3.15	<b>0.003**</b>	<b>0.020*</b>	2.72	<b>RG1</b>
logFGM	46.7	-1.69	0.098.	0.490	357	RG2
FPMsqr	53.0	0.90	0.371	1.000	175	RG1
<b>logFEM</b>	56.6	-8.30	<b>0.000***</b>	<b>0.000***</b>	6592	<b>RG2</b>

<sup>+</sup> are Wilcoxon rank sum test results, using the test statistic *U*.

<sup>#</sup> Mean differences are differences between non-transformed means in the two rain groups for all variables. Units for mean differences are eggs per gram of feces for GIP; log<sub>2</sub> titer for log<sub>2</sub>PA; number of ticks for Ecto; cells/μl of blood for WBC, Neut, and Lymph; percent for HCT; ng/g of fecal dry weight for FGM and FPM; and pg/g of fecal dry weight for FPM.

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$



**Table 8.** Results of paired two-tailed *t* tests and Wilcoxon signed rank tests comparing variables between rain groups for first and second **captured zebra samplings of the same individuals**. Significant tests ( $p \leq 0.05$ ) are in bold.  $N=28$  (and  $df=27$ ) for all variables in both rain groups.

Variable	<i>t</i> or <i>T</i>	<i>p</i> -value before correction	Holm's corrected <i>p</i> -value	Mean Difference <sup>#</sup>	Higher Group
<b>GIPsqr</b>	4.88	<b>0.000***</b>	<b>0.000***</b>	1926	<b>RG1</b>
log2PA	136 <sup>+</sup>	0.698	1.000	0.18	RG2
<b>Ecto</b>	48.5 <sup>+</sup>	<b>0.004**</b>	<b>0.019*</b>	2.61	<b>RG2</b>
<b>WBCsqr</b>	3.59	<b>0.001***</b>	<b>0.010**</b>	1836	<b>RG1</b>
<b>logNeut</b>	3.54	<b>0.002**</b>	<b>0.010**</b>	1046	<b>RG1</b>
<b>Lymphsqr</b>	3.25	<b>0.003**</b>	<b>0.019*</b>	629	<b>RG1</b>
HCTsqr	2.47	<b>0.020*</b>	0.080.	2.61	RG1
logFGM	-2.06	<b>0.049*</b>	0.147	649	RG2
FPMsqr	-0.62	0.539	1.000	118	RG2
<b>logFEM</b>	-5.67	<b>0.000***</b>	<b>0.000***</b>	5194	<b>RG2</b>

<sup>+</sup> are Wilcoxon signed rank test results, using the test statistic *T*.

<sup>#</sup> Mean differences are differences between non-transformed means in the two rain groups for all variables. Units for mean differences are eggs per gram of feces for GIP; log<sub>2</sub> titer for log<sub>2</sub>PA; number of ticks for Ecto; cells/μl of blood for WBC, Neut, and Lymph; percent for HCT; ng/g of fecal dry weight for FGM and FPM; and pg/g of fecal dry weight for FPM.

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 9.** Maximum likelihood estimates for the best fit generalized estimating equation pathogen models for **captured zebras**. Significant coefficients ( $p \leq 0.05$ ) are in bold.

Response	Coefficients	Estimate $\pm$ SE	Wald statistic	$p$ -value	% QIC reduced from full model
GIPsqr	<b>Intercept</b>	35.5 $\pm$ 2.36	227	<b>0.000***</b>	2.82
	<b>Rain2</b>	0.09 $\pm$ 0.01	52.3	<b>0.000***</b>	
	<b>Neut</b>	-0.01 $\pm$ 0.01	5.47	<b>0.019*</b>	
	<b>Lymph</b>	0.01 $\pm$ 0.01	11.1	<b>0.001***</b>	
	<b>log2PA</b>	-0.72 $\pm$ 0.34	4.47	<b>0.035*</b>	
	<b>Lact</b>	3.26 $\pm$ 0.94	12.0	<b>0.001***</b>	
PA	<b>Intercept</b>	-2.55 $\pm$ 0.71	12.5	<b>0.001***</b>	24.9
	Rain2	-6.92e-05 $\pm$ 6.13e-04	0.01	0.910	
	<b>Lymph</b>	1.36e-04 $\pm$ 6.15e-05	4.88	<b>0.027*</b>	
	<b>logFGM</b>	0.88 $\pm$ 0.20	20.5	<b>0.000***</b>	
	<b>FEM</b>	-2.55e-05 $\pm$ 8.95e-06	8.09	<b>0.005**</b>	
	GIP	-4.08e-05 $\pm$ 3.33e-05	1.50	0.221	
	<b>Rain2*FEM</b>	2.77e-07 $\pm$ 9.95e-08	7.77	<b>0.005**</b>	
Ectosqr	<b>Intercept</b>	1.55 $\pm$ 0.22	51.3	<b>0.000***</b>	9.09
	Rain2	3.78e-04 $\pm$ 1.02e-03	0.14	0.711	
	FEM	4.10e-05 $\pm$ 2.12e-05	3.75	0.053.	
	<b>Preg</b>	0.56 $\pm$ 0.20	7.72	<b>0.006**</b>	
	<b>Lact</b>	0.21 $\pm$ 0.08	7.04	<b>0.008**</b>	
	<b>Rain2*Preg</b>	-2.47e-03 $\pm$ 1.05e-03	5.53	<b>0.019*</b>	
	Rain2*FEM	-3.96e-07 $\pm$ 2.12e-07	3.48	0.062.	

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 10.** Maximum likelihood estimates for the best fit generalized estimating equation immunity and hormone models for **captured zebras**. Significant coefficients ( $p \leq 0.05$ ) are in bold.

Response	Coefficients	Estimate $\pm$ SE	Wald statistic	$p$ -value	% QIC reduced from full model
logNeut	<b>Intercept</b>	2.70 $\pm$ 0.14	361	<b>0.000***</b>	30.7
	<b>Rain2</b>	6.29e-04 $\pm$ 2.00e-04	9.94	<b>0.002**</b>	
	<b>Lymph</b>	1.51e-04 $\pm$ 1.80e-05	70.7	<b>0.000***</b>	
	<b>HCT</b>	7.14e-03 $\pm$ 3.25e-03	4.84	<b>0.028*</b>	
	<b>FPM</b>	6.56e-05 $\pm$ 1.45e-05	20.5	<b>0.000***</b>	
	<b>FEM</b>	-6.95e-06 $\pm$ 3.43e-06	4.11	<b>0.043*</b>	
	<b>GIP</b>	-2.65e-05 $\pm$ 8.26e-06	10.3	<b>0.001***</b>	
	<b>Ecto</b>	-5.78e-03 $\pm$ 2.64e-03	4.78	<b>0.029*</b>	
	<b>Lact</b>	0.03 $\pm$ 0.01	6.91	<b>0.009**</b>	
Lymphsqr	<b>Intercept</b>	-0.47 $\pm$ 6.41	55.4	<b>0.000***</b>	8.19
	<b>Rain2</b>	-0.01 $\pm$ 5.83e-03	5.41	<b>0.020*</b>	
	<b>logNeut</b>	0.29 $\pm$ 1.82	247	<b>0.000***</b>	
	<b>GIPsqr</b>	0.12 $\pm$ 0.04	11.3	<b>0.000***</b>	
	<b>Age</b>	-3.10e-03 $\pm$ 6.22e-04	24.9	<b>0.000***</b>	
	<b>Preg</b>	-1.72 $\pm$ 0.42	16.5	<b>0.000***</b>	
logFGM	<b>Intercept</b>	3.43 $\pm$ 0.03	12259	<b>0.000***</b>	52.7
	<b>Rain2</b>	-3.60e-04 $\pm$ 1.67e-04	4.66	<b>0.031*</b>	
	<b>FPM</b>	2.72e-05 $\pm$ 1.79e-05	2.31	<b>0.129</b>	
	<b>FEM</b>	1.08e-05 $\pm$ 2.70e-06	16.0	<b>0.000***</b>	
	<b>log2PA</b>	0.01 $\pm$ 4.10e-03	8.59	<b>0.003**</b>	
	<b>Rain2*FPM</b>	2.57e-07 $\pm$ 1.23e-07	1.23e-07	<b>0.037*</b>	
Agesqr	<b>Intercept</b>	51.3 $\pm$ 1.43	1278	<b>0.000***</b>	8.48
	<b>Lymph</b>	-0.01 $\pm$ 0.01	18.2	<b>0.000***</b>	
	<b>Foal</b>	0.37 $\pm$ 1.64	0.05	0.822	
	<b>Lact</b>	3.45 $\pm$ 0.78	19.4	<b>0.000***</b>	
	<b>Foal*Lact</b>	-2.61 $\pm$ 1.12	5.25	<b>0.022*</b>	

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 11.** Results of Tukey's HSD tests or two-tailed Wilcoxon rank sum tests comparing variables between rain, sex, and age groups for **non-captured zebra**. Significant tests ( $p \leq 0.05$ ) are in bold. For non-normal variables, interaction effect comparisons were only explored with Wilcoxon rank sum tests if preliminary explorations with ANOVA and Tukey HSD tests revealed a  $p \leq 0.1$ . For normalized variables examined with Type III ANOVAs and Tukey HSD tests, only significant interaction comparisons ( $p \leq 0.05$ ) are listed here. N's are listed as males first; then yearlings first; then RG1 first.

