AN ABSTRACT OF THE DISSERTATION OF

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Title: Trade-offs in a Pathogen-rich World: Genetic and Maternal Drivers of Variation in Costly Resistance and Immunity in African Buffalo (*Syncerus caffer*)

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Disease acts as a powerful selective force in natural systems, driving the rapid evolution of resistance in the host. In the face of a myriad of pathogenic challenges in natural systems, hosts must balance the energetic needs of maintenance and reproduction with costly resistance mechanisms. In this dissertation I will (1) characterize *Mycobacterium bovis* (bovine tuberculosis (bTB)) resistance strategies in African buffalo (*Syncerus caffer*) and determine associated costs that lead to the maintenance of variation in host response, (2) identify putative mechanisms of bTB infection resistance, and (3) determine the effects of maternal rearing environment on calf survival, growth, and immune development.

Here I demonstrate measureable costs of a highly heritable form of bTB resistance in free-ranging African buffalo. Buffalo able to delay infection to a later age show reduced condition throughout life and reduced survival following infection, but a higher overall reproductive rate. Taken together, the costs and benefits of infection resistance imply a "fast" life history strategy in these animals as they reproduce early, die after infection, and show evidence of investment in highly costly immunity to prevent infection with bTB. I also present evidence that variation in infection resistance is mechanistically tied to phagocyte activation, with measurable differences in IL-12 production among genotypes at a locus associated with a four-fold increase in bTB risk. Additionally, I reveal that milk fat content is highly conserved across mothers, but immune components of milk vary in a resource-dependent manner with mothers of higher condition, size, and age provisioning higher concentrations of immune-active constituents. However, although milk fat positively impacts calf survival, associations of immune components are less clear. Generally, lactoferrin (an anti-microbial peptide) and immunoglobulin G (IgG) in milk associated with higher growth rates, but lower immune function in the calf, especially before weaning. These findings agree with theoretical predictions that maternal passive immunity acts to 'free-up' neonatal energetic resources for growth.

Taken together, this body of work elucidates the roles of genetic background and maternal effects on disease susceptibility and early health and immunity and represents a unique validation of previously proposed theoretical and laboratory work. Additionally, patterns in disease response and maternal provisioning identified here contribute to our overall understanding of resource partitioning in stochastic systems.

Trade-offs in a Pathogen-rich World: Genetic and Maternal Drivers of Variation in Costly Resistance and Immunity in African Buffalo (*Syncerus caffer*)

by
Hannah F. Tavalire

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APPROVED:

Major Professor, representing Integrative Biology

Chair of the Department of Integrative Biology

Dean of the Graduate School

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

Hannah F. Tavalire, Author

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In chapters 2 and 3, Vanessa Ezenwa and Dr. Anna Jolles are the principal investigators on the NSF EEID grant which financially supported the field captures for longitudinal buffalo sampling from 2008-2012. In chapter 4, Anna Jolles is the principal investigator on the USDA-AFRI grant funding the semi-captive herd project.

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Chapter 4: Milk immune assays were conducted by Hannah Tavalire and Brianna Beechler, while other general immune assays and milk collection were part of the larger project team's protocol (see acknowledgements for chapter 4). All analyses were conducted by Hannah Tavalire.

All chapters were written by Hannah Tavalire. Anna Jolles provided help with the conceptualization of each chapter, grant editing, input on analyses, and manuscript editing throughout the writing process.

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CHAPTER 1

General Introduction

Wild animals face a multitude of challenges in natural environments where energetic demands and available resources fluctuate, and not always favorably or predictively. Two persistent sources of energetic demand are reproduction and immunity. Selective pressures put upon the host by ubiquitous pathogens create high demands for energetic resources, but the host must balance these needs with its ultimate fitness (McDade, 2005; Zuk and Stoehr, 2002). Resistance often evolves as a means to avoid the negative fitness effects of infection, but many resistance mechanisms come at a fitness cost (Boots and Haraguchi, 1999). Only when the fitness benefits of resistance outweigh the costs of its mechanism is resistance favored, though this is highly contingent upon characteristics of the pathogen as well (e.g., virulence, prevalence, or transmission route; Anderson and May, 1982; Antonovics and Thrall, 1994; Gandon and Vale, 2014). Therefore, understanding the underlying mechanisms of pathogen resistance is paramount to fully evaluating the delicate energetic trade-offs between host fitness and immunity.

Though often measured as offspring number or reproductive rate, reproductive investment in mammals does not cease at parturition. In fact, lactation is arguably the most costly aspect of reproductive investment in large mammals (Rogowitz, 1996). Not only must mothers allocate resources to their own maintenance and immunity, through milk they must also maximize neonatal growth and ‘train’ the immune system of the developing neonate (Carlier and Truyens, 1995; Grindstaff, 2008; Hasselquist and Nilsson, 2009). However, maximizing immune *and* energetic components of milk may not be adaptive in wild systems, where early growth may disproportionately increase survival and maternal resources are limited (Bernhart, 1961).

Here I use African buffalo (*Syncerus caffer*) as a wild model system to study the evolution of disease resistance and maternal effects of rearing environment. Specifically, I will: (1) characterize *Mycobacterium bovis* (bovine tuberculosis (bTB)) resistance strategies in African buffalo and determine associated costs that lead to the maintenance of variation in host response, (2) identify putative mechanisms of bTB infection resistance, and (3) determine the effects of maternal rearing environment on calf survival, growth, and immune development. I am fortunate to work with a truly unique dataset in the African buffalo of Kruger National Park, South Africa. In chapters 2 and 3, I use a four-year longitudinal study of bTB infection in free-ranging animals to characterize bTB resistance and identify putative mechanisms of infection resistance. As a broad host range zoonotic pathogen, bTB is managed in many areas of the world in both wildlife and domesticated animals. Here I have the unique opportunity to witness the natural history of bTB infection in a population of wild buffalo and hope that my findings will inform future management practices. In chapter 4, I use an on-going study of a smaller herd of semi-captive buffalo to determine the impacts of maternal rearing environment on calf survival, growth, and immune function. Together, these datasets comprise some of the most comprehensive immunological data available in a wild animal, and will serve as a model system for disease dynamics and resource allocation in a natural setting.

In chapter 2, I identify two distinct bTB resistance strategies, as some animals delay the onset of infection (infection resistance), while others limit pathogen growth rate and resulting damage once infected (proliferation resistance). Infection resistance came with a significant condition cost before infection and dramatically reduced survival once infected, but associated with a higher overall reproductive rate among converted animals before and after infection. These findings suggest that infection resistant buffalo are following a "fast" life history trajectory, with early investment in reproduction and immunity (Johnson et al., 2012; Previtali et al., 2012; Tieleman et al., 2005). Importantly, infection resistance is highly heritable in this population of buffalo and therefore represents a putative target of selection. On the other hand, proliferation resistance appears to be contingent upon host condition at the onset of infection. I did not have the power to assess whether this form of resistance is heritable.

Heritable traits must have a genetic basis, so in chapter 3 I set out to identify putative mechanism of infection resistance. I identify two loci associating with time to onset of infection. Each locus had a "risk" allele conferring a ~four fold increase in risk of bTB infection over time. Genomic mapping revealed one locus to be within a linkage block of a peroxisomal membrane protein that has been previously identified as a component of oxidative burst and toll-like receptor-mediated apoptosis. Interestingly, animals harboring the risk allele at this locus produced significantly less IL-12 (a pro-inflammatory cytokine) over the four-year study, regardless of bTB status. These findings suggest a breakdown in the activation of macrophages during bTB progression and resulting IL-12 production. This mirrors results seen in numerous human clinical studies (e.g., Cooper et al., 2007; Fulton et al., 1996; Handzel et al., 2007; Taha et al., 1997; Zhang et al., 1994) and provides novel support for the importance of the IL-12 pathway and macrophages in tuberculosis disease progression in a wild system.

In chapters 2 and 3, I demonstrate that genetic background has measureable effects on disease susceptibility. In chapter 4, I show that maternal effects of rearing environment affect survival, growth, and immune function in young buffalo. Here I found immune components of milk co-vary with maternal condition, age, and size, but energetic provisioning in the form of fat is uniform across mothers. Calf death risk decreased with increasing fat content in milk, while increased concentrations of immune active components generally associate with a delay in immune development. Lactoferrin associates with higher growth, as seen in domesticated cattle (Robblee et al., 2003), while the effects of IgG concentration are less clear. Taken together, my findings support theories that maternally-derived passive immunity provides immune protection in early life, allowing for increased partitioning of neonatal resources to growth (Grindstaff, 2008), perhaps demonstrating this concept for the first time in a wild system.

In conclusion, these chapters elucidate the roles of genetic background and maternal effects of rearing environment on disease susceptibility and early health and immune development. The work presented here represents a unique validation of previously proposed theoretical work and laboratory findings regarding costs of resisting, tuberculosis resistance mechanisms, and maternal effects in a wild system.

