ECOLOGY AND IMMUNE FUNCTION IN THE SPOTTED HYENA, *CROCUTA CROCUTA*

By

Andrew S. Flies

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ABSTRACT

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The immune system is one of the most complex physiological systems in animals. In light of this complexity, immunologists have traditionally tried to eliminate genetic and environmental variation by using highly inbred rodents reared in highly controlled and relatively hygienic environments. However, the immune systems of animals evolved in unsanitary, stochastic environments. Furthermore, socio-ecological variables affect the development and activation of immune defenses within an individual, resulting in a high degree of variation in immune defenses even among individuals with similar genetic backgrounds. The conventional immunology approach of eliminating these variables allows us to answer some questions with great clarity, but a fruitful complement is to quantify how the social and ecological factors impact the immune function of animals living in their natural, pathogen-rich environments.

Spotted hyenas (*Crocuta crocuta*) have recently descended from carrion feeding ancestors, and they routinely survive infection by a plethora of deadly pathogens, such rabies, distemper virus, and anthrax. Additionally, spotted hyenas live in large, complex societies, called clans, in which the effects of social rank pervade many aspects of hyena biology. High-ranking hyenas have priority of access to food resources, and rank is positively correlated with fitness. However, very little research has been done to understand basic immune function in spotted hyenas or how socio-ecological variables such as rank can affect immune function. Here we addressed three primary questions. First, why do spotted hyenas rarely die from infectious

disease? Second, is the spotted hyena immune system fundamentally different from that of other mammals? Third, how do socio-ecological variables affect immune function in spotted hyenas.

Here we show that two primary components of the hyena immune system, immunoglobulins and toll-like receptors (TLRs), are similar to those in domestic cats (*Felis catus*), the closest relative of hyenas that has been studied in depth immunologically. The structure and molecular weights of hyena immunoglobulins are similar to those of cats, and the dynamics of the hyena antibody response to immunization also follow the standard pattern observed in cats. DNA sequencing of hyena toll-like receptors revealed more than 90% sequence similarity between hyenas and cats.

Given the importance of rank in spotted hyena clans, we investigated the effects of social rank on immune function in wild hyenas. Social rank is significantly correlated with serum bacterial killing capacity and total IgM. Additionally, serum bacterial killing capacity and total IgM are lower in lactating than in pregnant females. The higher levels of immune defenses in high-ranking females than in low-ranking females, and the reduced immune defenses observed during the energy costly period of lactation, suggests that immune defenses may be traded-off with other physiological systems. Furthermore, we found that bacterial killing capacity is a significant predictor of annual reproductive success in wild female hyenas. Finally, we assessed the differences in immune function between wild hyenas inhabiting a relatively pathogen-rich environment and captive hyenas in a relatively hygienic environment. Here we show that free-living hyenas generally had higher levels of immune defenses than captive hyenas. The lack of mortality from infectious disease in the wild hyenas suggests that their immune defenses are robust, and that pathogen exposure may be important for the development of the immune system, as suggested by the hygiene hypothesis.

ACKNOWLEDGEMENTS

Many people I know seemed to know exactly what they wanted to do with their lives from an early age. As a youth I was very active, curious, goal-oriented, and competitive. In high school I tried to excel in everything I did, primarily sports and school, but I had no clear idea of what type of career I would like after high school. In college I majored in computer science because I knew it was a field in which I could earn a high salary soon after graduation. After a six month internship as a software engineer I realized that this career would not fulfill me and that a good salary was not the motivation I needed. Again I considered my options but was left with more questions than answers.

My brother Dallas had been working in an immunology lab for several years and was about to move on to a different job. He suggested that I try working in an immunology lab. I thought that sounded reasonable and on the strength of Dallas' recommendation I was hired as a lab technician. I believe the fact the Lieping hired me despite the fact that I had not taken a biology class since I was a sophomore in high school is quite a testament to Lieping's confidence in Dallas. I immediately immersed myself in immunology and really enjoyed the challenge of learning new things. One day while on a short break at work I read a short article about researchers studying wolves and moose in northern Minnesota. Soon after reading this article I had the epiphany that I could turn my love of the outdoors and passion for science and learning into a career.

I continued working in Lieping's lab for a few more years and began taking ecology classes at night, with the eventual goal of transitioning to a job that included field work. I began looking for graduate programs that would allow me to meld my interests in immunology, travel,

and wildlife. Again I was fortunate to find people that were willing to take a chance on me. Kay Holekamp and Jean Tsao agreed to co-advise me in a Ph.D. program at Michigan State University and I began my graduate research studying immunology and ecology in spotted hyenas. Kay has been extremely patient with me during my gradual development as a scientist. Despite a demanding work schedule, she always found time to meet with me and respond to my often naïve questions. I know of no other advisers that consistently return comments on grant applications and manuscripts to their graduate students within a day or two. Her harsh but fair criticism of my writing and research were critical to my development as a scientist. I think of Kay's mentorship over the past six years as "tough love" and I am forever indebted to her.

My co-adviser Jean Tsao's constant support and unending enthusiasm for life and research helped me to explore different paths of research and to stay afloat when I questioned my ability as a scientist. I believe such doubts and insecurities are very common among graduate students and I was very lucky to have Jean as a mentor and friend. In my second year of graduate school I succeeded in tracking down microbiologist and veterinary scientist Linda Mansfield, and she welcomed me into her lab. Her lab provided the perfect blend of microbiology and immunology and I feel that my research got started in earnest when I began working daily in her lab. I hope that in some way Linda, Julia Bell, and other members of the enteric disease lab benefitted from having me take up space in their lab, but I don't think it can compare to how much I gained from their help and support.

Chris Grant is another person that was absolutely critical to my research. I initially contacted Chris because he was one of the few people that had anti-cat antibodies. Soon after contacting Chris it became clear that he was very interested in hyena immunology and would be another great friend and mentor. He supplied many of his reagents free of cost, and even allowed

me to work and sleep in his lab in Sacramento. His knowledge of the feline immune system always steered me in the right direction and I am sincerely grateful to him.

Pam Fraker, Andrew McAdam, and Barry Williams also were members of my guidance committee. Pam was quick to propose interesting research ideas and also quick to find flaws in existing ones, saving me the time of finding them myself at a later time point. Andrew McAdam is an extremely thoughtful scientist, and he has many qualities which I have attempted to cultivate in myself. Barry Williams stepped in when Pam and Andrew were unable to continue on as committee members. Barry's research was not disease-, hyena-, or immunology-oriented and his outside views of my research were a nice complement to my other committee members.

When I first began graduate school I had no idea how important other graduate students would be to my research. Katy Califf, Leslie Curren, and Eli Swanson have become great friends and colleagues over the past six years, and have provided a metaphorical shoulder to cry on, particularly during this last stretch of my Ph.D. program. The last few months of graduate school have been more physically and mentally draining than any other period of my life and I don't know how I would have stayed on course without their advice and support. Matt Maksimoski, an undergraduate research assistant that has been working with me for more than two years, has absolutely amazed me with his work ethic and sharp intellect. His skills in the lab developed quickly and will probably exceed my own very soon if he continues on a research path. Eric Smith was a former research assistant that has now moved on and is excelling in medical school. Eris is one of the most down-to-earth and kind people I have met. Brandtly Yakey is another volunteer research assistant that excelled in the lab. Charlie Kolodziejski, Theresa Kowalski, and Katie Leatherman also volunteered their time to help with my research. Jessica St. Charles, Ankit Malik, and J.P. Jerome helped me maintain direction and was always willing to take time out of

their hectic schedule to help me in the lab. Sarah Hamer was also always willing to set aside her work to help me move my research forward. I am very grateful to the help I received from all members of the Holekamp, Mansfield, and Tsao labs.

My experience in Kenya was greatly enriched by Kasaine Sankan. His friendship, help, and guidance in Kenya were invaluable. Steven Karkar, Lesingo Nairoi, James Kerempe, and John Keshe were also great companions and made my time in Kenya much more productive and comfortable. Many other great friends have helped me along the way. I would also like to thank the Narok County Council, Kenya Wildlife Service, and Maasai community for allowing me to work in their amazing country. I would like to thank the Stephen E. Glickman, Mary Weldele, and the staff of the Field Station for Behavioral Research at UC Berkeley for their assistance with all of the work involved captive hyenas.

Above all my family (Emily included) deserves credit for my completion of my Ph.D. Without the unconditional love and support from my parents I am positive that I would not have completed this degree. They have always steered me in the right direction, while simultaneously giving me the freedom to make my own choices and mistakes. Their work ethic and dedication surely rubbed off on me and I think this is my greatest asset. I have been away from my home in Minnesota for ten years now, but I will always consider their home to be my home, a place where I can truly relax and enjoy their company. My competitive streak is in large part due to the healthy competition among with my brothers and sister when I was growing up. I was always trying to catch my older brothers and stay one step ahead of my sister. I don't think I ever caught my brothers and my sister passed me at a young age. My sister, Beckie, may not know this but her decision to start a family had a life-changing effect on me. I have always been a bit selfish, but the birth of her son Carson opened my eyes and made me think on grander scales. I initially

wanted to do research so that I could enjoy being out in nature and do something I enjoyed. Following my introduction to baby Carson, my goals changed from being able to enjoy nature myself, to wanting to preserve nature for the enjoyment of future generations. Beckie has been a great sister and still checks in on me to make sure I am doing ok. I have already stated what an enormous impact Dallas has had on me, but he was the instigator in another turning point in my life. I don't remember exactly when, but at some point he gave me a book called *My Family and other Animals*. This book reminded me of things I liked doing as a kid, but had gotten away from as I grew up. In high school and college I thought of my brother Pete as a great athlete and intelligent guy. In the years since I have realized that he is one of the deepest thinkers I know. He is able to explain complex topics in simple terms and the depth of his knowledge and interests amazes me.

Finally, the greatest thing to come out of my time at Michigan State University is my relationship with Emily Johnston. When I first describe Emily to someone that knows me well, but does not know her, I often describe her as a female version of myself. This is not true though; her intellect, compassion, and competitive spirit far exceed my own. I believe that as a team, Emily and I can achieve anything that we set our collective mind to and I enthusiastically look forward to our future together. Her love drives me to succeed in the hope that I will be able to impress her and make her happy, despite knowing that her love is unconditional. Even though she was thousands of miles away while I was writing the final chapters of this dissertation, she was able to prop me up and keep me going by editing my manuscripts, providing critical research feedback, and being the best friend I have ever known. I could not have done this without her and the rest of my wonderful, supportive, encouraging family.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

The immune system is one the most complex systems in vertebrates, comparable to that of the nervous system, but with the additional caveat that cells of the immune system are mobile and move throughout the body (Flajnik and Kasahara 2010; Nei et al. 1997). The task of the immune system is complicated by the fact that many microorganisms are beneficial to host survival, such as bacteria in the guts of animals that aid in digestion of food. On the other hand, even beneficial or commensal microorganisms can become pathogenic given the appropriate circumstances, such as when bacteria spill from the gut into other host tissues due to injury. Additionally, the powerful tools of the immune system can themselves cause disease in the host when the immune defenses target healthy host tissue, a phenomenon known as autoimmunity.

In light of this complexity, traditional immunology studies have tried to eliminate as much variation as possible in the environment, host, and pathogen. This has led to the widespread use of genetically inbred mice as the standard model for immunological studies, with a primary focus on using simple animal models to understand human disease. The use of standard animal models for studying the immune system has led to great progress in understanding how the immune system fights or contributes to disease. Nevertheless, the highly controlled laboratory environment does not always translate well to the natural world, where environmental, host, and pathogen variation can lead to different immunological outcomes than those observed in the laboratory. This discontinuity between the lab and more natural settings has led some evolutionary ecologists and immunologists to attempt to understand how variation in environment, host, and pathogen can affect immunological outcomes, rather than trying to eliminate variation. This relatively new field of ecological immunology, or ecoimmunology,

attempts to understand variation in immune function by accounting for ecological differences, and by using a cost-benefit approach to understand how immune defenses are traded-off against other important physiological processes in response to ecological and within-host physiological changes (Sadd and Schmid-Hempel 2008).

Before proceeding further in this introduction, it is necessary to establish working definitions of words that will be used commonly throughout this dissertation. Immunology is the study of the physiological mechanisms that organisms use to defend host resources from exploitation (Parham 2009). Disease is defined as abnormal or impaired physiological function, and may be caused by infectious and non-infectious agents (Ostfeld et al. 2010). Pathogen refers to any replicating agent capable of causing disease in a host organism (Schmid-Hempel 2011); in this dissertation pathogens include the non-cellular entities viruses and prions, single-celled bacteria and protozoa, and multi-cellular organisms such as fungi, helminths, crustaceans, and parasitoid insects. Other commonly used terms include microparasites, which are agents that are organized at or below the cellular level, and macroparasites, which are multicellular organisms (Anderson and May 1979; Ewald 1994). Throughout this dissertation we will most commonly use the term pathogen, but may occasionally use the terms parasites, microparasite, or macroparasite when it will provide a more clear description of the pathogen or process being referred to. Finally, the term antibody will generally be used in instances where we are referring to an immunoglobulin that binds a known target molecule, however, immunoglobulin and antibody both describe the same type of protein and may be used interchangeably.

Why study ecoimmunology in spotted hyenas?

Spotted hyenas in East Africa that have been studied intensely for more than two decades have been observed to die very rarely from infectious disease, despite well-documented

exposure to pathogens such as rabies, canine distemper virus, and anthrax (Alexander et al. 1995b; East et al. 2001a; Harrison et al. 2004b; Lembo et al. 2011). Little or no clinical signs of disease or mortality were observed in spotted hyenas during periods of high mortality from infectious diseases occurring in sympatric species, such as African lions (*Panthera leo*) and wild dogs (*Lycaon pictus*) (Alexander and Appel 1994; Cleaveland et al. 2000; Gascoyne et al. 1993; Goller et al. 2010; Kat et al. 1996b; Munson et al. 2008). High levels of pathogen exposure are expected for highly social animals, animals that kill and consume other animals, and animals that routinely scavenge on decaying carrion (Altizer et al. 2003; Ogada et al. 2012). Their documented exposure to deadly pathogens, in addition to their rfecent descent from carrion feeding ancestors, provides the impetus for our first research question: why do spotted hyenas rarely die from infectious disease? Additionally, because so little was known about immune function in hyenas, we also asked another basic question: is the spotted hyena immune system fundamentally different from that of other mammals?

Spotted hyena behavioral ecology is unique in many ways. First, spotted hyenas live in the largest social groups of any mammalian carnivore, with groups, also known as clans, ranging up to 120 individuals. The number of social contacts an individual has is often correlated with the number pathogens they are exposed to (Rifkin et al. 2012). Furthermore, pathogen virulence is often correlated with the number of susceptible hosts in a population (Anderson and May 1982; Day et al. 2007). Second, clans are structured by strict linear dominance hierarchies, in which females are dominant to all males that immigrate into the clan (Frank 1986; Holekamp and Smale 1990; Smale et al. 1993). Dominant individuals have priority of access to food resources, and most measures of fitness are influenced by social rank (Hofer and East 2003; Holekamp et al. 1996; Kruuk 1972a; Swanson et al. 2011). These unique aspects of hyena

behavioral ecology led us to our next research question: how do socio-ecological variables affect immune function in spotted hyenas.

Vast amounts of time, money, and effort have been put into understanding variation in disease susceptibility in other species, including plants, humans, and other animals, but a definitive answer often remains elusive even in the most intensively studied systems. One of the principal benefits of studying immunology in well-defined animal models is that a plethora of immunological tools are already available, including monoclonal antibodies (mAbs) and full genomes. This is not the case for hyenas, as only a few studies have attempted to document immune function in hyenas. Highly specific antibodies are a primary tool for cellular and molecular immunology, but no anti-hyena antibodies have been developed yet.

This lack of immunological reagents available for studying immune function in hyenas led to the first chapter of this dissertation. Chapter two examines how social rank, reproductive status, and sex affect immune function in wild hyenas. Additionally, in chapter two we investigate if immune function is related to fitness in wild hyenas. Chapter three is a comparative study that tests if immune function is different in captive and wild hyenas. Finally, chapter four examines important signaling molecules of the innate immune system, known as toll-like receptors.

CHAPTER 1

A. S. Flies, C. K. Grant, L. S. Mansfield, E. J. Smith, M. L. Weldele and K. E. Holekamp. 2012. Development of a hyena immunology toolbox. Veterinary Immunology and Immunopathology 145(1–2): 110-119.

CHAPTER 1

DEVELOPMENT OF A HYENA IMMUNOLOGY TOOLBOX

Introduction

Wildlife disease outbreaks can have major impacts on conservation efforts and lasting effects on ecosystem processes (Claude 1996). For example, rabies and canine distemper virus (CDV) epizootics were associated with the extirpation of wild dogs (*Lycaon pictus*) in the Maasai Mara National Reserve (MMNR) in Kenya (Alexander and Appel 1994; Kat et al. 1995; Kat et al. 1996). Additionally, a CDV outbreak in East Africa killed more than 1000 lions (*Panthera leo*) (Munson et al. 2008; Roelke-Parker et al. 1996).

Animals that hunt and scavenge are likely exposed to a broad array of pathogens (Schulenburg et al. 2009). Although most carnivores, including lions and wild dogs, scavenge to some extent (Houston 1979), theory predicts that the immune systems of carnivores exhibiting morphological specializations for carrion-feeding should have been molded by selective pressures associated with surviving microbial assaults from their food (Blount et al. 2003; Mendes et al. 2006; Schulenburg et al. 2009). Spotted hyenas (*Crocuta crocuta*) are capable hunters that have descended within the last million years from carrion feeding ancestors (Lewis and Werdelin 2000; Werdelin 1989). Despite documented exposure to anthrax, rabies, CDV and several other pathogens, spotted hyenas in East Africa have exhibited extremely low mortality rates due to infectious diseases, even when epizootics decimated sympatric carnivore populations (Alexander et al. 1995; East et al. 2004; East et al. 2001; Harrison et al. 2004; Lembo et al. 2011; Watts and Holekamp 2009). Spotted hyenas are the most abundant large carnivores in Africa,

and may play a critical role in the ecology of disease in African wildlife and domestic animals throughout the continent (Hofer 1998).

In light of the extreme disease resistance manifested by hyenas and their potential importance for overall disease dynamics in African ecosystems, we set out to identify tools available for studying immune function in the spotted hyena. The two specific aims of this study were to identify antibodies that cross-react with hyena immunoglobulins and to assess the dynamics of the hyena humoral immune response to immunization with a non-pathogenic antigen. Domestic cats (*Felis catus*) were the closest phylogenetic relatives of hyenas that had been studied in detail immunologically (Bininda-Emonds et al. 1999; O'Brien and Johnson 2005), and we hypothesized that anti-cat isotype-specific antibodies would cross react with hyena immunoglobulin (Ig) epitopes.

We used ELISAs to test isotype-specific anti-feline antibodies for cross-reaction to hyena Ig epitopes and to assess temporal dynamics of hyena immunoglobulins in response to immune challenge. We used Western blots to confirm cross-reactivity and to estimate the molecular weight of hyena immunoglobulins. Reverse transcriptase polymerase chain reaction (RT-PCR), serum neutralization tests, western blots, and agglutination tests have been used previously to document pathogen exposure in spotted hyenas (East et al. 2004; East et al. 2001; Honer et al. 2006; Speck et al. 2008), but only a few studies have gone beyond documenting exposure and examined the immune response itself (East et al. 2008; Hanley et al. 2005; Honer et al. 2006; Van Helden et al. 2008). The current paper represents the first report of antibodies capable of detecting spotted hyena immunoglobulins other than IgG, and the first to document the temporal dynamics of the humoral immune response of the major isotypes with a defined antigen.

Materials and Methods

Captive spotted hyenas, sample collection, and immunization

All captive spotted hyenas were born and housed in the Field Station for Behavioral Research (FSBR) of the University of California, Berkeley (UCB). Berger et al. (1992) describe the husbandry conditions at this facility. Eight healthy adult (4 female, 4 male) spotted hyenas were subjected to an immunization protocol approved by both the University of California, Berkeley (Animal Use Protocol # R091-0609R) and Michigan State University (MSU) Institutional Animal Care and Use Committees (IACUC) (AUF # 07/08-099-00). Captive hyenas ranged from 4 to 17 years of age when immunizations began. Animals were immobilized with blow dart delivered i.m. injections of ketamine (4–6 mg/kg) and xylazine (1 mg/kg) for immunization and blood sampling.

Blood samples for serum analysis were collected on days 0, 14, 28, 180 and 365 from the jugular vein using Vacutainer tubes (BD, Franklin Lakes, NJ, cat# 366430), allowed to clot at ambient temperature, and then centrifuged. Serum was aliquoted into cryovials and stored at -80 ºC. Following blood collection on day 0, four animals were immunized with 250 μg of 2,4-dinitrophenol conjugated to keyhole limpet hemocyanin (DNP-KLH) (Calbiochem/EMD Biosciences, Germany, cat #324121) in sterile water, and four animals were immunized with 250 μg of DNP-KLH emulsified in TiterMax Gold (TiterMax USA, Inc., Norcross, Georgia, cat# G1). Each animal received one subcutaneous 250 μl injection on each side of the neck for a total of 500 μl. A booster, consisting of 250 μg of DNP-KLH in sterile water, was administered on day 14. Blood was collected on day 14 prior to administration of booster injections.

Samples from wild spotted hyenas

Serum samples from wild spotted hyenas were collected as part of the long-term research project in the Maasai Mara National Reserve, Kenya, started in 1988. Wild spotted hyenas were immobilized using tiletamine-zolazepam (6.5mg/kg Telazol; Fort Dodge Animal Health, Fort Dodge Iowa) in a plastic dart fired from an air rifle (Telinject Inc., Saugus, California) (Holekamp and Sisk 2003). Whole blood samples from wild hyenas were collected from the jugular vein using Vacutainer tubes and allowed to clot at ambient temperature, then centrifuged, aliquoted into cryovials, and snap frozen in liquid nitrogen. All immobilization protocols were approved by the MSU IACUC (AUF # 07/08-099-00) and the Kenya Wildlife Service.

Purification of spotted hyena immunoglobulins

Pooled serum samples from wild spotted hyenas were used for purification of hyena IgG, IgM, and IgA. Immunoglobulins from a 25 ml serum pool were first precipitated in 50% ammonium sulphate solution (Steward and Petty 1972), redissolved in phosphate buffered saline (PBS), and then extensively dialyzed against water. The dialysate was centrifuged and the precipitate was redissolved in PBS as a crude IgM preparation; this was then sized over Sephacryl S-300 using a 50 cm x 2.5 cm column and the peak fraction collected as partially-purified hyena IgM. Meanwhile, the clarified dialysate was passed over a DEAE Bio-Gel A column (12 cm x 2.5 cm) equilibrated with 0.1 M Tris pH 8.0, and a semi-purified IgG fraction was eluted using 0.1 M Tris pH 8.0 containing 45 mM sodium chloride; this peak was then sized over Sephacryl S-300 and the appropriate sized IgG fraction was collected. A further fraction was eluted from the DEAE column using 0.1M Tris pH 8.0 containing 70 mM sodium chloride. This peak was then sized over Sephacryl S-300, and Ig which eluted at a position behind the IgM peak but ahead of the IgG peak was collected as a hyena IgA enriched fraction. Protein

concentrations were determined with a UV spectrophotometer reading at 280 nm. Further details about the purification of immunoglobulins are available in Grant (1995).

Western blot verification of cross-reactive antibodies and molecular weight of hyena

Purified hyena and feline (*Felis catus*) IgG, IgM, and IgA fractions were subjected to SDS-PAGE and Western blot analysis under denaturing conditions to verify cross-reactivity of anti-feline antibodies to hyena Ig epitopes and to compare the molecular weight of Ig heavy and light chains between the two species. Purified hyena immunoglobulins were diluted in Laemli sample buffer (Laemmli 1970) with 0.1 M dithiothreitol, and were denatured by heating at 95ºC for 5 minutes. IgG, IgM and IgA samples were then loaded at 2 μg/well, 1 μg/well, and 2.5 μg/well, respectively, into a 10 % Tris-HCl polyacrylamide gel. Prestained protein standards were added to each gel to assess molecular weight of the target proteins. Running conditions (150V for 45 minutes) and running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) were used in accordance with manufacturer's instructions for the Bio-Rad Mini Protean II System.

Hyena and feline serum samples diluted 1:10 in Laemli sample buffer were used for analysis of cross-reactivity and molecular weight comparison of both heavy and light chains. Initial assays using serum samples exhibited migration patterns that appeared to be influenced by large quantities of albumin. AlbuminOut (GBiosciences, cat# 786-251) was therefore used to remove albumin from the serum samples, and this allowed us to estimate the molecular weight of target proteins more accurately.

Prior to transfer, the nitrocellulose membranes were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 15 minutes. Proteins were transferred at 100V for 75 minutes at 4ºC. After transfer, membranes were place directly into 5% non-fat dry milk (NFDM) in Tris buffered saline (TBS) (5% NFDM-TBS; 420mM Tris-HCl, 80mM Tris, 1.5 M

NaCl, 5 g/L blotting grade NFDM) and incubated overnight at 4ºC. See Table 1.1 for a comprehensive list of antibodies tested.

Staining was done using a Bio-Rad Multiscreen Apparatus. Primary monoclonal detection antibodies were added at 1 μg/ml in 1% NFDM-TBS and incubated for 90 minutes at ambient temperature on a shaking platform. The membrane was then washed 3 times with 0.05% Tween-20 in TBS (TBS-T). Secondary biotin-F(ab')$_2$ fragment goat anti-mouse IgG (H+L) was used with mAbs and was diluted 1:5000 in 1% NFDM-TBS and incubated on a shaker for 60 minutes. The membrane was again washed 3 times with TBS-T. Extravidin-peroxidase was diluted 1:1000 in TBS-T and incubated with the membrane for 30 minutes. Color change was developed using a CN/DAB substrate kit. Color change reaction was stopped by washing with distilled water after approximately 10 minutes. The staining process used with polyclonal HRP conjugated anti-IgG, anti-IgM, and anti-IgA involved the following process: a blocking step, wash, incubation of antibodies at a 1 μg/ml, a final wash step, and followed by color development. Images were captured using the Bio-Rad VersaDoc Molecular Imaging system, and molecular weight was determined using Bio-Rad Quantity One software package.

Table 1.1. Antibodies tested by ELISA and Western blot (WB).

Target	Catalog #	Type	Assays confirmed[a]	Supplier
IgA	IgA5-3B	mono	ELISA (cat, hyena)	CMIC[b]
IgA	CDA2-43	mono	ELISA(cat, hyena)	CMIC
IgA	NB7264	poly	ELISA (cat, hyena), WB (cat, hyena)	Novus Biologicals
IgE	E6-71	mono	ELISA (cat, hyena)	CMIC
IgE	E2-19	mono	ELISA (cat, hyena)	CMIC
IgG	GPB2-2	mono	ELISA (cat, hyena), WB (cat)	CMIC
IgG	04-20-02	poly	ELISA (cat, hyena), WB (cat, hyena)	KPL[c]
IgG(H+L)	102-065-003	poly	ELISA (cat, hyena), WB (cat, hyena)	Jackson ImmunoResearch
IgG(H+L)	OB-680-05	poly	ELISA (cat, hyena), WB (cat, hyena)	Southern Biotechnology
IgM	CM7	mono	ELISA (cat, hyena), WB (cat, hyena)	CMIC
IgM	CM6E	mono	ELISA (cat, hyena), WB (cat, hyena)	CMIC
IgM	04-20-03	poly	ELISA (cat, hyena), WB (cat, hyena)	KPL
K light chain	FIG1-7A	mono	ELISA (cat, hyena), WB (cat, hyena)	CMIC
λ light chain	CAG8-7C	mono	ELISA (cat, hyena), WB (cat)	CMIC

[a]Parentheses contain the species for which antibody binding has been conrmed.

[b]Custom Monoclonals International Corp.

[c]Kirkegaard & Perry Laboratories, Inc.

Identification of cross-reactive anti-feline antibodies and quantification of humoral response using an enzyme-linked immunosorbent assay (ELISA)

To further examine the cross-reactivity of commercially available anti-feline immunoglobulins with spotted hyena immunoglobulins and to quantify spotted hyena humoral response to immunization, we tested spotted hyena sera using ELISAs. ELISA plates were coated with either 50 µl of bovine serum albumin (BSA) as a control or DNP-BSA at 5 µg/ml in 50 µl of carbonate buffer (0.1 M, pH 9.5) and stored overnight at 4 ºC. BSA coated wells were used as a reference for background binding.

Plates were washed with PBS containing 0.05% Tween-20 (PBS-T). Plates were then blocked for one hour with 5% NFDM-PBS. This and all subsequent incubations were run at ambient temperature on a shaking platform. Plates were then washed with PBS-T. Monoclonal anti-DNP (Silver Lakes Research, cat# CH1911) was subjected to serial two fold dilutions to generate a standard curve. Negative control wells were incubated with PBS only, and pre-immune sera (day 0) also served as an additional control. See Table 1.2 for dilutions of serum samples and anti-isotype detection antibodies. All serum samples were incubated for 90 minutes before washing and each sample was tested in duplicate. Plates were then incubated with primary anti-isotype antibodies for 90 minutes and washed with PBS-T. Next, 50 µl of anti-mouse IgG-HRP was diluted 1:5000 and added to each well with mAbs, including the standard wells. Plates were then incubated for 45 minutes and washed with PBS-T. Color change was developed using 50 µl/well of 3,3',5,5'-tetramethylbenzidine and reaction was stopped using 50 µl of 0.5 M H_2SO_4 after 20-60 minutes, depending on the primary antibody (Ab). Absorbance was then read at 450nm on a standard plate reader.

Statistical analysis

All analyses were performed in R (R Development Core Team 2011). Results from ELISAs are expressed as anti-DNP equivalent concentrations in order to create a relative measure that can be used for all isotypes. The Calib package in R was use to create a logistic regression based on the absorbance values of the standards in each plate. The regression was then used to calculate the relative anti-DNP concentration for each sample. The mean of duplicate anti-DNP equivalent concentrations for each time point for each individual were used in all analyses. Using serum diluted 1:1000 with polyclonal IgM detection Ab or serum diluted 1:4000 with polyclonal IgG detection Ab, we calculated a standardized percent of IgG and IgM out of the sum total of IgG and IgM (percent IgG = IgG / (IgG + IgM)).

In the ELISAs using polyclonal anti-IgG and monoclonal anti-κ, the absorbance values from the two individuals with the highest titers exceeded the linear range of the standard curve. We were unable to further optimize the assay conditions to fall within the linear range of the standard curve because doing so would render undetectable the absorbance values of the two individuals with the lowest concentrations of anti-DNP antibodies. Instead of extrapolating beyond the linear range of the standard curve for the two individuals with the highest absorbance values, we assigned to these two individuals the maximum values within the linear range of the standard curve.

Table 1.2. Peak anti-DNP equivalent concentrations.

Subject	Age	Adjuvant	IgA	IgE	IgG	IgM	K chain	λ chain	poly IgG	poly IgM
A	4.7	YES	0.464	0.561	1.623	2.104	6.250[a]	0.392	12.500[a]	0.722
B	6.8	NO	0.139	0.138	0.123	6.361	4.035	0.077	5.365	2.502
C	11.2	YES	0.322	0.35	0.684	2.361	6.250[a]	0.235	12.500[a]	1.044
D	11.3	NO	0.042	0.048	0.024	0.356	0.824	0.016	1.365	0.236
E	13.9	YES	0.011	0.024	0.009	0.192	0.371	0.006	0.82	0.101
F	15.1	NO	0.003	0.011	0.002	0.159	0.106	0.001	0.322	0.134
G	15.1	YES	0.034	0.035	0.018	0.307	0.608	0.012	1.183	0.175
H	17	NO	0.01	0.006	0.001	0.208	0.055	0.001	0.16	0.198
Detection Ab catalog #			IgA5-3B	E6-71	GPB2-2	CM7	CAG8-7C	FIG1-7A	04-20-02	04-20-03
Detection Ab conc. (μg/ml)			5	10	10	5	10	5	0.5	0.5
Serum dilution			1:100	1:100	1:100	1:100	1:100	1:100	1:400	1:1000

Age is in years. Concentrations are the mean of duplicate ELISA results.

[a] Indicates the concentration exceeded the linear range of the standard curve.

Results

Western blot verification of cross-reactive antibodies and molecular weight of hyena immunoglobulins

Hyena γ heavy chain was slightly smaller than feline γ heavy chain based on Western blot analysis (Fig. 1.1). We estimate feline γ heavy chain to be 55-56 kilodaltons (kDa), whereas hyena γ heavy chain was 53-54 kDa (Table 1.3). Note that the estimated molecular weight for each heavy or light chain we tested varied slightly depending on whether we used purified Ig or sera, and also based on the serum dilution used; for this reason, molecular weights are presented as ranges rather than as specific values. Also, variations in sample preparation may explain the slight differences between our results and previously published results (Grant 1995; Klotz et al. 1985; Yamada et al. 2007). Hyena μ chain, estimated at 77-82 kDa, was slightly larger than feline μ chain, which we estimated at 74-80 kDa. Feline and hyena α heavy chains were 54-60 kDa and 57-61 kDa, respectively. Neither feline nor hyena ε heavy chain could be detected in serum by Western blot; we were unable to obtain purified IgE.

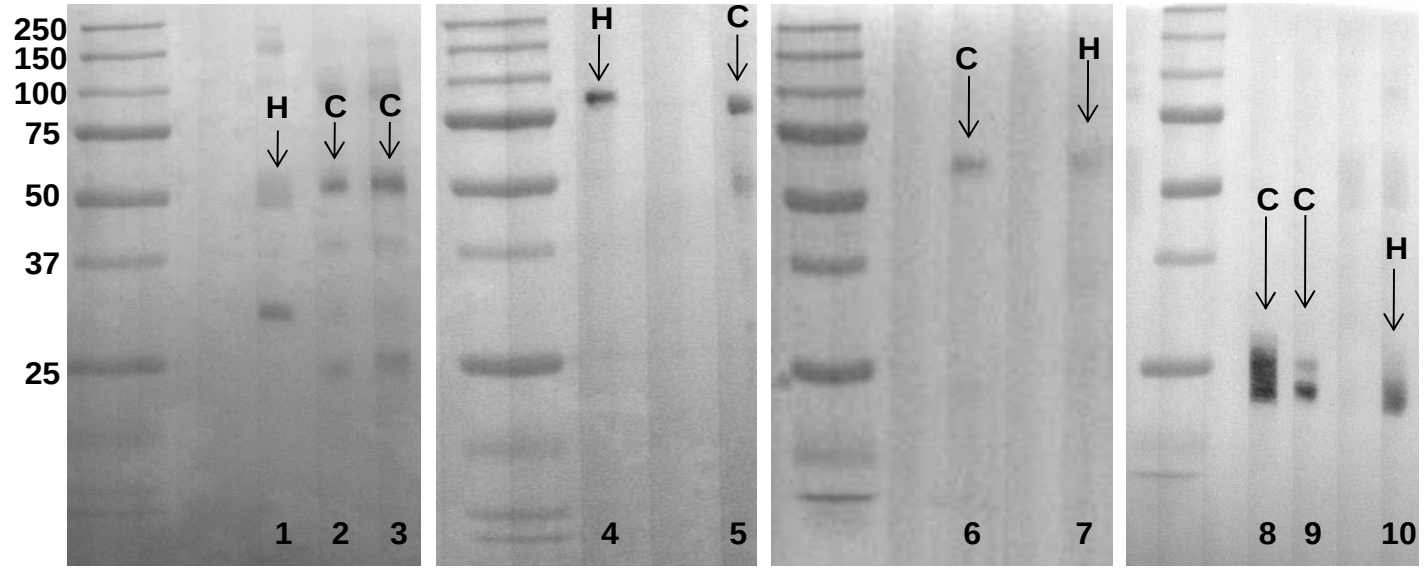

Fig. 1.1. Western blot of heavy and light chains in domestic cats (C) and spotted hyenas (H). Molecular weight standards in kilodaltons are indicated on the left.
Lane 1. Purified hyena IgG. Hyena γ chain detected with polyclonal anti-IgG (04-20-02)
Lane 2. Purified cat IgG. Cat γ chain detected with monoclonal anti-IgG (GPB2-2)
Lane 3. Purified cat IgG. Cat γ chain detected with polyclonal anti-IgG (04-20-02)
Lane 4. Purified hyena IgM. Hyena μ chain detected with monoclonal anti-IgM (CM7)
Lane 5. Purified cat IgM. Cat μ chain detected with monoclonal anti-IgG (CM7)
Lane 6. Purified cat IgA. Cat α chain detected with polyclonal anti-IgA (NB7264)
Lane 7. Purified hyena IgA. Hyena α chain detected with polyclonal anti-IgA (NB7264)
Lane 8. Cat serum with albumin removed. Cat light chain detected with monoclonal anti-λ light chain (CAG8-7C)
Lane 9. Cat serum with albumin removed. Cat light chain detected with monoclonal anti- κ light chain (FIG1-7A)
Lane 10. Hyena serum with albumin removed. Hyena light chain detected with monoclonal anti- κ light chain (FIG1-7A)

Anti-κ light chain mAb detected a 23-25 kDa light chain protein band in cat serum samples and a 22-25 kDa band in hyena serum samples. The molecular weight and staining patterns of the anti-κ light chain mAb are consistent with the previously reported molecular weight of carnivore light chains (Grant 1995; Klotz et al. 1985; Yamada et al. 2007). See Table 1.3 for a complete listing of molecular weights of feline and hyena heavy and light chains. Anti-λ light chain mAb detected a 23-28 kDa protein band in cat serum, but no bands were detectable in hyena serum or purified Ig.

Quantification of humoral response using ELISA

During the weeks after experimental immunization of captive hyenas, anti-DNP specific antibodies in serum samples increased from pre-immunization day 0 titers for all isotypes tested using monoclonal (Fig. 1.2) and polyclonal (Fig. 1.3) antibodies. As expected, day 14 serum samples were elevated above baseline, and peak anti-DNP concentrations in serum samples were attained on day 28 post-immunization for most individuals and isotypes. Individuals with the strongest anti-DNP response still had detectable titers at one year post-immunization.