Variable	Comparison	N	U <sup>+</sup>	Tukey's or Holm's corrected <i>p</i> - value	Mean Difference <sup>#</sup>	Higher Group
GIPsqr	<b>Rain Groups</b>	169, 143		<b>0.001***</b>	479	<b>RG1</b>
	<b>Sexes</b>	158, 154		<b>0.003**</b>	473	<b>males</b>
	Ages	33, 279		0.449	353	yearlings
	<b>MY, FY@</b>	22, 11		<b>0.012*</b>	1764	<b>MY</b>
	<b>MY, FA</b>	22, 143		<b>0.012*</b>	1095	<b>MY</b>
FGM	<b>Rain Groups</b>	165, 137	15910	<b>0.000***</b>	693	<b>RG1</b>
	<b>Sexes</b>	154, 148	8979	<b>0.016*</b>	320	<b>females</b>
	<b>Ages</b>	31, 271	3029	<b>0.022*</b>	369	<b>adults</b>
	<b>MY, FY</b>	22, 9	44	<b>0.022*</b>	960	<b>FY</b>
	<b>MY, MA</b>	22, 132	922	<b>0.019*</b>	528	<b>MA</b>
	<b>MY, FA</b>	22, 139	733	<b>0.000***</b>	761	<b>FA</b>
	<b>RG1M, RG2M</b>	80, 74	3901	<b>0.006**</b>	565	<b>RG1M</b>
	<b>RG1M, RG1F</b>	80, 85	2680	<b>0.047*</b>	388	<b>RG1F</b>
	<b>RG1M, RG2F</b>	80, 63	3042	<b>0.047*</b>	408	<b>RG1M</b>
	<b>RG2M, RG1F</b>	74, 85	1301	<b>0.000***</b>	953	<b>RG1F</b>
	<b>RG1F, RG2F</b>	85, 63	3978	<b>0.000***</b>	796	<b>RG1F</b>
	<b>RG1Y, RG1A</b>	20, 145	881	<b>0.029*</b>	618	<b>RG1A</b>
	<b>RG2Y, RG1A</b>	11, 145	346	<b>0.014*</b>	906	<b>RG1A</b>
	<b>RG1A, RG2A</b>	145, 126	13338	<b>0.000***</b>	756	<b>RG1A</b>
FPM	<b>Rain Groups</b>	103, 60	3901	<b>0.016*</b>	310	<b>RG1</b>
	<b>Sexes</b>	59, 104	2337	<b>0.023*</b>	295	<b>females</b>
	<b>Ages</b>	9, 154	377	<b>0.023*</b>	494	<b>adults</b>
	<b>RG2M, RG1F</b>	26, 70	500	<b>0.003**</b>	529	<b>RG1F</b>
FEM	<b>Rain Groups</b>	103, 60	1739	<b>0.000***</b>	1961	<b>RG2</b>
	<b>Sexes</b>	59, 104	3062	0.984	1574	females
	<b>Ages</b>	9, 154	424	0.102	1886	adults
	<b>RG1M, RG2F</b>	33, 34	288	<b>0.002**</b>	3962	<b>RG2F</b>
	<b>RG2M, RG1F</b>	26, 70	1225	<b>0.038*</b>	235	<b>RG2M</b>
	<b>RG2M, RG2F</b>	26, 34	309	0.143	3728	RG2F
	<b>RG1F, RG2F</b>	70, 34	557	<b>0.000***</b>	3395	<b>RG2F</b>
FTMsqr	<b>Rain Groups</b>	64, 39		0.535	2.18	RG2
	<b>Ages</b>	15, 88		<b>0.000***</b>	22.4	<b>adults</b>

@MY=male yearlings; MA=male adults; FY=female yearlings; FA=female adults

+ Comparisons listed with a *U* test statistic were analyzed with Wilcoxon rank sum tests. *p* values for these tests were adjusted for familywise error rates using the Holm's Bonferroni correction.

# Mean differences are differences between non-transformed means for all variables. Units for mean differences are eggs per gram of feces for GIP; ng/g of fecal dry weight for FGM and FPM; and pg/g of fecal dry weight for FPM. .  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 12.** Results of Tukey's HSD tests or two-tailed Wilcoxon rank sum tests comparing variables between rain, sex, and age groups for **springbok**. Significant tests ( $p \leq 0.05$ ) are in bold. For non-normal variables, interaction effect comparisons were only explored with Wilcoxon rank sum tests if preliminary explorations with ANOVA and Tukey HSD tests revealed a  $p \leq 0.1$ . For normalized variables examined with Type III ANOVAs and Tukey HSD tests, only significant interaction comparisons ( $p \leq 0.05$ ) are listed here. *N*'s are listed as males always first; then yearlings first; then RG1 first.

Variable	Comparison	<i>N</i>	<i>U</i> <sup>+</sup>	Tukey's or Holm's corrected <i>p</i> -value	Mean Difference <sup>#</sup>	Higher Group
GIP	<b>Rain Groups</b>	156, 113	14155	<b>0.000***</b>	873	<b>RG1</b>
	Sexes	128, 141	8714	0.626	115	females
	Ages	56, 213	7102	0.084.	394	yearlings
	MY, FY	22, 34	502	0.084.	662	MY
	<b>MY, MA</b>	22, 106	1722	<b>0.002**</b>	935	<b>MY</b>
	<b>MY, FA</b>	22, 107	1587	<b>0.041*</b>	658	<b>MY</b>
GIS	<b>Rain Groups</b>	156, 113	14031	<b>0.000***</b>	405	<b>RG1</b>
	Sexes	128, 141	8743	0.655	29.7	females
	<b>Ages</b>	56, 213	7142	<b>0.043*</b>	200	<b>yearlings</b>
GIC	<b>Rain Groups</b>	156, 113	14210	<b>0.000***</b>	6617	<b>RG1</b>
	Sexes	128, 141	9468	0.483	1289	males
	<b>Ages</b>	56, 213	7729	<b>0.002**</b>	2397	<b>yearlings</b>
	<b>MY, FA</b>	22, 107	1678	<b>0.003**</b>	2561.13	<b>MY</b>
FGM	Rain Groups	151, 111	8147	0.701	971	RG2
	Sexes	127, 135	9696	0.137	459	males
	Ages	55, 207	4694	0.137	1128	adults
	MY, MA	22, 105	767	0.054.	1722	MA
	<b>RG1M, RG1F</b>	70, 81	3735	<b>0.005**</b>	976	<b>RG1M</b>
	RG1F, RG2F	81, 54	1742	0.183	1604	RG2F
FPM4	<b>Rain Groups</b>	63, 49		<b>0.020*</b>	134	<b>RG1</b>
	Sexes	24, 88		0.543	21.5	males
	Ages	22, 90		0.228	36.6	adults
FEM4	<b>Rain Groups</b>	63, 49		<b>0.000***</b>	1053	<b>RG2</b>
	Sexes	24, 88		0.101	613	males
	Ages	22, 90		0.302	197	adults
	<b>RG2M, RG1F</b>	18, 56		<b>0.000***</b>	1123	<b>RG2M</b>
	<b>RG1F, RG2F</b>	56, 32		<b>0.000***</b>	1045	<b>RG2F</b>
FTMsqrt	Rain Groups	45, 32		0.847	4.62	RG2

@ MY=male yearlings; MA=male adults; FY=female yearlings; FA=female adults

+ Comparisons listed with a *U* test statistic were analyzed with Wilcoxon rank sum tests. *p* values for these tests were adjusted for familywise error rates using the Holm's Bonferroni correction.

# Mean differences are differences between non-transformed means for all variables. Units for mean differences are eggs per gram of feces for GIP and GIS; oocysts per gram of feces for GIC; ng/g of fecal dry weight for FGM and FPM; and pg/g of fecal dry weight for FPM.

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 13.** Maximum likelihood estimates for the best fit generalized linear models for **non-captured zebras**. Significant coefficients ( $p \leq 0.05$ ) are in bold.