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CHAPTER 2

There is no cure that does not cost: fitness trade-offs and multiple forms of resistance to bovine tuberculosis in a wild mammal

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ABSTRACT

Disease acts as a powerful driver of evolution in natural host populations. When pathogen virulence is high, resistance should evolve to combat the negative fitness consequences of infection. However, though resistance often evolves, variation in pathogen susceptibility is often maintained in the host population. Maintenance of variation in host response is often credited in the theoretical literature to costs of resisting, though empirical examples of costs outside of the laboratory are limited. Here we observe multiple, distinct resistance phenotypes to bovine tuberculosis (bTB), an important zoonotic pathogen, in a free-ranging African buffalo population. We characterize these phenotypes as 'infection resistance' which delays the onset of infection, and 'proliferation resistance', which limits the spread of lesions caused by the pathogen once the host is infected. Infection resistance and proliferation resistance were not correlated in our study population: some animals were classified as both infection and proliferation resistant, others had one of the two resistance traits, and some had neither. Importantly, we found that infection resistance to bTB is highly heritable in this African buffalo population, and it comes at the cost of reduced body condition, and, once infected with bTB, drastically curtailed survival. On the other hand, infection resistance was associated with higher reproductive rates, regardless of infection status. Infection resistant animals thus appear to follow a "faster" life history, in that they reproduce more quickly, but die earlier, than infection susceptible buffalo. In contrast, proliferation resistance was associated with higher body condition and higher reproductive rate in both bTB-infected and healthy buffalo, suggesting that the proliferation resistance phenotype is perhaps more contingent upon general host health than genetic factors. This study provides a novel example of fitness trade-offs associated with multiple resistance phenotypes in a wild host-pathogen system.

INTRODUCTION

Disease can be a powerful driver of evolution through many mechanisms, from the maintenance of otherwise deleterious traits to broad selective sweeps. The expectation in co-evolutionary systems is that selective pressures from either organism will drive the evolution of resistance in the host and the evolution of virulence in the pathogen (Boots and Haraguchi, 1999; Roy and Kirchner, 2000). However, though host resistance often evolves in natural systems (e.g., Blanchet et al., 2010; Bonneaud et al., 2011; Hasu et al., 2009; Hayward et al., 2011), many resistance traits remain unfixed in the host population. This maintenance of variation for host response to pathogens suggests that, though resistance confers some advantages pertaining to infection, resistance may not maximize fitness under all circumstances in a stochastic system. A wealth of theoretical literature describes potential evolutionary mechanisms for the maintenance of variation in host response to pathogens (Best et al., 2011; Best et al., 2008; Miller et al., 2005), but evidence of these mechanisms in natural systems is sparse (however, see Auld et al., 2013; Hayward et al., 2014; Maze-Guilmo et al., 2014; Zhong et al., 2005). Two potential evolutionary mechanisms for maintenance in variation of host response are (1) multiple mechanisms of resistance that impart different fitness advantages and (2) context-dependent costs associated with these resistance mechanisms. Namely, costs and fitness benefits of resistance may fluctuate with resource availability (Boots, 2011), force of infection (Gandon and Vale, 2014), or the presence of competing pathogens in the system (Mideo et al., 2008). If infection resistance mechanisms come at a fitness cost, then there should be trade-offs involving reduced fitness while delaying infection, when the pathogen is absent, or perhaps reduced fitness once infection does occur. Average lifetime fitness may thus depend on the likelihood of infection, leading to ecological-evolutionary feedbacks between disease dynamics and the frequency of heritable resistance traits in the host population (Boots and Haraguchi, 1999).

Infection resistance is most commonly defined as the ability of a host to prevent infection by a pathogen (Simms and Triplett, 1994). Though binary by definition, infection resistance operates on a continuum, with some animals succumbing to infection early in life, while others delay infection for longer periods of time. Variation in age at infection is likely primarily due to genetic variation and resulting differences in immune function, but is also contingent upon infectious dose and host health at the time of exposure, especially in wild systems (Anderson and May, 1982; Boots, 2011). For example, avian malarial parasites reached larger population sizes and produced more sexual stages within canaries fed a nutrient-poor diet relative to supplemented birds (Cornet et al., 2014). Additionally, parasite production in snails experimentally infected with schistosomes increased relative to host size and snails challenged with a higher dose of parasites exhibited signs of infection sooner, though this was partially dependent upon host genetic background (Tavalire et al., 2016).

Multiple underlying mechanisms have been proposed for infection resistance across systems, as each co-evolutionary relationship is unique to the populations involved. However, though diverse, many of these mechanisms can be traced back to key components of pathogen recognition or presentation involving pathogen recognition receptors (PRRs) in vertebrates. For example, innate pathogen recognition involves highly conserved toll-like receptors (TLRs; Takeda and Akira, 2003), while adaptive pathogen presentation relies on the highly polymorphic major histocompatibility complex (MHC; Trowsdale, 2011). In fact, the evolutionary history of genes involved in pathogen recognition and presentation is highly indicative of strong diversifying selection in the case of MHC (Apanius et al., 1997) and strong purifying selection in the case of TLRs (Mukherjee et al., 2009; Tschirren et al., 2011). The MHC is one of the most diverse and duplicated regions of any vertebrate genome due to the evolutionary advantages of variation at this important component of T-cell-mediated pathogen recognition (Hughes and Yeager, 1998). Similarly, the TLRs have undergone multiple duplication events across evolutionary time to result in a diverse set of receptors identifying highly conserved molecular patterns of pathogens both within and outside of the host cell (Akira and Takeda, 2004). Therefore, it is not surprising that variation in infection resistance would associate with genetic variation within highly variable immune recognition genes or some component of their signaling pathways.

Another type of resistance is proliferation resistance, previously referred to as ‘control’ (Miller et al., 2005). Proliferation resistance describes the host’s ability to minimize the pathogen’s growth rate once infected. In acute infections, proliferation resistance corresponds to a high rate of pathogen clearance, while in chronic infections, proliferation resistance limits the spread of the pathogen to other tissues within the host, but fails to eliminate the pathogen completely. Proliferation resistance is similar to disease tolerance because it potentially limits pathogen damage to the host, but unlike tolerance, proliferation resistance limits the growth rate of the pathogen, making these two host strategies evolutionarily distinct (Boots, 2008). Mechanisms of proliferation resistance are less well-characterized than those of infection resistance, but likely involve mechanisms of immune containment. For example, many infections of the lung, including tuberculosis, elicit fibrous granuloma formation to wall off infected tissue and prevent spread (Keane et al., 1997; Mukhopadhyay et al., 2012; Sandler et al., 2003). Less obvious mechanisms of proliferation resistance may involve variation in rates of pathogen degradation due to intrinsic differences in immunity or nutritional limiting of the pathogen. For example, transferrin has evolved in primates to sequester iron, a limiting nutrient for most bacterial pathogens, therefore limiting bacterial growth rates (Barber et al., 2016).

Since infection and proliferation resistance likely arise from discrete mechanisms that carry unique fitness costs and benefits, we would expect these distinct strategies could evolve within the same population. Here, we use the *Mycobacterium bovis*- African buffalo (*Syncerus caffer*) co-evolutionary system to assess the presence of multiple resistance strategies and identify associated costs in a wild host-pathogen system. *Mycobacterium bovis* (the causative agent of bovine tuberculosis- bTB) is a broad host range zoonotic bacterial pathogen that causes chronic infection with high morbidity and eventual mortality in mammals (Ayele et al., 2004; de la Rua-Domenech, 2006; Welburn et al., 2015). bTB is most commonly transmitted through inhalation, colonizing lung and associated lymph tissues by infecting resident macrophages (Kaufmann, 1991; Kornfeld et al., 1999; Raja, 2004). The host immune system forms granulomas around infected tissue, often resulting in large areas of necrosis in the lungs (Russell, 2007). Since the spread of bTB into Southern Africa in the 1970s, African buffalo have served as a maintenance host of bTB in their native range, sustaining a relatively high prevalence of bTB and facilitating infection of other hosts in the savanna ecosystem (Cross et al., 2009; Rodwell et al., 2001). bTB has been previously shown to reduce survival, pregnancy rates, and condition in African buffalo (Ezenwa and Jolles, 2015; Jolles et al., 2005). Given these previous findings of negative fitness effects of bTB infection, we ask the following questions in this system: (1) How do buffalo vary in their ability to prevent infection and limit the spread of *M. bovis* if they do become infected, (2) is phenotypic variation in the host response to bTB heritable and therefore representative of genetic variation upon which selection can act, and (3) are there fitness costs associated with resistance to bTB?

METHODS

Study Area and Field Data Collection

Two hundred sub-adult and young adult female African buffalo (*Syncerus caffer*; initial ages 2-7 years) were captured every six months in the southern part of Kruger National Park, South Africa between June 2008 and August 2012 as part of a longitudinal study of co-infection and the consequences of anti-helminthic treatment (for more detail, see Ezenwa and Jolles, 2011 and Ezenwa and Jolles, 2015). These buffalo were sampled from two distinct herds occurring in the Crocodile Bridge and Lower Sabie areas of the park. The Crocodile Bridge herd includes buffalo in the area around the Crocodile River in the south-east extent of the park, while north of this the Lower Sabie herd includes buffalo near the eastern reaches of the Sabie River. These herds will hereafter be referred to as “CB” and “LS.” Estimated herd sizes during the study period for CB and LS were 2,100 and 1,100 buffalo, respectively.

Each buffalo was fitted with either a radio (n=193) or satellite (n=7) collar with a high frequency VHF transmitter upon first capture, that was then used to locate them for subsequent captures at roughly six month intervals. Individuals lost to death or emigration during the study period were replaced to maintain a constant sample size of 200 animals spread equally among the two herds (n_{total} =306). Of these animals, half (n=50 per herd) were randomly chosen to receive an anti-helminthic bolus (slow-release fenbendazole (Panicur, Hoechst Roussel)) as part of the study design outlined in Ezenwa and Jolles (2015).