Table 1.3. Molecular weights (kDa) of heavy and light chains

Target	Hyena	Cat
α	57-61[a]	54-60[a], 54-60[b]
ε	not detected[a]	not detected[a]
γ	53-54[a]	55-56[a], 50[b], 50[c], 59[d]
μ	77-82[a]	74-80[a], 74[b], 72[c]
κ	22-25[a]	24-25[a], 23-29[b], 22[c], 27[d]
λ	not detected[a]	23-28[a], 24-27[b], 22[c], 27[d]

[a] Results from this study

[b] Grant (1995)

[c] Klotz et al. (1985)

[d] Yamada et al. (2007)

The anti-DNP temporal dynamics for IgG and IgM were found to be similar regardless of whether we used polyclonal Abs or mAbs as anti-isotype probes. Individuals that had the highest IgG or IgM titers using mAb detection antibodies also had the highest titers using polyclonal antibodies for detection, and peak titers were reached on day 28 in most cases. Anti-IgM had the highest anti-DNP equivalent concentration for all individuals on day 0, suggesting that natural IgM antibodies to DNP were present prior to immunization (Ochsenbein and Zinkernagel 2000b). Among the anti-Ig mAbs tested, the anti-κ light chain detection antibody produced the highest anti-DNP equivalent concentrations; light chains are associated with all isotypes, so light chains are represented in higher concentrations than heavy chains.

Not surprisingly, use of adjuvant tended to elevate anti-DNP equivalent concentrations over those obtained with no adjuvant, although no statistical tests on adjuvant effect were performed due to small sample sizes. Interestingly, however, the four youngest individuals immunized attained the four highest anti-DNP titers across all isotypes tested, regardless of

whether or not they received adjuvant with the initial immunization (Table 1.2). The oldest hyena, individual H, was 17 years old at the initial immunization and did not receive adjuvant. Individual H did not produce a detectable response at a 1:100 serum dilution using mAb detection antibodies for IgG and λ, but did produce detectable responses for all other isotypes tested.

Temporal dynamics of the IgG and IgM relationship

Using serum diluted 1:1000 with polyclonal IgM detection Ab or serum diluted 1:4000 with polyclonal IgG detection Ab, we calculated a standardized percent of IgG and IgM out of the sum total of IgG and IgM (percent IgG = IgG / (IgG + IgM)). On day 0, anti-DNP IgG accounted for roughly 10% of the sum of IgG and IgM (Fig. 1.4). However, by days 14 and 28, IgG accounted for approximately 70% and 80% of the total, respectively. At days 180 and 365, IgG continued to account for more than 50% of the total anti-DNP IgG and IgM.

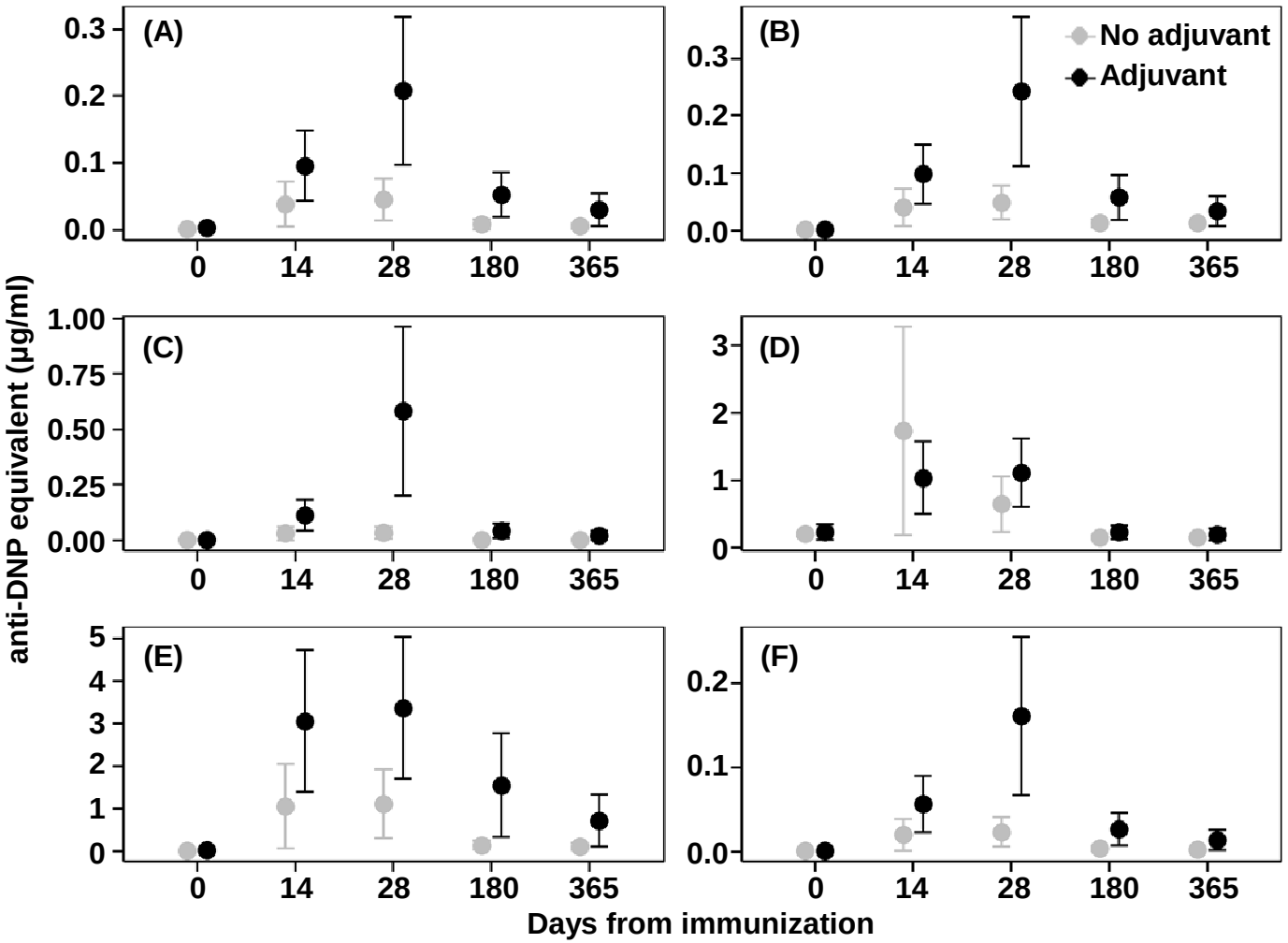

Fig. 1.2. ELISA results from DNP-KLH immunization using monoclonal detection antibodies for (A) IgA, (B) IgE, (C) IgG, (D) IgM, (E) κ light chain, and (F) λ light chain. Each point corresponds to the mean anti-DNP equivalent concentration (µg/ml) and error bars represent the SEM (n = 4). Black points are the means from individuals immunized with adjuvant and gray points are from individuals immunized without adjuvant. Day 0 represents pre-immune sera.

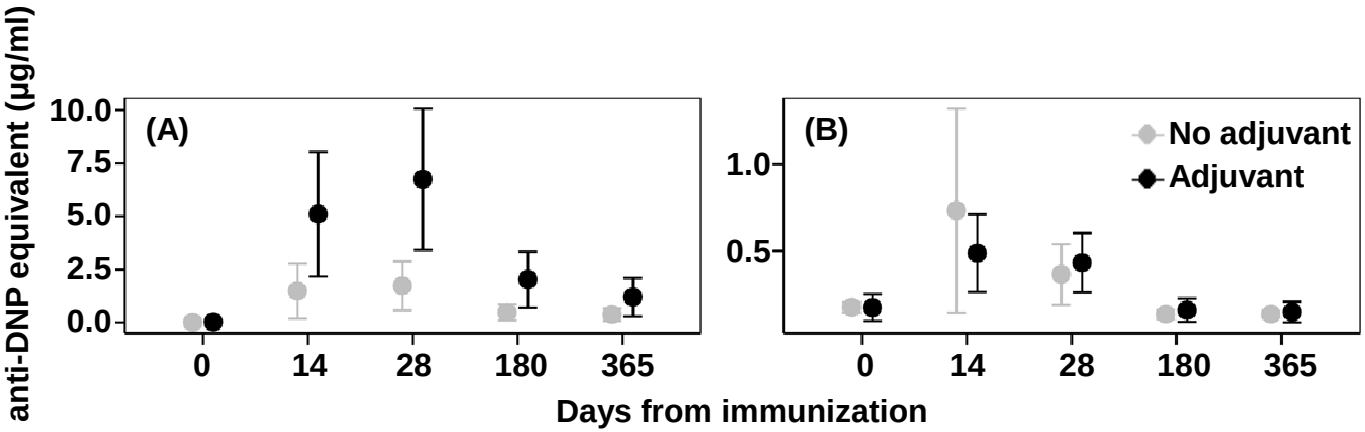

Fig. 1.3. ELISA results from DNP-KLH immunization using polyclonal detection antibodies for (A) IgG and (B) IgM. Each point corresponds to the mean anti-DNP equivalent concentration (µg/ml) and error bars represent the SEM (n = 4). Black points are the means from individuals immunized with adjuvant and gray points are from individuals immunized without adjuvant. Day 0 represents pre-immune sera.

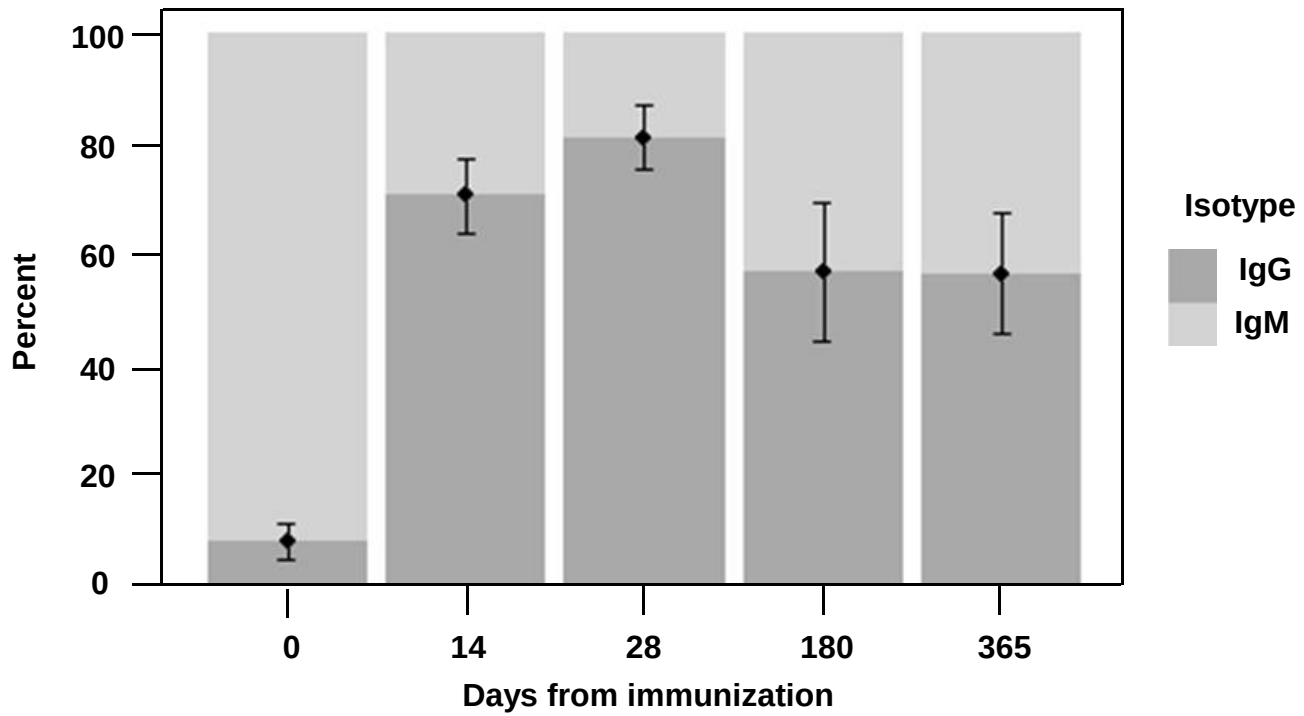

Fig. 1.4. Percent of total anti-DNP IgG and anti-DNP IgM. Percent IgG = IgG / (IgG + IgM). Percent IgM = IgM / (IgG + IgM). Serum was diluted 1:4000 for IgG and 1:1000 for IgM. Polyclonal detection antibodies were used at 0.5μg/μl for both IgG and IgM. Error bars represent the SEM of the percents for each day in the time course (n = 8). Individuals were included regardless of adjuvant status.

Discussion

Here we identified cross-reactive antibodies that specifically bind hyena epitopes of the four major secreted heavy chain isotypes found in carnivores, as well as two cross-reactive anti-light chain antibodies. The specific cross-reactivity of anti-cat Ig isotypes suggests a high level of homology between cats and hyenas. These antibodies can potentially be used for broad, cost effective monitoring of pathogen infections in spotted hyenas, and to establish baseline parameters of health and disease among wild members of this species throughout sub-Saharan Africa and among captive hyenas at zoos and research facilities. Additionally, these antibodies can likely be used to detect immunoglobulins in other species in the Hyaenidae family.

We confirmed binding of the antibodies to hyena immunoglobulins here with Western blots, and molecular weights were found to be close to that of cat immunoglobulins for each isotype examined. When we then used the antibodies to monitor the magnitude and temporal dynamics of humoral responses directed against the hapten-carrier complex DNP-KLH in immunized captive spotted hyenas, we observed the common pattern of an increased Ig concentration for all isotypes by day 14, with IgM concentration tapering off quickly and a gradual decrease in other isotypes over the course of one year. Thus, insofar as neither the magnitude nor temporal dynamics of the humoral response were unusual in spotted hyenas, these responses are unlikely to account for the unusual disease resistance observed in this species.

We were only able to identify one anti-IgG mAb that cross-reacted with hyena epitopes, despite identifying several cross-reacting polyclonal anti-IgG antibodies. Furthermore, this anti-IgG mAb showed either weak affinity for hyena IgG or was specific for an IgG subclass that is less abundant in hyenas than in cats. However, it will now be possible to develop anti-hyena IgG subclass-specific mAbs using the purified hyena IgG. Variation in Ab structure among species is

often most pronounced in subclasses of the major isotypes (Grant 1995), and differentiation between IgG_{2a} and IgG_1 would aid in the study of Th1 and Th2 subsets (Mosmann and Coffman 1989; Steinman 2007).

Interestingly, both ELISA and Western blot detected κ light chains at much higher concentrations than λ light chains in hyena sera. Generally, carnivores have approximately 90% λ and 10% κ light chains (Tizard 2009); More specifically, cats have been reported to have a 3:1 ratio of λ:κ using the same antibodies we used in this study (Grant 1995). It is possible that the reversed light chain ratio we observed in hyenas in this study is due to low affinity of the anti-λ light chain cat antibody for hyena immunoglobulins. However, the alternative possibility, that hyenas have a reversed light chain ratio compared to that found in most carnivores, merits further investigation.

To our knowledge, only three previous studies have reported cross-reacting antibodies that recognize immunoglobulins from any hyena species. First, anti-human IgA and IgM were found to cross-react with IgA and IgM from striped hyenas (*Hyaena hyaena*) using a gel diffusion assay (Neoh et al. 1973). Second, a polyclonal anti-cat IgG was used in an indirect immunofluorescence assay for diagnosis of coronavirus infection in spotted hyenas in Tanzania (East et al. 2004). Finally, polyclonal anti-cat IgG was used for Western blot detection of feline immunodeficiency virus (FIV) in spotted hyenas (Troyer et al. 2005). Two previous studies also used commercially available, Ab based test kits for confirmatory diagnosis of coronavirus and FIV infections (East et al. 2004; Harrison et al. 2004).

There are significant advantages of using hyena specific antibodies over other existing techniques for evaluating pathogen-specific responses. Most studies of pathogens infecting hyenas have relied on serum neutralization tests, agglutination assays, RT-PCR, or competitive

ELISAs that do not require species-specific antibodies (Alexander et al. 1995; Cronwright-Snoeren 2010; East et al. 2001; Harrison et al. 2004). Each of these existing techniques is limited in its usefulness by various factors, and use of the newly discovered antibodies described here would avoid many of these limitations. Serum neutralization tests rely on cell culture, which can be labor- and resource-intensive (Wellehan Jr et al. 2009). Positive RT-PCR results for pathogens provide clear evidence of infection, but offer little information about duration of the infection or the nature of the immune response. Additionally, RT-PCR can only detect active infections. Agglutination tests are rapid and cost-effective, but are most sensitive to pentameric IgM and less sensitive to other isotypes (Cohen et al. 1967). Competitive ELISAs and serum neutralization tests requiring no species-specific antibodies are limited because they permit no analysis of specific immunoglobulin isotypes. Rapid diagnosis using ELISA techniques can aid wildlife managers and veterinarians by permitting them to quickly diagnose disease outbreaks, and allowing them to develop better-informed responses to such outbreaks.

Potential uses of the new antibodies

We found that hyena antibodies can be detected in serum at least one year after exposure, long after many pathogens would be cleared from the host. Testing for both IgG and IgM has been used previously to stage infections; this method was used to assess whether infections are in earlier or later stages for tularemia (Carlsson et al. 1979), dengue (Innis et al. 1989), Rift Valley Fever (Pepin et al. 2010), myxomatosis in rabbits (*Oryctolagus cuniculs*) (Kerr 1997), and West Nile virus in equids (Durand et al. 2002). Pathogens to which wild spotted hyenas are known to be exposed include CDV, FIV, feline panleukopenia virus/canine parvovirus, feline coronavirus/feline infectious peritonitis virus, feline calicivirus, rabies, and bluetongue (Alexander et al. 1994; East et al. 2001; Harrison et al. 2004; Troyer et al. 2005). The temporal

dynamics apparent in the changing Ig ratios (Fig. 1.4) in our hyena subjects might be carefully exploited to stage infections. However, we emphasize that samples positive for IgM, but negative for IgG, must be interpreted cautiously, as this might indicate either an early stage infection or simply the presence of natural antibodies (Ochsenbein and Zinkernagel 2000a). The results of our experimental immunizations of captive hyenas show that all individuals had readily detectable anti-DNP IgM in pre-immune sera. Pre-infection sera are seldom available in serological studies of wildlife, so natural IgM might be misinterpreted as an active infection based solely on agglutination tests. Thus, ELISA or Western blots for IgG, IgA, and/or IgE in addition to assays for IgM are more effective for serological studies than agglutination tests alone.

In addition to facilitating the monitoring of pathogen exposure in spotted hyenas, the cross-reacting antibodies identified in our study will also provide researchers with tools for assessing maternal immunoglobulins in milk. Hyenas cubs are weaned roughly 14 months after birth, a lactation interval far longer than that of most other carnivores (Hofer and East 1995; Watts et al. 2009). Within 2-5 weeks of birth, spotted hyena cubs are transferred from an isolated natal den to a communal den where they live with up to 20 other cubs, and frequently engage in playful and aggressive interactions (Holekamp and Smale 1998; Kruuk 1972; Tanner et al. 2007). The frequent social interactions at communal dens create a situation that can lead to high rates of transmission of infectious pathogens (Altizer et al. 2003). Maternal IgG and IgA transferred from mother to offspring in milk could provide critical protection from pathogens during early life stages (Bourne and Curtis 1973; Brambell 1966; Claus et al. 2006; Mason et al. 1930).

IgG and IgM are commonly the focus of immunological studies, however, IgA is the primary Ab on mucosal surfaces, and in humans more IgA is produced than all other isotypes combined (Brandtzaeg et al. 1999; Fagarasan and Honjo 2003; Kerr 1990; van Egmond et al. 2001). Rabies virus has been detected in saliva of spotted hyenas expressing no clinical signs of disease (East et al. 2001); salivary IgA might play an important role in both intra- and inter-specific transmission of rabies by neutralizing the virus in saliva before the virus has a chance to infect new individuals. Furthermore, fecal samples are the most readily available and least invasive samples available in most studies of free-living carnivores, and these typically contain large quantities of IgA. Analysis of fecal IgA can thus potentially provide a glimpse into the gut and mucosal immune systems of wild hyenas.

Despite the common use of fecal parasite counts in wildlife studies (e.g. Engh et al. 2003; Gompper et al. 2003; Patton et al. 1986; Watve and Sukumar 1995), few studies have complemented these counts with analysis of fecal IgA concentrations or serum IgE concentrations (Devalapalli et al. 2006; Gompper et al. 2003). IgE is the primary isotype involved in defense against parasitic worms. Our study has identified two cross-reacting mAb against hyena IgE in ELISAs (Table 1.1) that might be used to assess effects of parasites on Ig concentrations and ratios. However, the specific binding of the anti-IgE mAbs could not be confirmed here by Western blot, possibly due to the low concentration and short half-life of IgE in serum. IgE and IgG_1 are associated with Th2 response (Mosmann and Coffman 1989; Mosmann and Sad 1996; Romagnani 1997), a topic that is ripe for a comparative study. For example, it would be interesting to compare Th1 and Th2 markers in captive hyenas living in relatively clean environments with wild hyenas that face a continual assault from food-borne and socially-transmitted pathogens.

In summary, we have identified Ig-class specific antibodies that specifically recognize hyena epitopes. The antibodies identified here can be used for monitoring the health of both wild and captive hyenas. We have shown that the cross-reacting antibodies can be used to assess specific antibody titer by ELISA; it is probable that these antibodies can also be used in Western blots for detecting antibodies against specific pathogens, which is commonly done to confirm ELISA or RT-PCR results. Finally, these basic immunology tools open the door to more advanced studies of immune function in a species that has demonstrated the remarkable ability to survive disease outbreaks that have decimated wild populations of other carnivore species living sympatrically with spotted hyenas.

LITERATURE CITED

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Alexander, K., and Appel, M. (1994). "African wild dogs (Lycaon pictus) endangered by a canine distemper epizootic among domestic dogs near the Masai Mara National Reserve, Kenya." *Journal of Wildlife Diseases*, 30(4), 481-485.

Alexander, K. A., Kat, P. W., Frank, L. G., Holekamp, K. E., Smale, L., House, C., and Appel, M. J. G. (1995). "Evidence of canine distemper virus infection among free-ranging spotted hyenas (*Crocuta crocuta*) in the Masai Mara, Kenya." *Journal of Zoo and Wildlife Medicine*, 26, 201-206.

Alexander, K. A., Maclaughlin, N. J., Kat, P. W., House, C., O'Brien, S. J., Lerche, N. W., Sawyer, M., Frank, L. G., Holekamp, K. E., Smale, L., McNutt, J. W., Laurenson, M. K., M.G.L. Mills, and Osburn, B. I. (1994). "Evidence of natural bluetongue virus infection among African and Indian carnivores." *American Journal of Tropical Medicine and Hygiene*, 51(568-576).

Altizer, S., Nunn, C. L., Thrall, P. H., Gittleman, J. L., Antonovics, J., Cunningham, A. A., Cunnningham, A. A., Dobson, A. P., Ezenwa, V., and Jones, K. E. (2003). "Social organization and parasite risk in mammals: integrating theory and empirical studies." *Annual Review of Ecology, Evolution, and Systematics*, 34, 517-547.

Berger, D., Frank, L., and Glickman, S. "Unraveling ancient mysteries: biology, behavior, and captive management of the spotted hyena, *Crocuta crocuta*." *Presented at Proceedings of the Joint Conference of the American Association of Zoo Veterinarians and the American Association of Wildlife Veterinarians*, Oakland, CA.

Bininda-Emonds, O. R. P., Gittleman, J. L., and Purvis, A. (1999). "Building large trees by combining phylogenetic information: a complete phylogeny of the extant Carnivora (Mammalia)." *Biological Reviews*, 74, 143-175.

Blount, J., Houston, D., Moller, A., and Wright, J. (2003). "Do individual branches of immune defence correlate? A comparative case study of scavenging and non-scavenging birds." *Oikos*, 102(2), 340-350.

Bourne, F., and Curtis, J. (1973). "The transfer of immunoglobulins IgG, IgA and IgM from serum to colostrum and milk in the sow." *Immunology*, 24(1), 157.

Brambell, F. (1966). "The transmission of immunity from mother to young and the catabolism of immunoglobulins." *Lancet*, 2(7473), 1087.

Brandtzaeg, P., Baekkevold, E. S., Farstad, I. N., Jahnsen, F. L., Johansen, F.-E., Nilsen, E. M., and Yamanaka, T. (1999). "Regional specialization in the mucosal immune system: what happens in the microcompartments?" *Immunology Today*, 20(3), 141-151.

Carlsson, H. E., Lindberg, A. A., Lindberg, G., Hederstedt, B., Karlsson, K.-A., and Agell, B. O. (1979). "Enzyme-Linked Immunosorbent Assay for Immunological Diagnosis of Human Tularemia." *J. Clin. Microbiol.*, 10(5), 615-621.

Claude, C. (1996). "Parasites, biodiversity and ecosystem stability." *Biodiversity and Conservation*, 5(8), 953-962.

Claus, M. A., Levy, J. K., MacDonald, K., Tucker, S. J., and Crawford, P. C. (2006). "Immunoglobulin concentrations in feline colostrum and milk, and the requirement of colostrum for passive transfer of immunity to neonatal kittens." *Journal of Feline Medicine & Surgery*, 8(3), 184-191.

Cohen, I. R., Norins, L. C., and Julian, A. J. (1967). "Competition Between, and Effectiveness of, IgG and IgM Antibodies in Indirect Fluorescent Antibody and Other Tests." *The Journal of Immunology*, 98(1), 143-149.

Cronwright-Snoeren, A. (2010). *The prevalence of canine distemper virus antibodies in wild carnivores in the Kruger National Park and Marakele National Park*, University of South Africa, Pretoria.

Devalapalli, A. P., Lesher, A., Shieh, K., Solow, J. S., Everett, M. L., Edala, A. S., Whitt, P., Long, R. R., Newton, N., and Parker, W. (2006). "Increased levels of IgE and autoreactive, polyreactive IgG in wild rodents: implications for the hygiene hypothesis." *Scandinavian Journal of Immunology*, 64(2), 125-36.

Durand, B., Chevalier, V., Pouillot, R., Labie, J., Marendat, I., Murgue, B., Zeller, H., and Zientara, S. (2002). "West Nile virus outbreak in horses, southern France, 2000: results of a serosurvey." *Emerging Infectious Diseases*, 8(8), 777.

East, M., Moestl, K., Benetka, V., Pitra, C., Höner, O. P., Wachter, B., and Hofer, H. (2004). "Coronavirus infection of spotted hyenas in the Serengeti ecosystem." *Veterinary Microbiology*, 102(1-2), 1-9.

East, M. L., Hofer, H., Cox, J. H., Wulle, U., Wiik, H., and Pitra, C. (2001). "Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas." *Proceedings of the National Academy of Sciences of the United States of America*, 98(26), 15026-15031.

East, M. L., Wibbelt, G., Lieckfeldt, D., Ludwig, A., Goller, K., Wilhelm, K., Schares, G., Thierer, D., and Hofer, H. (2008). "A *Hepatozoon* species genetically distinct from *H. canis* infecting spotted hyenas in the Serengeti Ecosystem, Tanzania." *J Wildl Dis*, 44(1), 45-52.

Engh, A., Nelson, K., Peebles, R., Hernandez, A., Hubbard, K., and Holekamp, K. (2003). "Coprologic survey of parasites of spotted hyenas (*Crocuta crocuta*) in the Masai Mara National Reserve, Kenya." *J Wildl Dis*, 39(1), 224-227.

Fagarasan, S., and Honjo, T. (2003). "Intestinal IgA synthesis: regulation of front-line body defences." *Nat Rev Immunol*, 3(1), 63-72.

Gompper, M. E., Goodman, R. M., Kays, R. W., Ray, J. C., Fiorello, C. V., and Wade, S. E. (2003). "A survey of the parasites of coyotes (*Canis latrans*) in New York based on fecal analysis." *Journal of Wildlife Diseases*, 39(3), 712.

Grant, C. K. (1995). "Purification and characterization of feline IgM and IgA isotypes and three subclasses of IgG", B. J. Willett and O. Jarrett, (eds.), *Feline immunology and immunodeficiency.* City, pp. 95-107.

Hanley, C. S., Simmons, H. A., Wallace, R. S., and Clyde, V. L. (2005). "Erythema Multiforme in a spotted hyena (*Crocuta crocuta*)." *Journal of Zoo and Wildlife Medicine*, 36(3), 515-519.

Harrison, T. M., Mazet, J. K., Holekamp, K. E., Dubovi, E., Engh, A. L., Nelson, K., Van Horn, R. C., and Munson, L. (2004). "Antibodies to canine and feline viruses in spotted hyenas (*Crocuta crocuta*) in the Masai, Mara National Reserve." *Journal of Wildlife Diseases*, 40(1), 1-10.

Hofer, H. (1998). "Spotted hyaena (*Crocuta crocuta*)", in M. G. L. Mills, G. Mills, and H. Hofer, (eds.), *Hyaenas: Status Survey and Conservation Action Plan*. Gland, Switzerland: World Conservation Union, pp. 29-38.

Hofer, H., and East, M. (1995). "Population dynamics, population size, and the commuting system of Serengeti spotted hyenas." *Serengeti II: dynamics, management, and conservation of an ecosystem*, 332–363.

Holekamp, K. E., and Sisk, C. L. (2003). "Effects of dispersal status on pituitary and gonadal function in the male spotted hyena." *Hormones and Behavior*, 44(5), 385-394.

Holekamp, K. E., and Smale, L. (1998). "Behavioral development in the spotted hyena." *BioScience*, 48(12), 997-1005.

Honer, O., Wachter, B., Speck, S., Wibbelt, G., Ludwig, A., Fyumagwa, R., Wohlsein, P., Lieckfeldt, D., Hofer, H., and East, M. (2006). "Severe Streptococcus infection in spotted hyenas in the Ngorongoro Crater, Tanzania." *Veterinary Microbiology*, 115(1-3), 223-228.

Houston, D. C. (1979). "The Adaptations of Scavengers", A. R. E. Sinclair and M. Norton-Griffiths, (eds.), *Serengeti: Dynamics of an Ecosystem*. City: University of Chicago Press: Chicago, pp. 263–286.

Innis, B. L., Nisalak, A., Nimmannitya, S., Kusalerdchariya, S., Chongswasdi, V., Suntayakorn, S., Puttisri, P., and Hoke, C. H. (1989). "An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate." *The American journal of tropical medicine and hygiene*, 40(4), 418.

Kat, P. W., Alexander, K. A., Smith, J. S., and Munson, L. (1995). "Rabies and African Wild Dogs in Kenya." *Proceedings: Biological Sciences*, 262(1364), 229-233.

Kat, P. W., Alexander, K. A., Smith, J. S., Richardson, J. D., and Munson, L. (1996). "Rabies among African wild dogs (*Lycaon pictus*) in the Masai Mara, Kenya." *Journal of Veterinary Diagnostic Investigation*, 8(4), 420.

Kerr, M. (1990). "The structure and function of human IgA." *Biochemical journal*, 271(2), 285.

Kerr, P. (1997). "An ELISA for epidemiological studies of myxomatosis: persistence of antibodies to myxoma virus in European rabbits (*Oryctolagus cuniculus*)." *Wildlife Research*, 24(1), 53-65.

Klotz, F., Gathings, W., and Cooper, M. (1985). "Development and distribution of B lineage cells in the domestic cat: analysis with monoclonal antibodies to cat mu-, gamma-, kappa-, and lambda-chains and heterologous anti-alpha antibodies." *J Immunol*, 134(1), 95-100.

Kruuk, H. (1972). *The Spotted Hyena: A Study of Predation and Social Behaviour*: University of Chicago Press.

Laemmli, U. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature*, 227(5259), 680-685.

Lembo, T., Hampson, K., Auty, H., Beesley, C. A., Bessell, P., Packer, C., Halliday, J., Fyumagwa, R., Hoare, R., Ernest, E., Mentzel, C., Mlengeya, T., Stamey, K., Wilkins, P. P., and Cleaveland, S. (2011). "Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009." *Emerging Infectious Diseases*, 17(3), 387-94.

Lewis, M., and Werdelin, L. (2000). "The evolution of spotted hyenas (*Crocuta crocuta*)." *IUCN Hyaena Specialist Group Newsletter*, 7, 34-36.

Mason, J., Dalling, T., and Gordon, W. (1930). "Transmission of maternal immunity." *The Journal of Pathology and Bacteriology*, 33(3), 783-797.

Mendes, L., Piersma, T., Hasselquist, D., Matson, K. D., and Ricklefs, R. E. (2006). "Variation in the innate and acquired arms of the immune system among five shorebird species." *J Exp Biol*, 209(2), 284-291.

Mosmann, T., and Coffman, R. (1989). "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties." *Annual Review of Immunology*, 7(1), 145-173.

Mosmann, T. R., and Sad, S. (1996). "The expanding universe of T-cell subsets: Th1, Th2 and more." *Immunology Today*, 17(3), 138-146.

Munson, L., Terio, K. A., Kock, R., Mlengeya, T., Roelke, M. E., Dubovi, E., Summers, B., Sinclair, A. R. E., and Packer, C. (2008). "Climate extremes promote fatal co-infections during canine distemper epidemics in African lions." *PloS one*, 3(6), e2545.

Neoh, S. H., Jahoda, D. M., Rowe, D. S., and Voller, A. (1973). "Immunoglobulin classes in mammalian species identified by cross-reactivity with antisera to human immunoglobulin." *Immunochemistry*, 10(12), 805-813.

O'Brien, S. J., and Johnson, W. E. (2005). "Big cat genomics." *Annual Review of Genomics and Human Genetics*, 6, 407-429.

Ochsenbein, A., and Zinkernagel, R. (2000a). "Natural antibodies and complement link innate and acquired immunity." *Trends in immunology*, 21(12), 624-629.

Ochsenbein, A. F., and Zinkernagel, R. M. (2000b). "Natural antibodies and complement link innate and acquired immunity." *Immunology Today*, 21(12), 624-630.

Patton, S., Rabinowitz, A., Randolph, S., and Johnson, S. S. (1986). "A coprological survey of parasites of wild neotropical felidae." *The Journal of Parasitology*, 517-520.

Pepin, M., Bouloy, M., Bird, B. H., Kemp, A., and Paweska, J. (2010). "Rift Valley fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention." *Veterinary Research*, 41(6).

R Development Core Team. (2011). "R: A Language and Environment for Statistical Computing", R. F. f. S. Computing, (ed.). City: Vienna, Austria.

Roelke-Parker, M. E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S. J., Pospischil, A., Hofmann-Lehmann, R., and Lutz, H. (1996). "A canine distemper virus epidemic in Serengeti lions(Panthera leo)." *Nature*, 379(6564), 441-445.

Romagnani, S. (1997). "The Th1/Th2 paradigm." *Immunology Today*, 18(6), 263-266.

Schulenburg, H., Kurtz, J., Moret, Y., and Siva-Jothy, M. T. (2009). "Introduction. Ecological immunology." *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1513), 3-14.

Speck, S., Höner, O. P., Wachter, B., and Fickel, J. (2008). "Characterization of Streptococcus equi subsp. ruminatorum isolated from spotted hyenas (Crocuta crocuta) and plains zebras (Equus burchelli), and identification of a M-like protein (SrM) encoding gene." *Veterinary microbiology*, 128(1-2), 148-159.

Steinman, L. (2007). "A brief history of TH17, the first major revision in the TH1/TH2 hypothesis of T cell–mediated tissue damage." *Nature Medicine*, 13(2), 139-145.

Steward, M., and Petty, R. (1972). "The use of ammonium sulphate globulin precipitation for determination of affinity of anti-protein antibodies in mouse serum." *Immunology*, 22(5), 747.

Tanner, J., Smale, L., and Holekamp, K. (2007). "Ontogenetic variation in the play behavior of spotted hyenas." *Journal of Developmental Processes*, 2(2), 5-30.

Tizard, I. (2009). *Veterinary Immunology:An Introduction*: Elsevier Inc.

Troyer, J., Pecon-Slattery, J., Roelke, M., Johnson, W., VandeWoude, S., Vazquez-Salat, N., Brown, M., Frank, L., Woodroffe, R., and Winterbach, C. (2005). "Seroprevalence and genomic divergence of circulating strains of feline immunodeficiency virus among Felidae and Hyaenidae species." *The Journal of Virology*, 79(13), 8282-8294.

van Egmond, M., Damen, C. A., van Spriel, A. B., Vidarsson, G., van Garderen, E., and van de Winkel, J. G. J. (2001). "IgA and the IgA Fc receptor." *Trends in immunology*, 22(4), 205-211.

Van Helden, P. D., van Pittius, N. C. G., Warren, R. M., Michel, A., Hlokwe, T., Morar, D., Godfroid, J., Du Plessis, E. C., and Bengis, R. (2008). "Pulmonary infection due to Mycobacterium goodii in a spotted hyena (Crocuta crocuta) from South Africa." *Journal of Wildlife Diseases*, 44(1), 151-154.

Watts, H. E., and Holekamp, K. E. (2009). "Ecological Determinants of Survival and Reproduction in the Spotted Hyena." *Journal of Mammalogy*, 90(2), 461-471.

Watts, H. E., Tanner, J. B., Lundrigan, B. L., and Holekamp, K. E. (2009). "Post-weaning maternal effects and the evolution of female dominance in the spotted hyena." *Proceedings of the Royal Society B: Biological Sciences*, 276(1665), 2291-2298.

Watve, M. G., and Sukumar, R. (1995). "Parasite abundance and diversity in mammals: correlates with host ecology." *Proceedings of the National Academy of Sciences*, 92(19), 8945-8949.

Wellehan Jr, J. F. X., Green, L. G., Duke, D. G., Bootorabi, S., Heard, D. J., Klein, P. A., and Jacobson, E. R. (2009). "Detection of specific antibody responses to vaccination in variable flying foxes (*Pteropus hypomelanus*)." *Comparative Immunology, Microbiology and Infectious Diseases*, 32(5), 379-394.

Werdelin, L. (1989). "Constraint and adaptation in the bone-cracking canid Osteoborus (Mammalia: Canidae)." *Paleobiology*, 15(4), 387-401.

Yamada, O., Tamura, K., Yagihara, H., Isotani, M., Azakami, M., Sawada, S., Ono, K., Washizu, T., and Bonkobara, M. (2007). "Light-chain multiple myeloma in a cat." *J Vet Diagn Invest*, 19(4), 443-447.

CHAPTER 2

CHAPTER 2

CHARACTERIZATION OF TOLL-LIKE RECEPTORS 1-10 IN SPOTTED HYENAS

Introduction

Pathogens are ubiquitous in most environments, and organisms at all trophic levels have evolved to defend themselves against exploitation. Identification and eradication of pathogens is necessary to maintain host health, and individuals who cannot quickly identify and eliminate pathogens will succumb to infectious disease. However, not all microorganisms are pathogenic, and the ability of an organism to distinguish friend from foe is critical for survival and reproduction. Excessive or unnecessary responses against non-pathogenic microorganisms can waste critical host resources, inflict collateral damage on the host itself, and kill potentially helpful microorganisms. Further complicating the issue, some microorganisms are beneficial when they are sequestered in one organ system, such as digestion-aiding bacteria in the gut, whereas the same microorganism can cause life-threatening damage in other organ systems, such as the nervous system.