Response	Coefficients	Estimate $\pm$ SE	z Value	Pr(> z )	N	$\Delta$ AIC
GIP	<b>Intercept</b>	8.12 $\pm$ 0.12	69.0	<b>0.000***</b>	302	2.00
	<b>Rain2</b>	2.19e-03 $\pm$ 3.84e-04	5.71	<b>0.000***</b>		
	<b>Sex</b>	-0.16 $\pm$ 0.07	-2.17	<b>0.030*</b>		
	<b>FGM</b>	-1.57e-04 $\pm$ 4.31e-05	-3.63	<b>0.000***</b>		
GIP	<b>Intercept</b>	7.70 $\pm$ 2.31e-03	17.5	<b>0.000***</b>	163	13.7
	Rain2	2.79e-03 $\pm$ 2.31e-03	1.21	0.228		
	FGM	2.35e-04 $\pm$ 1.21e-04	1.94	0.052.		
	<b>logFEM</b>	-0.35 $\pm$ 0.12	-2.80	<b>0.005**</b>		
	<b>rain2*FGM</b>	-1.69e-06 $\pm$ 5.64e-07	-3.00	<b>0.003**</b>		
	<b>rain2*logFEM</b>	1.80e-03 $\pm$ 6.43e-04	2.80	<b>0.005**</b>		
GIP (males)	<b>Intercept</b>	8.15 $\pm$ 0.17	48.6	<b>0.000***</b>	103	2.30
	<b>Rain2</b>	3.34e-03 $\pm$ 5.05e-04	6.612	<b>0.000***</b>		
	<b>FGM</b>	-2.55e-04 $\pm$ 6.26e-05	-4.07	<b>0.000***</b>		
FGM	<b>Intercept</b>	5.20e-04 $\pm$ 5.42e-05	9.59	<b>0.000***</b>	302	8.30
	<b>Rain2</b>	-3.25e-07 $\pm$ 9.06e-08	-3.59	<b>0.000***</b>		
	<b>AgeC</b>	-7.77e-05 $\pm$ 2.63e-05	-2.95	<b>0.003**</b>		
	<b>Sex</b>	-2.27e-04 $\pm$ 7.55e-05	-3.01	<b>0.003**</b>		
	<b>GIP</b>	2.29e-08 $\pm$ 6.82e-09	3.36	<b>0.001***</b>		
	<b>AgeC*Sex</b>	1.08e-04 $\pm$ 3.89e-05	2.78	<b>0.006**</b>		
	Rain2*GIP	-6.74e-11 $\pm$ 3.43e-11	-1.96	0.051.		
FGM	<b>Intercept</b>	4.52e-04 $\pm$ 5.21e-05	8.68	<b>0.000***</b>	163	3.30
	<b>Rain2</b>	-4.21e-07 $\pm$ 5.79e-08	-7.28	<b>0.000***</b>		
	Sex	2.22e-05 $\pm$ 1.17e-05	1.91	0.058.		
	<b>GIP</b>	4.00e-08 $\pm$ 1.92e-08	2.08	<b>0.039*</b>		
	<b>FPM4</b>	-1.60e-05 $\pm$ 5.88e-06	-2.72	<b>0.007**</b>		
	logFEM	-6.04e-06 $\pm$ 1.69e-05	-0.36	0.721		
	GIP*logFEM	-1.24e-08 $\pm$ 6.98e-09	-1.78	0.077.		
FGM (males)	<b>Intercept</b>	3.64e-04 $\pm$ 2.99e-05	12.2	<b>0.000***</b>	103	6.50
	<b>Rain2</b>	-2.52e-07 $\pm$ 1.20e-07	-2.10	<b>0.038*</b>		
	<b>GIP</b>	4.98e-08 $\pm$ 1.15e-08	4.34	<b>0.000***</b>		
	<b>FTM</b>	-1.27e-06 $\pm$ 3.15e-07	-4.04	<b>0.000***</b>		
	<b>Rain2*GIP</b>	-1.46e-10 $\pm$ 4.97e-11	-2.94	<b>0.004**</b>		

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Table 14.** Maximum likelihood estimates for the best fit generalized linear models for **springbok** helminths. Significant coefficients ( $p \leq 0.05$ ) are in bold.

Response	Coefficients	Estimate $\pm$ SE	z Value	Pr(> z )	N	$\Delta$ AIC
GIP	<b>Intercept</b>	4.50 $\pm$ 0.22	20.6	<b>0.000***</b>	26 7	18.8
	<b>Rain2</b>	0.01 $\pm$ 1.16e-03	8.93	<b>0.000***</b>		
	<b>GISsqrt</b>	0.08 $\pm$ 0.012	6.73	<b>0.000***</b>		
	FGM	4.46e-05 $\pm$ 2.30e-05	1.94	0.052.		
	<b>Rain2*GISsqrt</b>	-3.67e-04 $\pm$ 7.87e-05	-4.67	<b>0.000***</b>		
GIP	<b>Intercept</b>	4.74 $\pm$ 0.24	19.4	<b>0.000***</b>	11 4	10.9
	<b>Rain2</b>	0.01 $\pm$ 0.01	5.75	<b>0.000***</b>		
	<b>GISsqrt</b>	0.09 $\pm$ 0.02	3.74	<b>0.000***</b>		
	<b>Rain2*GISsqrt</b>	-4.49e-04 $\pm$ 1.43e-04	-3.14	<b>0.002**</b>		
GIP (males)	<b>Intercept</b>	4.75 $\pm$ 0.26	18.3	<b>0.000***</b>	77	44.2
	<b>Rain2</b>	8.49e-03 $\pm$ 1.89e-03	4.50	<b>0.000***</b>		
	<b>GISsqrt</b>	0.10 $\pm$ 0.03	4.09	<b>0.000***</b>		
	<b>Rain2*GISsqrt</b>	-3.87e-04 $\pm$ 1.44e-04	-2.69	<b>0.007**</b>		
GIS Counts	<b>Intercept</b>	6.64 $\pm$ 0.21	32.4	<b>0.000***</b>	26 7	41.7
	<b>Rain2</b>	-3.00e-03 $\pm$ 9.78e-04	-3.06	<b>0.002**</b>		
	<b>GIC4</b>	0.07 $\pm$ 0.02	3.50	<b>0.001***</b>		
	<b>FGM</b>	-1.63e-05 $\pm$ 1.13e-06	-40.1	<b>0.000***</b>		
GIS Zeros	<b>Intercept</b>	2.80 $\pm$ 0.56	5.01	<b>0.000***</b>		
	<b>Rain2</b>	-9.30e-03 $\pm$ 3.10e-03	-3.00	<b>0.003**</b>		
	<b>GIPsqrt</b>	-0.08 $\pm$ 0.02	-3.6	<b>0.000***</b>		
	<b>GIC4</b>	-0.30 $\pm$ 0.09	-3.19	<b>0.001***</b>		
GIS Counts	<b>Intercept</b>	6.65 $\pm$ 0.16	43.0	<b>0.000***</b>	11 4	4.33
	<b>FEM</b>	-2.13e-04 $\pm$ 8.12e-05	-2.62	<b>0.009**</b>		
	<b>Intercept</b>	3.16 $\pm$ 1.09	2.89	<b>0.004**</b>		
	<b>GIPsqrt</b>	-0.15 $\pm$ 0.04	-3.66	<b>0.000***</b>		
GIS Zeros	<b>GIC4</b>	-0.61 $\pm$ 0.17	-3.58	<b>0.000***</b>		
	<b>FEM</b>	1.05e-03 $\pm$ 4.34e-04	2.42	<b>0.016*</b>		
GIS (males) Zeros	<b>Intercept</b>	-2.97 $\pm$ 0.73	-4.05	<b>0.000***</b>	77	2.30
	<b>GIPsqrt</b>	0.11 $\pm$ 0.03	3.53	<b>0.000***</b>		
	<b>GIC4</b>	0.29 $\pm$ 0.10	2.90	<b>0.004**</b>		

.  $p \leq 0.1$ ; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

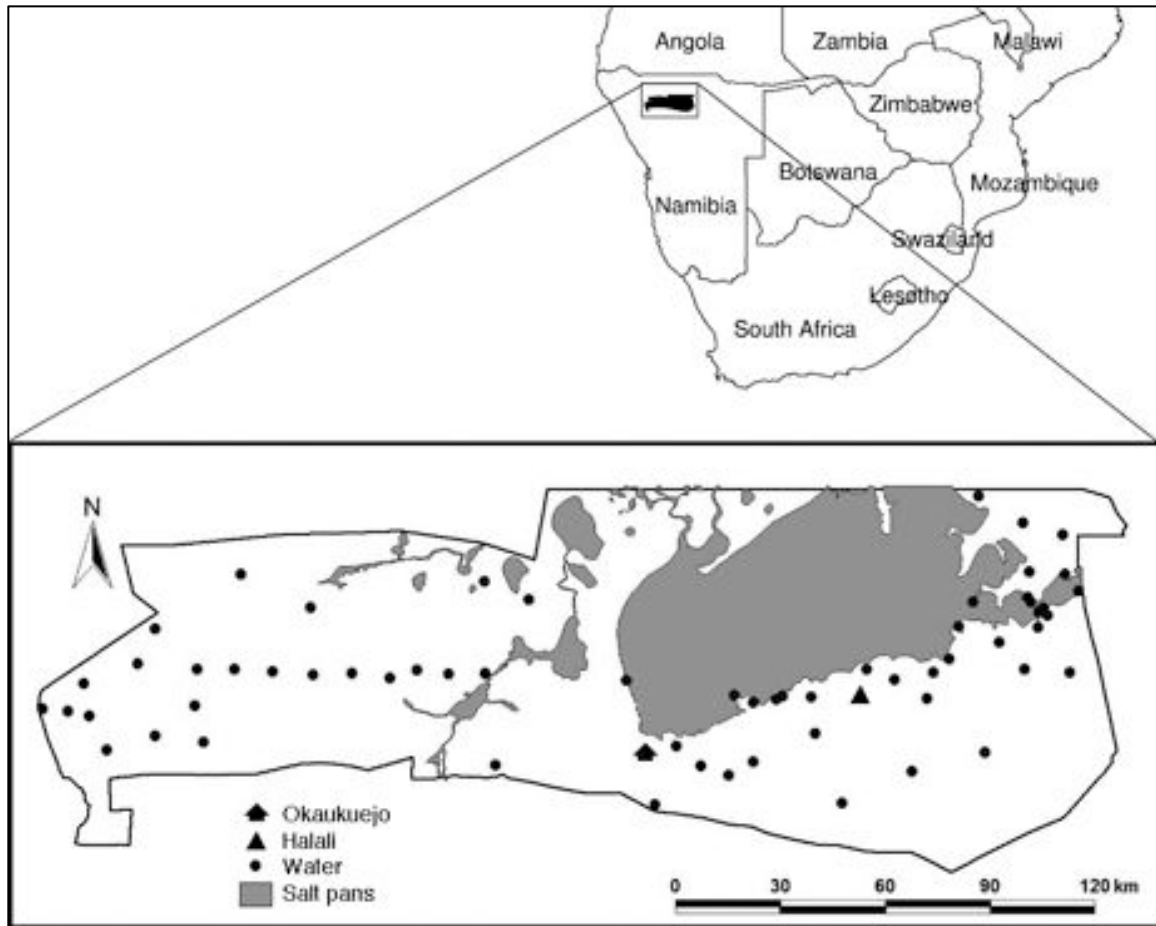
**Table 15.** Maximum likelihood estimates for the best fit generalized linear models for **springbok** *Eimeria* and stress hormones. Significant coefficients ( $p \leq 0.05$ ) are in bold.