At each capture, animals were immobilized by dart from a helicopter or truck using etorphine hydrochloride (M99, Captivon, Karino, South Africa). Following data collection, immobilization was reversed using diprenorphine (M5050) or naltrexone (40 mg/ml, Kyron). Animals were kept under observation until fully recovered and all immobilizations were conducted by a veterinarian according to the South African National Parks Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. All animal work for this study was approved by the institutional animal care and use committee at Oregon State University (ACUP #3267).

Age at each capture was determined in young animals by tooth emergence and in older animals by wear pattern per established methods in this species (Jolles, 2007). Pregnancy status and stage were determined by rectal palpation done by a wildlife veterinarian. This method shows 100% sensitivity in Egyptian buffalo after 51 days of gestation (*Bos bubalis*; Karen et al., 2011). The presence of a calf at heel was detected visually or by evidence of lactation (manual milking of all four teats; Jolles et al., 2005), and calves were aged by body size.

Body condition was assessed on a five-point scale through palpation and visual inspection of four areas where buffalo deposit fat: spine, hips, ribs, and base of tail. Condition ranged from 1 (very poor) to 5 (excellent) at each area, and was then averaged across these areas for an overall estimate of condition (Ezenwa et al., 2009). This method has been shown to correlate with fat deposits in the liver (Ezenwa et al., 2009).

All blood samples for disease diagnostics were collected within fifteen minutes of sedation and held on ice before bovine tuberculosis (bTB) testing. bTB infection was determined with the commercially available whole-blood gamma interferon (IFNγ) assay (BOVIGAM, Prionics, Switzerland). This assay measures the difference in IFNγ production of whole blood in response to incubation with bovine versus avian tuberculin antigens, while controlling for differences in background IFNγ levels. Individual samples were called as bTB positive or negative based on absorbance thresholds optimized for bTB infection in African buffalo (Michel et al., 2011). We obtained a time series of 2-9 bTB tests for each animal and used the full time series to more confidently assign bTB status. Animals with at least two consecutive positive bTB tests were assigned as bTB positive. Since bTB is chronic in buffalo and there is no evidence that they recover once infected we assumed animals stay positive until death (Bengis, 1999). Animals with alternating test results or short observation periods (<3 captures) were discarded.

At the completion of the study, bTB positive animals were culled as well as a random subset of bTB negative controls (n_{pos}=78, n_{neg}=57). These animals were necropsied and counts were taken of all tuberculosis-associated lesions in the lungs and lymph tissue to assess differences in disease-related pathology. A subset of 33 of these animals had known conversion ages during the study period and were used to characterize proliferation resistance in subsequent analyses.

For all subsequent analyses, we used only the data from buffalo that had been caught at least three times, had been tested for bTB at least twice, and had reached reproductive maturity before death or the end of the study (4 years of age; n=190). At the beginning of the study, prevalence of bTB infection was 0.14 and did not differ significantly among the two herds (X_1=0.987, p=0.32) or anti-helminthic treatment groups (X_1=0, p=1.0).

SNP Genotyping and filtering

We extracted 100-200ng/ml genomic DNA from dried ear tissue samples (DNeasy blood & tissue kit, Qiagen) and prepared individual libraries for sequencing using type IIB restriction associated DNA (2bRAD) methods, detailed in Wang et al. (2012). Briefly, this method used a type IIB restriction endonuclease (Alf1; Thermo Scientific #ER1801) to produce hundreds of thousands of 36bp reads from across the genome. We prepared genotyping libraries using reduced tag representation (RTR) as described in Wang et al. (2012) by ligating adaptors with 3' overhangs ending in NC and NG. Samples were sequenced on an Illumina HiSeq 3000 sequencer at the Oregon State University Center for Genome Research and Biocomputing. We excluded terminal tag positions, ambiguous base calls, long homopolymer regions, and excessively low quality reads (>5 positions with quality <10). After trimming, the remaining high quality reads were retained for all subsequent mapping and genotyping. Using custom Perl scripts, we extracted all AlfI sites (n=480,162) from the recently available *Syncerus caffer* genome (Glanzmann et al., 2016). We then used SHRiMP to map each individual sample to these sites and filter the resulting matches for statistically weak or ambiguous alignments using parameters similar to those described by the software authors (Rumble et al., 2009). We determined genotypes at each AlfI site with >10x coverage, then filtered out any monomorphic loci. We allowed for 10% missing data at any given locus and one polymorphism per tag. Animals that were genotyped at 5000 or fewer loci were removed from the dataset. We used custom scripts to extract scaffold and position information of single nucleotide polymorphisms (SNPs) for population structure analysis. Markers were discarded if they were not biallelic, violated Hardy Weinberg Equilibrium ($p<0.0001$), or had a minor allele frequency less than 5%. Quality filtering yielded 187 usable samples genotyped at 1999 SNPs.

SNP-based population structure and relatedness

To test whether the two herds sampled were genetically distinct, we calculated global F_{ST} using filtered markers in the R packages *hierfstat* (Goudet, 2005). We observed a global F_{ST} value of 0.0003, leading us to conclude that these herds are not genetically distinct. This result agrees with previously reported behavioral observations of frequent herd switching and long distance dispersal in African buffalo (Caron et al., 2016; Halley et al., 2002; Naidoo et al., 2014). We therefore consider any effect of 'herd' in subsequent analyses as environmental and not reflective of any differences in genetic background.

We determined pairwise relatedness (r) using the R package *related* (Pew et al., 2015) using the estimator calculated in the method of Wang et al. (2002). We used these estimates to create a clade-based dendrogram of relatedness. These data present a unique problem for the calculation of relatedness and therefore heritability, since we observed few closely related pairs by chance within our subset (we sampled between 5 and 9% of the total population in each herd, yielding 21 half-sibling pairs within 187 animals). To circumvent a large reduction in sample size, we used the program COLONY (Jones and Wang, 2010) to identify sibling pairs and used these pairs to create a bottom-up hierarchical grouping scheme based on most recent common ancestors (MRCAs). Base clade members share a MRCA within the same evolutionary timescale of confirmed siblings, then higher level clades group progressively less related individuals from there. The trade-off is that clades grouping more distantly related animals include more animals in each clade, providing a larger within-family sample size but also a potentially large within-family variance, while clades with more closely related animals potentially reduce within-family variance, but at the cost of sample size. We then asked the biological question: at what level of relatedness (MRCA clade level) is time to onset of bTB predicted in a group of relatively distantly related animals? We determined the clade level at which time to onset of bTB is most significantly predicted using Cox proportional hazards models with the different MRCA clade levels as fixed effects. We then used this clade level for all subsequent analyses of heritability (for more information on clade structure and time to onset analysis, see Appendix Table 1 and Figure 1).

Variation in Resistance

We evaluated two types of bTB resistance in the buffalo: infection resistance describes differences in time to onset of infection (i.e., conversion age), and proliferation resistance describes differences in lung pathology relative to time since onset of infection. Per-capita risk of bTB has been previously shown to be equal across herds (Ezenwa and Jolles, 2015) and those animals that became bTB positive later in life or never converted are considered to be more infection resistant. We expect stochastic variation in time to infection, but on average more resistant animals should become infected later in life. Proliferation resistance was assessed using a non-linear regression of total number of lung lesions over time since onset of infection. Those animals with positive residuals had more pathology than would be predicted for the time since onset of bTB, and are therefore proliferation susceptible, while those animals with negative residuals relative to the regression line had lower pathology than expected, and are therefore proliferation resistant. Here we use total lung lesion count as a proxy for total pathology. The number of lymph nodes affected, and the total lung area affected give similar results in our models, and correlate tightly with the number of lung lesions ($r=0.70$; $p<0.0001$). We assessed linear association of time to onset of bTB (infection resistance) and lesion over time residuals (proliferation resistance) using a Pearson product moment correlation.

Heritability of Infection Resistance

We used a restricted maximum likelihood (REML) linear mixed effect ‘animal model’ approach in R to estimate the narrow sense heritability of time to onset of bTB, and therefore degree of infection resistance. We included herd and treatment as fixed effects and MRCA clade as a random effect in the best-fit model. Our best-fit model was selected based on diagnostic checks for heteroscedasticity (residual plots), conditional R^2 (Nakagawa and Schielzeth, 2013; Wang and Schaalje, 2009), and Akaike's Information Criterion (AIC; Gurka, 2006). Total phenotypic (V_P) and additive genetic (V_A) variances were quantified using the variance output for the random effects of the best-fit REML model. V_A was assigned as the within-clade variance and V_P was assigned as the sum of the within-clade variance and residual variance. We calculated narrow sense heritability as the ratio of additive genetic variance to the total phenotypic variance: $h^2=V_A/V_P$. We computed the standard error of the heritability estimates using established methods (Klein et al., 1973). Since REML models cannot account for censored data and many animals in the study never converted to bTB positive, we conducted a sensitivity analysis in which negative animals under a certain age were excluded from the estimation of heritability. Using half-year increments, we tested the age cutoff for truncation of negative animals at which standard error of the heritability estimate was minimized, while maximizing sample size (i.e., where the cutoff age most likely represents the "true" minimum conversion age for each clade; see Appendix Table 2 and Figure 2). We found that time to conversion was significantly predicted by relatedness for age cut-offs between 6 and 11 years, with the lowest confidence interval for heritability at a cut-off of 6 years. Animals that remained bTB negative during the study period were assigned a "conversion age" of their minimum conversion age, i.e. their age at the end of the study, for the purpose of the heritability analysis. We found that including only negative animals six years of age or older minimized the standard error of our heritability estimate, while also retaining 75 of 99 usable animals (those that had at least 2 members in their base clade: i.e., at least one sibling pair). Animals that were positive at first capture were also excluded from these analyses, as their conversion age was unknown.