Toll-like receptor (TLR) genes are highly conserved across vertebrate genomes and the proteins for which they code function as sentinels of the immune system. Recognition of conserved pathogen-associated molecular patterns (PAMPs) by TLRs is often the first step in initiating both innate and adaptive immune responses (Barton and Kagan 2009). Due to the variable immune challenges confronted by different tissue types, TLR expression is also variable among host tissues (Siegemund and Sauer 2012). Furthermore, TLR expression is modulated in response to exposure levels. For example, TLR4, the receptor for lipopolysaccharides (LPS) derived from gram-negative bacteria, is expressed at low levels in the gut, where the immune

system is tolerant to the abundance of gram-negative bacteria (Abreu et al. 2001), but at higher levels in other types of tissues. This trade-off of resistance and tolerance extends more broadly to immune function as a whole. Resistance, the limiting of pathogen burden in a host, and tolerance, the limiting of health impacts of pathogens, are two common immune defense strategies (Schneider and Ayres 2008).

Chronic exposure to pathogens may lead to the induction of immune tolerance in order to avoid excessive inflammation. The behavioral ecology of spotted hyenas (*Crocuta crocuta*) suggests that exposure to pathogens from scavenging on putrid carcasses and suffering wounds inflected by conspecifics, competitors, and prey animals should be commonplace, and so the development of immune tolerance may be adaptive in this species. By contrast, the human immune system is extremely sensitive to microbial antigens, and autoimmune and other immunopathological disorders are commonplace in humans (Opal and Huber 2002). It has been well-documented that spotted hyenas are capable of surviving exposure to pathogens that are lethal to sympatric species (East et al. 2001; Harrison et al. 2004; Lembo et al. 2011), but little is known about the underlying mechanisms of immune function in spotted hyenas (Flies et al. 2012).

In order to begin elucidating the mechanisms of disease resistance and tolerance in spotted hyenas, here we characterized the sequences of TLR genes in spotted hyenas. Here we report partial sequences for hyena TLRs 1-10, and assess homology between TLRs in hyenas and other mammals. We also quantify relative TLR expression in four hyena tissues as a first step in mapping TLR expression in this species.

Materials and Methods

Tissue collection

The captive spotted hyenas used in this study were born and housed at the Field Station for Behavioral Research (FSBR) of the University of California, Berkeley (UCB). Tissue samples were opportunistically collected from two hyenas that were euthanized as part of a different study at UCB. Hyena 1 was 18 years old and hyena 2 was 13 years old at euthanization; both hyenas were in overall good health. Prior to euthanization, the animals were immobilized by blow dart delivered via intramuscular injections of ketamine (4–6 mg/kg) and xylazine (1 mg/kg). Following immobilization, hyenas were heavily sedated with diazepam, and euthanized with 1 mL / 4.5 kgs of Euthasol (Virbac AH, Inc, Ft. Worth, Texas). All tissues were excised by a clinical veterinarian from the Office of Laboratory Animal Care at the UCB, then rinsed with phosphate-buffered saline to remove red blood cells, and immersed into RNAlater solution. Tissue samples were stored overnight at ambient temperature, then cooled to 4 ˚C, and frozen at -80°C until further use.

Genomic DNA (gDNA) from wild spotted hyenas that had been collected as part of a long-term study in the Maasai Mara National Reserve, Kenya was used for primer testing and as a control for cDNA testing. Immobilization, blood collection, and gDNA extraction were completed as previously described (Engh et al. 2002; Van Horn et al. 2004). Bobcat (*Lynx rufus*) gDNA was provided by Kim Scribner (Michigan State University) and used as a positive control during initial primer testing.

RNA isolation

RNAlater-preserved samples were cut into small pieces (< 0.5 cm) to preserve excess tissue and improve processing efficiency. RNA was extracted following the manufacturer's instructions for the PrepEase RNA Spin Kit (USB, #78767) except for the cell lysis procedure.

For cell lysis, tissues were homogenized using 0.3 g of homogenizing beads (MO BIO Laboratories, #13113-50) and a bead beater homogenizer (Biospec Products, #693) for 45 seconds, then centrifuged at 10,000 RPM for 30 seconds, and the supernatant collected. Turbo DNAse (life Technologies # AM2238) was used in addition to the DNAse step provided in the PrepEase protocol to ensure digestion of all residual DNA.

Extracted RNA concentrations were determined using a Nanodrop spectrophotometer, and only samples with 260/280 ratios greater than 1.8 were included in the study. RNA solutions were then diluted to 1 ng/μL using RNase free water and frozen at -80 ˚C until further use. RNA integrity was assessed using an Agilent 2100 Bioanalyzer. Only samples with RNA integrity numbers (RINs) over 7.5 were used for cDNA creation and quantification of gene expression.

Primers and sequences

Felids are the closest phylogenetic relatives to hyenas for which TLRs have previously been sequenced (Ignacio et al. 2005), so we first attempted to use feline TLR primers with hyena gDNA. Bobcat gDNA was used as a positive control during our initial primer testing. New primers were designed as needed using the Integrated DNA Technologies' primer design tools based on the genome of the domestic cat (*Felis catus*). TLR10 primer sequences were obtained from Mercier, Peters et al. (2012) and feline primer sequences for the reference gene HPRT were obtained from Penning *et al*. (2007). PCR products for each gene were purified using a PCR clean-up system (Promega, #A9281) and sequenced at the MSU Research Technology Support Facility. Consensus sequences were created using CLC Sequence Viewer version 6.7.1 (Knudsen et al. 2012). The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) was used to calculate percent identity between hyena sequences and sequences from domestic cats,

domestic dogs (*Canis lupus familiaris*), humans (*Homo sapiens*), and house mice (*Mus musculus*).

Real-time quantitative reverse transcription PCR (qRT-PCR)

RNA (0.5 μg) was reverse transcribed to cDNA using random and oligo(dT) primers following the manufacturer's instructions for the qScript cDNA synthesis kit (VWR, # 101414-098) to a final volume of 20 μL. cDNA was then stored at 80˚C until use. All qPCR reactions were carried out using a Bio-Rad iQ5 iCycler and iQ5 software. PCR-grade water, PerfeCTa SYBR Green Fastmix (VWR, # 101414-262), primers, and cDNA template were aliquoted into 96-well plates and sealed. Each combination of tissue sample and target gene was assayed in triplicate. Primers were used at 200 nM with 2 μl of cDNA product per 25 μl reaction. We used a no template control and a no reverse transcriptase RNA control for each tissue sample tested. All qRT-PCR reactions used the following conditions: 2 minutes at 95˚C; followed by 45 cycles of 15 seconds at 95˚C, 30 seconds at T_a, and 60 seconds at 72˚C. Following the final extension of 120 seconds at 72˚C, a stepwise melt curve from 45˚C to 95˚C was performed. HPRT was used as a reference gene to normalize expression of target genes using the ΔΔCt method (Livak and Schmittgen 2001). Amplification efficiency was assessed using 10,000-fold serial dilutions of purified PCR products, and efficiency was calculated using Bio-Rad iQ5 software.

Results

DNA and amino acid (AA) sequence identity

We were able to amplify and sequence cDNA for TLRs 1-10 and the reference gene HPRT. See Table 2.1 for primer sequences, amplicon base pair lengths, and annealing temperatures. Hyena DNA sequences exhibited a high degree of sequence similarity to domestic

cats, with sequence identity ranging from 92-98% (Table 2.2). Nucleotide sequence identity between hyenas and other mammals in order of highest to lowest sequence similarity was: domestic cat, domestic dogs, humans, and mice. The only exception to this order was that the hyena TLR5 was more similar to the human TLR5 than it was to the domestic dog TLR5. Domestic cat TLR5 was more similar to human TLR5 than it was to domestic dog TLR5.

Table 2.1. Details of primers, amplicons, and reaction conditions.

Gene	Primer sequence 5' - 3' Forward / Reverse	Amplicon length (bp)	Ta (°C)
TLR1	AGTCAGCACAGCAGTAAACCTGGA GCTTGTATGCCAAACCAACTGGATG	190	54
TLR2	AGACTCTACCAGATGCCTCCTTCT GCGTGAAAGACAGGAATTCACAGG	168	56
TLR3	GACCTGTCAAGCCATTACCTCTGT CAAACTGCTCTGGCTGTCTGTCTA	255	55
TLR4	GCTGGCAATTCTTTCCAGGACAAC TCTGGAGGGAGTGAAGAGGTTCAT	208	57
TLR5	TTCCTTCCGCCAGGAGTATTTAGC GGAGTTCGCACTCACAGATGAACT	217	55
TLR6	CTCTCAAACATGTGGAAACAACTCGG GCTTGATGTCTGAGGACAAAGCAT	296	56
TLR7	TGGTGGGTTAACCATACAGAGGTG GAGAAAGAGCCACCGATACGGAAA	172	57
TLR8	GGACCGCTACCAACCTAACCATTT ACGATGCTCTTCCCTCTTTGATCC	169	55
TLR9	CTGGAGGAGCTGAACCTGAG GCGGGCAGGGGTTCTTATAG	100	52
TLR10	TGCCAACAACACATCCTTG GCAAGCACCTGAAAACAGAA	143	52
HPRT	ACTGTAATGACCAGTCAACAGGGG TGTATCCAACACTTCGAGGAGTCC	209	51

Hyena AA sequence identity to other species (Table 2.2) was much more variable than DNA sequence identity. Hyenas again generally show highest sequence similarity to domestic

cats; however, hyena TLR3 was more similar to domestic dogs than it was to that of domestic cats. Hyena TLR4, TLR5, and TLR6 AA sequences were more similar to human sequences than to dog sequences. Mouse AA similarity with hyenas was the lowest in all cases. HPRT AA sequence identity was greater than 95% for all species tested.

Table 2.2. DNA and amino acid (AA) sequence identity among *C. crocuta* and other mammals.

	F. catus		*C. lupus*		*H. sapiens*		*M. musculus*	
Gene	DNA	AA	DNA	AA	DNA	AA	DNA	AA
TLR1	97	92	89	79	85	70	74	63
TLR2	98	98	85	89	85	82	76	65
TLR3	95	89	92	92	89	82	81	78
TLR4	94	91	80	74	77	68	72	60
TLR5	93	93	76	70	77	77	70	67
TLR6	93	84	85	66	80	69	70	57
TLR7	98	98	91	83	89	85	83	80
TLR8	96	98	88	93	82	80	77	74
TLR9	92	91	91	88	89	88	80	78
TLR10	95	88	94	83	82	67	*	*
HPRT	96	97	95	95	93	97	90	95

* *M. musculus* do not possess a functional TLR10 gene

Tissue specific TLR expression

We assessed TLR expression in eight tissues from two hyenas: spleen (2x), lung (2x), pancreas (2x), inguinal lymph node, and sternal lymph node. Both lymph node samples were from the same hyena. Transcripts for TLRs 1-10 and HPRT were detected in all 8 tissues, although some occurred at relatively low levels. No amplification of non-reverse-transcribed RNA was observed in any of the samples we used, indicating there was no gDNA contamination. Additionally, the HPRT primer set spanned an intron, but only band sizes that corresponded to the mRNA transcript were observed.

Due to the small sample size (n = 2), we did not perform any statistical analysis, and have graphically presented the results from the eight tissues (Fig. 2.1). TLR5 was highly expressed in both pancreas samples, but was only minimally expressed in lymph nodes and spleens. TLR2 was the most abundant transcript in lungs from both hyenas. TLR3 was expressed only at modest levels in all tissues. TLR4, the primary receptor for lipopolysaccharide from gram-negative bacteria was expressed at low levels in most tissues.

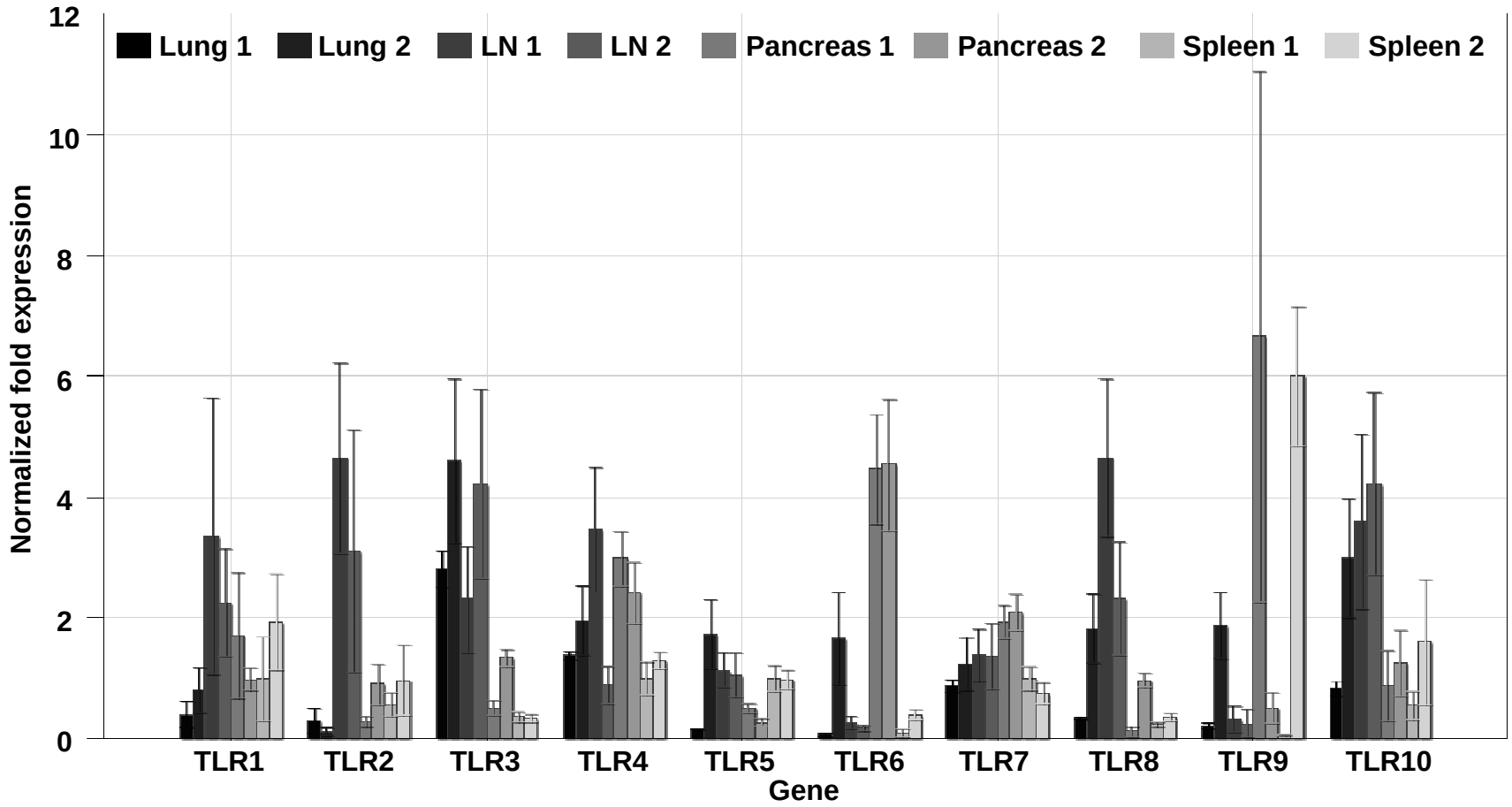

Fig. 2.1. Normalized expression of TLRs 1-10 in four tissues from two hyenas. Lymph node 1 (LN 1) is an inguinal lymph node from hyena 1 and lymph node 2 (LN2) is a sternal lymph node from hyena 1. Tissue samples 1 and 2 are from different hyenas for lung, pancreas, and spleen.

Discussion

Prior to this study, it was unknown which, if any, TLRs were expressed in hyena tissues. We have confirmed the expression of TLRs 1-10 in tissues collected from both spotted hyenas sampled here. All hyena sequences were more similar to sequences from domestic cats than those from domestic dogs; this was expected because both domestic cats and hyenas belong to the Feliformia suborder of the Carnivora order, whereas dogs belong to the Caniformia suborder.

Our data show that hyena TLRs are highly conserved relative to those of other mammals. Roach *et al*. (2005) suggest this is because the PAMPs, such as flagellin, that are recognized by TLRs are functionally important and cannot be easily mutated without reducing host fitness. The TLR1 family, which includes TLRs 1, 2, 6, and 10, form extracellular heterodimers that recognize lipopeptides (Roach et al. 2005). Roach, *et al*. (2005) suggested that TLR2 in most species may be under strong stabilizing selection, whereas TLR2 heterodimer mates, such as TLR6 may be under fewer evolutionary constraints than TLR2 itself. Our data support this hypothesis, as hyena TLR6 had the lowest AA sequence similarity (84%) with domestic cats of the ten TLRs we assessed, whereas TLR2 AA sequence identity (98%) was the highest, suggesting selection favoring conservation of this TLR.

Of particular interest in this context is how spotted hyenas survive regular exposure to rabies virus, whereas survival after rabies virus infection is extremely rare in most other mammals, with the notable exception of bats (Cowled et al. 2011). Rabies virus is a negative-sense single-stranded RNA virus that replicates primarily in neurons, eventually making its way to the salivary glands, where it can be secreted with saliva and thus transferred to new hosts. TLR3 is an intracellular transmembrane protein that recognizes double-stranded RNA (dsRNA) and host mRNA, and is a negative regulator of axonal growth (Cameron et al. 2007). $TLR3^{-/-}$

mice are more resistant to rabies virus than mice with functional TLR3 (Ménager et al. 2009). TLR3 was expressed at medium levels relative to other TLRs in all hyena tissues tested in this study. In black flying foxes (*Pteropus alecto*), TLR3 is highly expressed in the liver, moderately expressed in salivary glands, and minimally expressed in the brain (Cowled et al. 2011). It would be interesting to assess TLR3 expression in hyena neurons and salivary glands, as these are the tissues in which the rabies virus is most likely to be detected. Artificially inoculating long-lived animals with virulent pathogens such as rabies virus or anthrax are not feasible, but expression dynamics in response to protective rabies vaccines or attenuated anthrax strains could shed light on how hyenas are able to survive infection with these pathogens. Standard protocols for preservation of RNA in tissue samples opportunistically collected in the field, particularly from animals that have died of disease (East et al. 2001), would facilitate greater understanding of disease resistance mechanisms operating in hyenas.

One of the primary functions of TLRs is to help regulate the trillions of microorganisms that colonize mammals (Ley et al. 2008; Turnbaugh et al. 2007). Despite the vast number of microorganisms inhabiting most mammals, most sites within the body are free of microorganisms in healthy individuals, with the exception of the gut, skin, and mucous membranes. Systems with no direct exposure to the host's external environment, such as the brain and pancreas, should remain free of microorganisms. TLR5, which recognizes the bacterial protein flagellin, was expressed at higher levels in the two hyena pancreas samples than in the other six tissues we tested. Stimulation of TLR5 by flagellin in pancreatic islet cells of mice leads to reduced insulin secretion (Weile et al. 2011), which is hypothesized to allow increased circulating glucose for the immune system (Mizock 1995). The high expression of TLR5 in the

pancreas suggests that hyenas may respond in a similar way when threatened with a systemic infection.

TLRs, and immune function in general, can be regulated within a host by the presence or absence of microorganisms. Previous research shows that active infections, exposure to TLR agonists, or even common molecules such as glucose can significantly affect TLR expression levels (Franchini et al. 2010; Kwon et al. 2010; Mercier et al. 2012; Weile et al. 2011). For example, dogs infected with sino-nasal aspergillosis had significantly different expression levels of TLRs 1-4, and 6-10 in infected tissues (Mercier et al. 2012). No clinical signs of disease were observed in the hyenas used in this study, but our data show that TLR expression in several cases was variable between individuals within tissue types. For example, TLR8 was highly expressed in spleen 2, but expressed at a very low level in spleen 1, whereas the reverse pattern was observed in pancreas.

Chapter three of this dissertation shows that several measures of inducible immune function are higher in wild hyenas than in captive hyenas, and it is hypothesized that this difference is mediated by greater pathogen exposure in wild hyenas than in captives. The serum used in chapter three was obtained from peripheral blood samples, which are obtained via a relatively non-invasive form of tissue collection compared to collecting solid, internal organs. Because peripheral blood sample collection does not rely on euthanized animals or animals that have recently died of natural causes, it would be feasible to compare TLR expression in leukocytes isolated from the blood of wild and captive hyenas. If the hypothesis that pathogen exposure is the primary mediator of the observed differences between captive and wild hyenas, then we should see clear differences in the expression of TLRs between captive and wild hyenas.

LITERATURE CITED

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Abreu, M. T., Vora, P., Faure, E., Thomas, L. S., Arnold, E. T., and Arditi, M. (2001). "Decreased expression of toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide." *The Journal of Immunology*, 167(3), 1609-1616.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Research*, 25(17), 3389-3402.

Barton, G. M., and Kagan, J. C. (2009). "A cell biological view of toll-like receptor function: regulation through compartmentalization." *Nature reviews. Immunology*, 9(8), 535-42.

Cameron, J. S., Alexopoulou, L., Sloane, J. A., DiBernardo, A. B., Ma, Y., Kosaras, B., Flavell, R., Strittmatter, S. M., Volpe, J., Sidman, R., and Vartanian, T. (2007). "Toll-like receptor 3 is a potent negative regulator of axonal growth in mammals." *The Journal of Neuroscience*, 27(47), 13033-13041.

Cowled, C., Baker, M., Tachedjian, M., Zhou, P., Bulach, D., and Wang, L. F. (2011). "Molecular characterisation of Toll-like receptors in the black flying fox Pteropus alecto." *Developmental and comparative immunology*, 35(1), 7-18.

East, M. L., Hofer, H., Cox, J. H., Wulle, U., Wiik, H., and Pitra, C. (2001). "Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas." *Proceedings of the National Academy of Sciences of the United States of America*, 98(26), 15026-31.

Engh, A. L., Funk, S. M., Horn, R. C. V., Scribner, K. T., Bruford, M. W., Libants, S., Szykman, M., Smale, L., and Holekamp, K. E. (2002). "Reproductive skew among males in a female-dominated mammalian society." *Behavioral Ecology*, 13(2), 193-200.

Flies, A. S., Grant, C. K., Mansfield, L. S., Smith, E. J., Weldele, M. L., and Holekamp, K. E. (2012). "Development of a hyena immunology toolbox." *Veterinary Immunology and Immunopathology*, 145(1–2), 110-119.

Franchini, M., Zini, E., Osto, M., Jablonski, K., Kaufmann, K., Lutz, T. A., Reusch, C. E., and Ackermann, M. (2010). "Feline pancreatic islet-like clusters and insulin producing cells express functional Toll-like receptors (TLRs)." *Veterinary immunology and immunopathology*, 138(1-2), 70-8.

Harrison, T. M., Mazet, J. K., Holekamp, K. E., Dubovi, E., Engh, A. L., Nelson, K., Van Horn, R. C., and Munson, L. (2004). "Antibodies to canine and feline viruses in spotted hyenas (*Crocuta crocuta*) in the Masai, Mara National Reserve." *Journal of Wildlife Diseases*, 40(1), 1-10.

Ignacio, G., Nordone, S., Howard, K. E., and Dean, G. A. (2005). "Toll-like receptor expression in feline lymphoid tissues." *Veterinary immunology and immunopathology*, 106(3–4), 229-237.

Knudsen, B., Knudsen, T., Flensborg, M., Sandmann, H., Heltzen, M., Andersen, A., Dickenson, M., Bardram, J., Steffensen, P. J., Mønsted, S., Lauritzen, T., Forsberg, R., Thanbichler, A., Bendtsen, J. D., Görlitz, L., Rasmussen, J., Tordrup, D., Værum, M., Ravn, M. N., Hachenberg, C., Fisker, E., Dekker, P., de Meza, J., Hein, A.-M. K., Sinding, J. B., Quorning, J., Hvam, K., Mikkelsen, S., Liboriussen, P., Grydholt, J., Handberg, H., Bundgaard, M., Joecker, A., Simonsen, M., Nielsen, P. R. L., Joecker, A., Fleischer, P., Jakobsen, J., Juul, S., Appelt, U., Fejes, A., and Christensen, A. S. (2012). "CLC Sequence Viewer". City: CLC bio.

Kwon, S., Vandenplas, M. L., Figueiredo, M. D., Salter, C. E., Andrietti, A. L., Robertson, T. P., Moore, J. N., and Hurley, D. J. (2010). "Differential induction of Toll-like receptor gene expression in equine monocytes activated by Toll-like receptor ligands or TNF-α." *Veterinary immunology and immunopathology*, 138(3), 213-217.

Lembo, T., Hampson, K., Auty, H., Beesley, C. A., Bessell, P., Packer, C., Halliday, J., Fyumagwa, R., Hoare, R., Ernest, E., Mentzel, C., Mlengeya, T., Stamey, K., Wilkins, P. P., and Cleaveland, S. (2011). "Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009." *Emerging Infectious Diseases*, 17(3), 387-94.

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., Schlegel, M. L., Tucker, T. A., Schrenzel, M. D., Knight, R., and Gordon, J. I. (2008). "Evolution of mammals and their gut microbes." *Science*, 320(5883), 1647-51.

Livak, K. J., and Schmittgen, T. D. (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCt method." *Methods*, 25(4), 402-408.

Ménager, P., Roux, P., Mégret, F., Bourgeois, J.-P., Le Sourd, A.-M., Danckaert, A., Lafage, M., Préhaud, C., and Lafon, M. (2009). "Toll-like receptor 3 (TLR3) plays a major role in the formation of rabies virus negri bodies." *PLoS pathogens*, 5(2), e1000315.

Mercier, E., Peters, I. R., Day, M. J., Clercx, C., and Peeters, D. (2012). "Toll- and NOD-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis." *Veterinary immunology and immunopathology*, 145(3–4), 618-624.

Mizock, B. A. (1995). "Alterations in carbohydrate metabolism during stress: A review of the literature." *The American Journal of Medicine*, 98(1), 75-84.

Opal, S., and Huber, C. (2002). "Bench-to-bedside review: Toll-like receptors and their role in septic shock." *Critical Care*, 6(2), 125 - 136.

Penning, L. C., Vrieling, H. E., Brinkhof, B., Riemers, F. M., Rothuizen, J., Rutteman, G. R., and Hazewinkel, H. A. W. (2007). "A validation of 10 feline reference genes for gene

expression measurements in snap-frozen tissues." *Veterinary immunology and immunopathology*, 120(3–4), 212-222.

Roach, J., Glusman, G., Rowen, L., Kaur, A., Purcell, M., Smith, K., Hood, L., and Aderem, A. (2005). "The evolution of vertebrate Toll-like receptors." *Proc Natl Acad Sci USA*, 102, 9577 - 9582.

Schneider, D. S., and Ayres, J. S. (2008). "Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases." *Nature reviews. Immunology*, 8(11), 889-895.

Siegemund, S., and Sauer, K. (2012). "Balancing pro- and anti-inflammatory TLR4 signaling." *Nature immunology*, 13(11), 1031-1033.

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., and Gordon, J. I. (2007). "The Human Microbiome Project." *Nature*, 449(7164), 804-810.

Van Horn, R. C., Engh, A. L., Scribner, K. T., Funk, S. M., and Holekamp, K. E. (2004). "Behavioural structuring of relatedness in the spotted hyena (Crocuta crocuta) suggests direct fitness benefits of clan-level cooperation." *Molecular ecology*, 13(2), 449-458.

Weile, C., Josefsen, K., and Buschard, K. (2011). "Glucose activation of islets of Langerhans up-regulates Toll-like receptor 5: possible mechanism of protection." *Clinical & Experimental Immunology*, 166(2), 251-257.

CHAPTER 3

CHAPTER 3

IMMUNE FUNCTION, SOCIAL RANK, AND FITNESS AMONG WILD SPOTTED HYENAS

Introduction

Little research has addressed how the immune system is modulated by socio-ecological factors in free-living animals, or how immune function impacts fitness (Graham et al. 2010; Graham et al. 2011). Previous work with captive animals has documented the effects of social structure on immune function by manipulating social rank (Hawley et al. 2006; Lindström 2004; Zuk and Johnsen 2000). An elegant study by Tung *et al.* (2012) revealed that expression levels of immune-related genes were a strong predictor of social status within groups of captive female rhesus macaques (*Macaca mulatta*) in which social structure was experimentally controlled. Even the simple presence of conspecifics is capable of altering a wide array of physiological and immunological parameters in captive settings (Schapiro et al. 2000). Among wild baboons (*Papio cynocephalus*) high-ranking males are known to recover from illnesses and wounds more quickly than their low-ranking counterparts (Archie et al. 2012), suggesting the possibility of rank-related variation in immune function among free-living animals.

Social hierarchies are associated with skewed resource distribution and varying levels of chronic stress in humans, laboratory animals, and wildlife. In all of these, high-ranking individuals enjoy the highest priority of access to food resources, which are important for immune function in a wide range of taxa (Diamond and Kingsolver 2011; French et al. 2007; Love et al. 2008; Ruiz et al. 2010). In order to procure food, mates, and social position, high-ranking individuals in many species exhibit higher rates and intensities of aggressive behavior than do their low-ranking counterparts (Creel et al. 2012; Smith et al. 2006), and higher rates of

aggression in turn may lead to higher stress, greater risk of injury, and increased pathogen exposure. Increased pathogen exposure is commonly believed to be a primary cost of sociality, and a recent meta-analysis indicates that group size is positively correlated with most common modes of pathogen transmission (Rifkin et al. 2012).

Here we analyzed relationships among social rank, immune function and fitness in free-living mammalian carnivores living in large, complex, hierarchically-structured societies. The social groups, or clans, of spotted hyenas (*Crocuta crocuta*) are comprised of up to 120 individuals. Females typically remain in their natal clan for life, whereas males emigrate to new clans after puberty. Each clan is structured by a strict and stable linear dominance hierarchy in which natal animals are socially dominant to immigrants, and cubs assume ranks immediately below those of their mothers, and just above those of their older siblings (Holekamp and Smale 1993; Kruuk 1972; Smale et al. 1993). Social rank determines priority of access to food at kills, so the highest-ranking females enjoy the greatest quantity and quality of food from carcasses (Engh et al. 2000; Frank 1986). In this species, rank affects many aspects of endocrine physiology and reproduction, both of which can affect immune function (Deerenberg et al. 1997; Roberts et al. 2004). The highest-ranking females in a hyena clan begin reproducing at younger ages, have the shortest interbirth intervals, wean their cubs at the youngest ages (Holekamp et al. 1996), have the lowest circulating glucocorticoid levels when not lactating (Goymann et al. 2001), and their offspring are exposed to the highest androgen concentrations during pregnancy (Dloniak et al. 2006).

Here we quantified three general measures of immune function in a spotted hyena population that has been intensively and continuously studied since 1988: *in vitro* serum bacterial killing capacity (BKC), total serum IgG, and total serum IgM. A bacterial killing assay

provides a functional measure of the ability of hyena serum to kill bacteria (Liebl and Martin 2009). Serum BKC is mediated primarily by the complement system, one of the most ancient and integrated parts of the vertebrate immune system (Morgan et al. 2005; Taylor 1983). A primary function of complement is to kill bacteria, but complement also functions in removal of apoptotic cells, synaptogenesis, blood coagulation, and in initiation and resolution of inflammation (Ricklin et al. 2010). Constitutive defenses, such as complement, are maintained in the absence of direct pathogenic challenge and function as preventative defenses (Ricklin et al. 2010), although complement can be quickly up-regulated upon pathogen recognition (Carroll 1998). IgG is a primary acquired defense against pathogens; measurement of total serum IgG concentrations represents a basic tool for evaluating infection status and abnormalities in immune function (Barnard et al. 1996; Curno et al. 2009; Taylor et al. 2002). IgM is important for early stage defense against pathogens during the primary exposure and for removal of dead host cells (Ochsenbein and Zinkernagel 2000). Non-cellular protein components of the immune system, such as immunoglobulins and complement, are accessible to researchers in peripheral blood and they are more stable during handling and sample storage than are cellular components of the immune system (Beyer et al. 2011; Hartweg et al. 2007; Pai et al. 2002). The highly integrated nature of complement and immunoglobulins in host defense permits them to serve as reliable indicators of immune function in wild animals.

Our first goal in this study was to determine whether our three measures of immune function (i.e., complement, IgM, and IgG) vary with social rank or circulating hormone concentrations among wild spotted hyenas. If immune function is affected by dietary quality in wild hyenas as it is in other species (Diamond and Kingsolver 2011; Love et al. 2008; Martin et al. 2007; Pomeroy et al. 1997), then we expected to observe higher immunoglobulin

concentrations and superior BKC in high- than low-ranking hyenas. Second, because energetic resources are traded off between immune defenses and reproduction (French et al. 2007; Graham et al. 2010), we inquired whether immune defenses were compromised in females during lactation, which is the phase of female reproduction known to incur the highest energetic costs (Gittleman and Thompson 1988; Thomson et al. 1970). Third, we tested to see whether BKC and antibody concentrations in female hyenas are higher than in males. Higher immune levels of immune defenses in females than in males has been observed in many other mammals (Butterworth et al. 1967; Eidinger and Garrett 1972; Petro and Bhattacharjee 1980; Purtilo and Sullivan 1979; Terres et al. 1968), and it is hypothesized that females can increase fitness by investing in immunity to extend their reproductive lifespan (Rolff 2002). Finally, we used archived serum samples and long-term reproductive data to inquire whether immune function could predict fitness among adult females, using annual reproductive success (ARS) as a proxy.

Materials and Methods

Study population

Behavioral observations of individually identifiable wild spotted hyenas were collected from 1988-2012 as part of a long-term study in the Maasai Mara National Reserve in Kenya. Unique spot patterns were used to determine individual identities. Sex was determined from the dimorphic glans morphology of the phallus (Frank et al. 1990), and individual ages were determined as previously described (Holekamp and Smale 1990; Smale et al. 1993; Van Horn et al. 2003). Data documenting wins and losses as outcomes of agonistic interactions were extracted to generate rank matrices (Holekamp and Smale 1993; Smale et al. 1993). Here we used relative rank instead of absolute rank to account for variations in clan size. The individual

with a relative rank of 1 was the highest-ranking animal in the clan and the individual with a relative rank of 0 was the lowest-ranking.

Female hyenas were considered to be adults at their first parturition or at 36 months, whichever came first (Swanson et al. 2011). The reproductive lifespan of adult females was calculated as the number of years she was present in the clan after reaching adulthood. ARS was calculated by dividing the total number of offspring that survived to weaning by the length of the individual's reproductive lifespan. Only immigrant males were included in this study for our comparisons of adult females and males.

Sample collection

Wild spotted hyenas were immobilized with tiletamine-zolazepam (6.5mg/kg Telazol; Fort Dodge Animal Health, Fort Dodge Iowa) in a plastic dart fired from an air rifle (Telinject Inc., Saugus, California) (Holekamp and Sisk 2003). All immobilizations and sample collections were approved by the MSU Institutional Animal Care and Use Committee (AUF # 07/08-099-00). Briefly, whole blood was collected from the jugular vein of anesthetized hyenas, allowed to clot at ambient temperature, centrifuged, and the sera frozen in liquid nitrogen (-196°C). Samples were transported to MSU on dry ice and stored permanently at -80°C. Sample collection dates for hyena sera ranged from 1996 – 2009.

Laboratory Methods

We used serum samples obtained from 64 immobilizations of 53 adult spotted hyenas. Bacterial killing capacity was determined using a method similar to that used by Nizet *et al.* (2001). Iron is particularly critical for bacterial growth (Drakesmith and Prentice 2012), and lysed red blood cells could contribute excess iron to the assay, so all serum samples were passed through a 0.22 μm filter to remove cell debris and red blood cells. In a preliminary study using

human sera, we assessed effects of freezing, heating, and filtering serum samples. See the appendix of this chapter for a full description and validation of the bacterial killing assays.

We used two members of the *Enterobacteriaceae* family, *Escherichia coli* (ATCC# 8739) and *Proteus mirabilis* (ATCC# 35659), to assess serum bacterial killing capacity. Both bacterial species are common enteric bacteria (Thompson-Chagoyán et al. 2007) and are highly susceptible to complement-mediated killing. The minimum inhibitory concentration (MIC) was defined as the negative $\log_2$ of the highest dilution (i.e., lowest serum concentration) that exhibited over 90 percent inhibition compared to the mean of 6 control wells per plate (Fig. 3.1) (Tennessen et al. 2009; Waitz 1990). The mean of triplicates for each sample was used as the MIC. As we were interested in only a general measure of bacterial killing capacity and not measures against specific bacteria, we collapsed the MICs from the *E. coli* and *P. mirabilis* into a single mean for a more robust assessment of bacterial killing capacity. Total IgG and IgM were measured using a sandwich ELISA and reagents as described by Flies *et al.* (2012). We used competitive binding ELISA kits for cortisol (Neogen #D402710) and testosterone (Neogen #D402510) according to the manufacturer's instructions. See the appendix to this chapter for additional details about laboratory methods.