Response	Coefficients	Estimate $\pm$ SE	z Value	Pr(> z )	N	$\Delta$ AIC
GIC	<b>Intercept</b>	5.77 $\pm$ 0.68	8.44	<b>0.000***</b>	26 7	12.7
	<b>Rain2</b>	0.019 $\pm$ 1.924e-03	10.0	<b>0.000***</b>		
	<b>AgeC</b>	-0.74 $\pm$ 0.33	-2.25	<b>0.025*</b>		
	<b>GISqrt</b>	0.12 $\pm$ 0.02	6.08	<b>0.000***</b>		
	<b>Rain2*GISqrt</b>	-5.10e-04 $\pm$ 1.30e-04	-3.93	<b>0.000***</b>		
GIC	<b>Intercept</b>	4.50 $\pm$ 0.34	13.4	<b>0.000***</b>	11 4	8.90
	<b>Rain2</b>	0.014 $\pm$ 2.07e-03	6.54	<b>0.000***</b>		
	<b>GISqrt</b>	0.05 $\pm$ 0.02	2.64	<b>0.008**</b>		
GIC (males)	<b>Intercept</b>	3.17 $\pm$ 0.38	8.32	<b>0.000***</b>	77	7.10
	<b>Rain2</b>	0.02 $\pm$ 2.77e-03	8.37	<b>0.000***</b>		
	<b>GISqrt</b>	0.19 $\pm$ 0.04	5.13	<b>0.000***</b>		
	<b>Rain2*GISqrt</b>	-7.66e-04 $\pm$ 2.11e-04	-3.63	<b>0.000***</b>		
FGM	<b>Intercept</b>	2.30e-04 $\pm$ 3.35e-05	6.863	<b>0.000***</b>	26 3	27.9
	<b>Rain2</b>	2.91e-07 $\pm$ 9.60e-08	3.032	<b>0.003**</b>		
	<b>AgeC</b>	-3.58e-05 $\pm$ 1.63e-05	-2.205	<b>0.028*</b>		
	<b>GISqrt</b>	-1.51e-06 $\pm$ 4.41e-07	-3.420	<b>0.001***</b>		
	<b>GISqrt</b>	3.23e-06 $\pm$ 9.54e-07	3.390	<b>0.001***</b>		
	<b>Rain2*GISqrt</b>	-1.40e-08 $\pm$ 5.85e-09	-2.393	<b>0.017*</b>		
FGM	<b>Intercept</b>	2.62e-04 $\pm$ 3.87e-05	6.78	<b>0.000***</b>	11 3	14.1
	Sex	-5.61e-05 $\pm$ 4.16e-05	-1.35	0.181		
	<b>FEM</b>	-6.45e-08 $\pm$ 1.63e-08	-3.96	<b>0.000***</b>		
	<b>Sex*FEM</b>	4.94e-08 $\pm$ 1.90e-08	2.61	<b>0.011*</b>		
FGM (males)	<b>Intercept</b>	2.42e-04 $\pm$ 3.40e-05	7.11	<b>0.000***</b>	77	5.70
	GIC4	-3.43e-06 $\pm$ 5.07e-06	-0.68	0.501		
	<b>FTM</b>	-1.08e-06 $\pm$ 2.98e-07	-3.64	<b>0.001***</b>		
	GIC4*FTM	9.65e-08 $\pm$ 5.44e-08	1.78	0.080.		

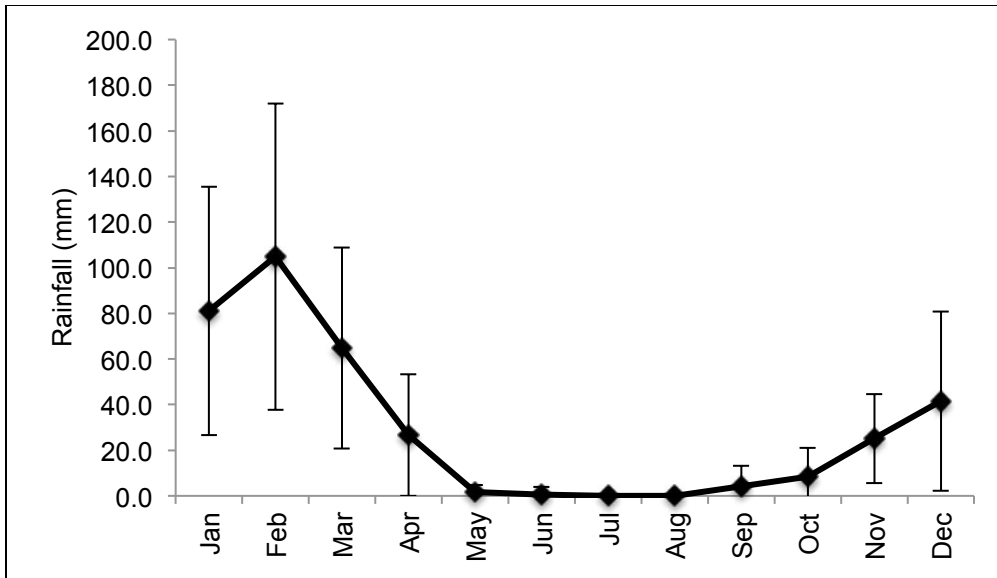
.  $p \leq 0.1$ ; \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$