Fitness Effects of bTB Infection and the Cost of Resistance

We assessed long-term health and fitness costs of infection between bTB positive and negative animals by comparing average body condition and number of calves during the study period (reproductive success). We used linear models to assess differences in body condition and generalized linear models with a Poisson distribution to compare calf counts over the study period, with years reproductively mature as an offset term. We initially included initial age and age^2 in these models, as condition is expected to decline naturally with age (Gorsich et al., 2011) and reproductive rate is expected to increase between age 4-6 (Jolles et al., 2005), though these terms were dropped during model selection if they were not part of the best-fit model. To assess short-term fitness effects of bTB, we compared converted animals before and after bTB onset to age, treatment, and herd matched bTB negative buffalo captured over the same time period using maximum likelihood mixed effect models.

To evaluate costs of either resistance strategy outlined above, buffalo that were observed for at least 36 months and acquired bTB during the study period (with comparable periods as bTB negative and positive) were assigned as either infection resistant, proliferation resistant, neither resistance type, or both. Animals were considered infection resistant if they converted after the mean conversion age of 5.5 and were considered proliferation resistant if they had a negative residual value for the total lesions over time since conversion nonlinear regression. We then compared body condition and reproductive rate before and after bTB conversion across these groups. Full maximum likelihood mixed effect models included herd, treatment, the two resistance types as binary factors, and animal ID as a random effect. Initially, interaction terms for both resistance types and an interaction of each type with time (‘before’ and ‘after’ conversion) were included in the models, but none were retained following model selection.

To assess survival costs of infection resistance, we compared post-conversion survival times of infection resistant and infection susceptible animals using a Cox proportional hazards survival analysis and Kaplan-Meijer curves for visualization. The full model contained age at infection, treatment, infection resistance, and herd, but age dropped out during model selection.

When applicable, normality was assessed in all models using data visualization and Shapiro-Wilks testing. All model selection was done using AIC and residual plot visualization, unless otherwise noted. All statistics were run in R version 3.2.4 (R Core Team, 2016).

RESULTS

Variation in host response to bTB

We investigated natural variation in the host-pathogen interaction between free-living African buffalo, and *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB). In this study, we followed 200 buffalo for a four-year period, replacing those that died or emigrated to maintain a constant sample size (n_{total}=306). Animals were distributed evenly between two neighboring herds in Kruger National Park, South Africa. Animals were captured every six months to test for bTB status, body condition, and reproductive status. Half of the animals were experimentally de-wormed, to test the effects of helminth infection on bTB incidence and progression. These results have been published elsewhere (Ezenwa and Jolles, 2011; Ezenwa and Jolles, 2015). At the beginning of the study period, prevalence of bTB infection was 0.14 and did not differ significantly among the two herds (X_1=0.987, p=0.32) or anti-helminthic treatment groups (X_1=0, p=1.0). For the work presented here, we included only those animals that were captured at least 3 times and had at least two consistent bTB tests. Of this subset of 190 animals, 26 were initially bTB positive, 108 remained bTB negative, and 56 acquired bTB infection during the study period. Within our subset, 33 buffalo that converted to bTB positive during the four years were culled at the end of the study, and the extent of bTB infection described by examining lung tissue for characteristic bTB lesions.

We found evidence for variation in both infection resistance and proliferation resistance in our study population. We found that the age at which buffalo become infected with *M. bovis* varies broadly, from two to greater than ten years of age, with age-specific incidence increasing to age four and then remaining relatively constant (Figure 2.1A-B). Mean age of conversion for all animals that converted during the study period was 5.5 years (n=72 of 306; 95%CI=(5.08, 5.87)). Among bTB-infected animals, the number of lung lesions generally increased with time (Figure 2.2), although almost a third of culled animals remained lesion-free despite a positive bTB test. We also found clear variation among animals in the rate of pathogen proliferation, with some showing many fewer lesions than expected from the time- lesion number regression, and others developing lesions much faster than expected.

Heritability of host resistance

Because the buffalo included in our study were relatively similar in age and come from large herds, many of them likely were not closely related, while others might have been half or full siblings. As such, before addressing heritability, we conducted a preliminary analysis to assess which level of relatedness best predicted time to onset of bTB infection. We found strong evidence for heritability of infection resistance across levels of relatedness. Hierarchical levels of shared ancestry back to the fourth most recent common ancestor (MCRA) explained significant variation in infection resistance (Appendix Table 1 and Figure 1). Family group was most predictive of time to onset of bTB infection when animals were grouped by their second shared MRCA (i.e., roughly, relatedness based on shared grandparents: 'clade 2') and bTB negative animals under six years of age at the end of the study were removed from the analysis (because at this age it was unclear whether they would truly acquire bTB late in life, or whether they might convert soon after our sampling period, which would put them in the early conversion group; see methods section for details). Using clade 2 as our family grouping variable (N_{clades}=30, $n_{per\ clade}$=2.5), and controlling for herd and treatment as fixed effects in a restricted maximum likelihood (REML) 'animal model', we found that time to onset of bTB infection had a high heritability of 0.37 (SE= 0.14, p=0.009) in this population of buffalo. We lacked power to assess the heritability of proliferation resistance, as only six animals were culled that had at least two members in their base clade (h^2=0.45, SE=0.70, p=0.52).

Long and short-term fitness effects of bTB infection

In order to assess how fitness costs and benefits of resistance strategies might lead to the maintenance of variation in host response in this system, we first assessed the negative fitness effects of bTB infection. A strong negative effect of bTB infection on survival in buffalo has been demonstrated for these populations previously (Ezenwa and Jolles 2015; Jolles et al., 2005). To assess both long and short-term health consequences of bTB infection, we compared body condition and calving success (i) among animals that were either bTB positive or bTB negative for the duration of the study period, and (ii) before and after bTB conversion in animals that acquired bTB during the study period relative to a group of age, herd, and treatment matched control animals over the same time period. We found that animals which were bTB positive throughout had an average body condition score that was 0.60 lower than bTB negative animals, after accounting for age (Table 2.1A). The effect of bTB on condition was strongest in young animals, and was much more pronounced in the dry season (Table 2.1B-C). We found no evidence for a reduction in condition when we compared animals that converted during the study, to age- and herd-matched bTB negative animals over the same time period (Table 2.2A). Detection of a loss in condition in bTB positive but not in recently converted animals suggests that condition loss due to infection happens on a longer time scale, or that those buffalo that acquire bTB are overall in poorer condition than those that do not.

We found no effect of bTB infection on overall reproductive success when comparing bTB positive and negative animals across the study period (Table 2.1D). We also observed no reduction in reproductive rate of converted animals following conversion when compared to age-matched negative animals over the same time period (Table 2.2B).

Fitness costs and benefits of resistance phenotypes

In order to compare costs associated with each resistance strategy, we categorized variation in infection resistance (time to onset of infection), and variation in proliferation resistance (number of bTB lesions relative to time since infection) into distinct resistance phenotypes. Animals that did not acquire bTB until at least 5.5 years of age are considered ‘infection resistant’ (Figure 2.1A). Animals with negative residuals in the regression of bTB lesion number on the time since infection are considered ‘proliferation resistant’, since they have fewer lesions than would be expected based on the time they have been infected (i.e., below the regression curve in Figure 2.2). The two types of resistance did not appear correlated among converted buffalo ($r=-0.05$, $n=33$, $p=0.60$); with some individuals only infection resistant or proliferation resistant, some both, and some neither (Figure 2.3A). The majority of buffalo were proliferation resistant ($n_p=11$)

or both infection and proliferation resistant (n_b=11). We might not have been able to detect a correlation between infection resistance and proliferation resistance if animals that became infected early in life (infection-susceptible) also died quickly from bTB (due to proliferation-susceptibility). We would not have observed these animals because they did not survive until the end of the study, when we culled bTB-positive animals. To account for this possibility, we tested whether age at infection was correlated with time until death in bTB-positive animals that died naturally during the study period. We found no correlation (r^2=0.002, p=0.99) and are therefore confident that these forms of resistance represent distinct strategies.

We found a cost of infection resistance in the form of lower body condition, regardless of bTB status (Figure 2.3B and Table 2.3). However, we saw an overall loss in body condition across all buffalo during this time period, likely due to stochastic variation in resources availability or drought. We found no evidence for a reproductive cost of infection resistance (Figure 2.3C). Due to the age-dependency of our infection resistance classification, comparing overall survival time (final age at death or last observation) between infection resistant and susceptible animals was not meaningful. However, following infection, we found converted infection resistant animals to have a 7-fold higher death rate than animals that succumbed to infection before 5.5 years of age (n=56, events=10; Figure 2.4 and Table 2.4). We accounted for initial age, herd, and anti-helminthic treatment in this analysis. In addition to our survival findings, low condition has been previously shown in this system to be a strong predictor of mortality (Glidden and Jolles, *in prep*). Therefore, the infection resistance phenotype appears to be advantageous only in terms of preventing or delaying bTB infection, but carries a dramatic survival cost if bTB does occur and an additional increased risk of death due to lower body condition.

We observed no costs associated with proliferation resistance, after accounting for age at conversion and treatment (Figure 2.3). In fact, proliferation resistance was associated with increased condition and calving rate both before and after disease onset, suggesting that this resistance phenotype associated with overall higher energetic resources and fitness (Table 2.3). Here we find both resistance strategies were associated with a higher reproductive rate, as animals that were categorized as both infection and proliferation resistant had the highest reproductive rates before and after infection.