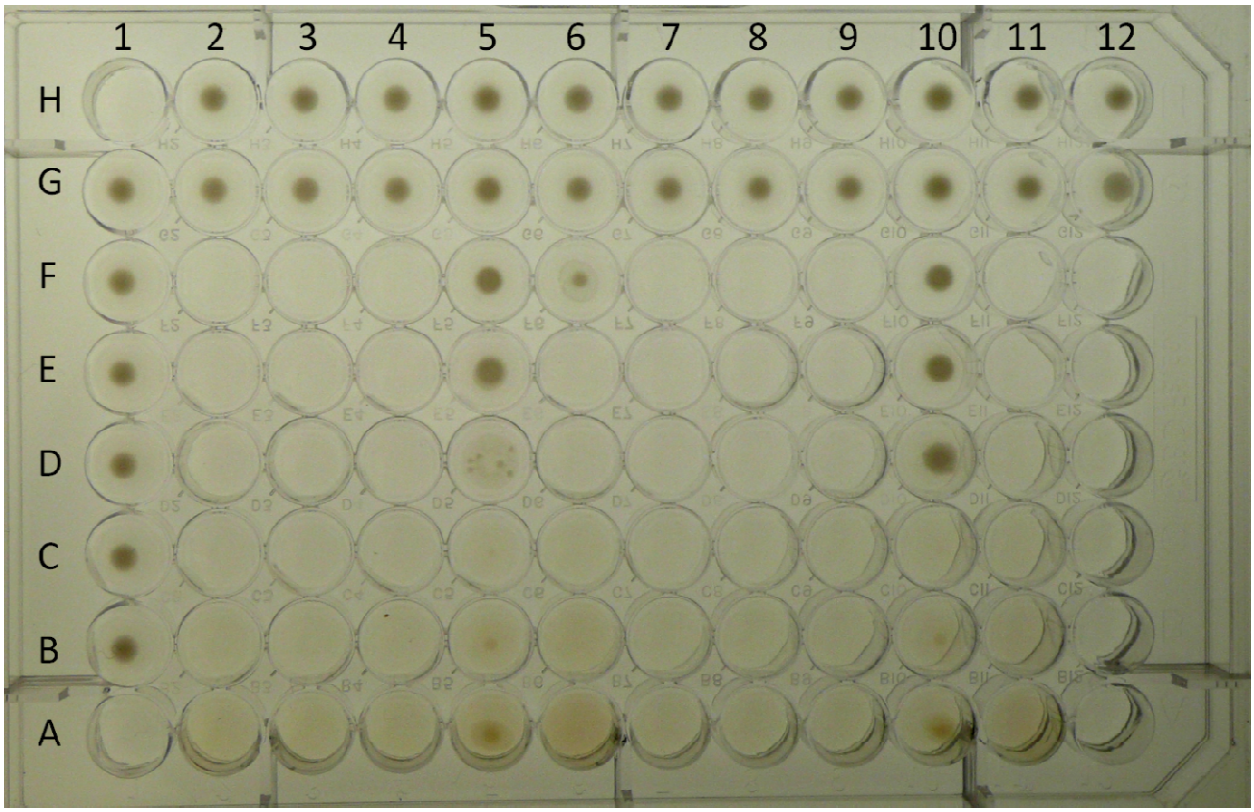

Fig. 3.1. Photo of 96-well plate bacterial killing assay. All columns except column 1 were serially diluted from row A to row H. The starting dilution in row A was 1:5 and the final dilution in row H were 1:640. The minimum inhibitory concentration (MIC) was the last dilution that fully inhibited bacterial growth. For instance, the MIC for column 2 is 1:160. For all analyses we used the $-\log_2$ of the MIC, which for 1:160 is 7.32. Column 1 contains 2 blank wells and 6 control bacteria wells. Columns 2 and 7 contain unfiltered sera. Columns 3, 4, 8 and 9 contain filtered sera. Columns 5 and 10 contain filtered sera supplemented with iron. Columns 6 and 11 contain filtered sera supplemented with unlysed sheep red blood cells. Column 12 contains serially diluted ampicillin. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Statistical analysis

When hyenas were sampled more than once, we used the mean of repeated measurements for all analyses, except for the analysis of immune measures in pregnant versus lactating females and for assessments of the effects of sample storage time and the time elapsed from darting to blood collection; in these analyses, we used only the first sample from each individual. Individuals that were neither pregnant nor lactating were excluded from the reproductive status

analyses. Three samples that were collected within nine days of parturition were removed from our data set due to major physiological and immunological changes associated with parturition and the ability of pregnancy-associated plasma proteins to inhibit complement activity (Bischof 1981; Bischof et al. 1984; Guidry et al. 1980; Markowska-Daniel et al. 2010; Smith et al. 1979). Two of the three samples removed had MICs that were nearly 4 standard deviations below the mean and exhibited large influence on analyses involving bacterial killing capacity.

We used version 2.14.2 of the software package R (R Development Core Team 2012) to create linear models to assess the relationships between response and predictor variables. Because no single model is a perfect representation of nature, we used an approach that involved information theoretic multimodel inference rather than choosing a single best model (Burnham and Anderson 2002; Moore and Borer 2012). Using this approach we specified our predictor variables in the full model, and explored all possible subset models for each dependent variable (Moore and Borer 2012). For the analysis of rank, we used BKC, total IgG, and total IgM as response variables, and rank, cortisol, and testosterone as predictor variables in the full model; this resulted in eight candidate models derived from the full model for each response variable, inclusive of the null model and all possible combinations of one, two, or three predictor variables. For the analysis of reproductive status and sex, we again used our three immune measures as response variables, but in addition to the covariates cortisol and testosterone, we included either reproductive status or sex as predictors instead of rank, which again resulted in eight candidate models for each response variable. The ARS analysis included BKC, total IgM, and total IgG as predictor variables, and ARS as the response variable; this resulted in 8 candidate models. Immune predictor variables that were significantly correlated with ARS were then tested again with rank as a covariate.

The full set of candidate models for each response variable were ranked according to Akaike's information criteria corrected for small sample size (AICc) (Burnham and Anderson 2002). Models with a difference in AICc of less than two (Δ AICc < 2) are considered to be equally good (Burnham and Anderson 2002). We used the 'MuMIn' package in R to produce weighted averages of regression parameter coefficients, 95% confidence intervals, and p-values for each input variable in models with Δ AICc < 2, hereafter referred to as the "top models" (R Development Core Team 2012). In cases where only a single model had Δ AICc < 2, we report the results directly from the linear model, rather than weighted averages. Backward stepwise regression model selection produced similar results to the multimodel inference approach outlined above. We also used the 'MCMCglmm' package in R (Hadfield 2010) to confirm the results obtained from our multimodel weighted averages.

Variables were tested for normality by visual inspection of histograms. Total IgG, total IgM, cortisol, and testosterone were log transformed prior to standardization and regression analysis. Before performing linear regressions using the 'stats' package in R (R Development Core Team 2012), all continuous variables were standardized by centering and dividing by the standard deviation to allow comparisons across tests and reduce the effects of potentially collinear predictor variables (Gelman 2008; Grueber et al. 2011; Kutner et al. 2005; Schielzeth 2010). Regression coefficients from standardized variables can be interpreted as the number of units of change in the response variable as a result of a one unit change in the predictor variable (Kim 2011; Schielzeth 2010). For example, if the regression coefficient for a standardized predictor was two, we would expect to see a two unit change in the response variable for every one unit change in the predictor variable. Coefficients from binary predictor variables are not

directly comparable to coefficients from continuous variables; for more information see Gelman (2008) and Schielzeth (2010).

Model fit was evaluated by examining residuals plots, quantile plots, and by plotting each predictor variable by the residuals of the models (Zuur et al. 2009). The Anderson-Darling and Shapiro-Wilk tests were also used to assess normality of residuals (Bolker et al. 2009; Larivée et al. 2010; Zuur et al. 2009). All figures were created using the 'graphics', 'ggplot', and 'scatterplot3d' packages (R Development Core Team 2012).

Results

Rank and immune function in adult females

When we assessed the relationship between social rank and immune function in 29 adult females, we found that serum BKC ($p < 0.014$) and total serum IgM ($p < 0.021$) were both positively correlated with social rank (Fig. 3.2A, B). However, total serum IgG was unrelated to rank in adult females ($p < 0.281$) (Fig. 3.2C). Serum cortisol concentration was positively correlated with total serum IgM concentration when included as a covariate of rank ($p < 0.035$) (Fig. 3.2D), but we observed no significant relationship between cortisol and either total IgG or BKC in adult females. We observed no relationship between testosterone and any of our immune measures in these models.

Table 3.1. Linear models to assess the relationship between social rank and immune function among adult females (n = 29). Models with Δ AICc < 2 were included in the top model set. The weights listed here are derived from the full set of candidate models.

Response	Predictors	df	logLik	AICc	Δ AICc	Weight
BKC	Rank	3	-37.27	81.49	0.00	0.40
	Rank + Cortisol	4	-36.51	82.69	1.20	0.22
	Rank + Testosterone	4	-36.65	82.96	1.47	0.19
IgM	Rank + Cortisol	4	-35.25	80.16	0.00	0.47
IgG	Intercept	2	-40.64	85.74	0.00	0.29
	Testosterone	3	-39.68	86.31	0.57	0.22
	Rank	3	-39.97	86.91	1.16	0.16
	Cortisol	3	-40.37	87.70	1.96	0.11

Table 3.2. Results of AICc based multimodel weighted-average analysis of the relationship between rank and immune function among adult females. In cases where only a single model had Δ AICc < 2, the results from the single linear model are reported.

Response	Predictor	β	SE	Lower CI	Upper CI	p	Importance
BKC	Intercept	0.000	0.168	-0.345	0.345	1.000	-
	Rank	0.443	0.173	0.089	0.798	0.014	1.000
	Cortisol	0.201	0.170	-0.149	0.551	0.261	0.271
	Testosterone	0.186	0.175	-0.173	0.546	0.309	0.237
IgM	Intercept	0.000	0.160	-0.329	0.329	1.000	-
	Rank	0.402	0.163	0.067	0.738	0.021	1.000
	Cortisol	0.363	0.163	0.028	0.698	0.035	1.000
IgG	Intercept	0.000	0.185	-0.379	0.379	1.000	-
	Testosterone	-0.254	0.186	-0.636	0.128	0.193	0.280
	Rank	-0.212	0.188	-0.598	0.174	0.281	0.208
	Cortisol	-0.135	0.191	-0.527	0.256	0.498	0.140

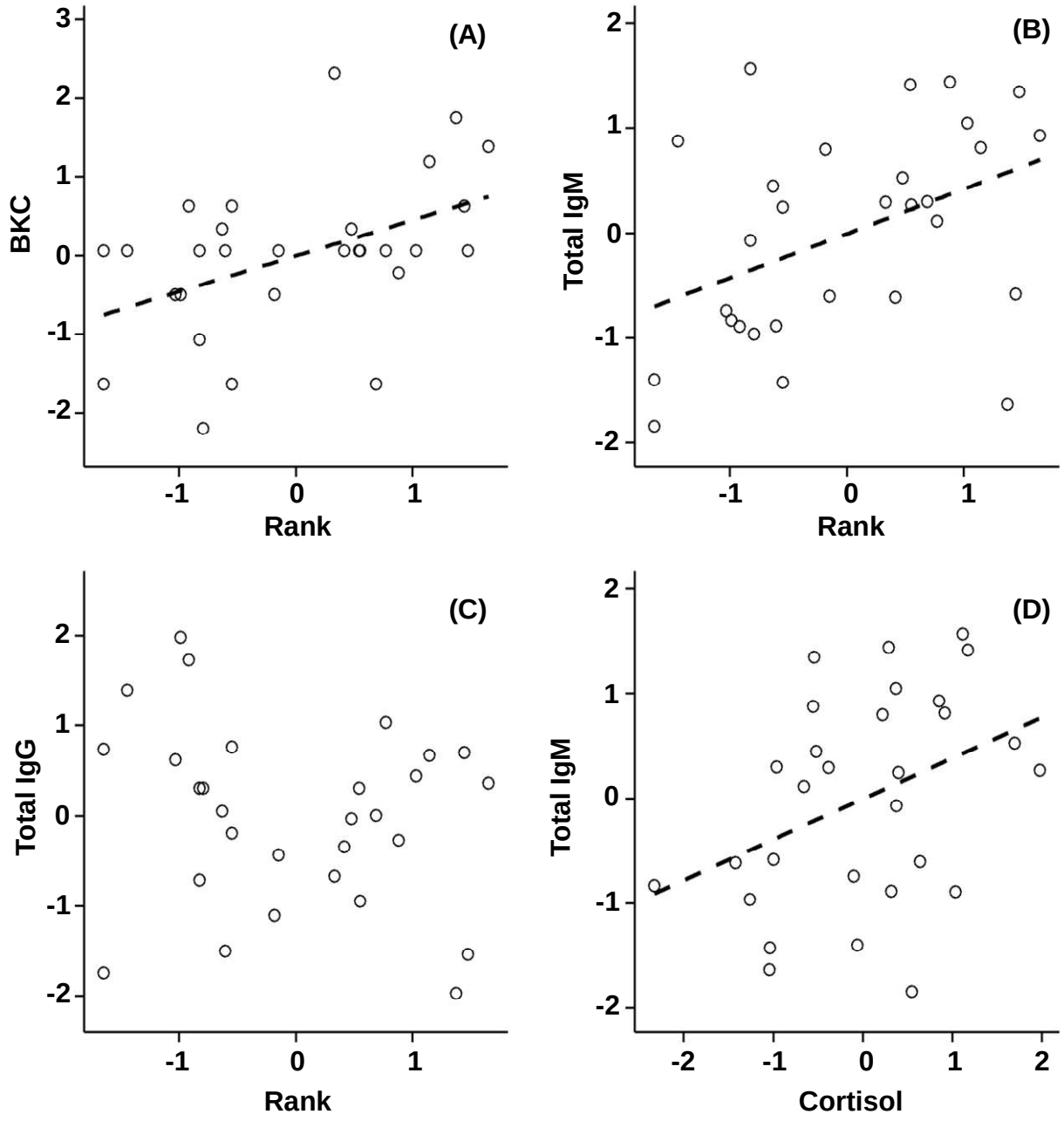

Fig. 3.2. Plots showing standardized relationships among immune defenses, social rank, and cortisol in adult female spotted hyenas (n = 29). (A) Social rank was a positively correlated with BKC ($p < 0.014$) and (B) total IgM ($p < 0.021$), but (C) was not correlated with total IgG ($p < 0.281$). (D) Cortisol was positively correlated with total IgM ($p < 0.035$).

Annual reproductive success (ARS) and immune function in adult females

For the analysis of annual reproductive success and immune function, we used data from 25 adult females. Here we found that BKC was significantly correlated with ARS ($p < 0.0015$). Previous research on spotted hyenas has demonstrated that rank is strongly correlated with ARS (Hofer and East 2003; Holekamp et al. 1996; Swanson et al. 2011), so we next included rank as a covariate of BKC in the linear regression analysis. Here we found that BKC was still significantly correlated with ARS ($p < 0.0158$) (Fig. 3.3), and our results also corroborate previous studies showing that rank is a strong predictor of fitness ($p < 0.0022$). Despite the correlations observed among rank and BKC in the previous results section, variance inflation factor (VIF) analysis indicated that collinearity did not affect any of the ARS models (all VIFs < 2).

Table 3.3. Linear models to assess the relationship between annual reproductive success (ARS) and immune function among adult females (n = 25). Models with Δ AICc < 2 were included in the top model set. The weights listed here are derived from the full set of candidate models.

Response	Predictors	df	logLik	AICc	Δ AICc	Weight
ARS	BKC	3	-29.4	65.9	0	0.636

Table 3.4. Results of AICc based multimodel weighted-average analysis of the relationship between annual reproductive success (ARS) and immune function among adult females.

Response	Predictor	β	SE	Lower CI	Upper CI	p	Importance
ARS	Intercept	0.000	0.163	-0.338	0.338	1.0000	-
	BKC	0.600	0.169	0.255	0.945	0.0015	1.000

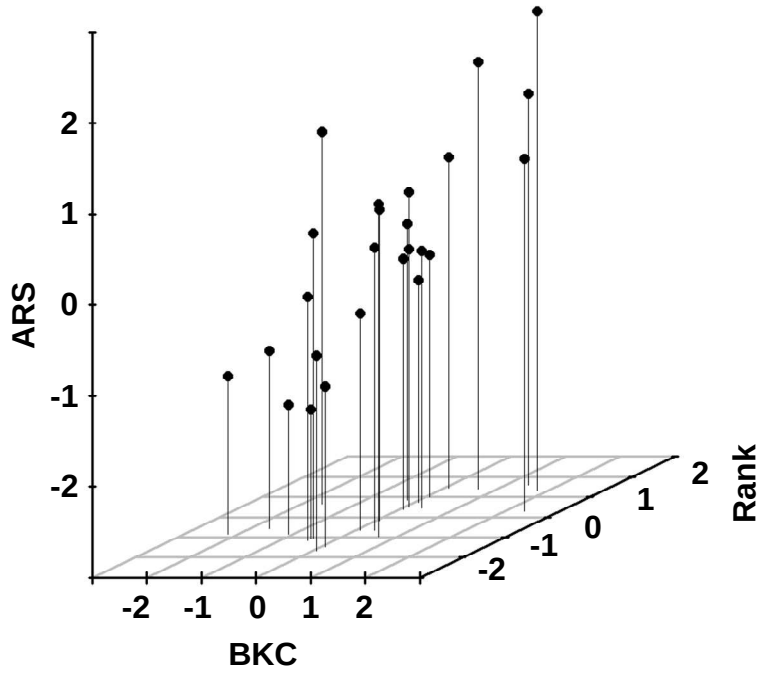

Fig. 3.3. Plot showing the standardized relationships among annual reproductive success (ARS), social rank, and bacterial killing capacity (BKC) for adult female spotted hyenas (n = 25). BKC ($p < 0.0158$) and rank ($p < 0.0022$) were significant predictors of ARS.

Reproductive status and immune function in adult females

Here we found that BKC and levels of total serum IgM were significantly higher in 14 pregnant than in 11 lactating females (BKC: $p < 0.038$; IgM: $p < 0.033$) (Fig. 3.4A, B). Mean total IgG levels were also higher in pregnant females, but not significantly ($p < 0.133$) (Fig. 3.4C). Cortisol and testosterone were included in the top models, but were not statistically significant predictors of immune function.

Table 3.5. Linear models to assess the relationship between reproductive status and immune function among adult females (n = 25). Models with Δ AICc < 2 were included in the top model set. The weights listed here are derived from the full set of candidate models.

Response	Predictors	df	logLik	AICc	Δ AICc	Weight
BKC	Reproductive status	3	-32.57	72.28	0.00	0.43
IgM	Reproductive status	3	-32.05	71.24	0.00	0.32
	Reproductive status + Testosterone	4	-31.21	72.42	1.18	0.18
	Reproductive Status + Cortisol	4	-31.21	72.42	1.18	0.18
IgG	Intercept	2	-34.96	74.47	0.00	0.31
	Reproductive status	3	-33.67	74.48	0.01	0.31

Table 3.6. Results of AICc based multimodel weighted-average analysis of the relationship between reproductive status and immune function among adult females. In cases where only a single model had Δ AICc < 2, the results from the single linear model are reported.

Response	Predictor	β	SE	Lower CI	Upper CI	p	Importance
BKC	Intercept	-0.462	0.280	-1.041	0.117	0.113	-
	Reproductive status	0.824	0.374	0.051	1.598	0.038	1.000
IgM	Intercept	-0.472	0.277	-1.046	0.101	0.107	-
	Reproductive status	0.843	0.375	0.067	1.619	0.033	1.000
	Cortisol	0.230	0.186	-0.156	0.616	0.243	0.263
	Testosterone	0.238	0.193	-0.161	0.637	0.243	0.263
IgG	Intercept	-0.173	0.305	-0.792	0.446	0.584	-
	Reproductive status	0.619	0.391	-0.189	1.428	0.133	0.499

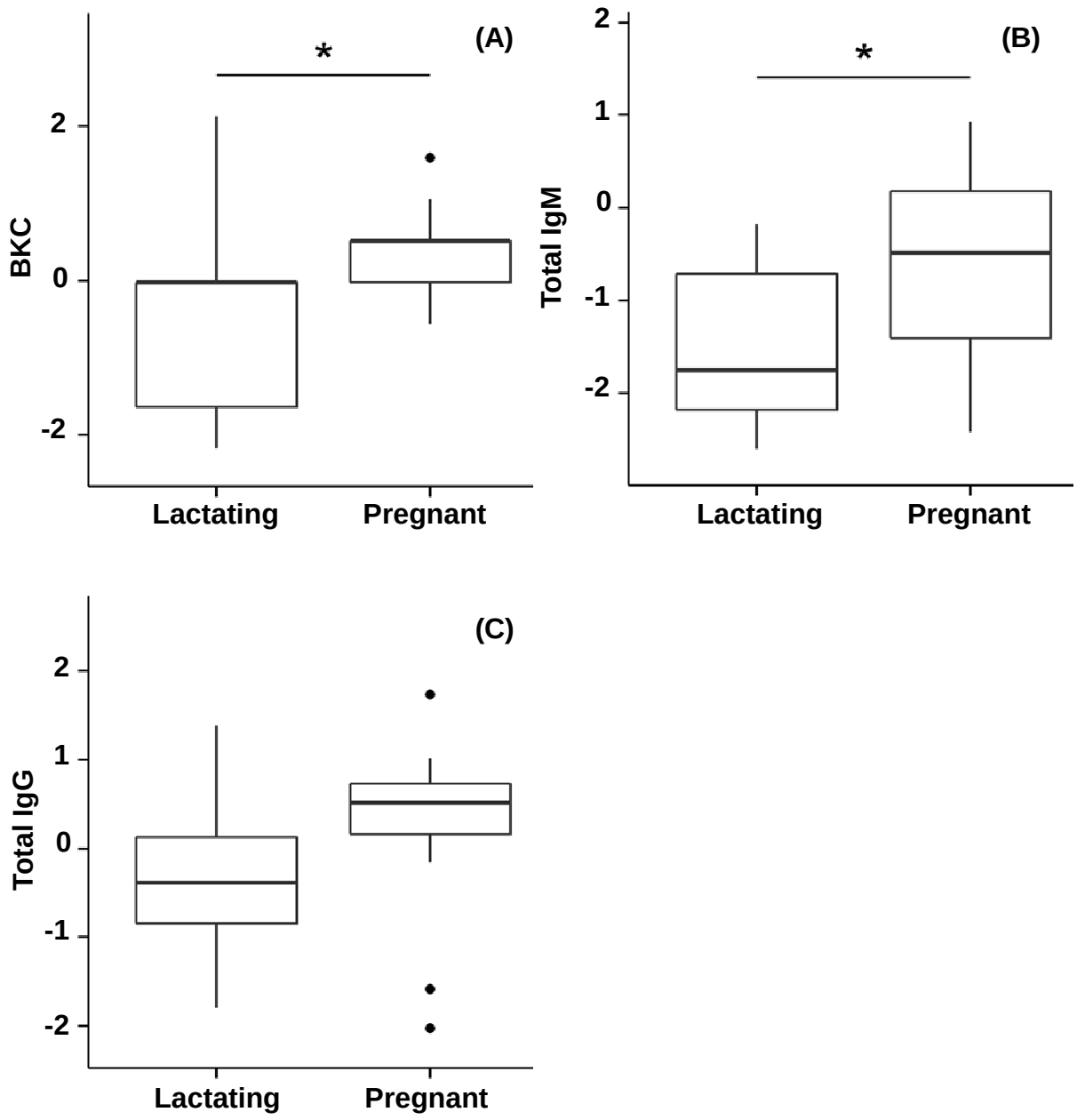

Fig. 3.4. Plots showing standardized comparisons of pregnant (n = 14) and lactating females (n = 11). Pregnant females had significantly greater (A) BKC ($p < 0.038$) and (B) IgM ($p < 0.033$) than lactating females. Pregnant females showed a trend for greater serum (C) IgG concentrations ($p < 0.133$).

Sex and immune function

In the analysis of differences in immune function between the 29 females and 15 males we found that serum from adult females had marginally higher BKC than serum from adult

immigrant males ($p < 0.034$) (Fig. 3.5A). Females also had significantly more total IgM than males ($p < 0.031$) (Fig. 3.5B). We found no relationship between sex and the serum concentration of total IgG (0.330) (Fig, 2.5C). We observed no relationship between cortisol or testosterone and any of our immune measures in these analyses.

Table 3.7. Linear models to assess the relationship between sex and immune function between adult females (n = 29) and males (n = 15). Models with Δ AICc < 2 were included in the top model set. The weights listed here are derived from the full set of candidate models.

Response	Predictors	df	logLik	AICc	Δ AICc	Weight
BKC	Sex	3	-59.19	124.98	0.00	0.33
	Sex + Testosterone	4	-58.45	125.93	0.94	0.21
	Sex + Cortisol	4	-58.58	126.19	1.20	0.18
IgM	Sex + Testosterone	4	-57.63	124.28	0.00	0.26
	Sex	3	-58.89	124.38	0.10	0.25
	Sex + Cortisol	4	-57.81	124.64	0.36	0.22
	Sex + Cortisol + Testosterone	5	-57.23	126.03	1.75	0.11
IgG	Intercept	2	-61.93	128.15	0.00	0.22
	Testosterone	3	-60.91	128.42	0.28	0.19
	Cortisol	3	-60.99	128.58	0.43	0.18
	Sex + Cortisol	4	-60.20	129.42	1.28	0.12
	Cortisol + Testosterone	4	-60.25	129.52	1.37	0.11
	Sex	3	-61.61	129.82	1.67	0.09

Table 3.8. Results of AICc based multimodel weighted-average analysis of the relationship between sex and immune function between adult females and males. In cases where only a single model had Δ AICc < 2, the results from the single linear model are reported.

Response	Predictor	β	SE	Lower CI	Upper CI	p	Importance
BKC	Intercept	0.259	0.186	-0.116	0.634	0.176	-
	Sex	-0.759	0.349	-1.461	-0.057	0.034	1.000
	Testosterone	0.162	0.151	-0.142	0.466	0.297	0.252
	Cortisol	0.207	0.174	-0.146	0.559	0.250	0.287
IgM	Intercept	0.284	0.191	-0.100	0.669	0.148	-
	Sex	-0.834	0.378	-1.592	-0.075	0.031	1.000
	Testosterone	0.246	0.180	-0.117	0.609	0.184	0.442
	Cortisol	0.189	0.157	-0.128	0.506	0.243	0.391
IgG	Intercept	0.026	0.166	-0.308	0.361	0.878	-
	Cortisol	-0.211	0.157	-0.526	0.105	0.191	0.444
	Testosterone	-0.201	0.152	-0.508	0.106	0.200	0.332
	Sex	-0.332	0.332	-1.000	0.336	0.330	0.232

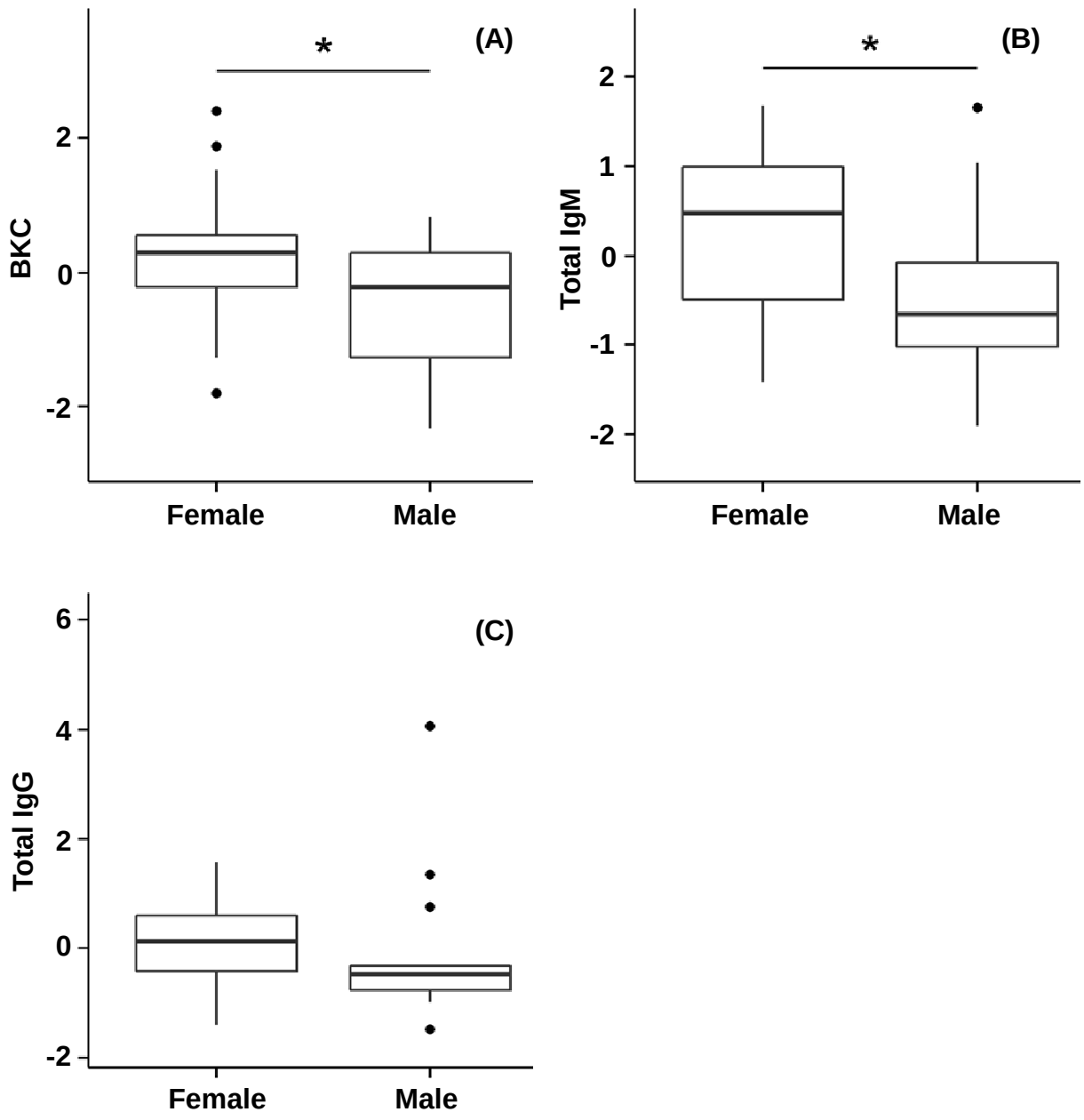

Fig. 3.5. Plots showing standardized comparisons of immune defenses in adult female (n = 29) and immigrant male (n = 15) spotted hyenas. Females had significantly higher (A) BKC ($p < 0.034$) and (B) total IgM ($p < 0.031$) than males; we did not observe a significant difference in (C) total IgG between males and females ($p < 0.330$).

Discussion

Here we have found that social rank is a strong predictor of immune defenses in wild spotted hyenas. It has been well-established that rank is a primary determinant of fitness in this species; one of the primary mediators of the increased fitness observed in high-ranking hyenas is their priority of access to food, which has obvious implications for producing and nurturing offspring. However, the influence of immune function on the fitness of these and other wild animals is poorly understood. Spotted hyenas exhibit remarkable resistance to pathogens. For example, spotted hyenas are known to survive exposure to pathogens such as rabies, canine distemper virus, and anthrax (Alexander et al. 1995; East et al. 2001; Harrison et al. 2004; Lembo et al. 2011). Our data indicate that rank and serum BKC are both positively correlated with ARS in this spotted hyena population. The positive correlation between BKC and ARS suggests that serum bacterial killing proteins may be important mediators of the fitness benefits associated with rank.

Prior research with both mammals and birds suggests that dominant individuals may experience lower susceptibility to infection than subordinates and faster recovery from infection (Archie et al. 2012; Cohen et al. 1997; Fairbanks and Hawley 2011; Hawley et al. 2007; Lindström 2004). The positive correlations we observed here among ARS, rank, and BKC are consistent with the hypothesis that the higher ARS observed among dominant animals than subordinate animals is mediated by first line immune defenses, such as complement, operating to prevent disease and to permit fast recovery when infection does occur. In primates, early response to infection is a major determinant of disease outcome (Estes et al. 2008), and dietary restriction or malnourishment are often associated with reduced immune function, increased frequency of infection, and higher rates of chronic infection than individuals with healthy diets

(Afacan et al. 2012; Cunningham-Rundles et al. 2005). As disease becomes more severe, a stronger immune response is needed and this diversion of energy from growth and reproduction to immune defense may have long-term consequences for the host in terms of loss of energy resources used to mount the immune response and collateral damage due to immunopathology (Blander and Sander 2012; Hasselquist and Nilsson 2012; Medzhitov et al. 2012).

Numerous studies have shown that heat treatment of serum or plasma inactivates the complement system (reviewed by Taylor, 1983); bacterial killing capacity in this study was abolished by heat treatment (Appendix - Table 3.9), supporting the idea that complement is the primary mediator of bacterial killing in our assays. The long evolutionary history of the complement system, extending all the way to the most primitive sponges (Dzik 2010), has led to complement proteins being integrated with many aspects of mammalian physiology and having many functions beyond the ability to lyse bacterial cells (Morgan et al. 2005); these functions include creating chemoattractant gradients followed by first responder cells, (Hawlisch and Kohl 2006; Ricklin et al. 2010), enhancing clotting at wound sites to prevent microbial dissemination to other sites within the host (Ricklin et al. 2010), and reducing immunopathology by marking cellular debris for phagocytosis (Gaipl et al. 2001; Trouw et al. 2005; Zwart et al. 2004). Long-term immunopathological damage, which is often associated with necrotic and apoptotic cellular debris, can be as detrimental to host fitness as pathogen-induced damage to the host (Medzhitov et al. 2012). Rank was found to be associated with rates of wound healing and recovery from disease among wild baboons, but the immunological mechanisms mediating healing and recovery are unknown (Archie et al. 2012). Wilcoxen *et al.* observed that the ability of serum to kill *E.* coli was under a positive selection gradient during a viral epizootic in scrub jays (*Aphelcoma coerulescens*) (Wilcoxen et al. 2010). That outbreak was caused by a virus,

indicating that BKC may have predictive power beyond bacterial infections (Wilcoxen et al. 2010). It would now be interesting to assess relationships among BKC, rank, and recovery from infection and wounds among wild hyenas.

Despite the positive selection on BKC observed during the scrub jay epizootic, the strongest immune response is not necessarily the best response (Graham et al. 2011). For instance, septic shock is the result of an inappropriate inflammatory response and can result in host death. In light of the potential negative consequences of immunopathology and the energy costs of producing excessive immune responses, individuals should achieve greater fitness when the magnitudes of immune defenses and disease risk are appropriately matched (Graham et al. 2011). Here we observed a positive correlation between BKC and ARS indicating that, in this case, stronger immune defenses may increase fitness.

Predictors of immune performance

A positive correlation between diet and immune function has been observed in a wide range of species: rats (*Rattus norvegicus*), mice (*Peromyscus maniculatus*), honeybees (*Apis mellifera*), and moths (*Manduca sexta*) (Alaux et al. 2010; Diamond and Kingsolver 2011; Kenney et al. 1968; Martin et al. 2007; Michalek et al. 1975). Among humans, complement components and immunoglobulin concentrations are lower in patients with severe nutrient and caloric intake deficiencies than those with normal nutrient and caloric intake (Hafez et al. 1977; Jelliffe and Chandra 1972; Pomeroy et al. 1997; Ricklin et al. 2010; Weyer et al. 2000). High social rank provides priority of access to resources among spotted hyenas, so low-ranking animals get less food and lower quality parts of a carcass during intense feeding competition (Frank 1986), and low-ranking hyenas are more likely than high-ranking individuals to feed on calorie-poor dried carcasses (Cooper et al. 1999). Energetic and nutritional resources are likely

important for complement production, as complement proteins can comprise up to 15% of the globular fraction of plasma (Dunkelberger and Song 2010; Walport 2001). A critical component of BKC, the complement protein C3, is produced by adipocytes and production is significantly increased in response to dietary lipids (Maslowska et al. 1997; Scantlebury et al. 1998). This is one potential mechanism through which nutritional status might mediate the rank-related variation in the immune measures reported here for hyenas. Furthermore, the constant "tick-over" of native C3 into hydrolyzed C3 allows the alternative complement pathway to provide a non-specific, non-induced first line immune defense that is continuously active (Bexborn et al. 2008; Lachmann and Halbwachs 1975). Neutralization of C3 by anti-C3 antibodies in bacterial killing assays has been shown to significantly reduce BKC (Merchant et al. 2005).

Effects of high social rank on immune function are likely mediated by a suite of physiological parameters, including hormones, neurochemical factors, direct sympathetic innervation of lymphoid tissue, and cytokines (Cohen et al. 2012; Demas et al. 2011; Tuchscherer et al. 1998). In many species glucocorticoids and testosterone suppress aspects of immune function, although other cases have been documented in which one or both of these hormone augment immune function (Dhabhar and McEwen 1997; Martin et al. 2006; Peters 2000; Roberts et al. 2004). Here we were surprised to find very few associations between these steroid hormones and our measures of immune function.

As expected based on earlier work with other species, our results indicate that female spotted hyenas have greater BKC and IgM concentrations than adult males. Although female spotted hyenas enjoy higher priority of access to food than males, many studies have shown that, even in other mammalian species in which males are dominant, females often have higher levels of immune defenses than males (Butterworth et al. 1967; Libert et al. 2010; Nunn et al. 2009).

Rolff (2002) proposed that the increased immune defenses observed in females might function to increase reproductive longevity, whereas males are more likely to enhance fitness by increasing their mating rates than by investing in immunity. We are not able to test the effects of immune defenses on reproductive longevity here, as most of our sampled subjects were still alive and reproductively active at the conclusion of this study.

Lactation in most mammals is more energetically costly than gestation (Gittleman and Thompson 1988). Our observation that immune defense levels are lower in lactating than pregnant females is consistent with the notion that energy resources are traded-off between immune defenses and reproduction (French et al. 2007; Graham et al. 2010). Although our results support the hypothesis that energetic trade-offs may lead to reduced immune function during lactation, female mammals also transfer antibodies to offspring via colostrum and milk, and this transfer might also have contributed to the differences observed here between pregnant and lactating females in immunoglobulin concentrations. For instance, 30% of the IgG and 10% of the IgM in milk from domestic pigs (*Sus scrofa domesticus*) is of serum origin rather than mammary origin (Bourne and Curtis 1973). Additionally, serum IgG concentrations progressively increase during lactation in domestic pigs, whereas serum IgM concentrations decrease during lactation (Klobasa et al. 1985). In domestic cows (*Bos taurus*), both serum IgG and IgM concentrations reach a minimum in the periparturient period, and IgM concentrations remain low during the onset of lactation (Herr et al. 2011). If lactating hyenas exhibit similar proportions of serum-derived antibodies in milk, it might lead to lower concentrations of antibodies in sera from lactating mothers, particularly in the early stages of lactation.

In summary, our data show that high-ranking hyenas have higher immune defenses than low-ranking hyenas for two of the three immune parameters we tested. Our work is one of the

first studies to demonstrate the positive relationship between increased immune defenses and annual reproductive success in free-living carnivores. Additionally, our data supported the hypotheses that immune defenses are costly, as BKC and total IgM were both lower in lactating than pregnant females. Finally, our results are similar to previous research showing that females often have higher levels of immune defenses than males.

APPENDIX

Appendix

Sample collection

Human sera for assessing the effects of freezing serum were collected by a professional phlebotomist from anonymous human donors. The use of samples from human subjects was approved by the Michigan State University (MSU) Institutional Review Board (IRB # 10-997). Fresh serum samples are defined as sera that were never frozen. All fresh samples were stored at 4°C and were used in the BKC within 36 hours of collection. Frozen human sera were stored at -80˚ C for 8-9 months prior to assay.

Bacterial killing assay

The bacterial killing assay we used was a modified version of the method developed by Nizet, *et al.* (2001). Briefly, all bacterial killing assays were conducted in 96-well round bottom plates (Fisher #08-772-54). Sera were treated as outlined below prior to adding to the 96-well plate. After treatment, sera were then diluted 1:2.5 in phosphate buffered saline (PBS) and added to row 1. Sera were then serially diluted in PBS, with dilutions ranging from 1:2.5 to 1:320 (NOTE: After the subsequent addition of 25 µl of bacteria-containing broth, the final dilutions became 1:5 - 1:640). Cation-adjusted Mueller-Hinton broth II was used as the bacterial growth medium for all assays, as recommended by Reller and Stratton (1977) and the Clinical and Laboratory Standards Institute protocols for broth microdilution assays (CLSI 2009).