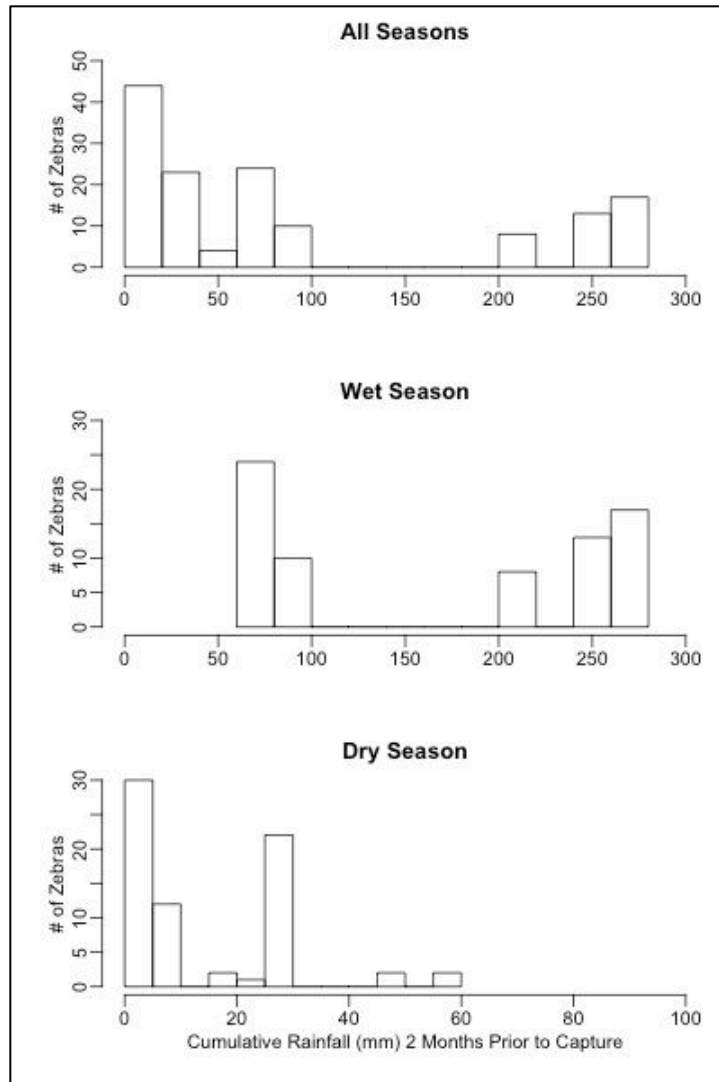
## Figures



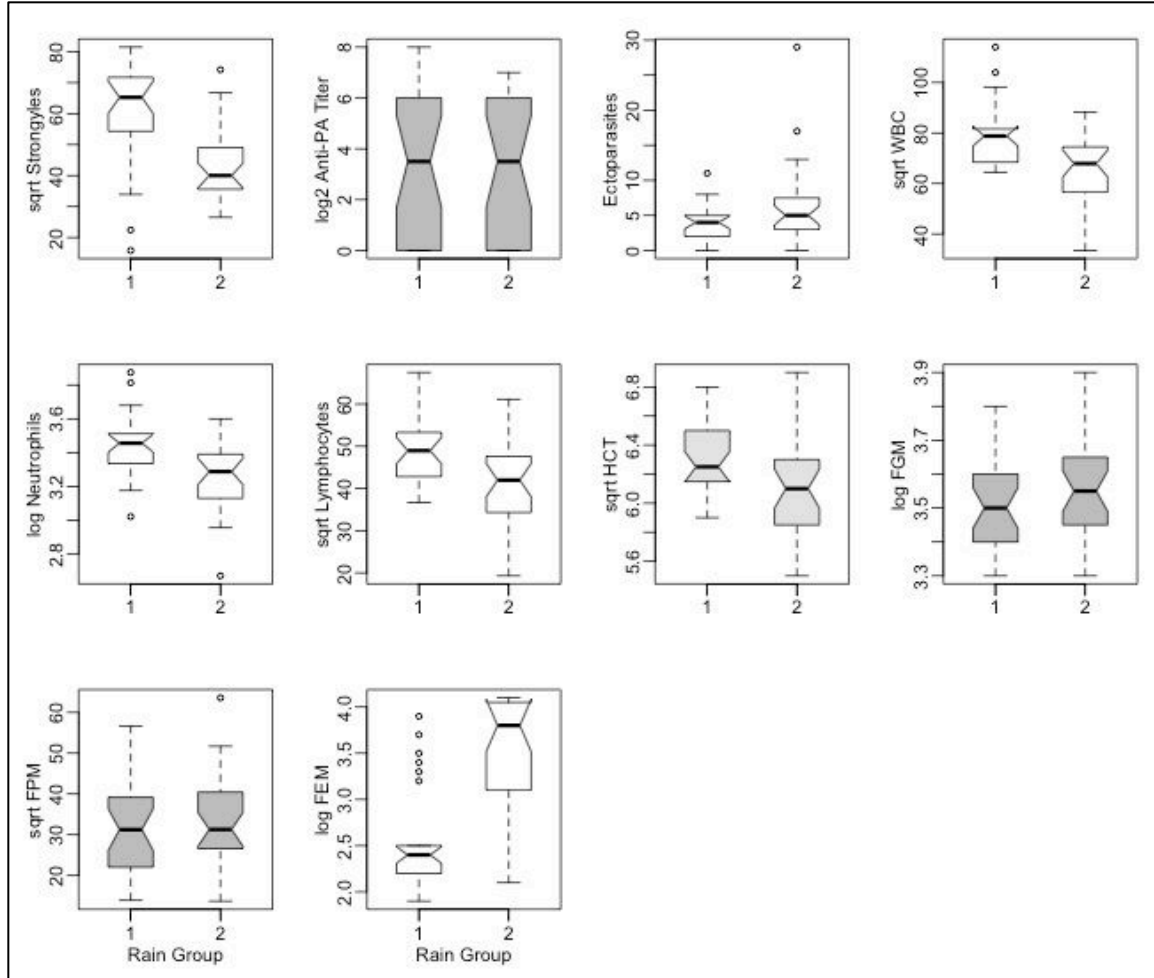
**Figure 1.** Etosha National Park in northern Namibia. The Etosha Ecological Institute is located in Okaukuejo in the center of the park; the majority of animal sampling for this study occurred in the nearby surrounding area, within a radius of approximately 20km (in the plains outside of the salt pans). During drier seasons, some sampling took place up to 100km to the east of Okaukuejo, around the Halali plains, and 15km south of Okaukuejo.



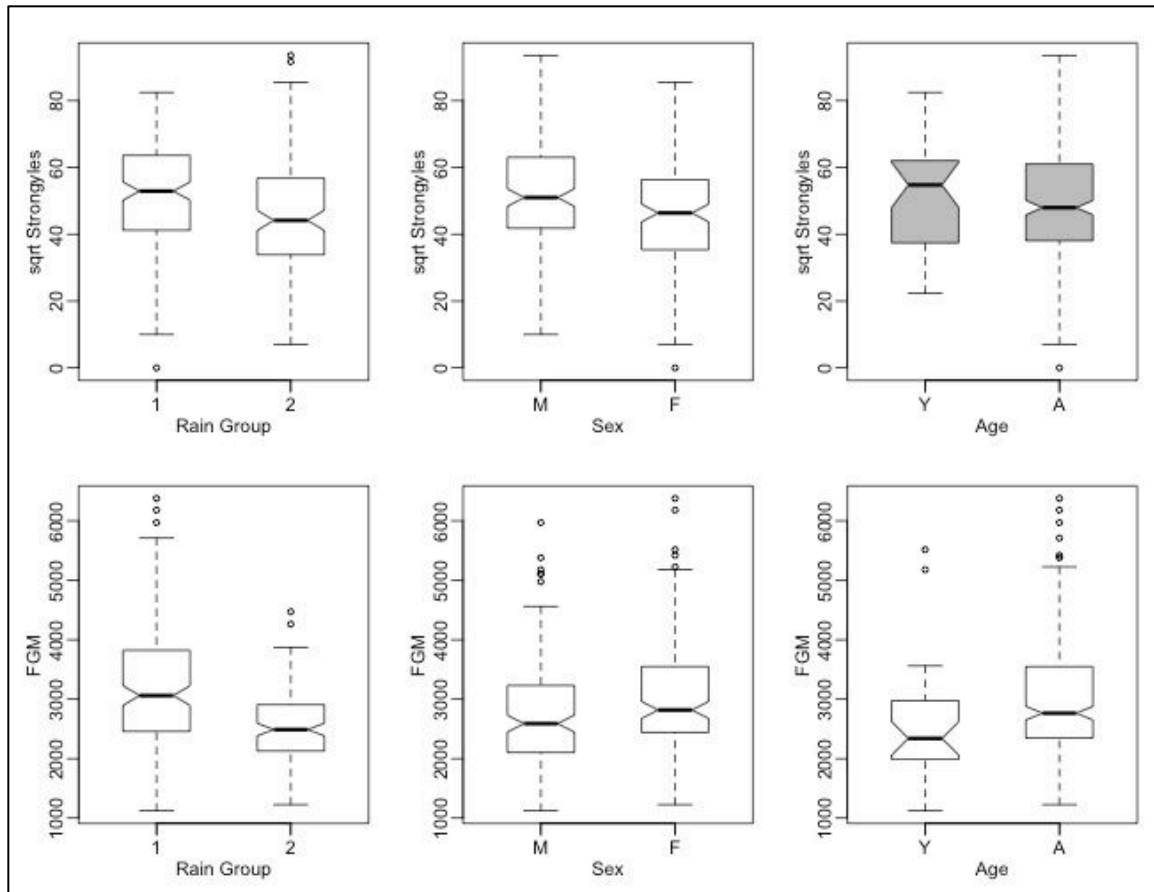
**Figure 2.** Mean ( $\pm$ SE) monthly Okaukuejo rainfall from 1974-2010. This encompasses the start of the most reliable anthrax sampling in ENP through the years of the current project (2008-2010).