DISCUSSION

Here we identify two distinct forms of host resistance in a natural host-pathogen system: infection resistance and proliferation resistance. We found infection resistance to be heritable, and therefore available for selection to act upon. We also provide evidence for multiple costs of infection resistance in the form of reduced condition before infection with bTB, as well as a delayed survival cost once infected. To our knowledge, this is one of few studies providing direct evidence for a cost of resistance in a natural animal system (but see Auld et al., 2013; Bonneaud et al., 2012; Graham et al., 2010; Zhong et al., 2005); extensive examples in plant systems are reviewed in Brown and Rant (2013) and Burdon and Thrall (2003)).

Several studies in recent years have invoked life history theory to explain relative investment in constitutively expressed, general immunity versus long-term immune memory (Ardia et al., 2011; Hanselmann and Jolles, 2011; Miller et al., 2007; Previtali et al., 2012; Sandmeier and Tracy, 2014). Namely, organisms following a “fast” pace of life invest disproportionately in constitutively expressed, general immune mechanisms, and reproduce earlier in life, while organisms exemplifying a “slow” pace of life invest in immune memory that will protect them throughout a longer lifespan (Previtali et al., 2012). Though a strong innate immune response may offer immediate protection from constant exposure to pathogens, innate immune mechanisms are often highly energetically costly and can produce severe collateral damage to host tissues (Goldszmid and Trinchieri, 2012). Here we see evidence of a “fast” life history strategy in the form of energetically costly, but reproductively advantageous, infection resistance. A reduction in condition before infection may suggest increased resource allocation to constitutively expressed immune mechanisms or repair of collateral damage, resulting in the delay or prevention of bTB infection, though we did not directly assess mechanisms of immunity here. Furthermore, infection resistant buffalo that converted to bTB later in life had an overall higher reproductive rate over the study period than animals that converted earlier, suggesting a reproductive advantage of resisting infection. Additionally, the fitness advantages of infection resistance cease upon infection, as these animals are at seven times higher risk of death once infected. Taken together, these findings provide convincing evidence for the disproportionate investment in pre-patent fitness exemplary of a "fast" pace of life. Interestingly, life history strategy is often assigned at the species or even population level, but here we provide novel evidence of heritable, intra-individual variation in life history strategy among conspecifics. Furthermore, a "fast" pace of life is often associated with small, short-lived species (e.g., birds and small rodents; Miller et al., 2007), but here we show variation in life history strategy in a large mammal.

Conversely, we found proliferation resistance does not come at a cost in this system and seems to be a trait of generally healthy animals. This finding suggests that at the onset of bTB, animals with higher initial fitness and condition suffer less pathology than animals in poor condition, which may lead to overall higher survival rates in this group, since condition is highly predictive of survival in buffalo (Glidden and Jolles, *in prep*). It has been demonstrated across taxa that organisms in better condition have higher available resources to allocate to immune coping mechanisms or tolerate resource leeching by the pathogens themselves, especially in environments where resources are seasonally limited (Martin et al, 2008). Furthermore, ruminants prioritize energetic allocation to growth, pregnancy, and lactation over immune function when resources are limited (Coop, 1999), suggesting that buffalo of lower condition would allocate fewer resources to proliferation resistance once infected. Unfortunately, because these animals were culled, we cannot directly address a survival advantage of proliferation resistance. We also could not assess heritability of this trait in the current study, so we cannot determine if proliferation resistance is due to genetic background or simply a surplus in energy reserves available to fight infection.

We also conclude that these resistance phenotypes are not correlated in this population, suggesting that they arise from distinct physiological mechanisms. This is to be expected due to differences in the nature of each defense strategy: infection resistance likely results from strong pathogen recognition or pathogen clearing, while variation in proliferation resistance likely arises from differences in pathogen containment strategies or degradation (e.g., granuloma formation in the lungs).

bTB infection patterns in this study likely do not purely result from resistance phenotypes and underlying immune mechanisms. Previous work has elucidated the roles of co-infecting pathogens in bTB susceptibility in this system, as certain concurrent infections increase bTB risk (Beechler et al., 2011; Ezenwa and Jolles, 2011; Gorsich et al., 2011). Furthermore, contact patterns and connectivity in other animal systems have been shown to influence disease exposure, incidence, and resulting spread (Jones et al., 2017; Lange and Thulke, 2017; Rushmore et al., 2013). Co-infecting pathogens along with seasonal fission-fusion dynamics in African buffalo herds could drive variable patterns in bTB spread in this system, contributing to the maintenance of variation in resistance phenotypes.

In order to assess the true fitness advantages, and resulting evolutionary dynamics, of these resistance phenotypes, we would need to quantify relative risk of infection and absolute risk of infection in the total population. Pathogen virulence has been shown to drive the evolution and maintenance of resistance phenotypes in many wild systems (Ferrandon, 2009; Little et al., 2010). For infection resistance to evolve in a system, pathogen virulence must be high such that the costs of resisting are less than the negative fitness effects of succumbing to infection over the lifetime of the animal (Boots and Haraguchi, 1999). Since condition is highly predictive of survival in buffalo, energetically costly infection resistance mechanisms, constitutively expressed regardless of force of infection, may confer negative fitness effects in the absence of bTB. However, these immune mechanisms, if general enough, could confer resistance to other pathogens as well, complicating the evolutionary dynamics of this trait. Furthermore, if bTB force of infection is high, and most animals get infected before reproductive age, we would expect strong directional selection for infection resistance in this system. Conversely, if bTB force of infection is low and infection resistance mechanisms do not confer resistance to other virulent pathogens, we would expect the condition costs of infection resistance to impact long-term reproductive rates and survival, resulting in directional selection away from this trait.

In conclusion, here we provide compelling evidence for multiple resistance phenotypes with unbalanced costs of resisting. Though environmental factors likely contribute to variation in time to infection, we find infection resistance to be highly heritable. Future work involving genetic association and quantitative genetic modeling will help pinpoint plausible mechanisms of resistance and project its evolutionary trajectory in this system.

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TABLES

Table 2.1: Linear models for measures of health and fitness relative to infection in animals that remained bovine tuberculosis (bTB) positive or negative throughout the study period (n=126). Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. Overall Average Body Condition	2.195	<0.0001
bTB positive	-0.600	0.019
Final Age	0.220	0.002
Final Age^2	-0.014	0.0005
Herd- Lower Sabie	0.246	<0.0001
BTB positive*Final Age	0.068	0.037
Adj. R^2=0.183		
B. Average Wet Season Condition	2.240	<0.0001
bTB positive	-0.232	0.030
Final Age	0.187	0.045
Final Age^2	-0.010	0.045
Adj. R^2=0.043		
C. Average Dry Season Condition	2.597	<0.0001
bTB positive	-0.582	0.033
Final Age	0.144	0.049
Final Age^2	-0.011	0.009
Herd- Lower Sabie	0.311	<0.0001
BTB positive*Final Age	0.069	0.048
Adj. R^2=0.210		
D. Total Number of Calves* (offset= number reproductive seasons in study)	0.0073	<0.0001
bTB positive	1.320	0.182
Final Age	1.160	<0.0001
Herd- Lower Sabie	1.535	<0.010

*Estimates were natural log back-transformed and represent a multiplicative effect on the trait median.

Table 2.2: Maximum likelihood mixed effect models for measures of health and fitness before and after bovine tuberculosis (bTB) infection in animals that converted to bTB positive during the study period compared to age and herd matched controls that remained bTB negative across the same time period. Here we see that converted animals did not significantly differ from age-matched controls for either fitness trait before conversion (n=66). Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. Average BCS Before & After Infection	3.656	<0.0001
Time (After)	-0.450	0.0002
Converted	0.045	0.719
Herd- Lower Sabie	0.423	0.0001
Initial Age	-0.091	0.011
Treatment (control)	-0.149	0.136
Time (After) * Converted	-0.236	0.142
B. Average Repro Rate Before & After Infection	-0.417	0.002
Time (After)	0.130	0.163
Converted	0.052	0.575
Herd- Lower Sabie	0.211	0.003
Initial Age	0.116	<0.0001
Time (After) * Converted	0.255	0.054

Table 2.3: Maximum likelihood mixed effect models comparing measures of health and fitness before and after bovine tuberculosis (bTB) infection in converted infection and proliferation resistant animals (n=32). Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. Average BCS Before & After Infection	3.242	<0.0001
Time (After)	-0.687	<0.0001
Infection Resistant	-0.378	0.015
Proliferation Resistant	0.302	0.042
Herd (Lower Sabie)	0.453	0.004
B. Average Repro Rate Before & After Infection	-0.632	0.002
Time (After)	0.381	0.0002
Infection Resistant	0.307	0.027
Proliferation Resistant	0.196	0.054
Treatment (control)	0.207	0.042
Herd (Lower Sabie)	0.264	0.013
Initial Age	0.089	0.051

Table 2.4: Cox proportional hazards model comparing death risk of converted infection resistant and susceptible animals following bovine tuberculosis (bTB) infection (n=56, events=10). The “treatment” variable refers to buffalo that were not treated for worms (control) versus buffalo that were treated for worms (bolus; reference group). Estimates are natural log back-transformed and represent a multiplicative increase in risk of death. Best-fit models were selected using AIC and residual plots.