We tested two strains of bacteria rather than relying on the results from a single strain. Ideally, several strains of bacteria can be tested when serum sample volume is not limiting. *E. coli* (ATCC# 8739) was chosen because it has been used as a standard bacterial strain in several other studies of bactericidal capacity (Liebl and Martin 2009); *P. mirabilis* (ATCC# 35659) was chosen because like *E. coli* it is a gram negative bacterium that is commonly found in the

gastrointestinal tract of many animals and was more susceptible to complement-mediated killing than most other strains in preliminary testing. One day prior to beginning the BKC, *E. coli* or *P. mirabilis* were inoculated into Mueller-Hinton II cation adjusted broth (MHB) and incubated overnight at 37°C while shaking at120 rpm. After overnight incubation, bacterial cell concentration was adjusted to approximately 10^8 CFU/ml by diluting bacteria in PBS until optical density at 600 nm matched the corresponding turbidity of the McFarland turbidity standard (BD Biosciences # 287298). Bacteria were then serially diluted in PBS to a concentration of 10^4 CFU/ml.

Control wells, serum wells and antibiotic wells were quickly loaded with 25 µl stock bacteria-containing broth, resulting in a final concentration of 250 CFU/well and total volume of 50 µl/well. 25 µl of sterile MHB were added to blank wells to bring the total volume of each well to 50 µl. Next, the plates were incubated at 37°C and 5% carbon dioxide and turbidity was measured after 18 hours on a Bio-Tek plate reader at 600nm. Percent inhibition was calculated by dividing the optical density of each sample well by the mean optical density of the control bacteria wells. Because the assay allows the bacteria to grow to saturation, there was a clear distinction among wells exhibiting bacterial growth and those without growth. The minimum inhibitory concentration (MIC) was defined as the negative $\log_2$ of the lowest dilution that exhibited over 90 percent inhibition compared to the mean of the control wells (Tennessen et al. 2009; Waitz 1990). For example, if 1:40 was the lowest dilution exhibiting complete growth inhibition, the response variable value would be: $-\log_2 (1/40) = 5.32$. Additionally, the plates were visually inspected to ensure there was no bacterial growth at the calculated MIC. The mean of triplicates for each sample was used as the MIC.

Total IgG and IgM ELISAs

Due to the lack of hyena-specific antibodies, we used anti-feline antibodies that have been shown to cross-react with hyena epitopes (Flies et al. 2012). Briefly, a sandwich ELISA format was used and 100 µl of either anti-cat IgG (Bethyl Labs, # A20-117A) or anti-cat IgM (Custom Monoclonals International, # CM7) capture antibodies were loaded into 96-well plates at 10 µg/ml and incubated at ambient temperature for 60 minutes. Plates were then washed 2X with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8), loaded with 175 µl of blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0), incubated at ambient temperature for 30 minutes, and then washed 3X with wash buffer. 100 µl of hyena serum previously diluted 1:100,000 and 1:500 for IgG and IgM, respectively, were then added to each well. Serial dilutions of purified hyena IgG or IgM were added to each plate and were used to create a standard curve for each plate. Plates were incubated for 60 minutes and then washed 3X. HRP-conjugated detection antibodies for IgG (KPL # 04-20-02) and IgM (KPL # 04-20-03) were diluted 1:2500 and 1:2000, respectively, and incubated for 60 minutes before washing 5X. *3,3′,5,5′*-Tetramethylbenzidine (TMB) substrate (Rockland Immunochemicals, # TMBE-1000) was warmed to ambient temperature and 100 µl were added to each well. Color change was stopped using 100 µl of 0.5 M H_2SO_4 after approximately 10 minutes and read at 450nm on a standard plate reader. IgG and IgM concentrations were calculated using the 'calibFit' package in the software package R (R Development Core Team 2012).

Hormone ELISAs

We used Neogen Cortisol (D402710) and Testosterone (D402510) competitive binding ELISA kits according to the manufacturer's instructions. Briefly, hormones were extracted

following the instructions of the Neogen kits, diluted 1:100, and 50 µl were aliquoted into pre-coated 96-well plates. 50 µl of enzyme conjugate was added and the plates were incubated for 60 minutes to allow competitive binding to occur. Plates were then washed 3X and substrate was then added to initiate the color change reaction. Absorbance values were read at 630nm with a reference wavelength of 450 nm after a 30 minute incubation step. All samples and standards were run in duplicates. Hormone concentrations were calculated using the 'calibFit' package in the software package R (R Development Core Team 2012).

Testing for effects of sample storage and collection

Previous studies have found that freezing and thawing plasma and serum samples can result in decreased bacterial killing capacity and also that long-term storage at -15°C or -40°C can lead to decreased bactericidal capacity (Liebl and Martin 2009; Skarnes 1978). However, Skarnes (1978) also reported no loss of bactericidal activity of serum through up to five freeze-thaw cycles. Mollnes *et al.* (1988) reported no change in the complement component C3 activation products after storage at 4°C for 10 days or at -70°C for three years and after up to four freeze-thaw cycles. Other studies have indicated that acute phase proteins, such as C-reactive protein, are stable for several days at 4°C and robust to the effects of freeze-thaw cycles (Pai et al. 2002; Zimmerman et al. 2012). Horrocks *et al.* (2011) reported that plasma ovatransferrin activity, an acute phase protein with iron-binding and immunomodulatory properties, is stable for up at least 6 years at ≤ 20°C.

We used linear regression to test for potential changes in immune parameters associated with capture and handling of hyenas in the field (n = 53); here we used only the first sample from 53 individuals. We found no association between time from darting to blood collection and BKC, ($p > 0.77$), total IgM ($p > 0.80$), or total IgG ($p > 0.44$,) (Fig. 3.6).

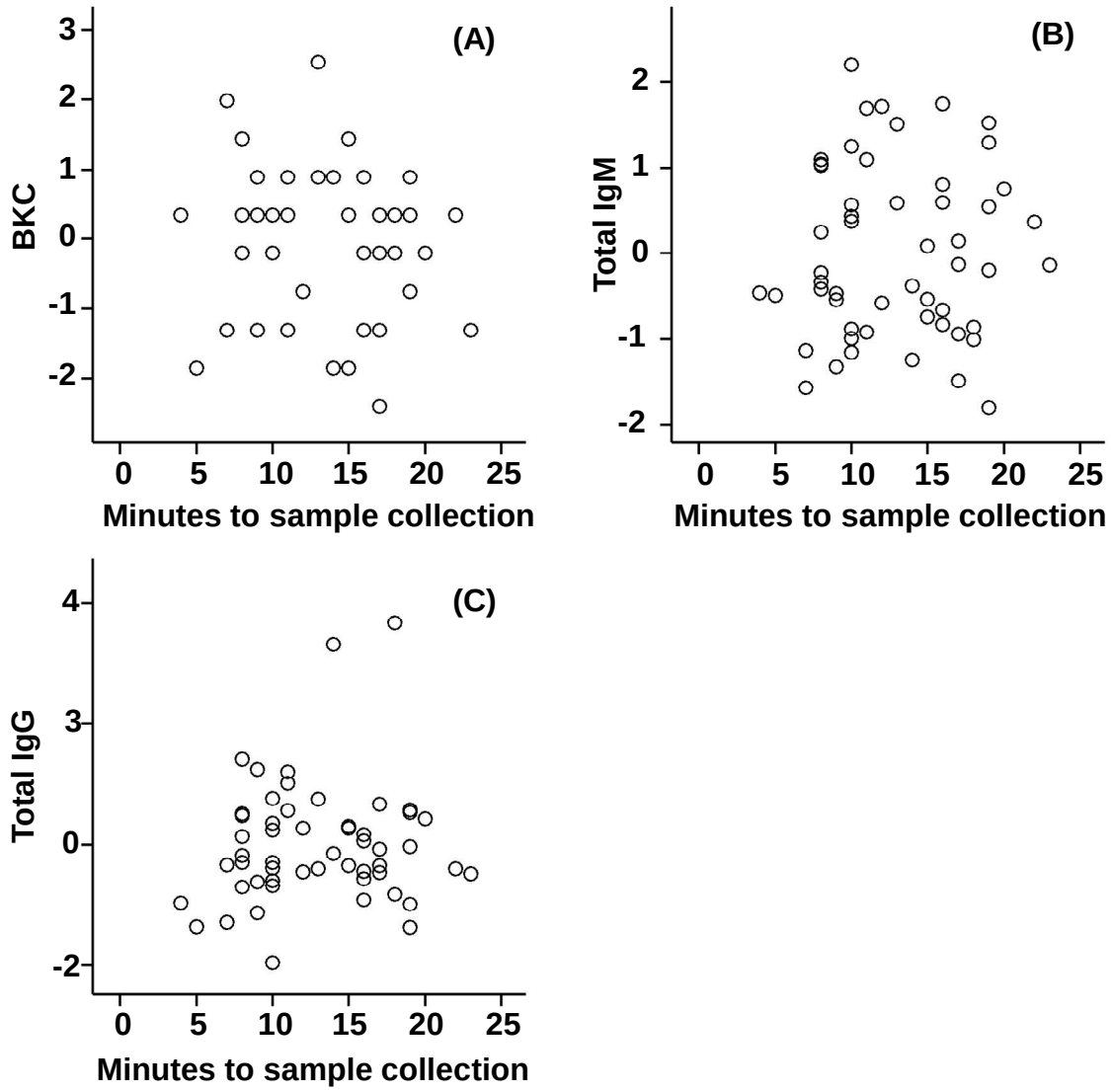

Fig. 3.6. Analysis of time from darting to blood collection and immune measures. Blood collection time was calculated as the time at which the blood sample was collected minus the time that the hyena was first darted. Here we tested 53 adult hyena serum samples collected between 4 and 23 minutes after darting. We found no significant effect of the latency to collect blood after darting on (A) bacterial killing capacity ($p > 0.771$), (B) total IgM ($p > 0.802$), or (C) total IgG ($p > 0.444$).

To assess the effects the freeze-thaw process on bacterial killing capacity, we compared the bacterial killing abilities of fresh human sera (collected and stored at 4°C for less than 36 hours) and human sera that had been stored at -80°C for more than 8 months (Appendix - Table 3.9). We were not able to compare fresh and frozen hyena sera. To assess the effects of long-

term storage at -80°C, we tested filtered hyena serum samples that were collected in the field over a period ranging from 1996 to 2009; here we used only the first sample collected from each individual. We found that duration of long-term storage at -80°C had no effect of on BKC ($p > 0.801$) (Fig. 3.7). Additionally, we tested the effects of heating sera at 56°C for 30 minutes; all bacterial killing capacity was abolished after heat treatment as previously reported (Taylor 1983) (Appendix - Table 3.9). Previous research has shown that IgG and IgM are stable over long periods of time, especially when stored at temperatures lower than -70°C (Elwell and Bethell 2001; Pinsky et al. 2003). We found no significant effects of storage time on either total IgM or IgG ($p > 0.101$; $p > 0.126$) (Fig. 3.7).

Table 3.9. Comparison of the effects of freezing, heating at 56°C for 30 minutes, and passing through a 0.22 μm filter on the minimum inhibitory concentration (MIC) using human and hyena serum samples in bacterial killing assays against *E. coli* (n = 10 per treatment).

Species	Matched Pairs	Frozen	Treatment	Mean	SD	Within-sample CV	Among-sample CV
Human	No	No	Filtered	7.07	0.791	0.0535	0.112
		No	Filtered + heat	1.37	0.158	0.038	0.115
		Yes	Filtered	7.52	0.753	0.0459	0.1
		Yes	Filtered + heat	1.32	0	0	0
Human	No	No	Unfiltered	7.07	0.717	0.083	0.101
		No	Unfiltered + heat	1.32	0	0	0
		Yes	Unfiltered	7.47	0.883	0.065	0.118
		Yes	Unfiltered + heat	1.32	0	0	0
Human	Yes	Yes	Filtered	7.47	0.242	0.0198	0.0323
		Yes	Filtered + iron	5.07	0.486	0.0712	0.0958
		Yes	Unfiltered	7.52	0.35	0.0132	0.0465
Hyena	Yes	Yes	Filtered	8.05	0.408	0.0121	0.0509
		Yes	Unfiltered	7.92	0.492	0.0255	0.0621

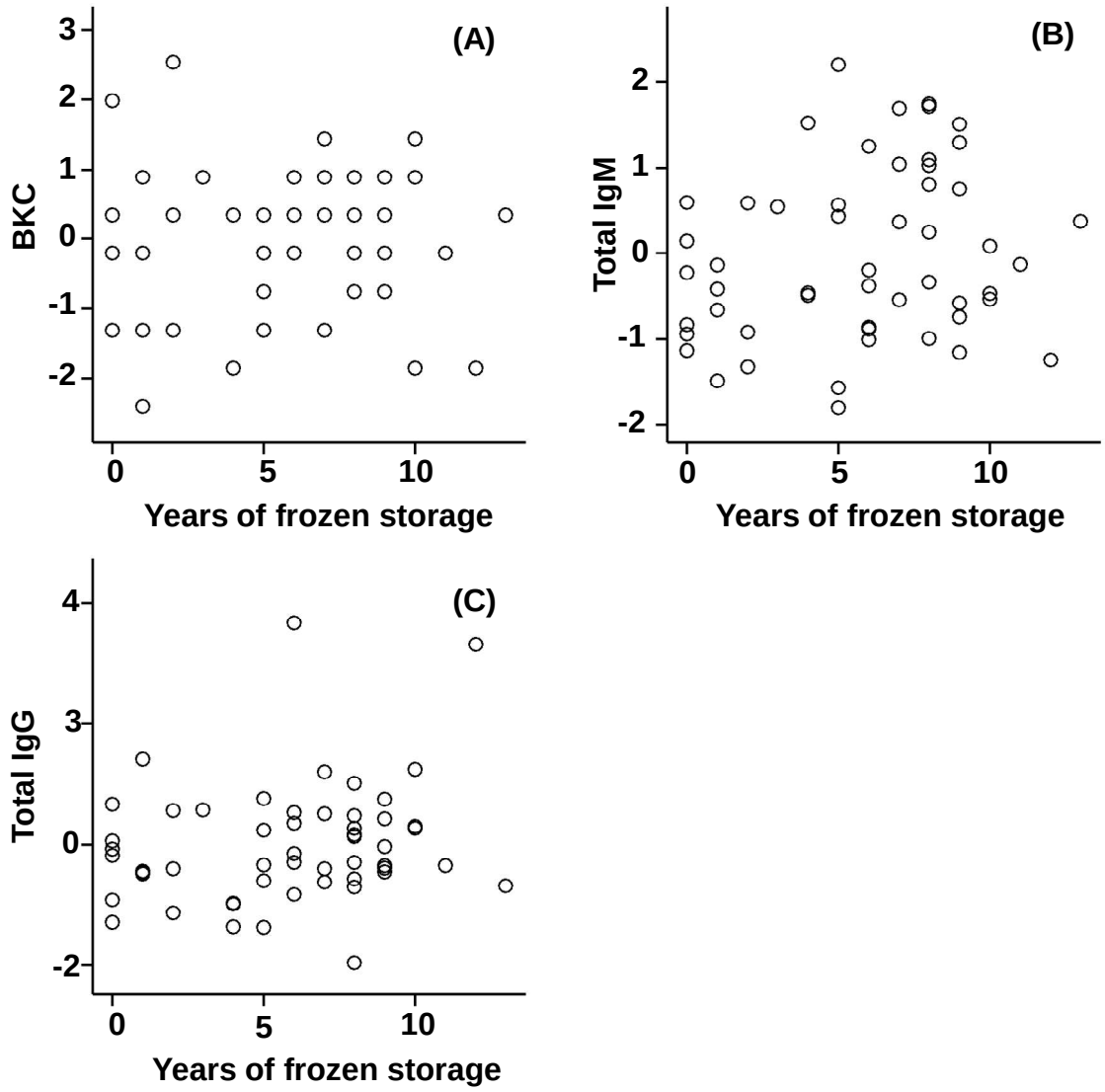

Fig. 3.7. Analysis of long-term storage and immune measures. We tested serum samples from 53 adult hyenas collected between 1996-2009. We found no significant effect of storage time at -80°C on (A) bacterial killing capacity ($p > 0.805$), (B) total IgM ($p > 0.101$), and (C) total IgG ($p > 0.126$).

Sample quality test

In preliminary assays using unfiltered sera, the majority of serum samples exhibited clear patterns of inhibition; for example, complete inhibition was observed at dilutions 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160, whereas no inhibition was observed at dilutions 1:320, and 1:640. This pattern will be referred to as the "standard pattern". However, a few samples exhibited unusual

patterns of bacterial growth inhibition; for example, complete inhibition was observed at dilutions 1:80 and 1:160, but partial inhibition observed at dilutions 1:5, 1:10, 1:20, 1:40 and no inhibition observed at dilutions 1:320, and 1:640 (Fig. 3.1). This second pattern will be referred to as the "noisy pattern". Additionally, we observed the noisy pattern more frequently using hyena samples from the field than in human samples collected by the professional phlebotomist. We suspected that variation in sample quality was leading to a complex interaction between bacterial growth rate and bacterial killing by serum components. This complex interaction is likely to be present, but not detected in assays that use only a single serum or plasma dilution, and could potentially lead to perplexing results.

To explain the noisy pattern, we hypothesized that nutrients from lysed RBCs in sera, iron in particular, provided additional nutrients to bacteria, thus allowing the bacterial growth rate to exceed bacterial killing capacity of the sera. From this hypothesis, we predicted that filtering out residual RBCs and other cellular debris would eliminate the noisy pattern. Second, we predicted that supplementing filtered sera with iron would result in lower MICs and produce the noisy pattern in the assay using samples that without iron supplementation produced the standard pattern.

To test prediction 1, we sterile-filtered sera using 0.22 μm low protein binding filters (Millipore, cat# SLGV033RS) to eliminate residual RBCs, lysed RBCs, and other cellular debris. Samples were then tested as matched pairs of filtered and unfiltered sera. Confirming prediction 1, the noisy pattern was eliminated by filtering. To test prediction 2, we diluted sera with 0.01 M $FeCl_3$-$6H_2O$ (Sigma # F-2877) in PBS and tested against matched sera diluted in PBS only (Spatafora and Moore 1998). Confirming prediction 2, lower MICs were observed and patterns resembling the noisy pattern were induced by iron supplementation (Fig. 3.9).

LITERATURE CITED

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Afacan, N. J., Fjell, C. D., and Hancock, R. E. W. (2012). "A systems biology approach to nutritional immunology – Focus on innate immunity." *Molecular aspects of medicine*, 33(1), 14-25.

Alaux, C., Ducloz, F., Crauser, D., and Le Conte, Y. (2010). "Diet effects on honeybee immunocompetence." *Biology letters*, 6(4), 562-5.

Alexander, K. A., Kat, P. W., Frank, L. G., Holekamp, K. E., Smale, L., House, C., and Appel, M. J. G. (1995). "Evidence of canine distemper virus infection among free-ranging spotted hyenas (*Crocuta crocuta*) in the Masai Mara, Kenya." *Journal of Zoo and Wildlife Medicine*, 26(2), 201-206.

Archie, E. A., Altmann, J., and Alberts, S. C. (2012). "Social status predicts wound healing in wild baboons." *Proceedings of the National Academy of Sciences*.

Barnard, C. J., Behnke, J. M., and Sewell, J. (1996). "Social status and resistance to risease in house mice (*Mus musculus*): status-related modulation of hormonal responses in relation to immunity costs in different social and physical environments." *Ethology*, 102(1), 63-84.

Bexborn, F., Andersson, P. O., Chen, H., Nilsson, B., and Ekdahl, K. N. (2008). "The tick-over theory revisited: Formation and regulation of the soluble alternative complement C3 convertase (C3(H2O)Bb)." *Molecular Immunology*, 45(8), 2370-2379.

Beyer, H. L., Hampson, K., Lembo, T., Cleaveland, S., Kaare, M., and Haydon, D. T. (2011). "Metapopulation dynamics of rabies and the efficacy of vaccination." *Proceedings. Biological sciences / The Royal Society*, 278(1715), 2182-90.

Bischof, P. (1981). "Pregnancy-associated plasma protein-A: An inhibitor of the complement system." *Placenta*, 2(1), 29-34.

Bischof, P., Geinoz, A., Herrmann, W. L., and Sizonenko, P. C. (1984). "Pregnancy-associated plasma protein a (PAPP-A) specifically inhibits the third component of human complement (C3)." *Placenta*, 5(1), 1-7.

Blander, J. M., and Sander, L. E. (2012). "Beyond pattern recognition: five immune checkpoints for scaling the microbial threat." *Nature reviews. Immunology*, 12(3), 215-225.

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H., and White, J. S. (2009). "Generalized linear mixed models: a practical guide for ecology and evolution." *Trends in ecology & evolution*, 24(3), 127-35.

Bourne, F. J., and Curtis, J. (1973). "The transfer of immunoglobulins IgG, IgA and IgM from serum to colostrum and milk in the sow." *Immunology*, 24.

Burnham, K. P., and Anderson, D. R. (2002). *Model selection and multimodel inference: a practical information-theoretic approach*: Springer Verlag.

Butterworth, M., McClellan, B., and Aklansmith, M. (1967). "Influence of sex on immunoglobulin levels." *Nature*, 214(5094), 1224-1225.

Carroll, M. C. (1998). "The role of complement and complement receptors in induction and regulation of immunity." *Annual Review of Immunology*, 16(1), 545-568.

CLSI. (2009). "Methods for dilution antimicrobial susceptibility tests for bacterial that grow aerobically; approved standard". City: Clinical and Laboratory Standards Institute.

Cohen, A. A., Martin, L. B., Wingfield, J. C., McWilliams, S. R., and Dunne, J. A. (2012). "Physiological regulatory networks: ecological roles and evolutionary constraints." *Trends in Ecology and Evolution*, 27(8), 428-435.

Cohen, S., Line, S., Manuck, S. B., Rabin, B. S., Heise, E. R., and Kaplan, J. R. (1997). "Chronic social stress, social status, and susceptibility to upper respiratory infections in nonhuman primates." *Psychosomatic Medicine*, 59(3), 213-21.

Cooper, S. M., Holekamp, K. E., and Smale, L. (1999). "A seasonal feast: long-term analysis of feeding behaviour in the spotted hyaena (Crocuta crocuta)." *African Journal of Ecology*, 37(2), 149-160.

Creel, S., Dantzer, B., Goymann, W., and Rubenstein, D. R. (2012). "The ecology of stress: effects of the social environment." *Functional Ecology*, 1-15.

Cunningham-Rundles, S., McNeeley, D. F., and Moon, A. (2005). "Mechanisms of nutrient modulation of the immune response." *The Journal of allergy and clinical immunology*, 115(6), 1119-28; quiz 1129.

Curno, O., Behnke, J. M., McElligott, A. G., Reader, T., and Barnard, C. J. (2009). "Mothers produce less aggressive sons with altered immunity when there is a threat of disease during pregnancy." *Proceedings of the Royal Society B: Biological Sciences*, 276(1659), 1047-1054.

Deerenberg, C., Arpanius, V., Daan, S., and Bos, N. (1997). "Reproductive effort decreases antibody responsiveness." *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 264(1384), 1021-1029.

Demas, G. E., Adamo, S. A., and French, S. S. (2011). "Neuroendocrine-immune crosstalk in vertebrates and invertebrates: implications for host defence." *Functional Ecology*, 25(1), 29-39.

Dhabhar, F. S., and McEwen, B. S. (1997). "Acute stress enhances while chronic stress suppresses cell-mediated immunity *in vivo*: a potential role for leukocyte trafficking." *Brain, behavior, and immunity*, 11(4), 286-306.

Diamond, S. E., and Kingsolver, J. G. (2011). "Host plant quality, selection history and trade-offs shape the immune responses of Manduca sexta." *Proceedings. Biological sciences / The Royal Society*, 278(1703), 289-97.

Drakesmith, H., and Prentice, A. M. (2012). "Hepcidin and the Iron-Infection Axis." *Science*, 338(6108), 768-772.

Dunkelberger, J. R., and Song, W. C. (2010). "Complement and its role in innate and adaptive immune responses." *Cell research*, 20(1), 34-50.

Dzik, J. M. (2010). "The ancestry and cumulative evolution of immune reactions." *Acta Biochim Polonica*, 57(4), 443-466.

East, M. L., Hofer, H., Cox, J. H., Wulle, U., Wiik, H., and Pitra, C. (2001). "Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas." *Proceedings of the National Academy of Sciences of the United States of America*, 98(26), 15026-31.

Eidinger, D., and Garrett, T. J. (1972). "Studies of the regulatory effects of the sex hormones on antibody formation and stem cell differentiation." *The Journal of experimental medicine*, 136(5), 1098-1116.

Elwell, K., and Bethell, D. R. (2001). "Antibody Storage", in G. C. Howard and D. R. Bethell, (eds.), *Basic methods in antibody production and characterization*. CRC Press, pp. 193-194.

Engh, A. L., Esch, K., Smale, L., and Holekamp, K. E. (2000). "Mechanisms of maternal rank 'inheritance' in the spotted hyaena, Crocuta crocuta." *Animal Behaviour*, 60(3), 323-332.

Estes, J. D., Gordon, S. N., Zeng, M., Chahroudi, A. M., Dunham, R. M., Staprans, S. I., Reilly, C. S., Silvestri, G., and Haase, A. T. (2008). "Early resolution of acute immune activation and induction of PD-1 in SIV-infected sooty mangabeys distinguishes nonpathogenic from pathogenic infection in rhesus macaques." *The Journal of Immunology*, 180(10), 6798-6807.

Fairbanks, B., and Hawley, D. M. (2011). "Interactions between host social behavior, physiology, and disease susceptibility", in G. E. Demas and R. J. Nelson, (eds.), *Ecoimmunology*. New York: Oxford University Press, pp. 440-468.

Flies, A. S., Grant, C. K., Mansfield, L. S., Smith, E. J., Weldele, M. L., and Holekamp, K. E. (2012). "Development of a hyena immunology toolbox." *Veterinary Immunology and Immunopathology*, 145(1–2), 110-119.

Frank, L. G. (1986). "Social organization of the spotted hyaena Crocuta crocuta. II. Dominance and reproduction." *Animal Behaviour*, 34(5), 1510-1527.

Frank, L. G., Glickman, S. E., and Powch, I. (1990). "Sexual dimorphism in the spotted hyaena (Crocuta crocuta)." *Journal of Zoology*, 221(2), 308-313.

French, S. S., Johnston, G. I. H., and Moore, M. C. (2007). "Immune activity suppresses reproduction in food-limited female tree lizards Urosaurus ornatus." *Functional Ecology*, 21(6), 1115-1122.

Gaipl, U., Kuenkele, S., Voll, R., Beyer, T., Kolowos, W., Heyder, P., Kalden, J., and Herrmann, M. (2001). "Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death." *Cell death and differentiation*, 8(4), 327.

Gelman, A. (2008). "Scaling regression inputs by dividing by two standard deviations." *Statistics in Medicine*, 27(15), 2865-2873.

Gittleman, J. L., and Thompson, S. D. (1988). "Energy allocation in mammalian reproduction." *American Zoologist*, 28, 863-875.

Goymann, W., East, M. L., Wachter, B., Honer, O. P., Mostl, E., Van't Hof, T. J., and Hofer, H. (2001). "Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyenas." *Proceedings. Biological sciences / The Royal Society*, 268(1484), 2453-9.

Graham, A. L., Hayward, A. D., Watt, K. A., Pilkington, J. G., Pemberton, J. M., and Nussey, D. H. (2010). "Fitness correlates of heritable variation in antibody responsiveness in a wild mammal." *Science*, 330(6004), 662-665.

Graham, A. L., Shuker, D. M., Pollitt, L. C., Auld, S. K. J. R., Wilson, A. J., and Little, T. J. (2011). "Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology." *Functional Ecology*, 25(1), 5-17.

Grueber, C. E., Nakagawa, S., Laws, R. J., and Jamieson, I. G. (2011). "Multimodel inference in ecology and evolution: challenges and solutions." *Journal of Evolutionary Biology*, 24(4), 699-711.

Guidry, A. J., Butler, J. E., Pearson, R. E., and Weinland, B. T. (1980). "IgA, IgG1, IgG2, IgM, and BSA in serum and mammary secretion throughout lactation." *Veterinary immunology and immunopathology*, 1(4), 329-341.

Hadfield, J. D. (2010). "MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package." *Journal of Statistical Software*, 33(2), 1-22.

Hafez, M., Aref, G., Mehareb, S., Kassem, A., El-Tahhan, H., Rizk, Z., Mahfouz, R., and Saad, K. (1977). "Antibody production and complement system in protein energy malnutrition." *The Journal of tropical medicine and hygiene*, 80(2), 36.

Harrison, T. M., Mazet, J. K., Holekamp, K. E., Dubovi, E., Engh, A. L., Nelson, K., Van Horn, R. C., and Munson, L. (2004). "Antibodies to canine and feline viruses in spotted hyenas (*Crocuta crocuta*) in the Masai, Mara National Reserve." *Journal of Wildlife Diseases*, 40(1), 1-10.

Hartweg, J., Gunter, M., Perera, R., Farmer, A., Cull, C., Schalkwijk, C., Kok, A., Twaalfhoven, H., Holman, R., and Neil, A. (2007). "Stability of soluble adhesion molecules, selectins, and C-reactive protein at various temperatures: implications for epidemiological and large-scale clinical studies." *Clinical Chemistry*, 53(10), 1858-1860.

Hasselquist, D., and Nilsson, J.-Å. (2012). "Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds?" *Animal Behaviour*, 83(6), 1303-1312.

Hawley, D. M., Jennelle, C. S., Sydenstricker, K. V., and Dhondt, A. A. (2007). "Pathogen resistance and immunocompetence covary with social status in house finches (Carpodacus mexicanus)." *Functional Ecology*, 21(3), 520-527.

Hawley, D. M., Lindström, K., and Wikelski, M. (2006). "Experimentally increased social competition compromises humoral immune responses in house finches." *Hormones and behavior*, 49(4), 417-424.

Hawlisch, H., and Kohl, J. (2006). "Complement and Toll-like receptors: key regulators of adaptive immune responses." *Molecular Immunology*, 43(1-2), 13-21.

Herr, M., Bostedt, H., and Failing, K. (2011). "IgG and IgM levels in dairy cows during the periparturient period." *Theriogenology*, 75(2), 377-385.

Hofer, H., and East, M. L. (2003). "Behavioral processes and costs of co-existence in female spotted hyenas: a life history perspective." *Evolutionary Ecology*, 17(4), 315-331.

Holekamp, K. E., and Sisk, C. L. (2003). "Effects of dispersal status on pituitary and gonadal function in the male spotted hyena." *Hormones and Behavior*, 44(5), 385-394.

Holekamp, K. E., and Smale, L. (1990). "Provisioning and food sharing by lactating spotted hyenas, *Crocuta crocuta* (Mammalia: Hyaenidae)." *Ethology*, 86(3), 191-202.

Holekamp, K. E., and Smale, L. (1993). "Ontogeny of dominance in free-living spotted hyaenas: juvenile rank relations with other immature individuals." *Animal Behaviour*, 46(3), 451-466.

Holekamp, K. E., Smale, L., and Szykman, M. (1996). "Rank and reproduction in the female spotted hyaena." *Journal of Reproduction and Fertility*, 108(2), 229-237.

Horrocks, N. P. C., Irene Tieleman, B., and Matson, K. D. (2011). "A simple assay for measurement of ovotransferrin - a marker of inflammation and infection in birds." *Methods in Ecology and Evolution*, 2(5), 518-526.

Jelliffe, D. B., and Chandra, R. K. (1972). "Immunocompetence in undernutrition." *The Journal of Pediatrics*, 81(6), 1194-1200.

Kenney, M. A., Roderuck, C. E., Arnrich, L., and Piedad, F. (1968). "Effect of protein deficiency on the spleen and antibody formation in rats." *The Journal of Nutrition*, 95(2), 173-178.

Kim, R. S. (2011). "Standardized regression coefficients as indices of effect sizes in meta-analysis."

Klobasa, F., Habe, F., Werhahn, E., and Butler, J. E. (1985). "Changes in the concentrations of serum IgG, IgA and IgM of sows throughout the reproductive cycle." *Veterinary immunology and immunopathology*, 10(4), 341-353.

Kruuk, H. (1972). *The spotted hyena: a study of predation and social behavior*: University of Chicago Press Chicago.

Kutner, M. H., Nachtsheim, C. J., Neter, J., and Li, W. (2005). *Applied linear statistical models* McGraw-Hill.

Lachmann, P. J., and Halbwachs, L. (1975). "The influence of C3b inactivator (KAF) concentration on the ability of serum to support complement activation." *Clinical and experimental immunology*, 21(1), 109-114.

Larivée, M. L., Boutin, S., Speakman, J. R., McAdam, A. G., and Humphries, M. M. (2010). "Associations between over-winter survival and resting metabolic rate in juvenile North American red squirrels." *Functional Ecology*, 24(3), 597-607.

Lembo, T., Hampson, K., Auty, H., Beesley, C. A., Bessell, P., Packer, C., Halliday, J., Fyumagwa, R., Hoare, R., Ernest, E., Mentzel, C., Mlengeya, T., Stamey, K., Wilkins, P. P., and Cleaveland, S. (2011). "Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009." *Emerging Infectious Diseases*, 17(3), 387-94.

Libert, C., Dejager, L., and Pinheiro, I. (2010). "The X chromosome in immune functions: when a chromosome makes the difference." *Nature reviews. Immunology*, 10(8), 594-604.

Liebl, A. L., and Martin, L. B. (2009). "Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry." *Functional Ecology*, 23(6), 1091-1096.

Lindström, K. M. (2004). "Social Status in Relation to Sindbis Virus Infection Clearance in Greenfinches." *Behavioral ecology and sociobiology*, 55(3), 236-241.

Love, O. P., Salvante, K. G., Dale, J., and Williams, T. D. (2008). "Sex-specific variability in the immune system across life-history stages." *The American naturalist*, 172(3), E99-112.

Markowska-Daniel, I., Pomorska-Mól, M., and Pejsak, Z. (2010). "Dynamic changes of immunoglobulin concentrations in pig colostrum and serum around parturition." *Polish Journal of Veterinary Sciences*, 13(1), 21-27.

Martin, L. B., 2nd, Weil, Z. M., and Nelson, R. J. (2006). "Refining approaches and diversifying directions in ecoimmunology." *Integrative and comparative biology*, 46(6), 1030-9.

Martin, L. B., Navara, K. J., Weil, Z. M., and Nelson, R. J. (2007). "Immunological memory is compromised by food restriction in deer mice Peromyscus maniculatus." *American*

Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 292(1), R316-R320.

Maslowska, M., Scantlebury, T., Germinario, R., and Cianflone, K. (1997). "Acute in vitro production of acylation stimulating protein in differentiated human adipocytes." *Journal of Lipid Research*, 38(1), 1-11.

Medzhitov, R., Schneider, D. S., and Soares, M. P. (2012). "Disease Tolerance as a Defense Strategy." *Science*, 335(6071), 936-941.

Merchant, M. E., Roche, C. M., Thibodeaux, D., and Elsey, R. M. (2005). "Identification of alternative pathway serum complement activity in the blood of the American alligator (Alligator mississippiensis)." *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology*, 141(3), 281-8.

Michalek, S. M., Rahman, A. F. R., and McGhee, J. R. (1975). "Rat Immunoglobulins in Serum and Secretions: Comparison of IgM, IgA and IgG in Serum, Colostrum, Milk and Saliva of Protein Malnourished and Normal Rats." *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, 148(4), 1114-1118.

Mollnes, T., Garred, P., and Bergseth, G. (1988). "Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation." *Clinical and experimental immunology*, 73(3), 484.

Moore, S. M., and Borer, E. T. (2012). "The influence of host diversity and composition on epidemiological patterns at multiple spatial scales." *Ecology*, 93(5), 1095-1105.

Morgan, B. P., Marchbank, K. J., Longhi, M. P., Harris, C. L., and Gallimore, A. M. (2005). "Complement: central to innate immunity and bridging to adaptive responses." *Immunology Letters*, 97(2), 171-9.

Nizet, V., Ohtake, T., Lauth, X., Trowbridge, J., Rudisill, J., Dorschner, R. A., Pestonjamasp, V., Piraino, J., Huttner, K., and Gallo, R. L. (2001). "Innate antimicrobial peptide protects the skin from invasive bacterial infection." *Nature*, 414(6862), 454-457.

Nunn, C. L., Lindenfors, P., Pursall, E. R., and Rolff, J. (2009). "On sexual dimorphism in immune function." *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 364(1513), 61-9.

Ochsenbein, A. F., and Zinkernagel, R. M. (2000). "Natural antibodies and complement link innate and acquired immunity." *Immunology Today*, 21(12), 624-630.

Pai, J. K., Curhan, G. C., Cannuscio, C. C., Rifai, N., Ridker, P. M., and Rimm, E. B. (2002). "Stability of novel plasma markers associated with cardiovascular disease: processing within 36 hours of specimen collection." *Clinical Chemistry*, 48(10), 1781-1784.

Peters, A. (2000). "Testosterone treatment is immunosuppressive in superb fairy–wrens, yet free–living males with high testosterone are more immunocompetent." *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 267(1446), 883-889.

Petro, T. M., and Bhattacharjee, J. (1980). "Effect of dietary essential amino acid limitations upon native levels of murine serum immunoglobulins, transferrin, and complement." *Infection and Immunity*, 27(2), 513-518.

Pinsky, N. A., Huddleston, J. M., Jacobson, R. M., Wollan, P. C., and Poland, G. A. (2003). "Effect of multiple freeze-thaw cycles on detection of measles, mumps, and rubella virus antibodies." *Clinical and diagnostic laboratory immunology*, 10(1), 19-21.

Pomeroy, C., Mitchell, J., Eckert, E., Raymond, N., Crosby, R., and Dalmasso, A. P. (1997). "Effect of body weight and caloric restriction on serum complement proteins, including Factor D/adipsin: studies in anorexia nervosa and obesity." *Clinical & Experimental Immunology*, 108(3), 507-515.

Purtilo, D. T., and Sullivan, J. L. (1979). "Immunological bases for superior survival of females." *Archives of Pediatrics & Adolescent Medicine*, 133(12), 1251.

R Development Core Team. (2012). "R: A language and environment for statistical computing". City: R Foundation for Statistical Computing.

Reller, L. B., and Stratton, C. W. (1977). "Serum dilution test for bactericidal activity. II. Standardization and correlation with antimicrobial assays and susceptibility tests." *Journal of Infectious Diseases*, 136(2), 196.