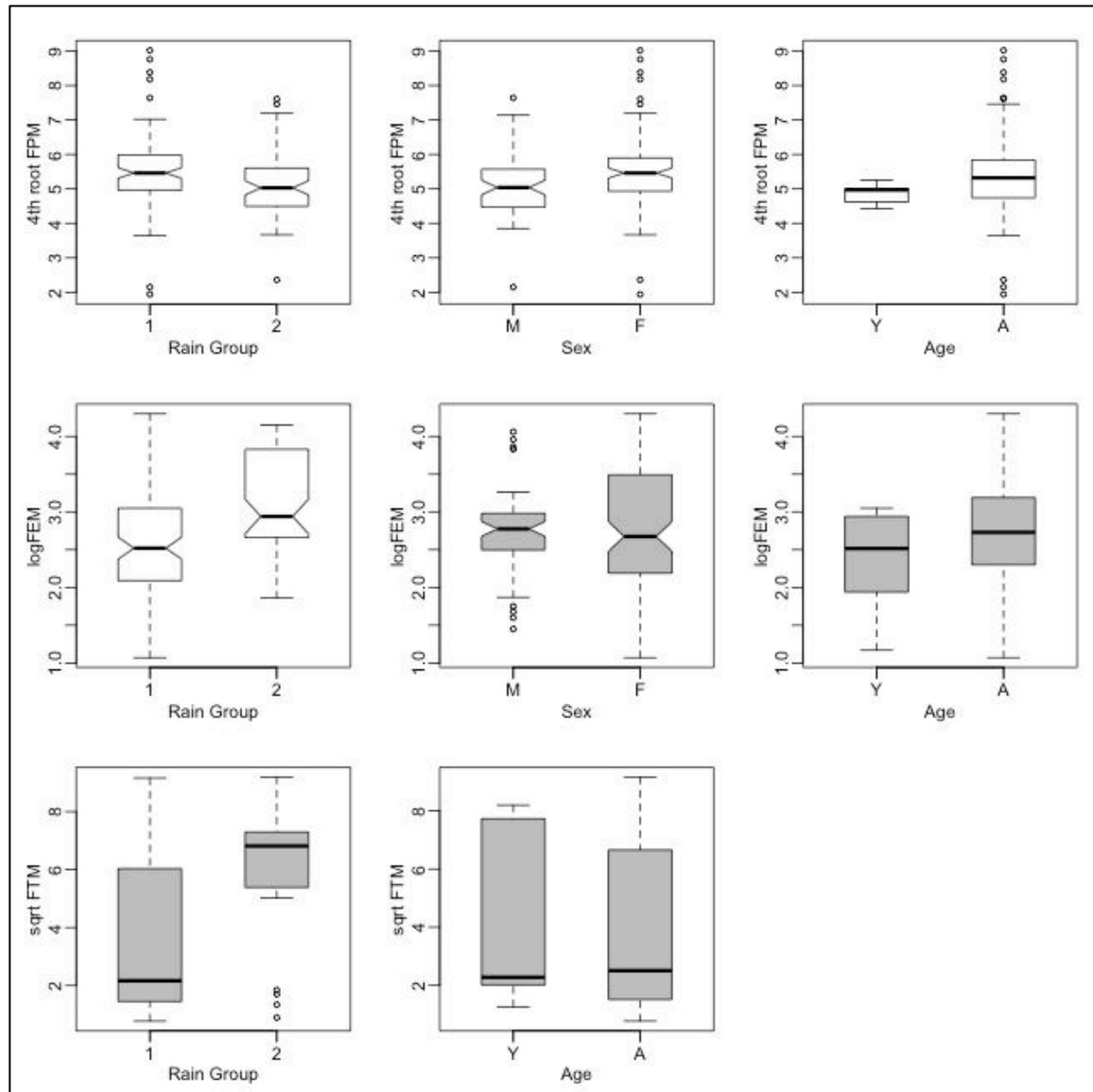
**Figure 3.** Cumulative rainfall 2 months prior to each zebra capture. The total rainfall in the 60 days prior to capture was determined for each individual zebra capture event, and that number was assigned to that individual-capture as its associated rainfall amount. While we sampled animals in nominally "wet" or "dry" seasons, we saw a clear bimodal pattern in rainfall amounts that did not necessarily align with seasons (particularly see the Wet Season data). We therefore used rainfall amounts to assign each individual-capture to a rain group: RG1, the high rainfall group, containing individuals that had experienced  $\geq 200\text{mm}$  rainfall two months prior to sampling; and RG2, the low rainfall group, containing individual samplings connected with  $\leq 100\text{mm}$  rainfall in the two months prior.



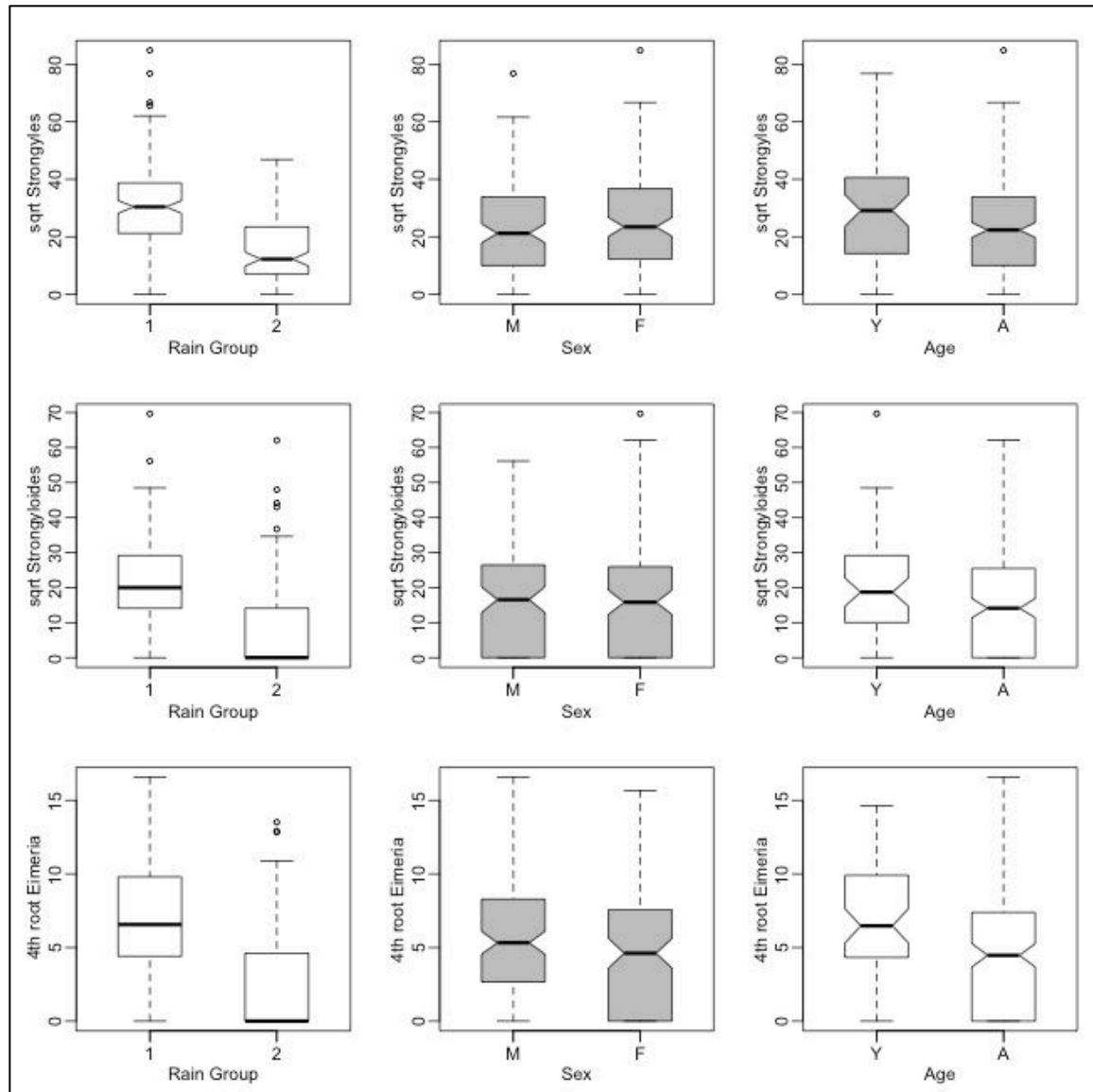
**Figure 4.** Pairwise comparisons of pathogens, immune factors, and hormones between rain groups for the **same captured zebra individuals** resampled twice. Center lines in boxplots represent medians, with notches extending to  $\pm 1.58 \text{ IQR} / \sqrt{N}$  where IQR is the interquartile range and  $N$  is the length of  $x$ . Notches that do not overlap provide strong evidence that the medians differ (Chambers et al. 1983) (not shown for log2PA because notches grossly overlapped the ends of the boxes). The box hinges represent nearly the first and third quartiles, with whiskers showing the largest and smallest observations 1.5x the box size from the nearest hinge. Points outside this range are shown as open circles. Boxplots in white are for variables that are significantly different from each other by  $t$  tests or Wilcoxon rank sum tests, whereas gray boxplots are not significantly different. sqrtHCT is light gray because the rain group for this variable was nearly significant ( $p=0.080$ ).