Model	Estimate	P-value
Death Risk		
Infection Resistant	6.99	0.021
Treatment (control)	13.0	0.007
Herd (Lower Sabie)	9.46	0.051

FIGURES

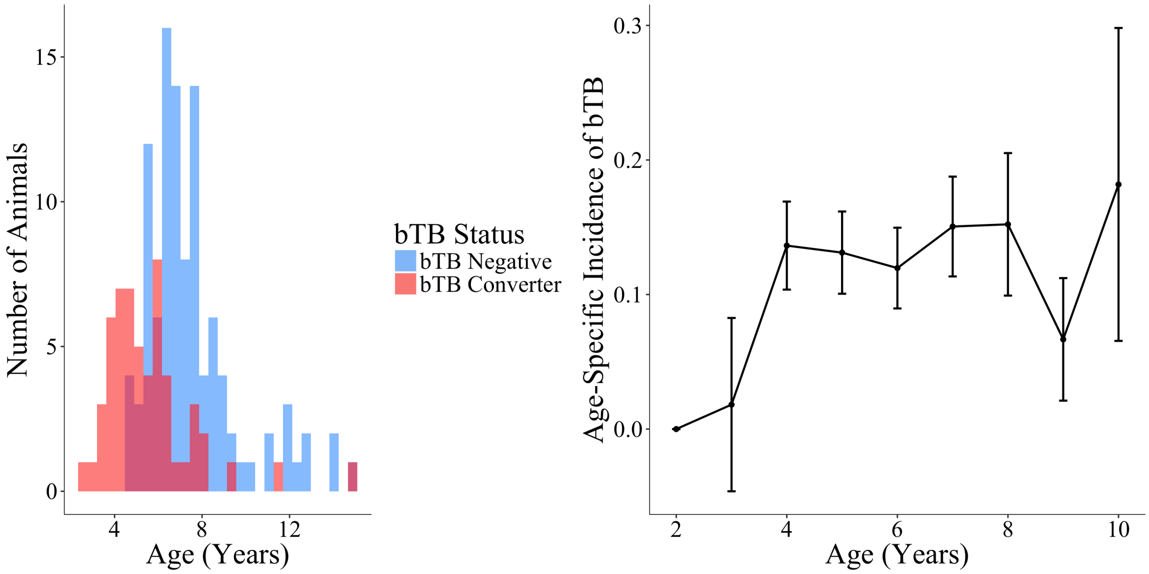

Figure 2.1: (A) Age distribution of animals that converted to bovine tuberculosis (bTB) positive (red) during the study period and animals that remained bTB negative (blue) throughout the study period (n=190). Animals that converted later in life or remained bTB negative to a later age are considered more infection resistant that those that converted at a young age. Animals that were bTB positive at first capture are not shown. (B) Age-specific incidence of bTB. Incidence of bTB increases to age four, then remains relatively constant (n=306).

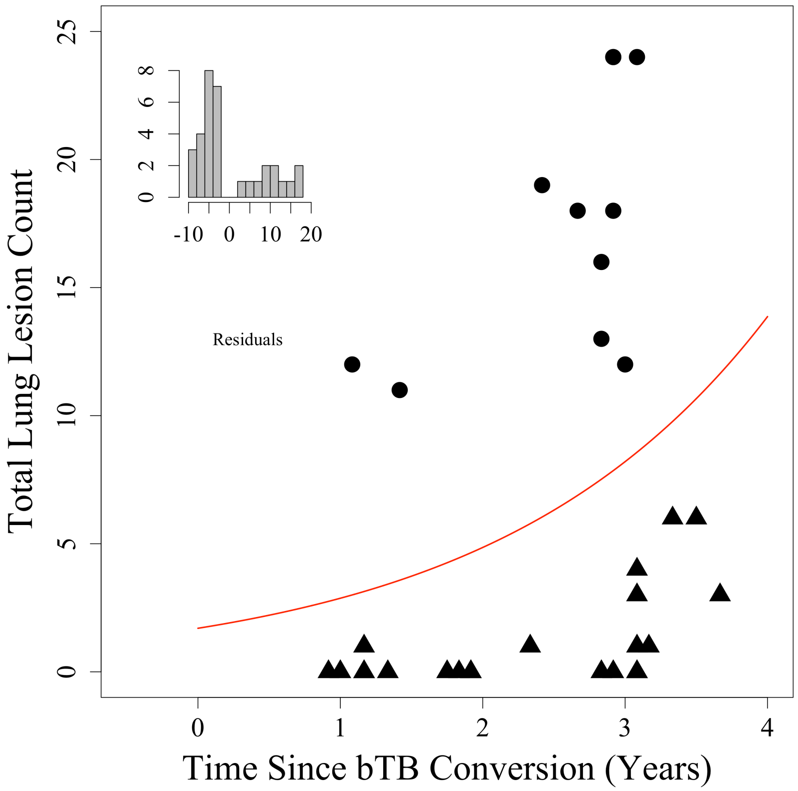

Figure 2.2: Number of bovine tuberculosis (bTB) lung lesions versus time since conversion to bTB positive. Here we see two distinct groups of animals on either side of the nonlinear prediction line. Those above the regression line have positive residuals and are considered proliferation susceptible (circles), since they have a higher number of lesions than would be expected give time since conversion. Those points below the regression line have negative residuals and are considered proliferation resistant (triangles) since they have less pathology than predicted by the time since conversion to bTB positive. The inset histogram of residual values shows these two distinct groups.

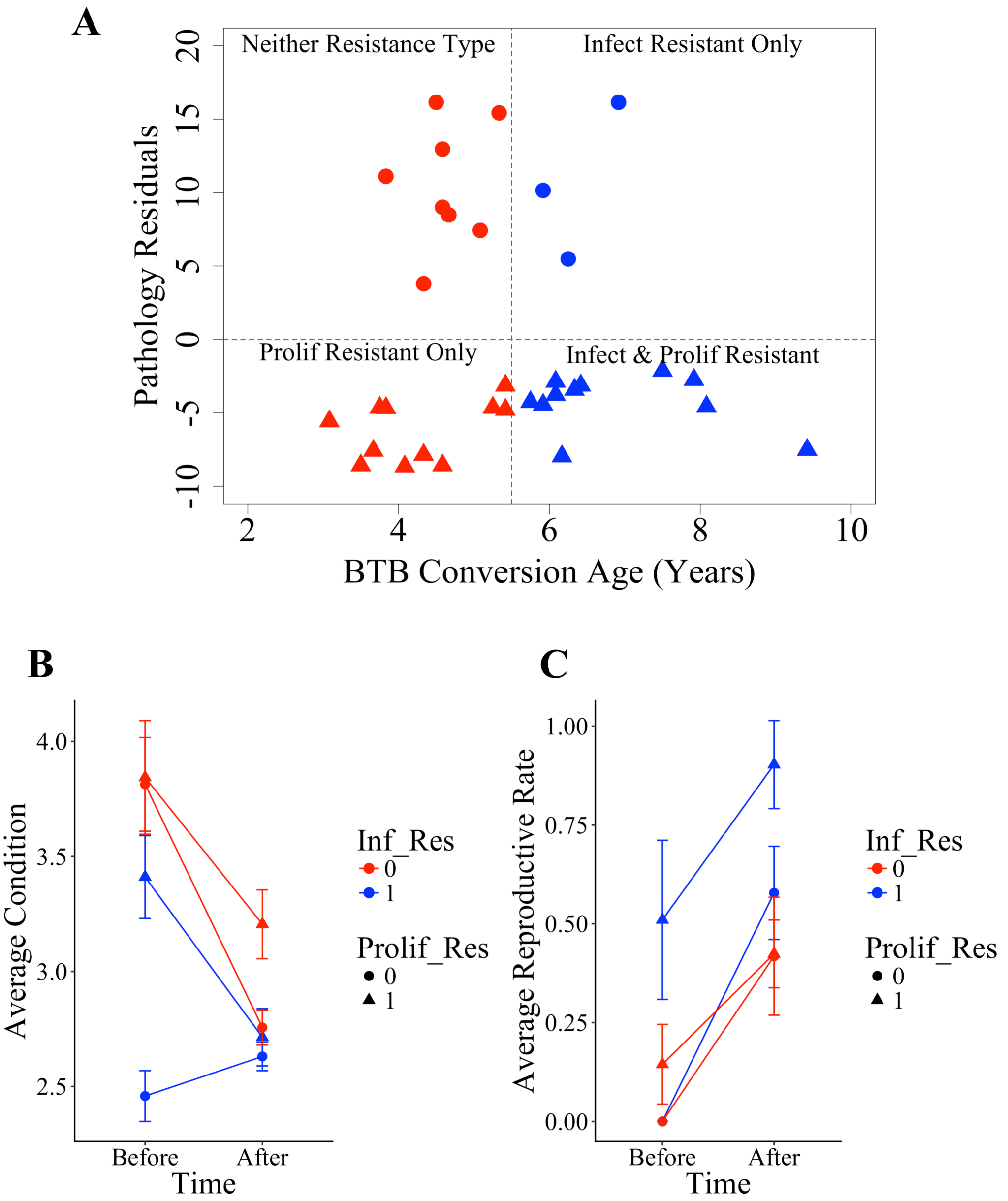

Figure 2.3: (A) Lung pathology residual score (proliferation resistance) over conversion age (infection resistance). Groupings used for cost analyses are based on residuals (a negative value implies less pathology than predicted and therefore proliferation resistance) and conversion age (those that converted after the mean conversion age of 5.5 years are considered infection resistant). Clockwise from upper left, groups include "Neither Resistance Type" (n_n=8), "Infection Resistant Only" (n_i=3), both "infection and proliferation resistant" (n_b=11), or "Proliferation Resistant Only" (n_p=11). Reaction norms depict the difference in condition (B) and reproductive rate (C) in our four resistance groups before and after bovine tuberculosis (bTB) conversion. We see a distinct cost of infection resistance in the form of lower body condition before the onset of infection. Animals that were both proliferation resistant and infection resistant had the highest overall reproductive rate.