Ricklin, D., Hajishengallis, G., Yang, K., and Lambris, J. D. (2010). "Complement: a key system for immune surveillance and homeostasis." *Nature immunology*, 11(9), 785-97.

Rifkin, J. L., Nunn, C. L., and Garamszegi, L. Z. (2012). "Do animals living in larger groups experience greater parasitism? A meta-analysis." *The American Naturalist*, 180(1), 70-82.

Roberts, M. L., Buchanan, K. L., and Evans, M. R. (2004). "Testing the immunocompetence handicap hypothesis: a review of the evidence." *Animal Behaviour*, 68(2), 227-239.

Rolff, J. (2002). "Bateman's principle and immunity." *Proceedings. Biological sciences / The Royal Society*, 269(1493), 867-72.

Ruiz, M., French, S. S., Demas, G. E., and Martins, E. P. (2010). "Food supplementation and testosterone interact to influence reproductive behavior and immune function in Sceloporus graciosus." *Hormones and behavior*, 57(2), 134-139.

Scantlebury, T., Maslowska, M., and Cianflone, K. (1998). "Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in ifferentiated human adipocytes." *Journal of Biological Chemistry*, 273(33), 20903-20909.

Schapiro, S. J., Nehete, P. N., Perlman, J. E., and Sastry, K. J. (2000). "A comparison of cell-mediated immune responses in rhesus macaques housed singly, in pairs, or in groups." *Applied Animal Behaviour Science*, 68(1), 67-84.

Schielzeth, H. (2010). "Simple means to improve the interpretability of regression coefficients." *Methods in Ecology and Evolution*, 1(2), 103-113.

Skarnes, R. C. (1978). "Humoral bactericidal systems nonspecific and specific mechanisms." *Infection and Immunity*, 19.

Smale, L., Frank, L. G., and Holekamp, K. E. (1993). "Ontogeny of dominance in free-living spotted hyaenas: juvenile rank relations with adult females and immigrant males." *Animal Behaviour*, 46(3), 467-477.

Smith, J. E., Memenis, S. K., and Holekamp, K. E. (2006). "Rank-related partner choice in the fission–fusion society of the spotted hyena (Crocuta crocuta)." *Behavioral ecology and sociobiology*, 61(5), 753-765.

Smith, R., Bischof, P., Hughes, G., and Klopper, A. (1979). "Studies on pregnancy-associated plasma protein A in the third trimester of pregnancy." *BJOG: An International Journal of Obstetrics & Gynaecology*, 86(11), 882-887.

Spatafora, G. A., and Moore, M. W. (1998). "Growth of Streptococcus mutans in an iron-limiting medium." *Methods in Cell Science*, 20(1), 217-221.

Swanson, E. M., Dworkin, I., and Holekamp, K. E. (2011). "Lifetime selection on a hypoallometric size trait in the spotted hyena." *Proceedings. Biological sciences / The Royal Society*, 278(1722), 3277-85.

Taylor, B. C., Brotheridge, R. M., Jessup, D. A., and Stott, J. L. (2002). "Measurement of serum immunoglobulin concentration in killer whales and sea otters by radial immunodiffusion." *Veterinary Immunology and Immunopathology*, 89, 187-195.

Taylor, P. (1983). "Bactericidal and bacteriolytic activity of serum against gram-negative bacteria." *Microbiological Reviews*, 47(1), 46.

Tennessen, J. A., Woodhams, D. C., Chaurand, P., Reinert, L. K., Billheimer, D., Shyr, Y., Caprioli, R. M., Blouin, M. S., and Rollins-Smith, L. A. (2009). "Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (Rana pipiens) populations suggest intraspecies differences in resistance to pathogens." *Developmental and comparative immunology*, 33(12), 1247-57.

Terres, G., Morrison, S. L., and Habicht, G. S. (1968). "A quantitative difference in the immune response between male and female mice." *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, 127(3), 664-667.

Thompson-Chagoyán, O., Maldonado, J., and Gil, A. (2007). "Colonization and impact of disease and other factors on intestinal microbiota." *Digestive Diseases and Sciences*, 52(9), 2069-2077.

Thomson, A., Hytten, F., and Billewicz, W. (1970). "The energy cost of human lactation." *British Journal of Nutrition*, 24(02), 565-572.

Trouw, L. A., Nilsson, S. C., Gonçalves, I., Landberg, G., and Blom, A. M. (2005). "C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation." *The Journal of experimental medicine*, 201(12), 1937-1948.

Tuchscherer, M., Puppe, B., Tuchscherer, A., and Kanitz, E. (1998). "Effects of social status after mixing on immune, metabolic, and endocrine responses in pigs." *Physiology & Behavior*, 64(3), 353-360.

Tung, J., Barreiro, L. B., Johnson, Z. P., Hansen, K. D., Michopoulos, V., Toufexis, D., Michelini, K., Wilson, M. E., and Gilad, Y. (2012). "Social environment is associated with gene regulatory variation in the rhesus macaque immune system." *Proceedings of the National Academy of Sciences*.

Van Horn, R. C., McElhinny, T. L., and Holekamp, K. E. (2003). "Age estimation and dispersal in the spotted hyena (*Crocuta crocuta*)." *Journal of Mammalogy*, 84(3), 1019-1030.

Waitz, J. A. (1990). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*: National Committee for Clinical Laboratory Standards.

Walport, M. (2001). "Complement. First of two parts." *The New England journal of medicine*, 344(14), 1058.

Weyer, C., Tataranni, P. A., and Pratley, R. E. (2000). "Insulin action and insulinemia are closely related to the fasting complement C3, but not acylation stimulating protein concentration." *Diabetes Care*, 23(6), 779-785.

Wilcoxen, T. E., Boughton, R. K., and Schoech, S. J. (2010). "Selection on innate immunity and body condition in Florida scrub-jays throughout an epidemic." *Biology letters*, 6(4), 552-554.

Zimmerman, L. J., Li, M., Yarbrough, W. G., Slebos, R. J. C., and Liebler, D. C. (2012). "Global stability of plasma proteomes for mass spectrometry-based analyses." *Molecular & Cellular Proteomics*, 11(6).

Zuk, M., and Johnsen, T. S. (2000). "Social environment and immunity in male red jungle fowl." *Behavioral Ecology*, 11(2), 146-153.

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., and Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*: Springer Verlag.

Zwart, B., Ciurana, C., Rensink, I., Manoe, R., Erik Hack, C., and Aarden, L. A. (2004). "Complement Activation by Apoptotic Cells Occurs Predominantly via IgM and is Limited to Late Apoptotic (Secondary Necrotic) Cells." *Autoimmunity*, 37(2), 95-102.

CHAPTER 4

CHAPTER 4

IMMUNE DEFENSES OF WILD AND CAPTIVE HYENAS: A COMPARATIVE ANALYSIS

Introduction

Evolutionary processes have shaped the vertebrate immune system over time, but socio-ecological factors mediate activation, duration, and intensity of immune responses (Viney et al. 2005). For example, sex, predation risk, diet, sociality, energetic trade-offs, life-history stage, and pathogen pressure can influence immune function (Folstad and Karter 1992; Hamilton and Zuk 1982; Horrocks et al. 2011; Joop and Rolff 2004; Schmid-Hempel 2003; Thomas and Rudolf 2010). In recent decades, the prevalence of allergy and autoimmune disease has been rapidly increasing in human populations (Palomares et al. 2010; Rook 2009). The hygiene hypothesis was originally proposed as an explanation for the commonly observed pattern of increased allergic disease in relatively hygienic environments (Strachan 1989) and in populations with access to modern medicine. The hygiene hypothesis has evolved since it was first proposed, but one of the basic tenets of the hypothesis is that lack of exposure to microbes, including both macro and microparasites, leads to dysregulated development of immune defense pathways, and subsequently increased risk of allergy and autoimmune disease. Many studies have found significant differences in immune function and prevalence of allergic disease between people who were raised on farms and people who grew up in urban environments or rural non-farm environments, as reviewed in (von Mutius and Vercelli 2010). Although debate about the implications and breadth of the hygiene hypothesis continues, it is clear that environmental exposure to micro and macroparasites can shape the development of the immune system (Wander et al. 2012; Yazdanbakhsh et al. 2002).

The use of highly controlled laboratory experiments in conventional immunology generally eliminates socio-ecological factors that might influence immune function. In an effort to assess genotypic effects, the results of experiments utilizing inbred or genetically modified, lab-reared animals are often compared to 'wild-type' animals that are used as proxies for their unmodified, free-living relatives. However, the 'wild-type' controls are usually bred and reared in the laboratory, which could potentially have a major effect on immune system development and maintenance.

Despite the widespread use of 'wild-type' animals in laboratory research, few studies have actually examined immune function in wild animals living in their natural environment, and fewer still have compared immune function between wild and captive conspecifics (Abolins et al. 2011; Buehler et al. 2008b; Devalapalli et al. 2006). Differences in immune function between captive and wild animals can be expected due to reduced energy expenditure in captive animals, regular cleaning of captive facilities, lower diversity of pathogens encountered in captivity, and a generally more stable diet and environment in captivity (Buehler et al. 2008a; Friend and Franson 1999; Joop and Rolff 2004). Indeed, two separate studies that compared immunoglobulin concentrations of laboratory rodents to wild rodents reported that wild rodents generally had higher levels of immunoglobulins (Abolins et al. 2011; Devalapalli et al. 2006). Similarly, Buehler *et al*. (2008b) found that immune defenses in wild birds were reduced after nine months in captivity.

Few studies to date have explicitly examined the effects of ecological variables on immune function in the mammalian order Carnivora (Schwartz et al. 2004; Van Loveren et al. 2000). Carnivores are particularly interesting with respect to immune function because they consume other animals, many of which harbor micro- and macroparasites capable of infecting

both prey and predator. For example, Harrison *et al*. (2006) found 28% of wildlife carcasses tested positive for *Salmonella spp*. In addition to the pathogens potentially acquired from their prey, carnivores that scavenge food are also exposed to toxins produced by microbes in decomposing meat (Burkepile et al. 2006); for example, more than 140 Shiga toxin-producing *Escherichia coli* strains were detected in game meat samples collected in Germany (Miko et al. 2009).

Spotted hyenas (*Crocuta crocuta*) obtain the majority of their food from fresh kills, but also routinely scavenge on carrion in various stages of decay (Cooper et al. 1999). These hyenas (*Crocuta crocuta*) consume entire carcasses, including bones, which can potentially cause lesions in the gastrointestinal tract (Flajnik and Kasahara 2009). This increases the risk of an infection acquired not only from the pathogens inhabiting the carcass, but also from the hyena's own gut microbiota spilling into other body tissues. Furthermore, wild spotted hyenas are known to survive exposure to rabies virus, canine distemper virus, and anthrax (East et al. 2001; Harrison et al. 2004; Lembo et al. 2011). The high pathogen exposure rates coupled with the low mortality rates from infectious disease suggest that immune function may be particularly robust in wild spotted hyenas.

Our primary objective in this study was to assess differences in immune function between wild spotted hyenas inhabiting a pathogen-rich and highly variable environment, and captive hyenas inhabiting a more hygienic, stable environment. We used the defense component model framework (Fig. 4.1), modified from Schmid-Hemp and Ebert (2003), to compare immune defenses in wild and captive hyenas along two continua: constitutive to induced and non-specific to specific. Constitutive defenses are present in the absence of infection and are fully functional without previous exposure to a pathogen (Schmid-Hempel and Ebert 2003). Induced

defenses are initiated after the immune system has recognized a potential pathogen (Schmid-Hempel and Ebert 2003). Defenses may be induced within minutes of pathogen detection, or may take more than a week for a functional response to be mounted, depending on the type of defense. Specific defenses are effective against only a limited range of pathogens and may be modified during a host's lifetime. Specific defenses are most effective upon repeated exposure to the same pathogen, because the specific defenses are maintained from the initial infection, allowing a much faster response. Non-specific defenses target a broad range of pathogens and are not modified over time.

Natural antibodies (NAbs) by definition are produced without known exposure to the antigens they bind (Baumgarth 2011), and are thus non-specific. NAbs are constitutively produced in the absence of microorganisms and do not undergo extensive somatic mutation of the variable region or affinity maturation (Baumgarth et al. 2005; Carroll 2004a), but can be rapidly induced to higher production levels by infection (Baumgarth 2011; Choi and Baumgarth 2008). By contrast, specific antibodies, primarily of the IgG isotype, are produced in response to infection and undergo somatic mutation and affinity maturation (Baumgarth et al. 2005). NAbs are primarily of the IgM isotype, but can also be IgG and IgA (Avrameas 1991; Lacroix-Desmazes et al. 1995). Natural IgM makes up the majority of total IgM in mice, and functions in concert with complement to provide first line defenses to inhibit pathogen invasion and replication (Baumgarth 2011). For instance, natural anti-influenza virus IgM and IgG2a concentrations do not increase in response to influenza infection, whereas specific IgM and IgG2a concentrations do increase (Baumgarth et al. 1999).

We hypothesized here that the relatively hygienic environment experienced by captive hyenas would lead to lower levels of immune defenses in captive than wild animals. More

specifically, we predicted higher levels of induced immune defenses in a pathogen-rich natural environment than in a relatively hygienic environment, whereas constitutive, non-specific immune defenses, which are less dependent on pathogen exposure and are maintained at baseline levels, should be similar in pathogen-rich wild and pathogen-poor captive environments (Abolins et al. 2011; Schmid-Hempel and Ebert 2003).

We measured relative concentrations of total IgG and IgM antibodies and natural IgG and IgM antibodies (NAbs) against keyhole limpet hemocyanin (KLH). Total IgG consists of primarily induced, specific antibodies that have been induced by infection and falls within quadrant I of the defense component model (Fig. 4.1). Total IgM is more likely than total IgG to be a relatively even mixture of specific and non-specific antibodies, so it should be located near the center of the constitutive-induced axis, and should thus be located near the convergence of the four quadrants in Fig. 4.1. Natural IgG and IgM to KLH are non-specific, but can be induced by infection. Finally, we assessed complement-mediated bacterial killing capacity (BKC) as a measure of non-specific, constitutive immune function.

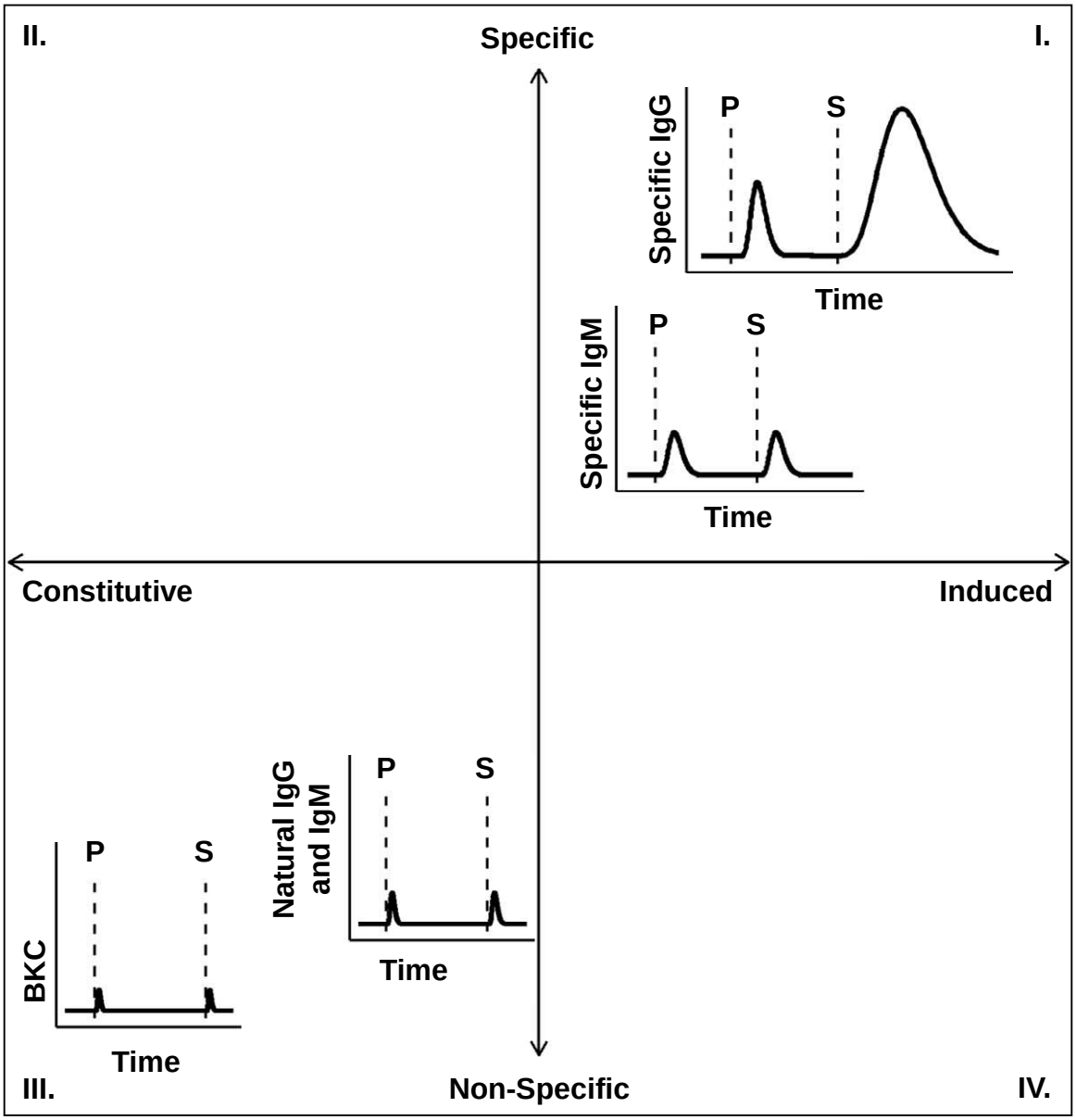

Fig. 4.1. Immune defense component model modified from Schmid-Hempel and Ebert (2003). Bacterial killing capacity (BKC) is the least specific defense and also falls at the constitutive end of the x-axis. Specific IgG falls near the induced end of the x-axis, and is the most specific defense represented here. Natural IgG and IgM are non-specific but can be induced to some extent. The dashed lines represent primary exposure (P) and secondary (S) exposure.

Materials and methods

Collection of serum samples from captive and wild hyenas

Captive spotted hyenas used in this study were born and housed at the Field Station for Behavioral Research at the University of California, Berkeley (UCB). The colony was founded

by importing 20 infant hyenas from Kenya in 1984-1985 (Glickman et al. 1992). All hyenas were treated with antihelminthic drugs prior to importation from Kenya and no parasitic worms have been detected in the colony since its founding, whereas a high diversity and abundance of parasitic worms have been detected in the feces of the wild hyenas (Engh et al. 2003). The captive hyena enclosures have cement floors that are hosed down each day with water, and steam cleaned annually. Fecal material is removed from medium-sized external enclosures (Fig. 4.2a) on a daily basis, and fecal material is removed from large outdoor enclosures on a monthly basis (Fig. 4.2b). No disease outbreaks have occurred in the captive population, but many virulent viral, bacterial, and protozoan pathogens have been documented in the wild population (Alexander et al. 1995; East et al. 2001; East et al. 2004; East et al. 2008; Harrison et al. 2004; Lembo et al. 2011). Ticks, fleas, tsetse flies, and other biting insects, any of which are capable of transmitting pathogens to hyenas, are commonly observed on wild hyenas, but not on captive hyenas. Additionally, the captive hyenas are fed only USDA approved meat and standard carnivore chow, whereas the wild hyenas feed on fresh ungulate carcasses and carrion (Cooper et al. 1999).

The captive hyenas used in our study have been in captivity for less than four generations and were derived directly from the same wild population under study here. The captive hyenas exhibit most of the same behaviors as wild hyenas, including scent marking, establishment of dominance hierarchies, greeting ceremonies, and mating (Glickman et al. 1992), however, the social density is much lower at the captive facility than in the wild. Pathogen transmission is often positively correlated with host density, and the effect becomes more pronounced in species that live in large aggregations (Rifkin et al. 2012). Wild hyenas live in social groups of up to 120 individuals. By contrast, captive hyenas are housed in groups of two or three. The low number of

hyenas in each enclosure reduces the possibility of direct transmission of pathogens among group members via social interactions. Interspecific pathogen transmission between captive hyenas and wild local animals is probably low because wild animals, such as skunks (*Mephitis mephitis*), are rarely observed straying into the hyena enclosures. Wild hyenas are regularly observed feeding on carcasses, from which lions, jackals, and vultures, have also fed, and wild hyenas often engage in fierce battles with other carnivores.

All sera from captive hyenas were collected with approvals from both the University of California, Berkeley (Animal Use Protocol # R091-0609R) and the Michigan State University Institutional Animal Care and Use Committees (Animal Use Form # 07/08-099-00). Captive hyenas were immobilized with blow dart-delivered intramuscular injections of ketamine (4–6 mg/kg) and xylazine (1 mg/kg). Blood was drawn from the jugular vein and allowed to clot at ambient temperature for approximately 2 hours. The clotted blood was then centrifuged and serum was collected, aliquoted, and frozen at -80°C until use.

Sera from wild spotted hyenas in the Maasai Mara, Kenya, were collected as part of the Michigan State University Hyena Research Project (Animal Use Form #07/08-099-00). Wild spotted hyenas were immobilized using Telazol (6.5 mg/kg; Fort Dodge Animal Health) in a plastic dart fired from an air rifle (Telinject Inc.) (Holekamp and Sisk 2003). Blood was collected as described above, but was snap frozen in liquid nitrogen in the field, then transported to Michigan on dry ice, and stored at -80°C until use.

(A) (B)

Fig. 4.2. Hyena enclosures at the University of California, Berkeley. Figure (A) shows the standard captive enclosure and (B) shows the open yard where 2-3 hyenas can range at a time.

Total IgG and IgM

Total IgG and IgM were quantified using sandwich ELISAs. All ELISA steps were carried out at 20-22˚C in a humidity chamber to avoid desiccation. Checkerboard dilutions of sera and detection antibodies were used to determine optimal dilutions the assays. First, capture antibodies (IgG: Bethyl Labs # A20-117A; IgM: Custom Monoclonals International # CM7) were diluted into coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) at 10 μg/ml, aliquoted into 96-well polystyrene plates (Santa Cruz Biotechnology # sc-204463), and allowed to bind to the plates for 60 minutes. The plates were then washed 2X with 175 μl of wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20) using a 12-channel pipet. Non-specific binding was then blocked by incubating with 150 μl/well of blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA) for 60 minutes. All standards, sera, and detection antibodies were diluted in dilution buffer

(50mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween-20). We used pooled sera from both captive and wild hyenas to create a standard curve on each plate; the same pooled sera were used to standardize relative concentrations from both captive and wild hyenas. Standard curves were run in duplicate on each plate. The starting dilutions of the pooled sera standard curve were 1:10,000 and 1:100 for total IgG and IgM, respectively. We performed 12 doubling dilutions to achieve final standard dilutions of 1:20,480,000 and 1:204,800 for IgG and IgM, respectively. Serum samples were diluted 1:100,000 for IgG and 1:1,000 for IgM and 100 µl were aliquoted into each well and incubated for 60 minutes. All samples were tested in triplicate. The plates were washed three times before adding 100 µl of horseradish peroxidase (HRP)-conjugated anti-IgG (Kirkegaard & Perry Laboratories # 04-20-02) or anti–IgM (Kirkegaard & Perry Laboratories # 04-20-03) detection antibodies to the plates at concentrations of 0.2 µg/ml and 0.25 µg/ml, respectively. The plates were then incubated 60 minutes and washed four times. 100 µl/well of 3,3',5,5'-tetramethylbenzidine (TMB) was used as the substrate for the HRP, and the color change reaction was stopped using 0.2 M H_2SO_4. Absorbance was read at 450 nm on a standard plate reader. Relative serum concentrations were calculated using the "calibFit" package in R (R Development Core Team 2012).

Detection of natural antibodies to keyhole limpet hemocyanin (KLH)

We used anti-KLH antibodies as our measure of NAbs (Parmentier et al. 2004). KLH is a standard immunogen in laboratory studies; it is unlikely that either captive or wild hyenas could ever have been exposed to KLH, which is derived from marine gastropods (Abolins et al. 2011). Natural anti-KLH is detectable in pre-immune human serum, and concentrations are relatively stable over time (Korver et al. 1984; Thornton et al. 1994). NAbs were quantified using the same protocol as above, but with the following changes. Instead of coating capture antibodies to the

plates, we coated KLH (EMD Millipore # 374805) at 10 μg/ml. The pooled sera standard starting dilutions were and 1:50 and 1:12.5 for IgG and IgM. We performed 12 doubling dilutions to achieve final standard dilutions of 1:102,400 and 1:25,600 for IgG and IgM, respectively. Serum samples were diluted 1:250 for IgG and 1:100 for IgM, and 100 μl were aliquoted into each well and incubated for 60 minutes. The horseradish peroxidase (HRP)-conjugated anti-IgG or anti–IgM detection antibodies were added to the plates at concentrations of 0.2 μg/ml and 0.25 μg/ml, respectively. Note that anti-KLH IgG and IgM represent subsets of total IgG and IgM, but they make up only a small portion of the total.

Bacterial killing capacity (BKC)

We used a modified version of the bacterial killing assay method described previously in chapter two of this dissertation. We tested two strains of bacteria: *E. coli* (ATCC# 8739) was chosen because it has been used as a standard bacterial strain in several other studies of bactericidal capacity (Liebl and Martin 2009); *P. mirabilis* (ATCC# 35659) was chosen because, like *E. coli,* it is a gram negative bacterium that is commonly found in the gastrointestinal tract of many animals and was more susceptible to complement-mediated killing than most other strains in preliminary testing.

All bacterial killing assays were conducted in 96-well round bottom plates (Fisher #08-772-54). All serum samples were passed through a 0.22 μm filter prior to adding to the 96-well plate to remove residual red blood cells and other cellular debris. Sera were then diluted 1:4 in phosphate buffered saline (PBS), added to row 1 and serially diluted in PBS, with dilutions ranging from 1:8 to 1:1,024 for *E. coli* 1:4 to 1:512 for *P. mirabilis* and (NOTE: After the subsequent addition of 50 μl of bacteria-containing broth, the final dilutions were 2X the initial dilutions). Cation-adjusted Mueller-Hinton broth II (MHBII) was used as the bacterial growth

medium for all assays (CLSI 2009; Reller and Stratton 1977). Control and blank wells were loaded with 50 µl of PBS.

One day prior to beginning the bacterial killing assay, *E. coli* or *P. mirabilis* were inoculated into MHBII and incubated overnight at 37°C while shaking at 120 rpm. After overnight incubation, bacterial cell concentration was adjusted to approximately 10^8 CFU/ml by diluting bacteria in PBS until optical density at 600 nm matched the corresponding turbidity of the McFarland turbidity standard (BD Biosciences # 287298). Bacteria were then serially diluted in PBS to a concentration of 10^4 CFU/ml. Control wells, serum wells and antibiotic wells were next quickly loaded with 50 µl stock bacteria-containing broth, resulting in a final concentration of 500 CFU/well and total volume of 100 µl/well. 50 µl of sterile MHB were added to blank wells to bring the total volume of each well to 100 µl. Plates were then placed in an incubator at 37˚C and turbidity was measured after 24 hours on a Bio-Tek plate reader at 600 nm. Percent inhibition was calculated by dividing the optical density of each sample well by the mean optical density of the control bacteria wells. Because the assay allows the bacteria to grow to saturation, there was a clear distinction among wells exhibiting bacterial growth and those without growth. The minimum inhibitory concentration (MIC) was defined as the negative $\log_2$ of the lowest dilution that exhibited over 90 percent inhibition compared to the mean of the control wells (Tennessen et al. 2009; Waitz 1990). For example, if 1:40 was the lowest dilution exhibiting complete growth inhibition, the response variable value would be: $-log_2\ (1/40) = 5.32$. Additionally, the plates were visually inspected to ensure there was no bacterial growth at the calculated MIC. The mean of triplicates for each sample was used as the MIC.

Statistical analysis

We used version 2.14.2 of the software package R (R Development Core Team 2012) to create linear regression models to assess the effects of captivity on immune function in spotted hyenas. Age and sex are known to affect aspects of immune function in some species, so we included age and sex as covariates in all our models. Because no single model is a perfect representation of nature (Burnham and Anderson 2004), we used an information theoretic multimodel inference approach rather than choosing a single best model (Moore and Borer 2012). The full model included captivity status, sex, and age as input variables, as well as all two-way interactions between input variables. We explored all possible subsets of the full model, which yielded 18 candidate models for each dependent variable (Moore and Borer 2012). All subset models were ranked according to Akaike's information criteria corrected for small sample size (AICc) (Burnham and Anderson 2002). Models with a difference in AICc of less than two (Δ AICc < 2) are considered to be equally good (Burnham and Anderson 2002). The top models, those with Δ AICc < 2, were then averaged using the 'MuMIn' in R package to produce weighted averages of regression parameter coefficients, 95% confidence intervals, and p-values for each input variable in the top models (R Development Core Team 2012). In cases where only a single model had Δ AICc < 2, we report the results directly from the linear model, rather than weighted averages.

The response variables total IgG, total IgM, anti-KLH IgG anti-KLH IgM, and MIC were normalized by log transformation; the input variable age was normalized using a square root transformation. All continuous response and predictor variables were then centered and standardized by subtracting the mean and dividing by the standard deviation; standardizing allows more interpretable comparisons of effect sizes by putting all effects on a common scale, and centering makes the main effects more biologically interpretable when interactions are

present (Gelman 2008; Schielzeth 2010). We performed model diagnostics on all of the top models using histogram plots of the residuals, Shapiro-Wilk tests for normality of residuals, and using qqnorm plots. We also checked for highly influential data points by assessing Cook's distance, covariance ratios, and hat statistics. We did not detect any outliers meeting criteria for removal (Zuur et al. 2010). Prior to the beginning of data analysis, we excluded four captive hyenas from the analysis of anti-KLH natural antibodies because they were immunized with KLH one year prior to the collection of the serum samples used in this study; anti-KLH IgG and IgM levels in these individuals had already returned to pre-immunization levels, but we excluded these animals nevertheless because their anti-KLH antibodies no longer met the definition of natural antibodies. One wild hyena was excluded from the study prior to beginning data analysis because its serum contained a large lipid globule and could not be filtered in a manner consistent with the other serum samples.

We also performed univariate non-parametric tests of the effects of captivity on immune function and the results were similar to the AICc model selection approach. Correlations among total IgG, total IgM, anti-KLH IgG, and anti-KLH IgM were assessed using Pearson product-moment correlation tests. Differences between immune measures in captive and wild hyenas were assessed using Levene's test for homogeneity of variance.

Results

Total IgG and IgM

We assessed the relative concentrations of total IgG and IgM in 15 captive and 14 wild hyenas. For total IgG, only a single model had Δ AICc < 2 (Table 4.1); captivity status was the only input variable in this model, indicating the wild hyenas had significantly higher serum

concentrations of IgG than did captive hyenas ($p < 0.0004$) (Fig. 4.3). Wild hyenas also had significantly more total IgM than captive hyenas ($p < 0.0001$) (Fig. 4.3); here age was non-significant ($p < 0.1003$), but was included in the top model set (Δ AICc < 2). (See Table 4.2 for full list of effect sizes, confidence intervals, and p-values for the analysis of total IgG and IgM)

Table 4.1. Linear models to assess the effects of captivity on total IgG and IgM in spotted hyenas (captive = 15, wild = 14). Models with Δ AICc < 2 were included in the top model set shown here. Models weights shown here were calculated from the full set of models. In cases where only a single model had Δ AICc < 2, we report only the results of that single linear model. CS = Captivity status.

Response	Predictors	df	logLik	AICc	Δ AICc	Weight
IgG	CS	3	-33.91	74.77	0.00	0.50
IgM	CS + Age	4	-22.68	55.03	0.00	0.36
	CS	3	-24.25	55.46	0.43	0.29

Table 4.2. Results of AICc based multimodel weighted-averages for total IgG and IgM. In cases where only a single model had Δ AICc < 2, the results from the single linear model are reported. CS = Captivity status.

Response	Predictor	β	SE	Lower CI	Upper CI	p	Importance
Total IgG	Intercept	-0.579	0.208	-1.006	-0.151	0.0099	-
	CS	1.198	0.300	0.583	1.814	0.0004	1.000
Total IgM	Intercept	-0.709	0.170	-1.056	-0.363	0.0001	-
	CS	1.469	0.275	0.909	2.029	0.0001	1.000
	Age	-0.228	0.132	-0.499	0.044	0.1004	0.553

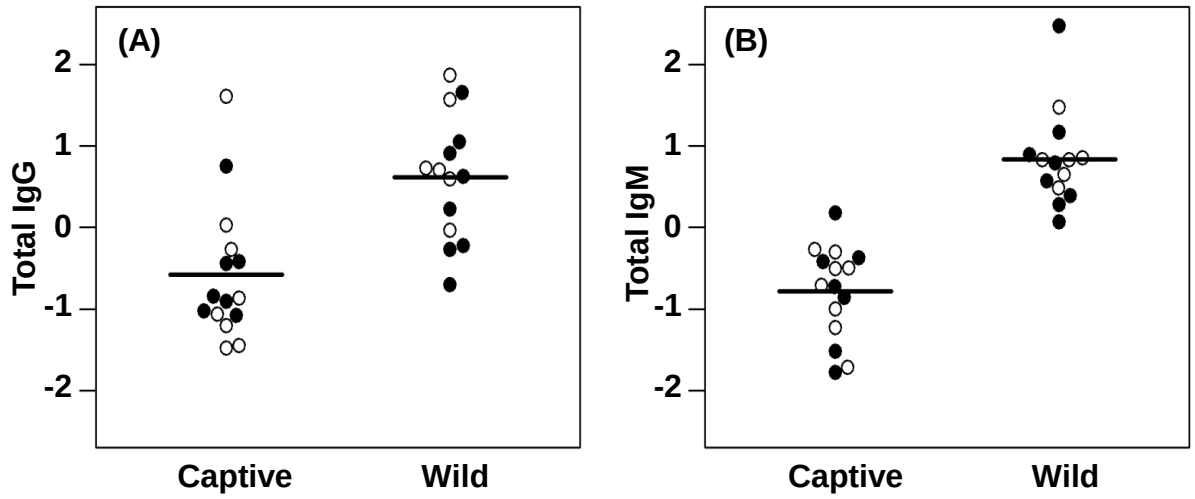

Fig. 4.3. Relative concentrations of (A) total IgG and (B) IgM in captive and wild hyenas. Females are indicated by open circles and males by filled circles.

Natural IgG and IgM against KLH

We measured natural antibodies to KLH in sera from 11 captive hyenas and 14 wild hyenas. As with total IgG, there was again only a single model with Δ AICc < 2, however, this time the model included captivity status, sex, and an interaction between captivity status and sex (Table 4.3). Wild hyenas had significantly higher concentrations of anti-KLH IgG than captive hyenas ($p < 0.0001$) (Fig 4.4a). There was also a significant interaction between captivity status and sex ($p < 0.0027$), with all wild female hyenas having higher concentrations of anti-KLH IgG than wild male hyenas (Fig. 4.4a). This difference between females and males was not observed in the captive hyenas. Four models fit the anti-KLH IgM data well according to the AICc values (Table 4.3). The weighted averages from these four models indicated a marginally non-significant interaction between sex and age ($p < 0.051$). The trend was for anti-KLH IgM to decrease with age in females, but not in males. There was also a trend for wild hyenas to have higher anti-KLH IgM than captive hyenas ($p < 0.12$) (Fig. 4.4b) and an overall trend for anti-

KLH IgM concentrations to decrease with age ($p < 0.1$). (See Table 4.4 for full list of effect sizes, confidence intervals, and p-values for the analysis of natural anti-KLH IgG and IgM)

Table 4.3. Linear models to assess the effects of captivity on natural anti-KLH IgG and IgM in spotted hyenas (captive = 11, wild = 14). Models with Δ AICc < 2 were included in the top model set shown here. Models weights shown here were calculated from the full set of models. In cases where only a single model had Δ AICc < 2, we report only the results of that single linear model. CS = Captivity status.

Response	Predictors	df	logLik	AICc	ΔAICc	Weight
anti-KLH IgG	Sex + CS + Sex * CS	5	-15.92	44.99	0.00	0.69
anti-KLH IgM	Age	3	-31.41	69.97	0.00	0.26
	CS	3	-31.91	70.96	0.98	0.16
	CS + Age	4	-30.66	71.32	1.34	0.13
	Sex + Age + Sex * Age	5	-29.08	71.33	1.35	0.13

Table 4.4. Results of AICc based multimodel weighted-averages for natural anti-KLH IgG and IgM. CS = Captivity status.

Response	Predictor	β	SE	Lower CI	Upper CI	p	Importance
anti-KLH IgG	Intercept	-0.974	0.204	-1.398	-0.550	0.0001	-
	CS	2.281	0.288	1.681	2.880	0.0001	1.00
	Sex	0.263	0.302	-0.366	0.891	0.3941	1.00
	Sex * CS	-1.374	0.405	-2.217	-0.532	0.0027	1.00
anti-KLH IgM	Intercept	-0.135	0.361	-0.863	0.593	0.716	-
	Age	-0.591	0.350	-1.295	0.114	0.100	0.77
	CS	0.737	0.449	-0.183	1.656	0.116	0.43
	Sex	-0.089	0.375	-0.870	0.691	0.822	0.19
	Sex * Age	0.823	0.397	-0.002	1.648	0.051	0.19

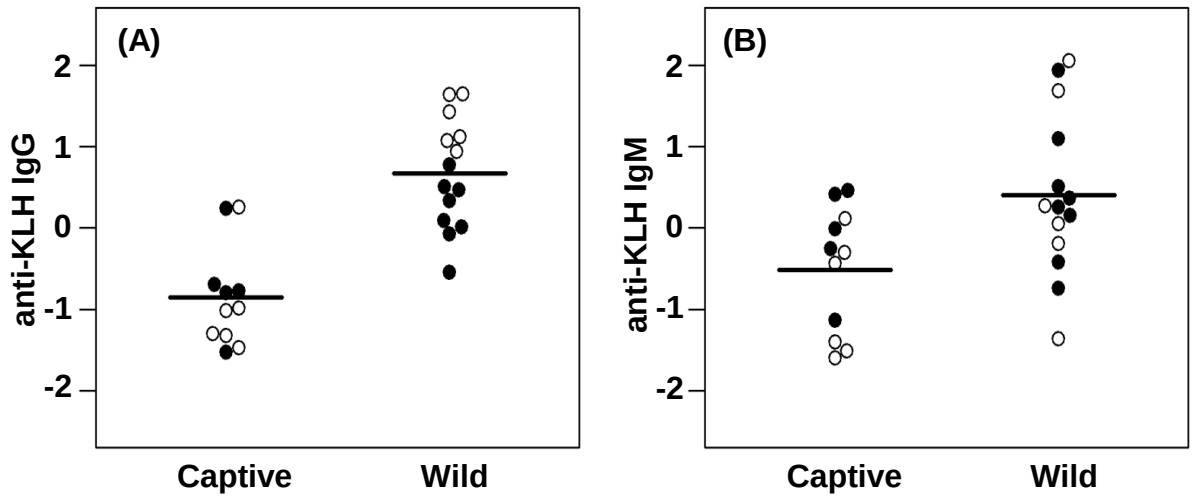

Fig. 4.4. Relative concentrations of (A) anti-KLH IgG and (B) IgM in captive and wild hyenas. Females are indicated by open circles and males by filled circles.