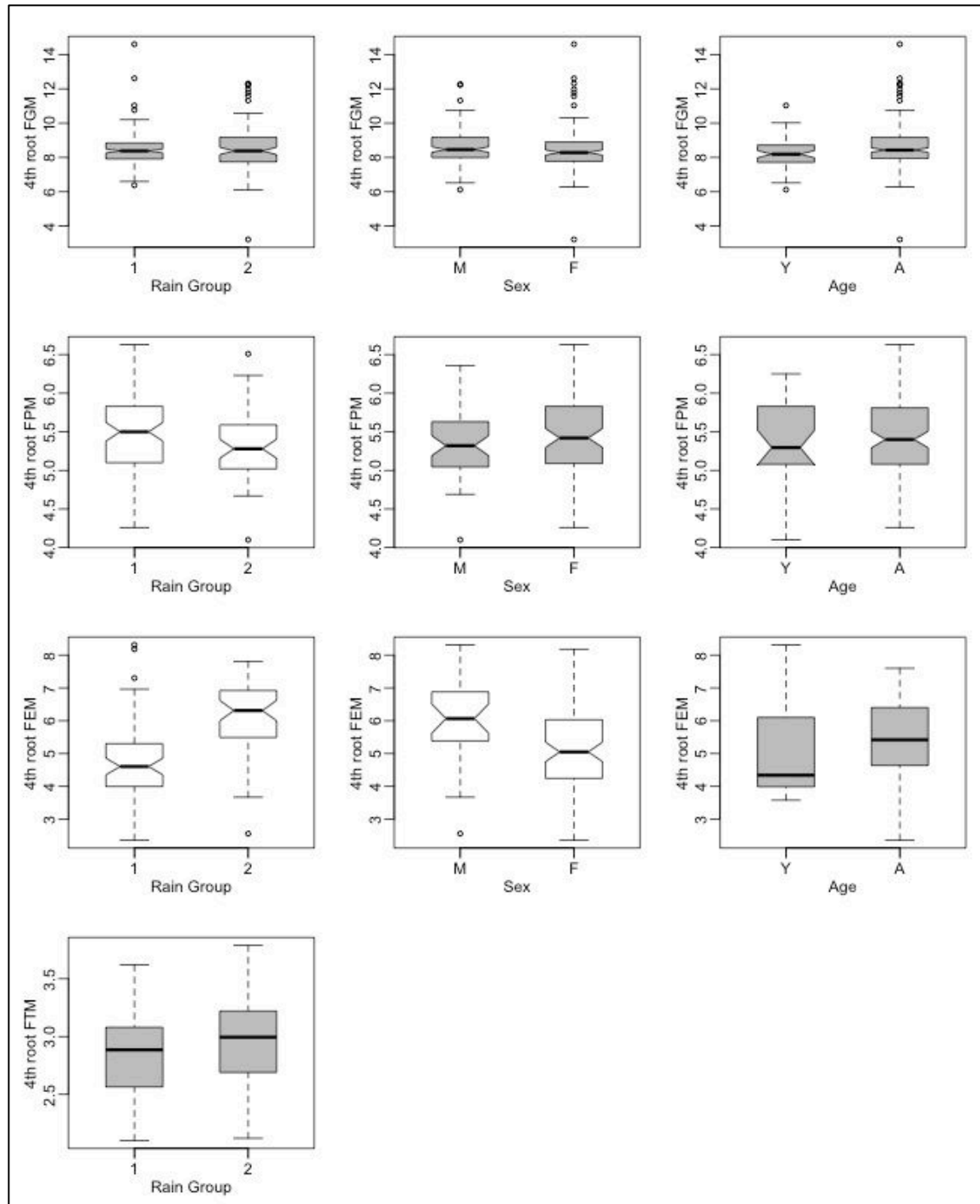
**Figure 5.** Comparisons of Strongyle counts and stress hormone metabolites (FGM) between rain groups, sexes, and ages for **non-captured zebra**. For clarity, comparisons between interaction effects are not shown. Boxplot statistics are interpreted as in Figure 4. Boxplots in white are for variables that are significantly different from each other by Tukey's *t* tests or Wilcoxon rank sum tests, whereas gray boxplots are not significantly different. Though non-transformed parasite counts (GIP) were compared with Wilcoxon rank sum tests, GIP is square root transformed here (as close to normality as possible) for clarity.



**Figure 6.** Comparisons of reproductive hormones between rain groups, sexes, and ages for **non-captured zebra**. For clarity, comparisons between interaction effects are not shown. Boxplot statistics are interpreted as in Figure 4. Boxplots in white are for variables that are significantly different from each other by Tukey's *t* tests or Wilcoxon rank sum tests, whereas gray boxplots are not significantly different. Though non-transformed FPM and FEM data were compared with Wilcoxon rank sum tests, they are fourth root and log transformed here (as close to normality as possible) for clarity.

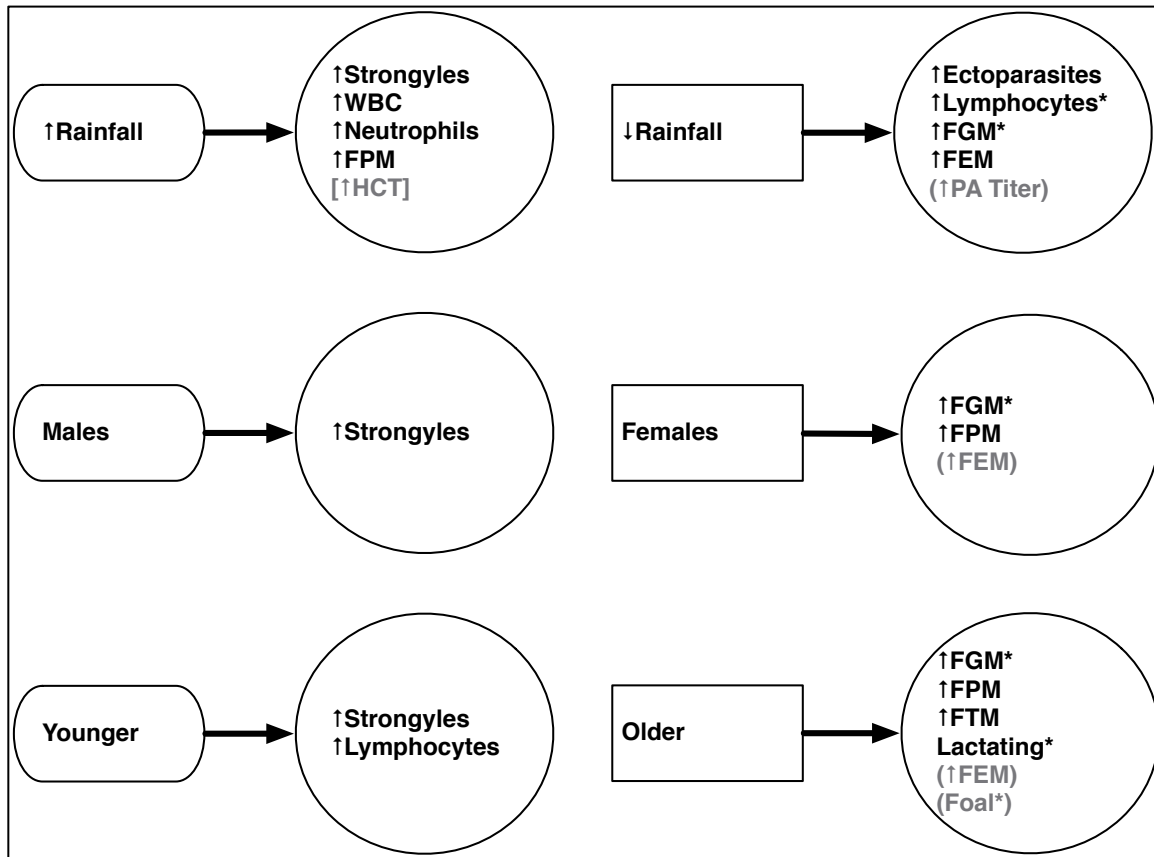


**Figure 7.** Comparisons of parasites between rain groups, sexes, and ages for **springbok**. For clarity, comparisons between interaction effects are not shown. Boxplot statistics are interpreted as in Figure 4. Boxplots in white are for variables that are significantly different from each other by Tukey's *t* tests or Wilcoxon rank sum tests, whereas gray boxplots are not significantly different. Though non-transformed parasite counts for all parasites were compared with Wilcoxon rank sum tests, they are fourth root and square root transformed here (as close to normality as possible) for clarity.