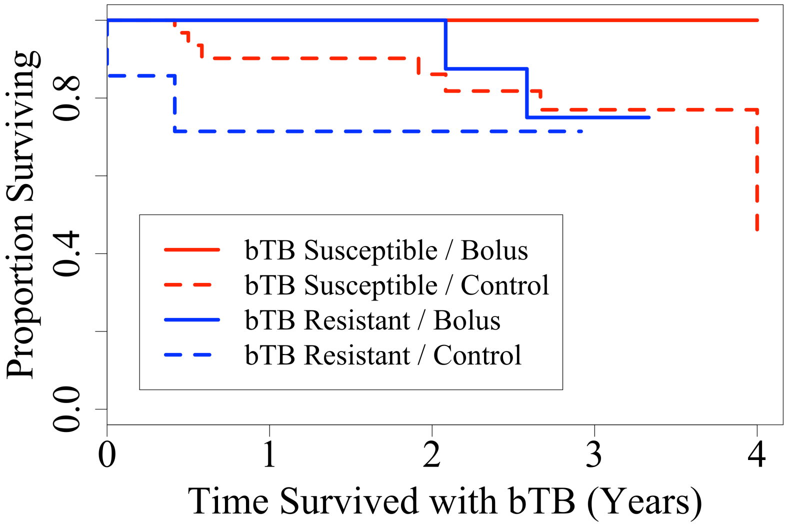

Figure 2.4: Survival curves for infection resistant and infection susceptible animals following bovine tuberculosis (bTB) infection grouped by anti-helminthic treatment group. Though bolus had significant, positive effects on lifespan in both resistance groups, infection resistance increased risk of death by seven fold. Though herd was a significant predictor of death risk in the model, for simplicity it is not included in this figure.

CHAPTER 3

From genes to inflammation: the genetic basis for resistance to bovine tuberculosis in African buffalo is reflected by innate cytokine production

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ABSTRACT

As the most general and conserved branch of the immune system, the innate response serves as a likely target for pathogen-mediated selection. Though innate receptors are highly conserved across taxa, many pathogens have evolved mechanisms to evade immune detection and resulting inflammatory cascades. *Mycobacterium bovis* is one of these pathogens, having evolved the ability to avoid recognition by alveolar macrophages. Here we use a genome-wide association study to identify candidate loci associating with variation in time to onset of bTB infection in African buffalo. We identify two loci near genes involved in oxidative burst and TLR4-mediated apoptosis that increase risk of bTB infection by 4 fold. Furthermore, genotype at one locus of interest is predictive of IL-12 production in whole-blood stimulation assays, with susceptible animals producing less IL-12 than those that converted later in life. Taken together, these results suggest high variation in phagocyte function and activation among resistance genotypes that corresponds directly to differences in bTB risk. Here we provide a unique example of measureable differences in inflammatory phenotype affecting resistant phenotype and a putative genetic basis for resistance in this system.

INTRODUCTION

Interactions among hosts and their parasites can be strong drivers of evolution. As stated in the Red Queen hypothesis, both parasite and host must aggressively participate in the co-evolutionary arms race in order to maintain the same relationship, and therefore remain extant (van Valen, 1973). Parasites and associated host defense mechanisms can dictate host resource partitioning, often having direct effects on life history traits, behavior, or sexual selection (Agnew et al., 2000; Hamilton and Zuk, 1982; Poulin, 1995; Zuk and Stoehr, 2002). Understanding the genetic basis for these host defense mechanisms is paramount to untangling complex co-evolutionary relationships among hosts and their parasites.

As the first line of host defense once a pathogen invades, the innate immune system represents a likely target for pathogen-mediated selective pressures within the co-evolutionary arms race, specifically pertaining to host infection resistance (i.e., the ability to prevent infection). Invading pathogens face an immediate onslaught of constitutively expressed innate immune components, including, but not limited to, antimicrobial peptides (AMPs), complement, and other pathogen-specific binding proteins, such as pathogen recognition proteins, C-reactive proteins, and anti-microbial lectins (Basset et al., 2003). Upon identification by cell-associated pattern recognition receptors (PRRs), pathogens face a secondary attack, usually resulting in phagocytosis and associated oxidative burst and degradation (Basset et al., 2003; Kumar et al., 2011; Mogensen, 2009). Though PRRs and other innate recognition mechanisms are based on pathogen components which are highly conserved and often integral to pathogen survival, many pathogens have evolved mechanisms by which they circumvent detection and degradation within host immune cells (Hornef et al., 2002). This evasion of detection often results in a sub-optimal or non-existent inflammatory response from innate effector cells and a breakdown in infection resistance, blocking the downstream integration of adaptive immune effectors, and leading to chronic disease (Finlay and McFadden, 2006).

Multiple species in the *Mycobacterium* genus have evolved complex evasion mechanisms that dramatically reduce host detection, resistance to infection, and resulting targeted immune responses (Raja, 2012). Initially, mycobacterial pathogens are bound by alveolar macrophage toll-like receptors (TLR2 or TLR4) and phagocytized. Binding of TLR2 or TLR4 normally results in macrophage activation and production of pro-inflammatory cytokines or apoptosis, but mycobacteria have evolved mechanisms to block activation of their host cells (Sanchez et al., 2010). Furthermore, once phagocytized, pathogenic strains of mycobacteria can block the maturation of the phago-lysosome, escaping into the cytosolic compartment to replicate, resulting in necrosis, instead of controlled apoptosis, thereby promoting bacterial spread (Kaufmann, 1991; Kornfeld et al., 1999; Welin and Lerm, 2012). Regardless of the specific evasion mechanism, mycobacterial infection often manifests as a reduction in alveolar macrophage activation and resulting pro-inflammatory signaling, specifically interleukin-12 expression (Cooper et al., 2007; Jouanguy et al., 1999).

The capacity of mycobacteria to evade host immune recognition and degradation make them both effective pathogens and interesting organisms within a co-evolutionary context. *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB) is a broad host range zoonotic pathogen capable of infecting most mammals (Ayele et al., 2004). African buffalo (*Syncerus caffer*) are gregarious, long-lived mammals that host a myriad of parasites, including *M. bovis*, likely making infection a strong driver of evolution in this system. Furthermore, bTB has been shown in previous buffalo studies to alter host infection patterns and dramatically impact host fitness (Beechler et al., 2011; Ezenwa and Jolles, 2011; Ezenwa and Jolles, 2015; Gorsich et al., 2011). Therefore, the African buffalo- bTB system offers a novel chance to study ecology and evolution of host-parasite interactions in a long-lived mammal. In Chapter 2 of this dissertation, we showed that there is high variation in resistance phenotype in this system, and that some of this variation is heritable. Since heritable traits must have a genetic basis, we conducted a genome-wide association (GWA) study to determine what genes associate with variation in resistance to bTB infection in African buffalo. Additionally, to tie bTB susceptibility to differences in inflammatory response and immune activation, we link infection resistance genotype to 'inflammatory phenotype' using whole blood stimulation tests for cytokine production.

METHODS

Study Area and Field Data Collection

Two hundred sub-adult and young adult female African buffalo (*Syncerus caffer*; initial ages 2-7 years) were captured every six months in the southern part of Kruger National Park, South Africa between June 2008 and August 2012 as part of a longitudinal study of co-infection and the consequences of anti-helminthic treatment (for more detail, see Beechler et al., 2011; Ezenwa and Jolles, 2015; Gorsich et al., 2011). Buffalo were sampled from two distinct herds occurring in the Crocodile Bridge and Lower Sabie areas of the park. The Crocodile Bridge herd includes buffalo in the area around the Crocodile River in the southeast extent of the park, while north of this the Lower Sabie herd includes buffalo near the eastern reaches of the Sabie River. These herds will hereafter be referred to as "CB" and "LS." Estimated herd sizes during the study period for CB and LS were 2,100 and 1,100 buffalo, respectively.

Each buffalo was fitted with either a radio (n=193) or satellite (n=7) collar with a high frequency VHF transmitter upon first capture, that was then used to locate them for subsequent captures at roughly six month intervals. Individuals lost to death or emigration during the study period were replaced to maintain a constant sample size of 200 animals spread equally among the two herds. Of these animals, half (n=50 per herd) were randomly chosen to receive an anti-helminthic bolus (slow-release fenbendazole (Panicur, Hoechst Roussel)) as part of the study design outlined in Ezenwa and Jolles (2015).

At each capture, animals were immobilized by dart from a helicopter or truck using etorphine hydrochloride (M99, Captivon, Karino, South Africa). Following data collection, immobilization was reversed using diprenorphine (M5050) or naltrexone (40 mg/ml, Kyron). Animals were kept under observation until fully recovered and all immobilizations were conducted by a veterinarian according to the South African National Parks Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. All animal work for this study was approved by the institutional animal care and use committee at Oregon State University (ACUP #3267).