Bacterial killing capacity (BKC)

We used the mean MIC for *E. coli* and *P. mirablis* for 15 captive and 14 wild hyenas as our measure of complement-mediated BKC. None of the input variables or interactions significantly affected the BKC in the hyenas we tested. Captivity status and sex were included in the best fitting model set (Δ AICc < 2) (Table 4.5), but neither captivity status ($p < 0.37$) nor sex ($p < 0.43$) significantly affected BKC (Fig. 4.5). The null model, which included only the intercept, had the lowest AICc value. (See Table 4.6 for full list of effect sizes, confidence intervals, and p-values for the analysis of BKC)

Table 4.5. Linear models to assess the effects of captivity on serum bacterial killing capacity (BKC) in spotted hyenas (captive = 15, wild = 14). Models with Δ AICc < 2 were included in the top model set shown here. Models weights shown here were calculated from the full set of models. CS = Captivity status.

Response	Predictors	df	logLik	AICc	ΔAICc	Weight
BKC	Intercept	2	-40.64	85.74	0.00	0.36
	CS	3	-40.11	87.17	1.43	0.17
	Sex	3	-40.28	87.52	1.78	0.15

Table 4.6. Results of AICc based multimodel weighted-averages for bacterial killing capacity (BKC). CS = Captivity status.

Response	Predictor	B	SE	Lower CI	Upper CI	p	Importance
BKC	Intercept	-0.081	0.241	-0.573	0.411	0.748	-
	Sex	0.307	0.374	-0.460	1.074	0.433	0.257
	CS	0.374	0.372	-0.388	1.136	0.336	0.216

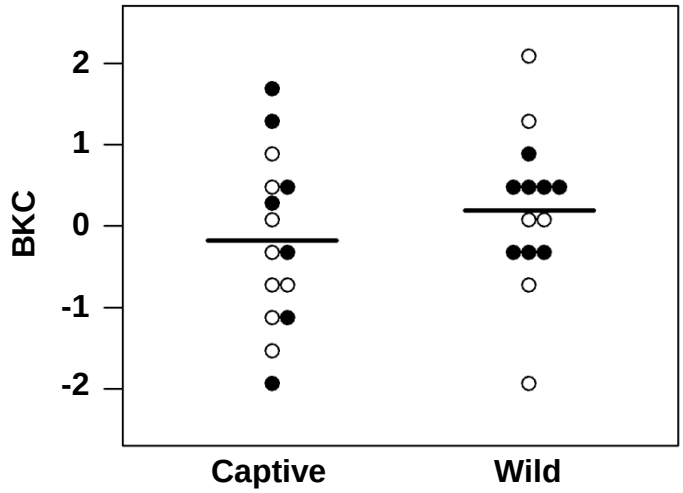

Fig. 4.5. Bacterial killing capacity (BKC) of serum from captive and wild hyenas. Females are indicated by open circles and males by filled circles.

Correlations among and variation within immune defenses

We observed significant correlations between total IgG and anti-KLH IgG (r = 0.42, d.f. = 23, p-value < 0.03611) and total IgM and anti-KLH IgM (r = 0.46, d.f. = 23, p-value =

0.01921). The correlation between total IgG and total IgM was marginally non-significant ($r = 0.34$, d.f. = 27, p-value < 0.068). We found no association between anti-KLH IgG and anti-KLH IgM or among BKC and any of our antibody measures. We used Levene's test to assess homogeneity of variance between captive and wild hyenas for each dependent variable, and found no difference between the groups ($p > 0.45$ in all cases).

Discussion

Antibodies and the B cells that produce them play critical effector and regulatory roles in the immune system. Tests of the hygiene hypothesis and efforts to unravel the mechanisms that drive allergy and autoimmunity currently focus exclusively on animals that are reared in environments far different than the environments in which they evolved. However these tests might in fact be better suited to comparative analysis of captive and wild populations. Our results indicate that wild spotted hyenas inhabiting a relatively pathogen-rich environment generally have higher levels of induced immune defenses than captive hyenas. Immune defenses that are primarily constitutive were not significantly different between wild and captive hyenas, but captivity status was included as a predictor variable in the top model set for all five immune measures we tested. Age and sex were only included as predictors in two and three of the top model sets, respectively. This indicates that captivity status is the most robust predictor of immune defenses, particularly for specific, induced defenses (total IgG, total IgM), and non-specific, induced defenses (natural IgG and IgM) as outlined in Fig. 4.1. Additionally, our results show that all wild females had higher anti-KLH IgG concentrations than all wild males, but this sex difference was not observed in captive hyenas.

The regular cleaning of the captive facilities, and the absence parasitic worms and viral pathogens such as rabies and canine distemper in the captive hyenas, suggest that the level of pathogen exposure is different between the captive and wild hyenas. Studies comparing rodents in antigen-free, germ-free, or specific pathogen-free environments detected no difference in total IgM concentrations or IgM repertoire, but total IgG concentrations were higher in the specific pathogen-free mice than in the antigen-free and germ-free mice (Bos et al. 1989; Haury et al. 1997; Hooijkaas et al. 1984). Devalapalli, Lesher, et al. (2006) compared immunoglobulin levels of inbred rats and mice with immunoglobulin levels in wild-caught rats and mice. They reported that total IgG was higher in wild rats and mice than in captives, and that total IgM was significantly higher in wild rats than in captives. We hypothesize that the higher total IgM in wild hyenas and rats compared to captives was due to the use of true wild animals rather than specific pathogen-free or germ-free inbred animals that were used in other studies (Bos et al. 1989; Devalapalli et al. 2006; Haury et al. 1997; Hooijkaas et al. 1984). Studies comparing immunoglobulin levels in humans also reported that both total IgG and total IgM were higher in populations where infectious disease prevalence was higher (Rowe et al. 1968; Turner and Voller 1966). Taken together, these studies indicate antibody concentrations are clearly affected by exposure to microbes.

Devalapalli, Lesher, et al. (2006) showed that both natural IgG and IgM were higher in wild-caught than captive-reared rats, suggesting that natural antibody production may be augmented by exposure to microbes. Additionally, wild mice had stronger anti-KLH responses than their captive counterparts following immunization with KLH (Abolins et al. 2011). Due to the logistics of immunizing and recapturing large, wild carnivores, we could not immunize the wild hyenas, but we were able to test natural anti-KLH antibodies. We observed significantly

greater natural anti-KLH IgG in the wild population than in the captive population, as well as a trend towards higher natural anti-KLH IgM in the wild population. In mice, natural antibody producing B-1 cells are unresponsive to B cell receptor signaling that induces proliferation in specific antibody-producing B-2 cells; B-1 cells do respond to signaling through toll-like receptors (TLRs) upon recognition of pathogen-associated molecular patterns (Baumgarth 2011). Stimulation of B-1 cells through TLRs may drive the augmented natural anti-KLH antibodies we observed in wild hyenas, although the mechanism for this is currently unknown.

Natural IgM makes up approximately 80% of total IgM in mice, but natural IgG constitutes only a small proportion of total IgG (Baumgarth et al. 1999). These ratios are consistent with our results, as the serum dilutions we used for quantifying total IgG were 400-fold higher than the dilutions for natural anti-KLH IgG, whereas the dilution for total IgM were only 10-fold higher than the dilution used for natural anti-KLH IgM.

We observed no difference in complement-mediated BKC between our captive and wild populations. First-line immune defenses such as complement are continually produced at low levels in the absence of pathogenic challenge, and function to prevent infection (Carroll 1998; Ricklin et al. 2010). Complement levels can increase quickly following infection, but the duration of the complement pulse is short compared to the rise in antibody levels following infection (Fig. 4.1) (Carroll 2004b). Specific IgM and IgG in response to infection typically peak around 7-10 days and 14-21 days, respectively; IgG molecules have a half-life of 2-3 weeks in the blood stream of human, whereas IgM molecules typically persist for less than one week (Mankarious et al. 1988; Sigounas et al. 1994). Furthermore, repeated exposure to a pathogen will often drive specific IgG titers to higher levels than the initial infection and specific IgG in hyenas has been detected one year after initial exposure (Flies et al. 2012). This combination of

protracted production time, long half-lives, and additive concentrations make IgG antibodies the most likely to be elevated in a pathogen-rich environment, with IgM falling between IgG and complement in sensitivity to pathogen exposure.

An alternative to the hypothesis that differences in pathogen exposure lead to the differences in induced immune function observed in wild and captive hyenas is that resource availability varies between the two populations. Mounting evidence suggests that immune defenses are energetically costly, as reviewed in (Ashley et al. 2012). The captive hyenas used in this study were fed a standard diet of 0.5 - 1 kg of carnivore chow and approximately 0.5 kg of bone daily, which is adequate for maintaining a stable mass. Several days may pass between meals for wild hyenas, and their body masses are highly variable, depending on the size and timing of the last meal. If resources are the primary mediator of immune defenses, then we should expect to see more variable immune defense levels in wild hyenas. Our results indicate that variance in immune defenses was similar for captive and wild hyenas, suggesting that resources are not responsible for the differences in immune function observed in this study.

Interestingly, wild females had higher anti-KLH IgG concentrations than did the wild males. Previous work has shown the NAbs, particularly natural autoantibodies, are important for the establishment of self-tolerance (Coutinho 1989; Lacroix-Desmazes et al. 1995; Lleo et al. 2010). Very little is known about self-tolerance and autoimmunity in wild animals, but in humans, autoimmune disease is far more common in women than men, and is often associated with hormonal changes associated with pregnancy and tolerance to fetal antigens (Whitacre 2001). In a well-studied population of soay sheep (*Ovis aries*) females had higher levels of IgG autoantibodies than did males, and autoantibody-positive females were more likely to survive harsh winters, had higher offspring survival, but reduced fecundity than did autoantibody-

negative females (Graham et al. 2010). It is possible that the observed difference in NAbs between wild female and male hyenas is associated with the complications of pregnancy.

In summary, our work shows that wild hyenas inhabiting a pathogen-rich environment have higher levels of induced specific and non-specific immune defenses than captive hyenas, whereas we observed no significant difference between wild and captive hyenas with respect to the constitutive, non-specific defense we tested. This has important implications for serological monitoring of disease in wildlife. Reference values for serological tests that are established in captive populations may be underestimated as compared to wild populations, and lead to an excess of false positives in wild populations. Furthermore, there is little evidence of disease-induced mortality in the wild hyena population, indicating that their immune defenses are robust. This is consistent with the notion that pathogen exposure is important for proper development and maintenance of the immune system, as suggested by the hygiene hypothesis.

LITERATURE CITED

LITERATURE CITED

Abolins, S. R., Pocock, M. J., Hafalla, J. C., Riley, E. M., and Viney, M. E. (2011). "Measures of immune function of wild mice, Mus musculus." *Molecular ecology*, 20(5), 881-92.

Alexander, K. A., Kat, P. W., Frank, L. G., Holekamp, K. E., Smale, L., House, C., and Appel, M. J. G. (1995). "Evidence of Canine Distemper Virus Infection among Free-Ranging Spotted Hyenas (Crocuta crocuta) in the Masai Mara, Kenya." *Journal of Zoo and Wildlife Medicine*, 26(2), 201-206.

Ashley, N. T., Weil, Z. M., and Nelson, R. J. (2012). "Inflammation: Mechanisms, Costs, and Natural Variation." *Annual Review of Ecology, Evolution, and Systematics*, 43(1), 385-406.

Avrameas, S. (1991). "Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'." *Immunology Today*, 12(5), 154-159.

Baumgarth, N. (2011). "The double life of a B-1 cell: self-reactivity selects for protective effector functions." *Nature reviews. Immunology*, 11(1), 34-46.

Baumgarth, N., Herman, O. C., Jager, G. C., Brown, L., Herzenberg, L. A., and Herzenberg, L. A. (1999). "Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system." *Proceedings of the National Academy of Sciences*, 96(5), 2250-2255.

Baumgarth, N., Tung, J. W., and Herzenberg, L. A. (2005). "Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion." *Springer Seminars in Immunopathology*, 26(4), 347-362.

Bos, N. A., Kimura, H., Meeuwsen, C. G., Visser, H. D., Hazenberg, M. P., Wostmann, B. S., Pleasants, J. R., Benner, R., and Marcus, D. M. (1989). "Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet." *European journal of immunology*, 19(12), 2335-2339.

Buehler, D., Piersma, T., and Irene Tieleman, B. (2008a). "Captive and free-living red knots Calidris canutus exhibit differences in non-induced immunity that suggest different immune strategies in different environments." *Journal of Avian Biology*, 39(5), 560-566.

Buehler, D. M., Piersma, T., and Irene Tieleman, B. (2008b). "Captive and free-living red knots Calidris canutus exhibit differences in non-induced immunity that suggest different immune strategies in different environments." *Journal of Avian Biology*, 39(5), 560-566.

Burkepile, D. E., Parker, J. D., Woodson, C. B., and *al., e.* (2006). "Chemically mediated competition between microbes and animals: microbes as consumers in food webs." *Ecology*, 87, 2821-2831.

Burnham, K. P., and Anderson, D. R. (2002). *Model selection and multimodel inference: a practical information-theoretic approach*: Springer Verlag.

Burnham, K. P., and Anderson, D. R. (2004). "Multimodel Inference." *Sociological Methods & Research*, 33(2), 261-304.

Carroll, M. C. (1998). "The role of complement and complement receptors in induction and regulation of immunity." *Annual Review of Immunology*, 16(1), 545-568.

Carroll, M. C. (2004a). "The complement system in B cell regulation." *Molecular Immunology*, 41(2–3), 141-146.

Carroll, M. C. (2004b). "The complement system in regulation of adaptive immunity." *Nature immunology*, 5(10), 981-986.

Choi, Y. S., and Baumgarth, N. (2008). "Dual role for B-1a cells in immunity to influenza virus infection." *The Journal of Experimental Medicine*, 205(13), 3053-3064.

CLSI. (2009). "Methods for dilution antimicrobial susceptibility tests for bacterial that grow aerobically; approved standard". City: Clinical and Laboratory Standards Institute.

Cooper, S. M., Holekamp, K. E., and Smale, L. (1999). "A seasonal feast: long-term analysis of feeding behaviour in the spotted hyaena (Crocuta crocuta)." *African Journal of Ecology*, 37(2), 149-160.

Coutinho, A. (1989). "Beyond Clonal Selection and Network." *Immunological Reviews*, 110(1), 63-88.

Devalapalli, A. P., Lesher, A., Shieh, K., Solow, J. S., Everett, M. L., Edala, A. S., Whitt, P., Long, R. R., Newton, N., and Parker, W. (2006). "Increased levels of IgE and autoreactive, polyreactive IgG in wild rodents: implications for the hygiene hypothesis." *Scandinavian Journal of Immunology*, 64(2), 125-36.

East, M. L., Hofer, H., Cox, J. H., Wulle, U., Wiik, H., and Pitra, C. (2001). "Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas." *Proceedings of the National Academy of Sciences of the United States of America*, 98(26), 15026-31.

East, M. L., Moestl, K., Benetka, V., Pitra, C., Honer, O. P., Wachter, B., and Hofer, H. (2004). "Coronavirus infection of spotted hyenas in the Serengeti ecosystem." *Veterinary microbiology*, 102(1-2), 1-9.

East, M. L., Wibbelt, G., Lieckfeldt, D., Ludwig, A., Goller, K., Wilhelm, K., Schares, G., Thierer, D., and Hofer, H. (2008). "A Hepatozoon species genetically distinct from *H. canis* infecting spotted hyenas in the Serengeti Ecosystem, Tanzania." *Journal of Wildlife Diseases*, 44(1), 45-52.

Engh, A. L., Nelson, K. G., Peebles, R., Hernandez, A. D., Hubbard, K. K., and Holekamp, K. E. (2003). "Coprologic survey of parasites of spotted hyenas (Crocuta crocuta) in the Masai Mara National Reserve, Kenya." *Journal of Wildlife Diseases*, 39(1), 224-227.

Flajnik, M., and Kasahara, M. (2009). "Origin and evolution of the adaptive immune system: genetic events and selective pressures." *Nature Reviews Genetics*.

Flies, A. S., Grant, C. K., Mansfield, L. S., Smith, E. J., Weldele, M. L., and Holekamp, K. E. (2012). "Development of a hyena immunology toolbox." *Veterinary Immunology and Immunopathology*, 145(1–2), 110-119.

Folstad, I., and Karter, A. J. (1992). "Parasites, bright males, and the immunocompetence handicap." *The American naturalist*, 139(3), 603-622.

Friend, M., and Franson, J. (1999). "Field Manual of Wildlife Diseases. General Field Procedures and Diseasesof Birds". City: Storming Media.

Gelman, A. (2008). "Scaling regression inputs by dividing by two standard deviations." *Statistics in Medicine*, 27(15), 2865-2873.

Glickman, S. E., Frank, L. G., Licht, P., Yalcinkaya, T., Siiteri, P. K., and Davidson, J. (1992). "Sexual differentiation of the female spotted hyena." *Annals of the New York Academy of Sciences*, 662(1), 135-159.

Graham, A. L., Hayward, A. D., Watt, K. A., Pilkington, J. G., Pemberton, J. M., and Nussey, D. H. (2010). "Fitness correlates of heritable variation in antibody responsiveness in a wild mammal." *Science*, 330(6004), 662-665.

Hamilton, W., and Zuk, M. (1982). "Heritable true fitness and bright birds: a role for parasites?" *Science*, 218(4570), 384-387.

Harrison, T., Harrison, S., Rumbeiha, W., Sikarskie, J., and McClean, M. (2006). "Surveillance for selected bacterial and toxicologic contaminants in donated carcass meat fed to carnivores." *Journal of Zoo and Wildlife Medicine*, 37(2), 102-107.

Harrison, T. M., Mazet, J. K., Holekamp, K. E., Dubovi, E., Engh, A. L., Nelson, K., Van Horn, R. C., and Munson, L. (2004). "Antibodies to canine and feline viruses in spotted hyenas (*Crocuta crocuta*) in the Masai, Mara National Reserve." *Journal of Wildlife Diseases*, 40(1), 1-10.

Haury, M., Sundblad, A., Grandien, A., Barreau, C., Coutinho, A., and Nobrega, A. (1997). "The repertoire of serum IgM in normal mice is largely independent of external antigenic contact." *European Journal of Immunology*, 27(6), 1557-1563.

Holekamp, K. E., and Sisk, C. L. (2003). "Effects of dispersal status on pituitary and gonadal function in the male spotted hyena." *Hormones and Behavior*, 44(5), 385-394.

Hooijkaas, H., Benner, R., Pleasants, J. R., and Wostmann, B. S. (1984). "Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered “antigen-free” diet." *European Journal of Immunology*, 14(12), 1127-1130.

Horrocks, N. P., Matson, K. D., and Tieleman, B. I. (2011). "Pathogen pressure puts immune defense into perspective." *Integrative and Comparative Biology*, 51(4), 563-76.

Joop, G., and Rolff, J. (2004). "Plasticity of immune function and condition under the risk of predation and parasitism." *Evolutionary Ecology Research*, 6(7), 1051-1062.

Korver, K., Zeijlemaker, W. P., Schellekens, P. T. A., and Vossen, J. M. (1984). "Measurement of primary in vivo IgM- and IgG-antibody response to KLH in humans: Implications of pre-immune IgM binding in antigen-specific ELISA." *Journal of Immunological Methods*, 74(2), 241-251.

Lacroix-Desmazes, S., Mouthon, L., Coutinho, A., and Kazatchkine, M. D. (1995). "Analysis of the natural human IgG antibody repertoire: life-long stability of reactivities towards self antigens contrasts with age-dependent diversification of reactivities against bacterial antigens." *European Journal of Immunology*, 25(9), 2598-2604.

Lembo, T., Hampson, K., Auty, H., Beesley, C. A., Bessell, P., Packer, C., Halliday, J., Fyumagwa, R., Hoare, R., Ernest, E., Mentzel, C., Mlengeya, T., Stamey, K., Wilkins, P. P., and Cleaveland, S. (2011). "Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009." *Emerging Infectious Diseases*, 17(3), 387-94.

Liebl, A. L., and Martin, L. B. (2009). "Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry." *Functional Ecology*, 23(6), 1091-1096.

Lleo, A., Invernizzi, P., Gao, B., Podda, M., and Gershwin, M. E. (2010). "Definition of human autoimmunity — autoantibodies versus autoimmune disease." *Autoimmunity Reviews*, 9(5), A259-A266.

Mankarious, S., Lee, M., Fischer, S., Pyun, K. H., Ochs, H. D., Oxelius, V. A., and Wedgwood, R. J. (1988). "The half-lives of IgG subclasses and specific antibodies in patients with primary immunodeficiency who are receiving intravenously administered immunoglobulin." *The Journal of Laboratory and Clinical Medicine*, 112(5), 634-640.

Miko, A., Pries, K., Haby, S., Steege, K., Albrecht, N., Krause, G., and Beutin, L. (2009). "Assessment of Shiga Toxin-Producing Escherichia coli Isolates from Wildlife Meat as Potential Pathogens for Humans." *Appl. Environ. Microbiol.*, 75(20), 6462-6470.

Moore, S. M., and Borer, E. T. (2012). "The influence of host diversity and composition on epidemiological patterns at multiple spatial scales." *Ecology*, 93(5), 1095-1105.

Palomares, O., Yaman, G., Azkur, A. K., Akkoc, T., Akdis, M., and Akdis, C. A. (2010). "Role of Treg in immune regulation of allergic diseases." *European journal of immunology*, 40(5), 1232-1240.

Parmentier, H. K., Lammers, A., Hoekman, J. J., Reilingh, G. D. V., Zaanen, I. T. A., and Savelkoul, H. F. J. (2004). "Different levels of natural antibodies in chickens divergently selected for specific antibody responses." *Developmental & Comparative Immunology*, 28(1), 39-49.

R Development Core Team. (2012). "R: A Language and Environment for Statistical Computing". City: R Foundation for Statistical Computing.

Reller, L. B., and Stratton, C. W. (1977). "Serum dilution test for bactericidal activity. II. Standardization and correlation with antimicrobial assays and susceptibility tests." *Journal of Infectious Diseases*, 136(2), 196.

Ricklin, D., Hajishengallis, G., Yang, K., and Lambris, J. D. (2010). "Complement: a key system for immune surveillance and homeostasis." *Nature immunology*, 11(9), 785-97.

Rifkin, J. L., Nunn, C. L., and Garamszegi, L. Z. (2012). "Do animals living in larger groups experience greater parasitism? A meta-analysis." *The American Naturalist*, 180(1), 70-82.

Rook, G. A. W. (2009). "Review series on helminths, immune modulation and the hygiene hypothesis: The broader implications of the hygiene hypothesis." *Immunology*, 126(1), 3-11.

Rowe, D., McGregor, I., Smith, S. J., Hall, P., and Williams, K. (1968). "Plasma immunoglobulin concentrations in a West African (Gambian) community and in a group of healthy British adults." *Clinical and Experimental Immunology*, 3(1), 63.

Schielzeth, H. (2010). "Simple means to improve the interpretability of regression coefficients." *Methods in Ecology and Evolution*, 1(2), 103-113.

Schmid-Hempel, P. (2003). "Variation in immune defence as a question of evolutionary ecology." *Proceedings. Biological sciences / The Royal Society*, 270(1513), 357-66.

Schmid-Hempel, P., and Ebert, D. (2003). "On the evolutionary ecology of specific immune defence." *Trends in Ecology & Evolution*, 18(1), 27-32.

Schwartz, J. A., Aldridge, B. M., Stott, J. L., and Mohr, F. C. (2004). "Immunophenotypic and functional effects of bunker C fuel oil on the immune system of American mink (Mustela vison)." *Veterinary Immunology and Immunopathology*, 101(3–4), 179-190.

Sigounas, G., Harindranath, N., Donadel, G., and Notkins, A. (1994). "Half-life of polyreactive antibodies." *Journal of clinical immunology*, 14(2), 134-140.

Strachan, D. P. (1989). "Hay fever, hygiene, and household size." *BMJ*, 299(6710), 1259-1260.

Tennessen, J. A., Woodhams, D. C., Chaurand, P., Reinert, L. K., Billheimer, D., Shyr, Y., Caprioli, R. M., Blouin, M. S., and Rollins-Smith, L. A. (2009). "Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (Rana pipiens)

populations suggest intraspecies differences in resistance to pathogens." *Developmental and comparative immunology*, 33(12), 1247-57.

Thomas, A. M., and Rudolf, V. H. W. (2010). "Challenges of metamorphosis in invertebrate hosts: maintaining parasite resistance across life-history stages." *Ecological Entomology*, 35(2), 200-205.

Thornton, B. P., Větvicka, V., and Ross, G. D. (1994). "Natural antibody and complement-mediated antigen processing and presentation by B lymphocytes." *The Journal of Immunology*, 152(4), 1727-37.

Turner, M. W., and Voller, A. (1966). "Studies on immunoglobulins of Nigerians. I. The immunoglobulin levels of a Nigerian population." *The Journal of Tropical Medicine and Hygiene*, 69(5), 99.

Van Loveren, H., Ross, P. S., Osterhaus, A. D. M. E., and Vos, J. G. (2000). "Contaminant-induced immunosuppression and mass mortalities among harbor seals." *Toxicology Letters*, 112–113(0), 319-324.

Viney, M. E., Riley, E. M., and Buchanan, K. L. (2005). "Optimal immune responses: immunocompetence revisited." *Trends in Ecology & Evolution*, 20(12), 665-669.

von Mutius, E., and Vercelli, D. (2010). "Farm living: effects on childhood asthma and allergy." *Nature reviews. Immunology*, 10(12), 861-8.

Waitz, J. A. (1990). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*: National Committee for Clinical Laboratory Standards.

Wander, K., O'Connor, K., and Shell-Duncan, B. (2012). "Expanding the hygiene hypothesis: Early exposure to infectious agents predicts delayed-type hypersensitivity to *Candida* among children in Kilimanjaro." *PloS One*, 7(5), e37406.

Whitacre, C. C. (2001). "Sex differences in autoimmune disease." *Nature Immunology*, 2(9), 777-780.

Yazdanbakhsh, M., Kremsner, P. G., and van Ree, R. (2002). "Allergy, parasites, and the hygiene hypothesis." *Science*, 296(5567), 490-494.

Zuur, A. F., Ieno, E. N., and Elphick, C. S. (2010). "A protocol for data exploration to avoid common statistical problems." *Methods in Ecology and Evolution*, 1(1), 3-14.

CHAPTER 5

CHAPTER 5

LIPOPOLYSACCHARIDE CHALLENGE EXPERIMENT IN SPOTTED HYENAS

Introduction

Mammals vary greatly in their ability to cope with pathogens. Disease risk varies with diet, and meat-eating mammals are known to contract pathogens directly from their prey; such pathogens include bacteria (e.g. *Brucella spp*, *Clostridium botulinum*) (Honer et al. 2006; McVey et al. 2002), viruses (e.g. bluetongue virus, African horse sickness virus) (Alexander et al. 1994), and protozoa (e.g. *Toxoplasma*, *Giardia*, *Eimeria*) (Murray et al. 1999; Nunn et al. 2003). Perhaps because of their evolved ability to feed on carrion heavily laden with pathogens, animals that make their living by scavenging appear to be more highly resistant to disease than species engaging primarily in other forms of trophic activity (Blount et al. 2003). It seems reasonable to expect that scavengers might be exposed to higher concentrations or greater diversity of pathogens than non-scavengers, and that natural selection would therefore have promoted the evolution of robust immune responses in scavengers to minimize deleterious effects of frequent and extensive pathogen exposure.

Most mammalian carnivores scavenge to some extent (Houston 1979), yet disease susceptibility varies widely among these species (Murray et al. 1999; Nunn et al. 2003). Relative to other carnivores, members of the Hyaenidae family appear to be remarkably resistant to many deadly viral and bacterial agents. Serological data from spotted hyenas (*Crocuta crocuta*) clearly indicate extensive exposure to disease-causing microbes (Alexander et al. 1995; East et al. 2001; Harrison et al. 2004; Lembo et al. 2011), yet the hyenas usually remain asymptomatic. For example, when distemper and rabies epizootics recently killed large numbers of lions (*Panthera*

leo) and wild dogs (*Lycaon pictus*) in east Africa, sympatric adult spotted hyenas were often seropositive for antibodies to these pathogens, indicating that they had been exposed, yet they showed no clinical signs of either disease (East et al. 2001; Kat et al. 1995; Packer et al. 1999). Whereas diseases often dramatically reduce other carnivore populations (Kissui and Packer 2004; Woodroffe and Ginsberg 1999), disease plays a trivial role in the population dynamics of wild hyenas (East et al. 2001; Watts and Holekamp 2009).

Spotted hyenas are highly successful predators that descended recently from carrion feeding ancestors (Lewis and Werdelin 2000); they often eat rotting carcasses passed over by other carnivores. In several different wild populations, hyenas have even been reported to ingest large quantities of flesh from carcasses of ungulates recently dead from tuberculosis (*Mycobacterium bovis*) or anthrax (*Bacillus anthracis*) infection without experiencing any ill effects (Gasaway et al. 1991; Lindeque and Turnbull 1994; Pienaar 1969). In addition, their ability to recover from multiple deep puncture wounds (e.g. inflicted by lions) without apparent inflammation suggests an unusual resistance to sepsis. These field observations all suggest that hyena immune function may exhibit characteristics that are not only unusual, but also potentially useful in the development of new therapeutic tools for fighting enteric disease and sepsis.

The mammalian immune system evolved under selective pressure imposed by microorganisms (Flajnik and Kasahara 2010). Carrion-feeding vertebrates and microbial decomposers compete intensively for animal carcasses, so the bacteria on rotting carcasses produce toxins to deter animal scavengers (Burkepile et al. 2006; DeVault et al. 2004; Janzen 1977). However, because hyenas often consume putrid carcasses so far decomposed that they are undergoing liquidification, it appears that these animals can somehow thwart microbial defenses. We suggest that hyenas may have evolved general and robust immunological mechanisms for

coping effectively with a broad array of pathogens. By contrast, the advent of cooking, cleaning, and refrigerating food prior to consumption by humans has reduced the microbial load ingested by humans (Erridge 2011). Humans are among the most sensitive animals to LPS and a strong inflammatory response can be induced from relatively low concentrations of LPS; LPS-induced endotoxin shock results in the death of nearly 200,000 people each year (Angus et al. 2001). Our goal here was evaluate inflammation in spotted hyenas in response to challenge with lipopolysaccharide (LPS), a major cell wall component of gram negative bacteria that stimulates inflammation through toll-like receptor 4 (Miller et al. 2005).

Materials and Methods

Sample and data collection in the field

Wild spotted hyenas in the Masai Mara National Reserve in Kenya were immobilized with tiletamine-zolazepam (6.5mg/kg Telazol; Fort Dodge Animal Health, Fort Dodge Iowa) in a plastic dart fired from an air rifle (Telinject Inc., Saugus, California) (Holekamp and Sisk 2003). All immobilizations and sample collections were approved by the MSU Institutional Animal Care and Use Committee (AUF # 07/08-099-00) and the United States Army Medical Research and Materiel Command Animal Care and Use Review Office (# R091-0609R). After immobilization, a standard thermometer was inserted into the rectum of the anesthetized hyena at approximately 15 minute intervals to determine body temperature. Pulse and mean arterial pressure (MAP) were recorded at approximately 15 minute intervals using a veterinary blood pressure monitor (MedVet # MD90). EDTA whole blood samples and blood without an anticoagulent were collected in vacutainer tubes at approximately 15-30 minute intervals.

After approximately 30 minutes of establishing baseline values for temperature, pulse, MAP, and collecting baseline blood samples, immobilized hyenas were injected with either phosphate buffered saline (PBS – control) or lipopolysaccharide (LPS). Six adult females received the control injections and eight adult females received LPS injections. LPS from E. coli strain O26:B6 was suspended in sterile saline at a concentration of 10 mg/ml, and each experimental animal was administered a dose via IV injection. Because no previous LPS challenge data existed for hyenas, we started with a dose that induces an inflammatory response and symptoms associated with sepsis in humans (5 ng/kg) (Kaneko et al. 2003; Schinkel et al. 2005), then doubled the dose for each subsequent hyena until the hyenas appeared to be show visible signs of an inflammatory response to the LPS injection. The first three individuals that received LPS injections were administered low doses (5 ng/kg, 10 ng/kg, and 20 ng/kg) to insure that we did not initially overdose the hyena. A dose of 50 ng/kg was used on the last five individuals that received LPS injections. In total, 8 adult females were injected with LPS and 6 adult females were injected with saline.

Whole blood collected in the presence of EDTA was used for calculating packed cell volume (PCV). Whole blood collected without anticoagulant was allowed to clot at ambient temperature, centrifuged, and the serum was collected. Total serum solids were measured using a refractometer, and sera were then frozen in liquid nitrogen (-196°C). Samples were transported to MSU on dry ice (-80°C) and stored permanently at -80°C.

Laboratory Methods

Bacterial killing capacity was determined using a method similar to the method outlined in detail in chapter 3 of this dissertation. We used three gram-negative bacteria (*Campylobacter jejuni* (ATCC# 700819), *Escherichia coli* (ATCC# 8739) and *Proteus mirabilis* (ATCC#

35659),) to assess serum bacterial killing capacity. All three enteric bacterial species were highly susceptible to complement-mediated bacterial killing in preliminary testing. All assays were conducted in 96-well round bottom plates (Falcon - VWR). All serum was sterile filtered using a Millipore 0.22 um GV low protein binding filter (cat#SLGV033RS) to eliminate residual red blood cells. Serum was then serially diluted in 50 µl of phosphate buffered saline (PBS) from a starting concentration of 1:2.5 to final concentration of 1:40; after the subsequent addition of 50ul of bacteria broth, the final concentrations became 1:5 - 1:80. Appropriate wells in the plate were serially diluted with ampicillin at a starting concentration of 5 mg/ml to 0.5 µg/ml. Blank wells and control wells were loaded with 50 µl of PBS alone. After all dilutions were complete, plates were frozen at -80°C until the assay was ready to begin. Plates were frozen in the interim period because it was not possible to test all bacterial species simultaneously due to constraints on the speed at which the serum, antibiotic and bacteria aliquots could be loaded into the plates. All serum and antibody dilutions were loaded on the same day to minimize the inter-assay variation for each bacteria species that would later be tested.

Bacteria were inoculated into 8 ml of Mueller-Hinton II cation-adjusted broth (MHB) in a 15 ml tube and incubated overnight at 37°C while shaking at125 rpm. *C. jejuni* was grown in 10% carbon dioxide atmosphere. The next morning, the bacteria concentration was adjusted by diluting with MHB until the optical density, as read at 600 nm, matched that of the McFarland Turbidity Standard No. 0.5. Bacteria were then further diluted to a concentration to be $1x10^6$ CFU/ml. Control wells, serum-containing wells and antibiotic-containing wells were then loaded with 50 µl stock bacteria broth, resulting in a final concentration of $5x10^4$ CFU/well and total volume of 100 µl /well. Blank wells were loaded with 50 µl of sterile MHB to bring the final volume of each well to 100 µl. Plates were then placed in incubators with 5% or 10% carbon

dioxide, depending on the growth requirements of the bacterial species. Turbidity was measured every six hours on a Bio-Tek plate reader at 600 nm using KC Junior software. Percent inhibition was then calculated by dividing the optical density of each well by the mean optical density of the control wells. Remaining bacteria stock was serially diluted and spread on tryptic soy agar (TSA) plates and incubated in either 5% or 10% carbon dioxide atmosphere. Colony counts were done at 24 or 48 hours depending on the bacteria species to verify the initial number of colony forming units in the bacteria stock.

We had hoped to next assay serum for concentrations of pyrogenic cytokines (TNF-alpha & IL-6) to assess their response kinetics as Forfia et al. (1998) have done previously in dogs. This would have allowed us to determine whether or not innate humoral responses in hyenas differ fundamentally from those observed in dogs. However, cytokine ELISA kits do not exist for hyenas, so we tried an anti-feline TNFa ELISA kit (RnD Systems # DY2586). This kit worked to some extent on cell culture supernatant from an *in vitro* hyena T cell line developed in collaboration with Dr. Christopher Grant at Custom Monoclonals International, but the ELISA kit was not reliable for serum-based assays. Because of the unreliability of this assay with serum, no additional cytokine kits were tested.

Results

We obtained a time series of physiological measures and raw data are reported graphically below (Figs. 5.1 - 5.5) and mean values are reported in Table 5.1. We also collected serum samples from each time course experiment, as well as snap-frozen and slowly-frozen EDTA whole blood at 10-15 time points in each experiment. Bacterial killing capacity of sera

from four time points from nine individuals was assessed using a bacterial killing assay. Individuals showed little variation in BKC over the time course of this experiment (Fig. 5.6).

Table 5.1. Mean and standard deviation for physiological parameters in hyenas.