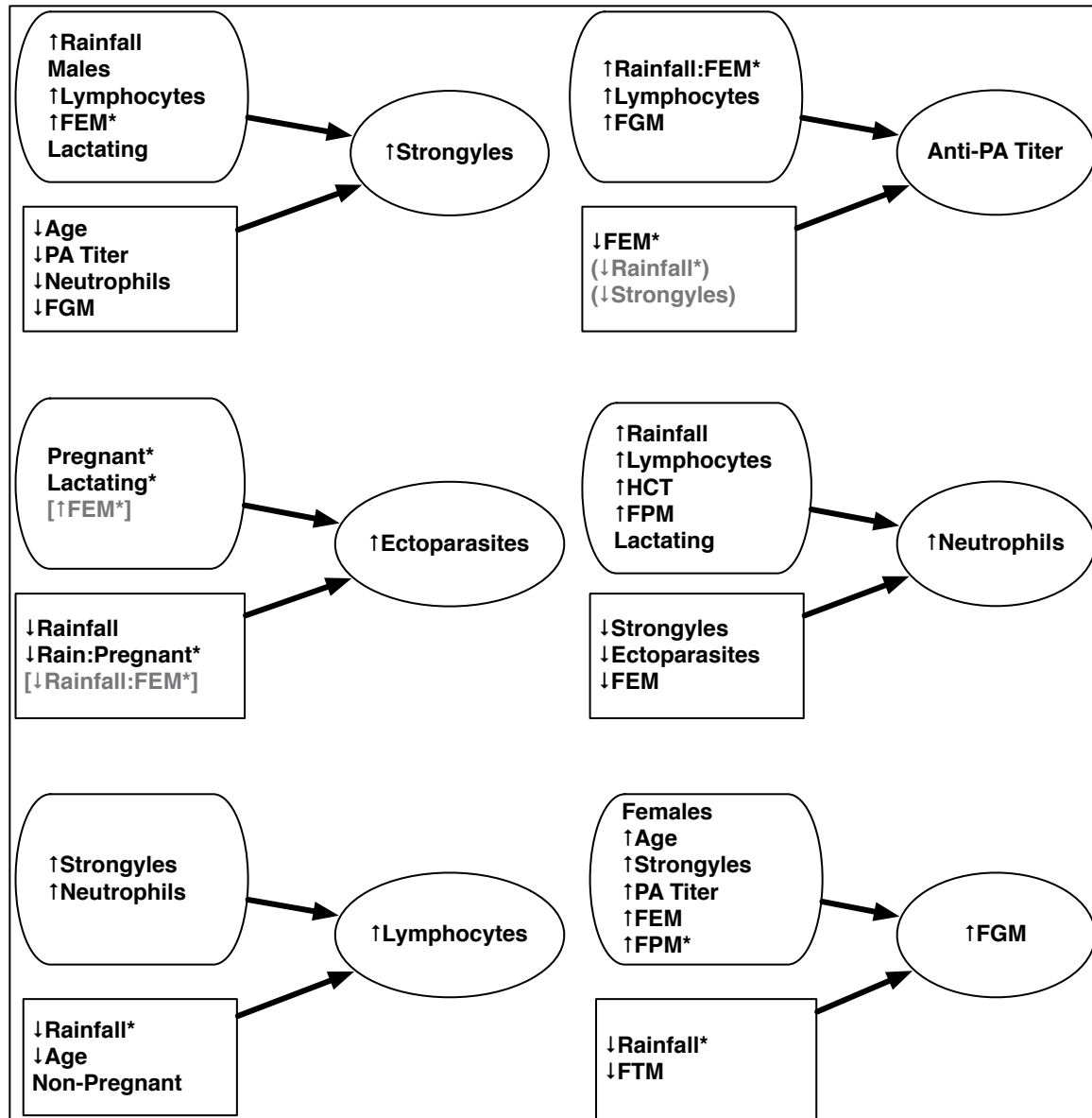


**Figure 8.** Comparisons of hormones between rain groups, sexes, and ages for **springbok**. For clarity, comparisons between interaction effects are not shown. Boxplot statistics are interpreted as in Figure 4. Boxplots in white are for variables that are significantly different from each other by Tukey's  $t$  tests or Wilcoxon rank sum tests, whereas gray boxplots are not significantly different. Though non-transformed FGM data were compared with Wilcoxon rank sum tests, they are fourth root transformed here (as close to normality as possible) for clarity.

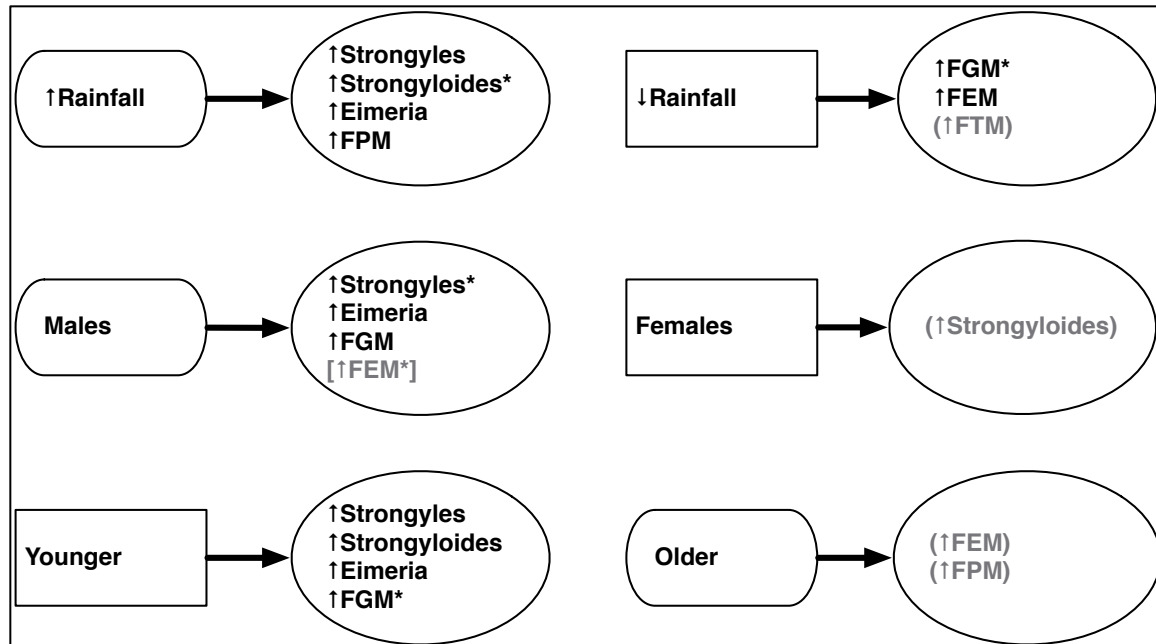




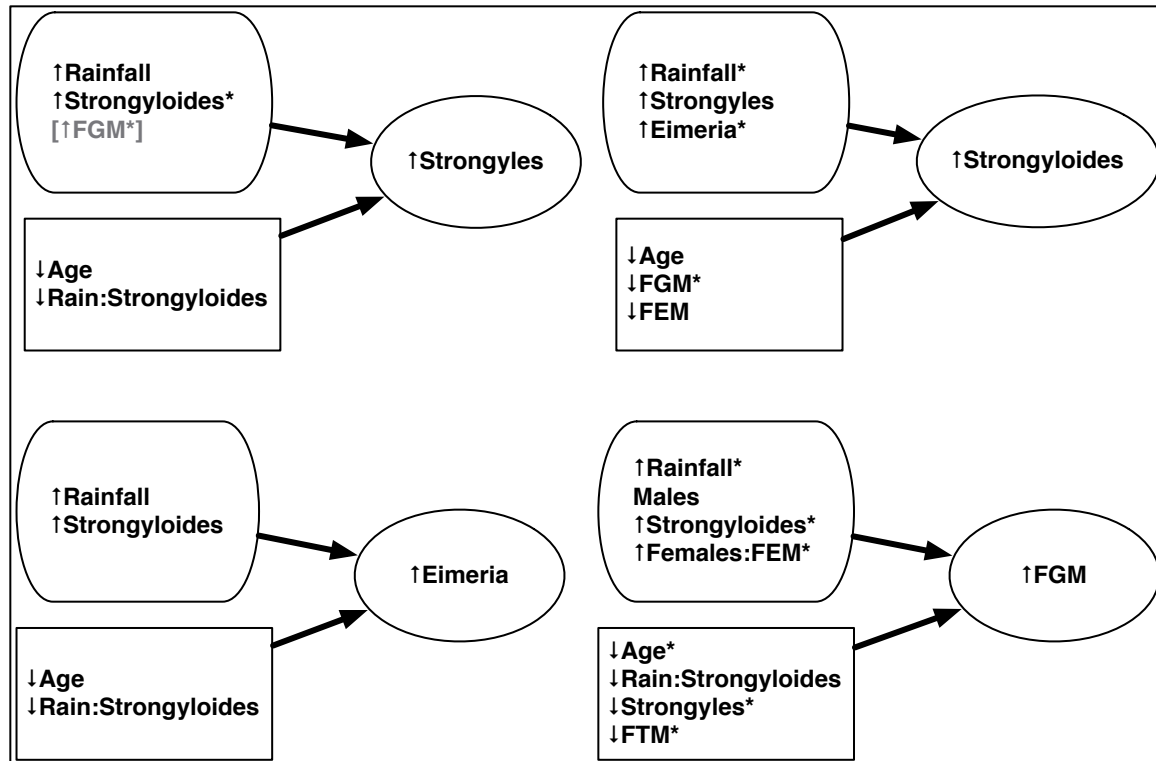
**Figure 9.** Illustrated relationships between rainfall, sex, and age and other variables for **all zebra** (captured and non-captured). These relationships are based on the overall trends observed for these variables across all group comparisons and linear models. The most consistent and strongest trends are shown; variable relationships for which there was a conflicting correlation in another model (in nearly all cases, in only one model) are marked with a \*. Response variables are contained in circles. Trends and correlations that were significant ( $p \leq 0.05$ ) at least once (and in most cases in several models) are in black print, while those variables that were nearly significantly different between groups ( $p \leq 0.1$ ) are in gray print within square brackets. Variables that were not significant ( $p > 0.1$ ) but showed consistently the same relationships and trends across several models are in gray print within parentheses.



**Figure 10.** Illustrated relationships between pathogens, immune factors, and FGM and other variables for **all zebra** (captured and non-captured). These relationships are based on the overall trends observed for these variables across all group comparisons and linear models. Explanatory variables showing a positive relationship to response variables are in pill-shaped boxes, while those showing a negative relationship to response variables are in rectangular boxes. Other relationships and symbols are interpreted as in Figure 10.



**Figure 11.** Illustrated relationships between rainfall, sex, and age and other variables for **springbok**. These relationships are based on the overall trends observed for these variables across all group comparisons and linear models. Relationships and symbols are interpreted as in Figure 10.



**Figure 12.** Illustrated relationships between parasites and FGM and other variables for all **springbok**. These relationships are based on the overall trends observed for these variables across all group comparisons and linear models. Explanatory variables showing a positive relationship to response variables are in pill-shaped boxes, while those showing a negative relationship to response variables are in rectangular boxes. Other relationships and symbols are interpreted as in Figure 10.

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