	Mean	SD
Temperature (°C)	36.1	2.3
Pulse	91.4	15.5
MAP (mm Hg)	114.9	16.1
PCV	35.7	4.7
Total solids (g/dL)	6.8	0.5

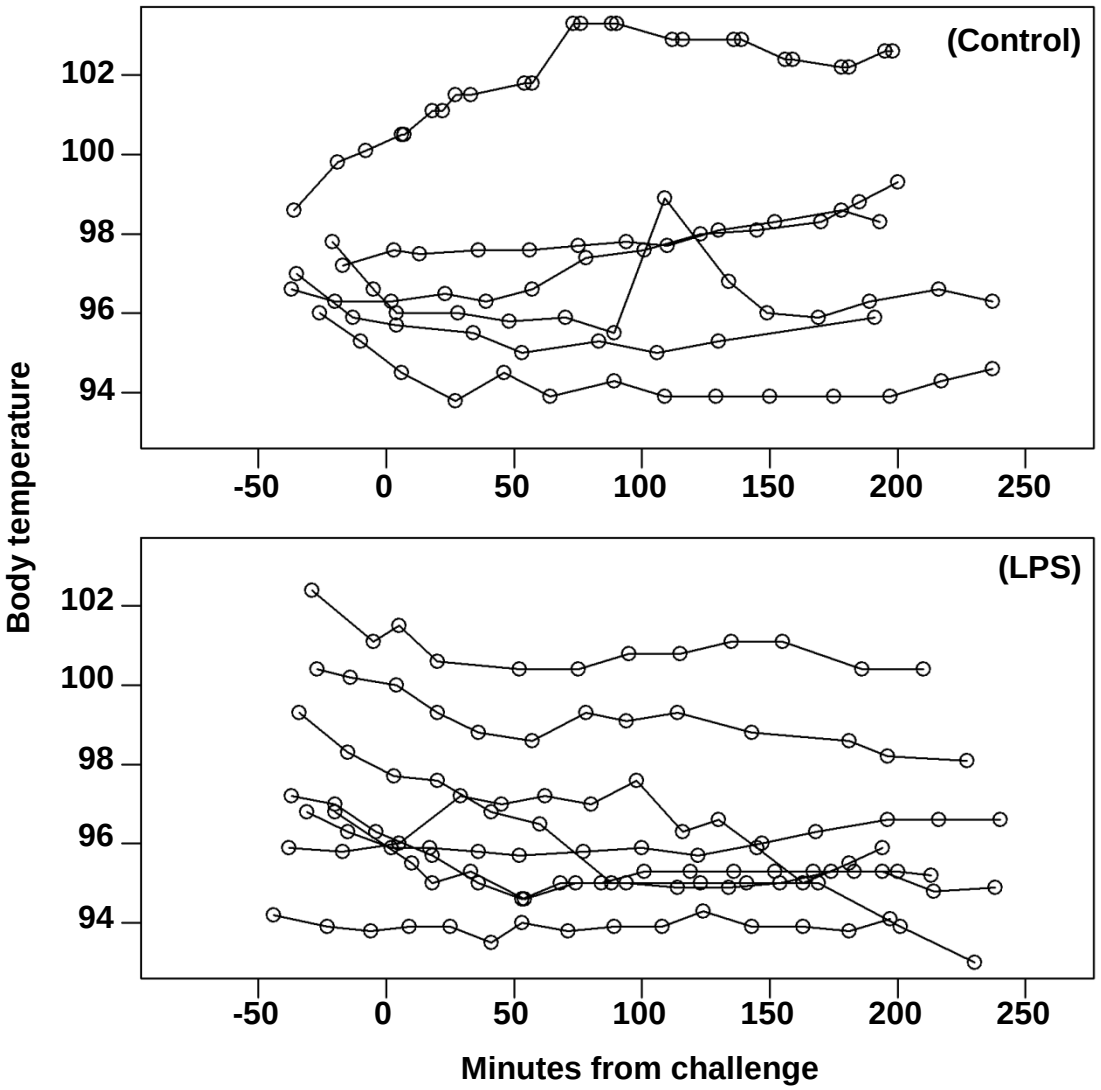

Fig. 5.1.Body temperature (°C) of hyenas pre- and post-injection. Body temperature was recorded approximately every 15 minutes from 6 individuals that were injected with phosphate buffered saline (control) and 8 individuals that were injected with lipopolysaccharide (LPS).

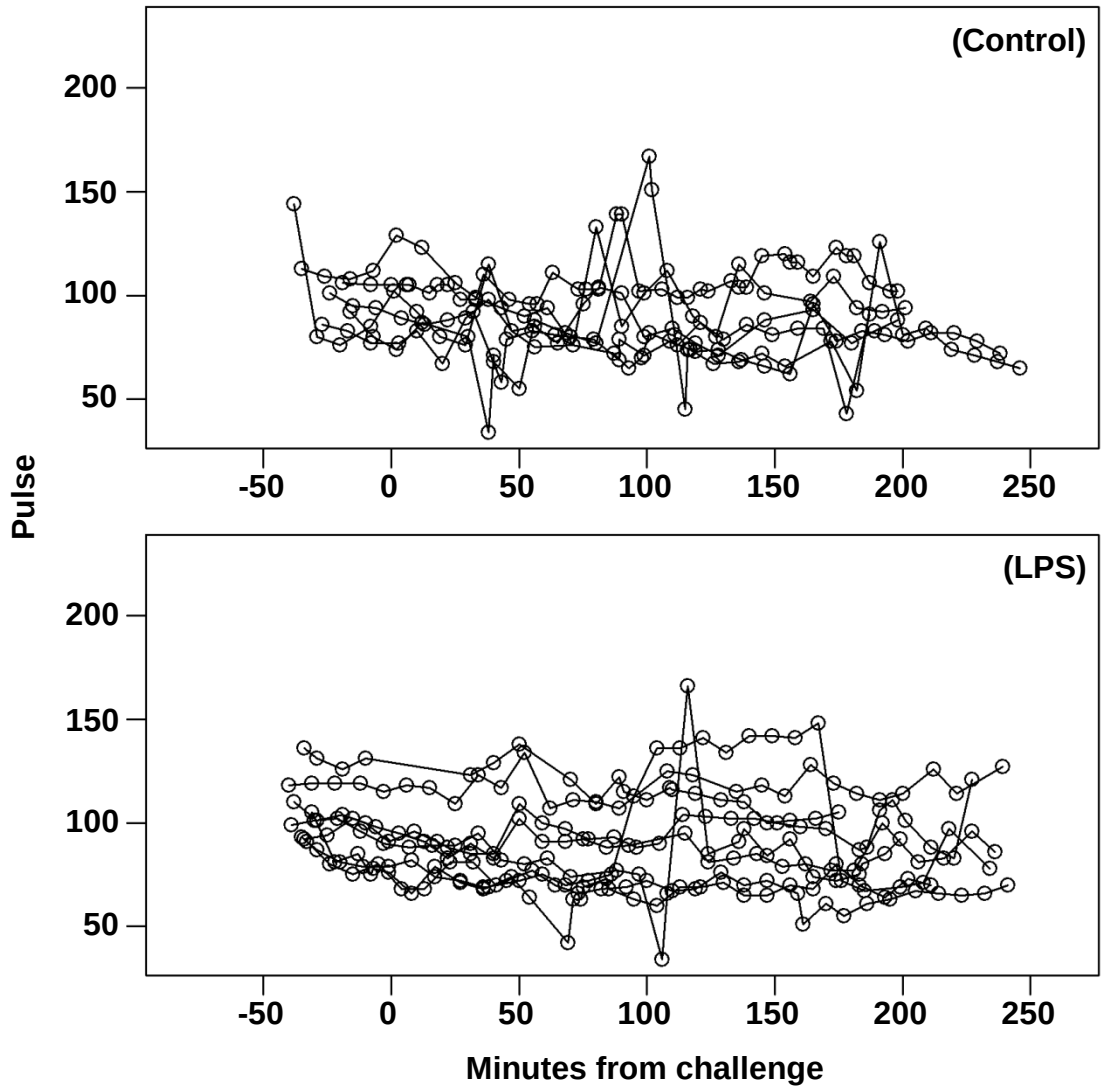

Fig. 5.2. Pulse of hyenas pre- and post-injection. Pulse was recorded approximately every 15 minutes from 6 individuals that were injected with phosphate buffered saline (control) and 8 individuals that were injected with lipopolysaccharide (LPS).

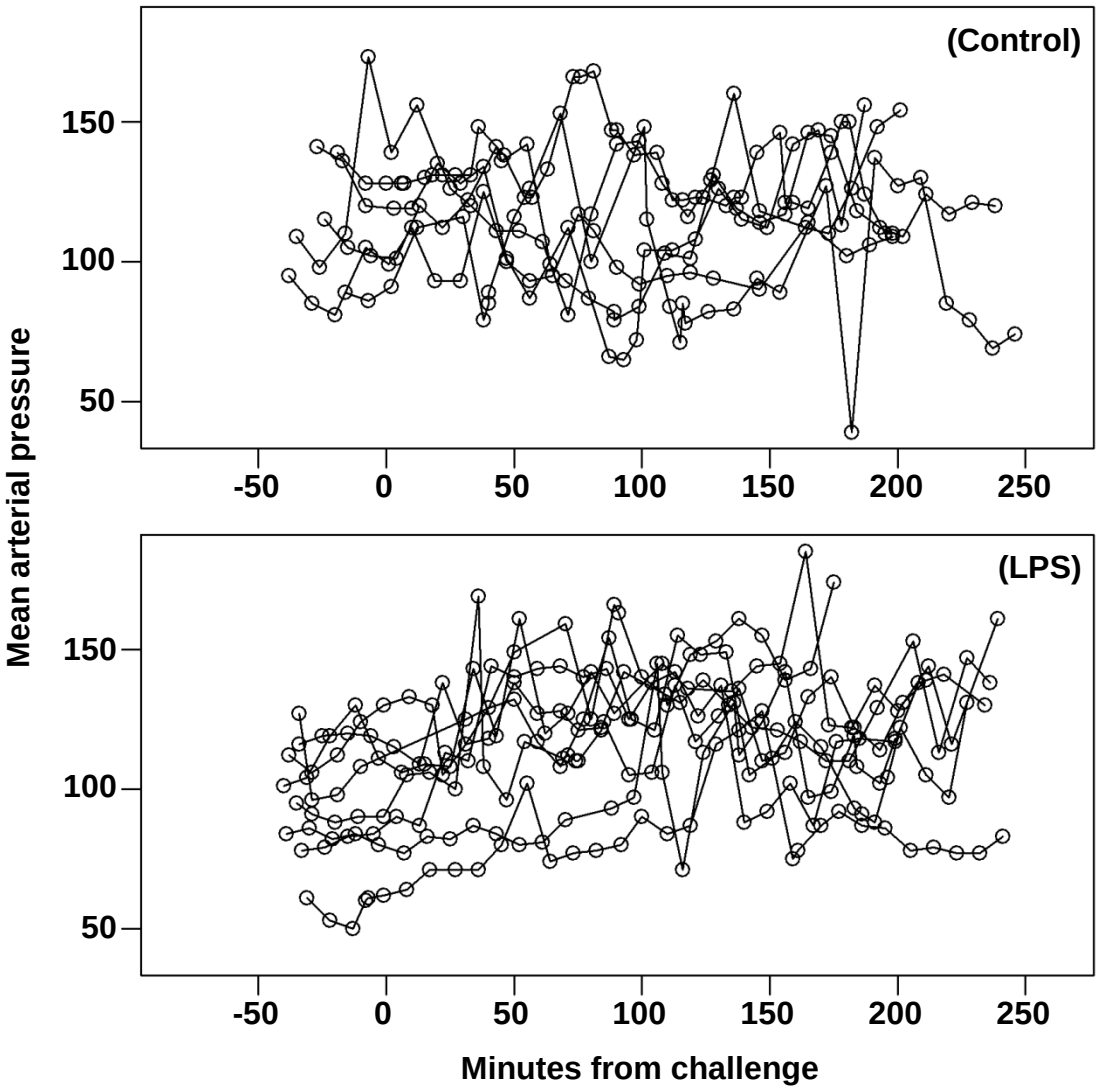

Fig. 5.3.Mean arterial pressure of hyenas pre- and post-injection. Mean arterial pressure was recorded approximately every 15 minutes from 6 individuals that were injected with phosphate buffered saline (control) and 8 individuals that were injected with lipopolysaccharide (LPS).

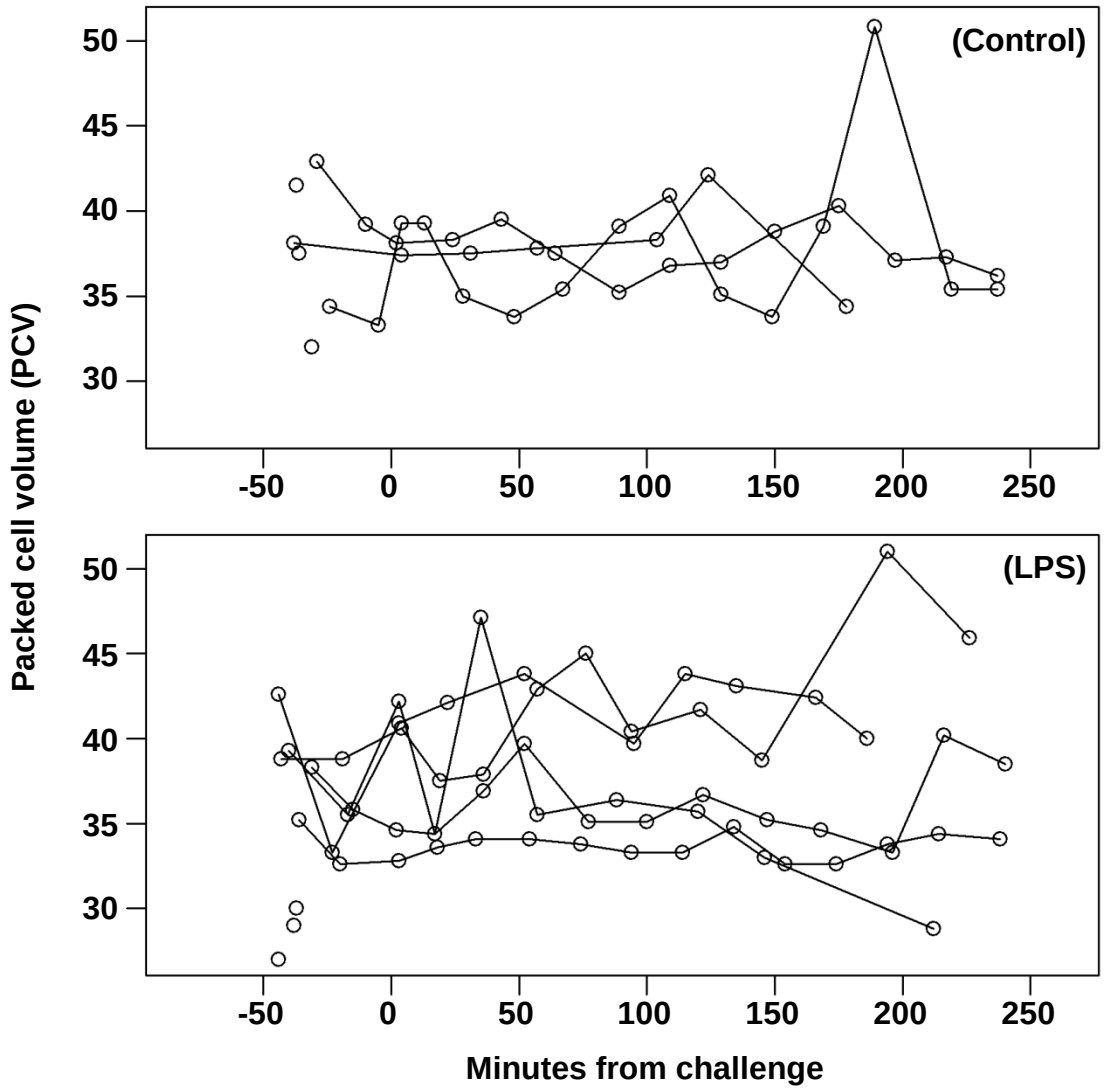

Fig. 5.4. Packed cell volume (PCV) of blood collected from hyenas pre- and post-injection. Whole blood samples were collected approximately every 15 minutes from 6 individuals that were injected with phosphate buffered saline (control) and 8 individuals that were injected with lipopolysaccharide (LPS).

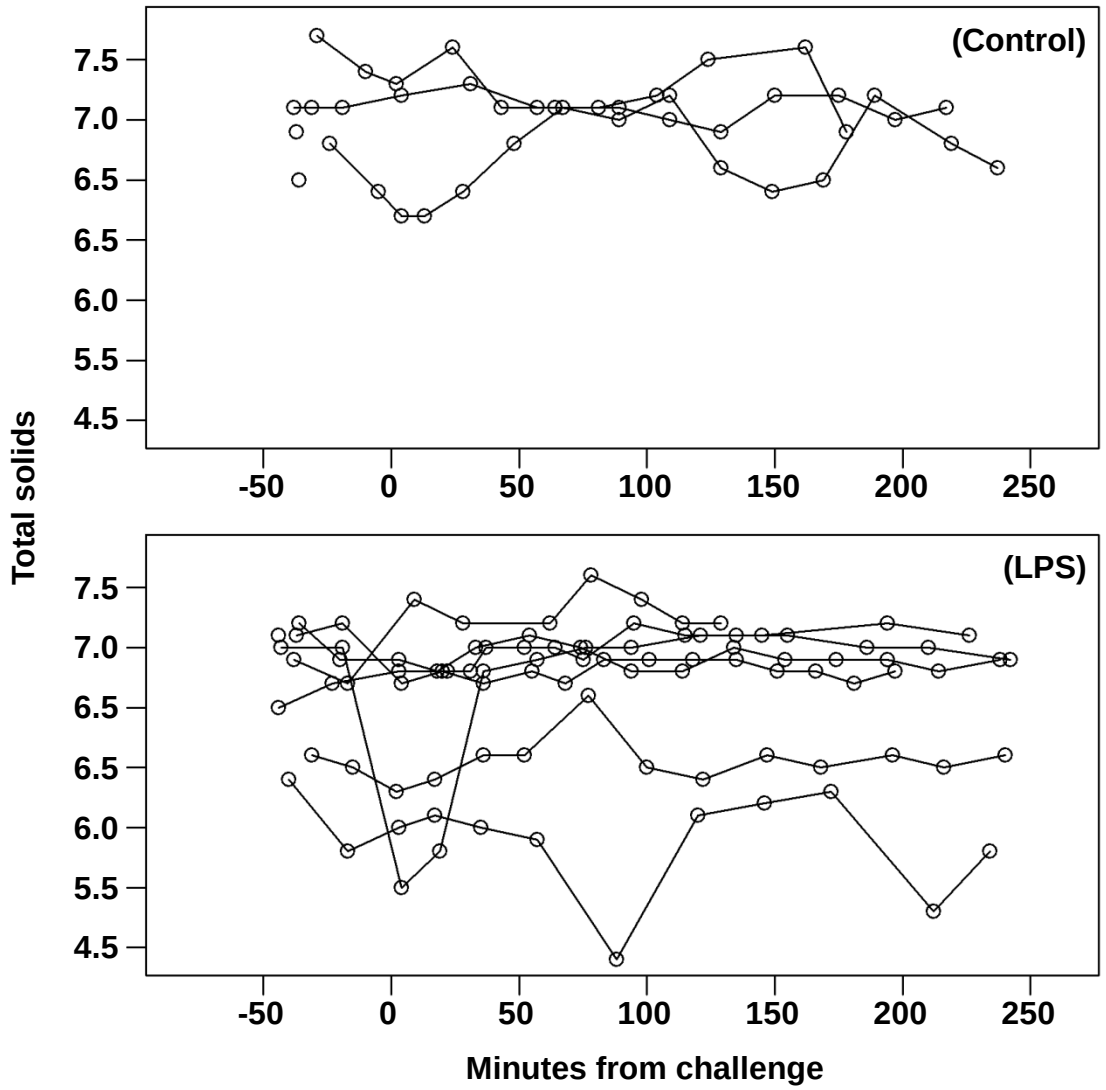

Fig. 5.5. Total solids in hyena sera pre- and post-injection. Serum samples were collected approximately every 15 minutes from 5 individuals that were injected with phosphate buffered saline (control) and 8 individuals that were injected with lipopolysaccharide (LPS).

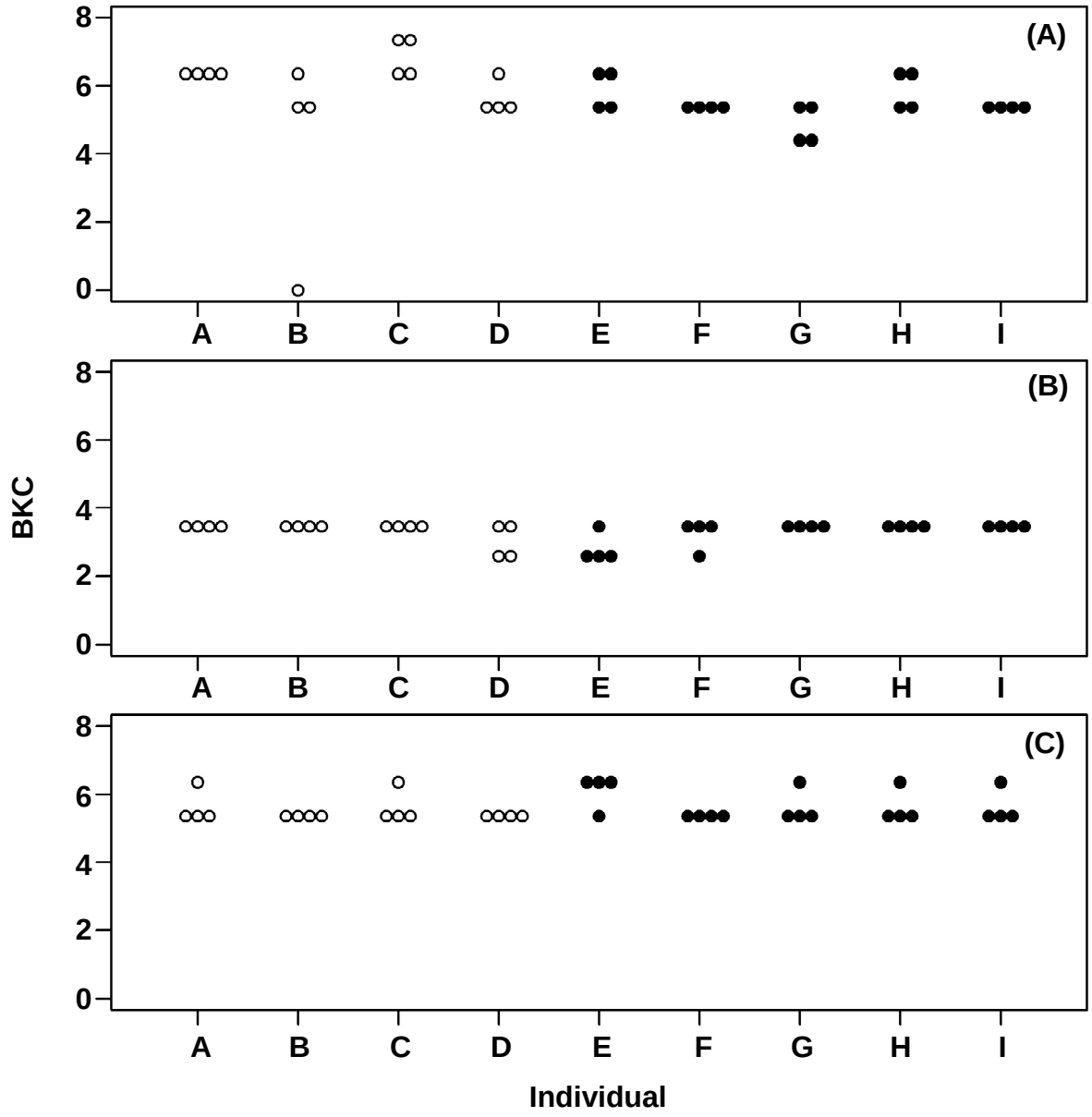

Fig. 5.6. Bacterial killing capacity (BKC) of hyena sera against (A) *Campylobacter jejuni*, (B) *Escherichia coli*, and (C) *Proteus mirablis*. Four serum samples from each individual were used in each assay. One sample was collected prior to intravenous injection with either phosphate buffered saline (control = open circles) or lipopolysaccharide (LPS = filled circles) and the other three samples were collected at various time points following the injection (range: 2 minutes to 200 minutes). Each circle in the figures represents the bacterial killing capacity of a single serum sample.

Discussion

The lack of physiological responses to LPS by hyenas at doses ranging from 5 ng/kg to 50 ng/kg indicates that the hyenas were either not responding to this LPS dosage or that the magnitude of the physiological responses was below our detection limits. The dose of LPS may have been too low to see an effect, but 50 ng/kg would likely have severe negative consequences for a human (Kaneko et al. 2003). Lethal endotoxin doses in cats (*Felis catus*) and baboons (*Papio anubis*) have been reported at 2 mg/kg (Al-Kaisi et al. 1977) and 6 mg/kg (Fletcher and Ramwell 1980), respectively.

Field conditions may have also hampered our ability to detect physiological responses to LPS challenge. All animals were immobilized before 1000 hours to decrease the risk of the animal overheating while anesthetized, but large fluctuations in environmental temperature occurred and the animals were repeatedly cooled by pouring water on them. Also, the effects of the Telazol anesthetic were highly variable and might have affected some physiological responses. Some individuals needed only a single booster injection, whereas others needed several doses of Telazol to maintain a proper level of anesthesia. Our results show that hyenas can tolerate at least 50 ng/kg of LPS without inducing septic response or shock.

LITERATURE CITED

LITERATURE CITED

Al-Kaisi, N., Parratt, J. R., Siddiqui, H. H., and Zeitlin, I. J. (1977). "Feline endotoxin shock: effects of methylprednisolone on kininogen-depletion, on the pulmonary circulation and on survival." *British Journal of Pharmacology*, 60(3), 471-476.

Alexander, K. A., Kat, P. W., Frank, L. G., Holekamp, K. E., Smale, L., House, C., and Appel, M. J. G. (1995). "Evidence of Canine Distemper Virus Infection among Free-Ranging Spotted Hyenas (Crocuta crocuta) in the Masai Mara, Kenya." *Journal of Zoo and Wildlife Medicine*, 26(2), 201-206.

Alexander, K. A., MacLachlan, N. J., Kat, P. W., House, C., O'Brien, S. J., Lerche, N. W., Sawyer, M., Frank, L. G., Holekamp, K., Smale, L., McNutt, J. W., Laurenson, M. K., Mills, M. G. L., and Osburn, B. I. (1994). "Evidence of natural bluetongue virus infection among African and Indian carnivores." *American Journal of Tropical Medicine and Hygiene*, 51, 568-576.

Angus, D. C., Linde-Zwirble, W. T., Lidicker, J., Clermont, G., Carcillo, J., and Pinsky, M. R. (2001). "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care." *Critical Care Medicine*, 29(7), 1303-1310.

Blount, J. D., Houston, D. C., Møller, A. P., and Wright, J. (2003). "Do individual branches of immune defence correlate? A comparative case study of scavenging and non-scavenging birds." *Oikos*, 102(2), 340-350.

Burkepile, D. E., Parker, J. D., Woodson, C. B., Mills, H. J., Kubanek, J., Sobecky, P. A., and Hay, M. E. (2006). "Chemically mediated competition between microbes and animals: microbes as consumers in food webs." *Ecology*, 87(11), 2821-2831.

DeVault, T. L., Brisbin Jr, I. L., and Rhodes Jr, O. E. (2004). "Factors influencing the acquisition of rodent carrion by vertebrate scavengers and decomposers." *Canadian Journal of Zoology*, 82(3), 502-509.

East, M. L., Hofer, H., Cox, J. H., Wulle, U., Wiik, H., and Pitra, C. (2001). "Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas." *Proceedings of the National Academy of Sciences of the United States of America*, 98(26), 15026-31.

Erridge, C. (2011). "Accumulation of Stimulants of Toll-Like Receptor (TLR)-2 and TLR4 in Meat Products Stored at 5 °C." *Journal of Food Science*, 76(2), H72-H79.

Flajnik, M. F., and Kasahara, M. (2010). "Origin and evolution of the adaptive immune system: genetic events and selective pressures." *Nature Reviews Genetics*, 11(1), 47-59.

Fletcher, J., and Ramwell, P. (1980). "Indomethacin treatment following baboon endotoxin shock improves survival." *Advances in shock research*, 4, 103.

Forfia, P. R., Zhang, X., Ochoa, F., Ochoa, M., Xu, X., Bernstein, R., Sehgal, P. B., Ferreri, N. R., and Hintze, T. H. (1998). "Relationship between plasma NOx and cardiac and vascular dysfunction after LPS injection in anesthetized dogs." *American Journal of Physiology-Heart and Circulatory Physiology*, 274(1), H193-H201.

Gasaway, W., Mossestad, K., and STANDERS, P. (1991). "Food acquisition by spotted hyaenas in Etosha National Park, Namibia: predation versus scavenging." *African Journal of Ecology*, 29(1), 64-75.

Harrison, T. M., Mazet, J. K., Holekamp, K. E., Dubovi, E., Engh, A. L., Nelson, K., Van Horn, R. C., and Munson, L. (2004). "Antibodies to canine and feline viruses in spotted hyenas (*Crocuta crocuta*) in the Masai, Mara National Reserve." *Journal of Wildlife Diseases*, 40(1), 1-10.

Holekamp, K. E., and Sisk, C. L. (2003). "Effects of dispersal status on pituitary and gonadal function in the male spotted hyena." *Hormones and Behavior*, 44(5), 385-394.

Honer, O. P., Wachter, B., Speck, S., Wibbelt, G., Ludwig, A., Fyumagwa, R. D., Wohlsein, P., Lieckfeldt, D., Hofer, H., and East, M. L. (2006). "Severe Streptococcus infection in spotted hyenas in the Ngorongoro Crater, Tanzania." *Veterinary microbiology*, 115(1-3), 223-8.

Houston, D. C. (1979). "The adaptations of scavengers." *Serengeti: dynamics of an ecosystem*, 263-286.

Janzen, D. H. (1977). "Why fruits rot, seeds mold, and meat spoils." *American Naturalist*, 691-713.

Kaneko, T., Stearns-Kurosawa, D., Taylor, F., Twigg, M., Osaki, K., Kinasewitz, G. T., Peer, G., and Kurosawa, S. (2003). "Reduced neutrophil CD10 expression in nonhuman primates and humans after in vivo challenge with E. coli or lipopolysaccharide." *Shock*, 20(2), 130-137.

Kat, P. W., Alexander, K. A., Smith, J. S., and Munson, L. (1995). "Rabies and African Wild Dogs in Kenya." *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 262(1364), 229-233.

Kissui, B. M., and Packer, C. (2004). "Top-down population regulation of a top predator: lions in the Ngorongoro Crater." *Proceedings. Biological sciences / The Royal Society*, 271(1550), 1867-74.

Lembo, T., Hampson, K., Auty, H., Beesley, C. A., Bessell, P., Packer, C., Halliday, J., Fyumagwa, R., Hoare, R., Ernest, E., Mentzel, C., Mlengeya, T., Stamey, K., Wilkins, P. P., and Cleaveland, S. (2011). "Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009." *Emerging Infectious Diseases*, 17(3), 387-94.

Lewis, M., and Werdelin, L. (2000). "The evolution of spotted hyenas (Crocuta)". City: Hyaena Specialist Group Newsletter, pp. 34-36.

Lindeque, P., and Turnbull, P. (1994). "Ecology and epidemiology of anthrax in the Etosha National Park, Namibia." *The Onderstepoort journal of veterinary research*, 61(1), 71.

McVey, D. S., Chengappa, M. M., Mosier, D. E., Stone, G. G., Oberst, R. D., Sylte, M. J., Gabbert, N. M., Kelly-Aehle, S. M., and Curtiss Iii, R. (2002). "Immunogenicity of χ4127 phoP− Salmonella enterica serovar Typhimurium in dogs." *Vaccine*, 20(11–12), 1618-1623.

Miller, S. I., Ernst, R. K., and Bader, M. W. (2005). "LPS, TLR4 and infectious disease diversity." *Nat Rev Micro*, 3(1), 36-46.

Murray, D. L., Kapke, C. A., Evermann, J. F., and Fuller, T. K. (1999). "Infectious disease and the conservation of free-ranging large carnivores." *Animal Conservation*, 2(4), 241-254.

Nunn, C. L., Gittleman, J. L., and Antonovics, J. (2003). "A comparative study of white blood cell counts and disease risk in carnivores." *Proceedings. Biological sciences / The Royal Society*, 270(1513), 347-56.

Packer, C., Altizer, S., Appel, M., Brown, E., Martenson, J., O'Brien, S. J., Roelke-Parker, M., Hofmann-Lehmann, R., and Lutz, H. (1999). "Viruses of the Serengeti: patterns of infection and mortality in African lions." *Journal of Animal Ecology*, 68(6), 1161-1178.

Pienaar, U. V. (1969). "Predator-prey relationships amongst the larger mammals of the Kruger National Park." *Koedoe*, 12, 108-176.

Schinkel, S., Schinkel, C., Pollard, V., Garofallo, R., Heberle, H., Reisner, P., Papaconstantinou, J., and Herndon, D. N. (2005). "Effects of endotoxin on serum chemokines in man." *European journal of medical research*, 10(2), 76.

Watts, H. E., and Holekamp, K. E. (2009). "Ecological Determinants of Survival and Reproduction in the Spotted Hyena." *Journal of Mammalogy*.

Woodroffe, R., and Ginsberg, J. R. (1999). "Conserving the African wild dog Lycaon pictus. I. Diagnosing and treating causes of decline." *Oryx*, 33(2), 132-142.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Is the spotted hyena immune system fundamentally different from that of other mammals?

In chapters one and two we performed descriptive studies on two fundamental components of the mammalian immune system, immunoglobulins and toll-like receptors (TLRs). The closest extant relative of hyenas that has been studied in any depth immunologically is the domestic cat (*Felis catus*), so we compared immunoglobulins and TLRs in hyenas with those in cats. Our data show that hyena immunoglobulins are similar to those in cats. We were able to detect hyena immunoglobulins in Western blots (IgA, IgG, IgM, κ-light chain, λ-light chain) using anti-cat antibodies that cross-reacted with hyena epitopes, confirming a high degree of structural similarity between hyena and cat immunoglobulins. We then used these cross-reactive antibodies to assess the hyena response to immunization. Antibody response dynamics in spotted hyenas were similar to those observed in most other mammals. These Western blot and ELISA data suggest that hyena immunoglobulins are not fundamentally different than those of other mammals. An interesting future research topic would to characterize immunoglobulin subclasses in hyenas or to look for a completely novel immunoglobulin isotype. Additionally, our research shows that hyenas might have a different ratio of λ:κ light chains than the ratio observed in cats; this could potentially be another interesting topic for future research.

We also showed that partial DNA sequences of hyena TLRs are similar to those of TLRs in cats and other mammals. All hyena TLR DNA sequences were greater than 92% similar to cat sequences. We showed that hyena TLRs 1-10 are expressed in several tissues collected from euthanized hyenas. These data show that hyena TLRs are unlikely to be fundamentally different from TLRs in other mammals, although we have not yet functionally tested hyena TLRs. If

differences between hyena TLRs and those of other mammals do play a role in the ability of hyenas to survive exposure to deadly pathogens, it is most likely to be due to different expression patterns of TLRs among hyenas and other mammals. Another interesting future research topic that will be feasible when the hyena genome is published is to search the genome for other TLRs that have not been detected in other mammals. Humans express 13 TLRs and several other TLRs have been detected in other vertebrates that have not been detected in humans or mice.

How do socio-ecological variables affect immune function in spotted hyenas.

Chapters three and four focus primarily on how socio-ecological variables can affect immune function in wild spotted hyenas. We observed a significant correlation among social rank and both bacterial killing capacity (BKC) and total IgM, whereas there was no correlation between rank and total IgG. We also documented that lactating females had higher BKC and total IgM than males, and that females generally had higher levels of BKC and total IgM than males. These data suggest that nutritional resources may be important for immune function in hyenas for three reasons. First, high-ranking hyenas have better access to food resources and two components of immune function were higher in high-ranking hyenas than in low-ranking hyenas. Second, lactation is more energetically costly than gestation, and the immune defenses measured here were lower during lactation than gestation. Third, females outrank immigrant males and female immune defenses were generally higher in females than in males. Additionally, there was no difference in IgG concentrations between high- and low-ranking hyenas, lactating and gestating females, and females and males; of the three types of immune defenses we tested, total IgG concentrations are the most likely to be affected by pathogen exposure, and thus the least likely to be primarily controlled by resource availability.

We also showed that BKC is a significant predictor of annual reproductive success (ARS) in adult females. This correlation was highly significant when rank was not included in our statistical models, and remained statistically significant when rank was included as a covariate. Our results confirmed the results of previous studies showing that rank was a significant predictor of annual reproductive success (Hofer and East 2003; Holekamp et al. 1996; Swanson et al. 2011). Here we suggest that high levels of complement-mediated BKC may function to prevent disease onset and to allow faster recovery from wounds and infection. This prevention of disease in its early stages could potentially allow female hyenas and other mammals to devote resources to reproduction rather than devoting energy resources to mounting a large adaptive immune response; this might be one mechanism by which females with high BKC levels are able to increase their ARS compared to females with low BKC levels.

An alternative explanation for the differences we observed between high- and low-ranking females in their BKC and total IgM is that high-ranking females may be exposed to more pathogens than low-ranking females, which could drive their immune defenses to higher levels. To test the effects of pathogen exposure on immune defenses, we compared immune defenses in wild and captive hyenas, two populations that are highly likely to differ in pathogen exposure. We observed that wild hyenas had higher levels of total IgG, natural IgG, total IgM, and a trend for higher natural IgM than the captive hyenas; there was no difference in BKC between the captive and wild hyenas. Of the five immune defenses we quantified, BKC is the least likely to be affected by pathogen exposure and total IgG is the most likely to be affected by pathogen exposure. Thus our results suggest that pathogen exposure may be a primary mediator of the differences we observed. The captive population inhabits an environment that is more hygienic than the wild environment; the captive enclosures are regularly cleaned, no parasitic worms have

been detected in the captive population, and no evidence of deadly pathogens such as rabies or canine distemper virus have been detected in the captive population. The captive hyenas used here were derived directly from the wild population and have been bred in captivity for fewer than four generations, so genetic differences between the wild and captive populations are unlikely to drive the differences we documented here. To our knowledge this is the first study to show that total IgM concentrations are significantly different in captive and wild populations with the same genetic background and could prove to be a useful model for testing predictions generated from the hygiene hypothesis.

Why do spotted hyenas rarely die from infectious disease?

Although we did not answer the primary question that initiated our research on immune function in spotted hyenas, we made great strides toward answering this question by excluding several possibilities. Our research suggests that no major differences exist between hyenas and cats in immunoglobulin structure, immunoglobulin dynamics, and TLR sequences. We also show that ecological variables are important for immune function in hyenas, and that future studies that attempt to determine why hyenas rarely die from disease will need to consider the role of socio-ecological variables when studying immune function in hyenas. Our research has opened the door for future studies to investigate why hyenas rarely die from disease, and it has also provided several of the necessary tools need to complete this research.

LITERATURE CITED

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Hofer, H., and East, M. L. (2003). "Behavioral processes and costs of co-existence in female spotted hyenas: a life history perspective." *Evolutionary Ecology*, 17(4), 315-331.

Holekamp, K. E., Smale, L., and Szykman, M. (1996). "Rank and reproduction in the female spotted hyaena." *Journal of Reproduction and Fertility*, 108(2), 229-237.

Swanson, E. M., Dworkin, I., and Holekamp, K. E. (2011). "Lifetime selection on a hypoallometric size trait in the spotted hyena." *Proceedings. Biological sciences / The Royal Society*, 278(1722), 3277-85.