Age at each capture was determined in young animals by tooth emergence and in older animals by wear pattern per established methods in this species (Jolles, 2007). Pregnancy status and stage were determined by rectal palpation done by a wildlife veterinarian. This method shows 100% sensitivity in Egyptian buffalo after 51 days of gestation (*Bos bubalis*; Karen et al., 2011). The presence of a calf at heel was detected visually or by evidence of lactation (manual milking of all four teats; Jolles et al., 2005), and calves were aged by body size.

Body condition was assessed on a five-point scale through palpation and visual inspection of four areas where buffalo deposit fat: spine, hips, ribs, and base of tail. Condition ranged from 1 (very poor) to 5 (excellent) at each area, and was then averaged across these areas for an overall estimate of condition. This method has been shown to correlate with fat deposits in the liver (Ezenwa et al., 2009).

bTB Testing and Cytokine Stimulations

All blood samples for disease diagnostics were collected within fifteen minutes of sedation and held on ice before bovine tuberculosis (bTB) testing. bTB infection was determined with the commercially available whole-blood gamma interferon (IFNγ) assay (BOVIGAM, Prionics, Switzerland). This assay measures the difference in IFNγ production of whole blood in response to incubation with bovine versus avian tuberculin antigens, while controlling for differences in background IFNγ levels. Individual samples were called as bTB positive or negative based on absorbance thresholds optimized for bTB infection in African buffalo (Michel et al., 2011). We obtained a time series of 2-9 bTB tests for each animal and used the full time series to more confidently assign bTB status (Ezenwa and Jolles, 2015). Animals with at least two consecutive positive bTB tests were assigned as bTB positive. Since bTB is chronic in buffalo and there is no evidence that they recover once infected we assumed animals stay positive until death (Bengis, 1999). Animals with alternating test results or short observation periods (<3 captures) were not included in our dataset.

At each capture we assessed host T_H1 and T_H2 immune function by measuring levels of interferon gamma (IFNγ), interleukin 12 (IL-12), and interleukin 4 (IL-4). These cytokines are indicative of each aforementioned immune branch, and INFγ and IL-12 have been identified as key components of effective immune responses during tuberculosis infection (Jouanguy et al., 1999; Trinchieri, 2003). Peripheral blood was collected from the jugular vein of immobilized buffalo and stored in heparinized tubes on ice in the field until processing that day. We used cytokine-specific ELISAs (Abd Serotec) to quantify levels of each cytokine following stimulation with pokeweed mitogen (*Phytolacca americana*; Sigma). Pokeweed is well established as a mitogen that illicits a T cell-dependent immune response in peripheral blood samples (Keightley et al., 1976; Moretta et al., 1979; Stanilova et al., 2005). Whole blood was incubated with 0.3mg/ml of pokeweed mitogen for twenty-four hours at 37 degrees Celsius, after which samples were spun, and the plasma supernatant was removed and stored at -20 degrees Celsius until ELISA testing. To control for variation in plasma storage time and ELISA plate effects, we included either storage time or plate as a random effect in subsequent mixed effect models.

SNP Genotyping and filtering

We extracted 100-200ng/ml genomic DNA from dried ear tissue samples (DNeasy blood & tissue kit, Qiagen) and prepared individual libraries for sequencing using type IIB restriction associated DNA (2bRAD) methods, detailed in Wang et al. (2012). Briefly, this method used a type IIB restriction endonuclease (Alf1; Thermo Scientific #ER1801) to produce hundreds of thousands of 36bp reads from across the genome. We prepared genotyping libraries using reduced tag representation (RTR) as described in Wang et al. (2012) by ligating adaptors with 3' overhangs ending in NC and NG. Samples were sequenced on an Illumina HiSeq 3000 sequencer at the Oregon State University Center for Genome Research and Biocomputing. We excluded terminal tag positions, ambiguous base calls, long homopolymer regions, and excessively low quality reads (>5 positions with quality <10). After trimming, the remaining high quality reads were retained for all subsequent mapping and genotyping. Using custom Perl scripts, we extracted all AlfI sites (n=480,162) from the recently available *Syncerus caffer* genome (Glanzmann et al., 2016). We then used SHRiMP to map each individual sample to these sites and filter the resulting matches for statistically weak or ambiguous alignments using parameters similar to those described by the software authors (Rumble et al., 2009). We determined genotypes at each AlfI site with >10x coverage, then filtered out any monomorphic loci. We allowed for 10% missing data at any given locus and one polymorphism per tag. Animals that were genotyped at 5000 or fewer loci were removed from the dataset. We used custom scripts to extract scaffold and position information of single nucleotide polymorphisms (SNPs) for population structure analysis. Markers were discarded if they were not biallelic, violated Hardy Weinberg Equilibrium (p<0.0001), or had a minor allele frequency less than 5%. To assess independence of each marker, we used r^2 to quantify linkage disequilibrium (LD) among all pairwise combinations of SNPs and removed markers in high LD. r^2 is a robust LD metric in SNP datasets with highly variable minor allele frequencies because it accounts for this variation in allele frequencies between markers, where D’ does not (VanLiere and Rosenberg, 2008). Of the

SNPS tested, 15 pairs were in significant LD ($r^2>0.5$), 13 of which were within 100kb on the same scaffold. We therefore removed the four pairwise SNPs in high LD that were not physically linked, since these markers are not independent. Ultimately, filtering yielded 187 usable buffalo samples genotyped at 1480 SNPs.

To test whether the two herds sampled were genetically distinct, we calculated global F_{ST} and performed a principal components analysis (PCA) using filtered markers in the R packages *hierfstat* (Goudet, 2005) and *adegenet* (Jombart and Ahmed, 2011), respectively.

Genome-wide Association (GWA)

Here we evaluated bTB infection resistance in African buffalo as variation in time to onset of infection (i.e., conversion age). Since per-capita incidence was previously shown to be equal among herds, we assume uniform exposure in this system (Ezenwa and Jolles, 2015). One would expect stochastic variation in time to infection, but on average, more resistant animals should become bTB positive later in life.

We used right-censored Cox proportional hazards models to identify SNPs associating with variation in time to onset of bTB, and therefore infection resistance. Buffalo that converted during the study or never converted to bTB positive were included in the analysis (n=160), while animals that were bTB positive at first capture were excluded since their conversion age could not be determined. We tested associations with each SNP genotype and allele separately using custom scripts and the R package *survival* (Therneau, 2015). Best-fit models for time to onset of bTB conversion included genotype or allelic main effects, as well as bolus treatment, herd, and initial age as covariates. We also controlled for the effects of background relatedness in our models by including the first five axes of the relatedness PCA (above) as fixed effects (Price et al., 2006). Resulting p-values for SNP genotypes or alleles were corrected for false discovery rate within each model using established methods (Benjamini and Hochberg, 1995; n=1480). Resulting corrected q values were considered significant at $q<0.1$ and highly significant at $q<0.05$.

Since none of the significantly associating SNPs fell within gene coding regions, we calculated mean linkage block size to determine an appropriate window around each SNP within which to search for putative candidate genes. We calculated r^2 values for all physically linked pairwise SNPs that occurred within 100kb on the same scaffold, then determined the mean distance at which r^2 was greater than 0.9, and therefore reflected the average linkage block size for markers in high linkage disequilibrium. We found, on average, linkage persisted within a 29kb block.

Inflammatory Phenotypes

Here we define variation in 'inflammatory phenotype' as any measurable difference in cytokine production among individuals. IFNγ and IL-12 are important signal cytokines during the T_H1 and phagocyte response to invading *Mycobacterium spp* (Jouanguy et al., 1999). Furthermore, one SNP in this study associating with variation in time to bTB occurred near two genes with potential function in phagocyte apoptosis and innate pathogen degradation. We therefore compared SNP genotype at this locus with IFNγ, IL-4, and IL-12 production to determine if variation in immune function could be linked to bTB infection resistance genotype. If SNP genotype is indicative of variation in phagocytic response, we would expect that IL-12 production would differ among SNP genotypes since activated phagocytes release IL-12 (Fulton et al., 1996). In contrast, we would expect no difference in INFγ or IL-4 production among genotypes, since these cytokines are a proxy for T_H1 and T_H2 immune function, respectively, and can be produced independently of phagocyte activation (Constant and Bottomly, 1997).

When applicable, normality was assessed in all models using data visualization and Shapiro-Wilks testing. All model selection was done using Akaike's Information Criterion (AIC), conditional R^2 (mixed models), and residual plot visualization (Burnham and Anderson, 2002), unless otherwise noted. All statistics were run in R version 3.2.4 (R Core Team, 2016).

RESULTS

Summary statistics

Observed and expected heterozygosity for each of the 1480 SNPs ranged from 0.0604 to 0.5839 (median 0.2177) and 0.0950 to 0.500 (median 0.2188), respectively. A global F_{ST}, value of 0.0003 suggests population genetic differentiation among the two herds is very low. Furthermore, principle components analysis (PCA) revealed no obvious clustering among individuals that would be indicative of genetic groups (Figure 3.1). These results agree with previously reported behavioral observations of frequent herd switching and long distance dispersal in African buffalo (Caron et al., 2016; Halley et al., 2002; Naidoo et al., 2014), including in our herds (Spaan, 2015). Therefore, we consider any effects of herd in subsequent analyses to be environmental.

GWA

GWA yielded consistent support for strong associations between time to onset of bTB and two SNPs on two separate scaffolds (Figure 3.2). These SNPs remained highly significant ($q<0.05$) after false discovery rate correction in models with genotype as a main effect, and moderately significant ($q<0.10$) in models with allele as a main effect (Table 3.1). We found the rare allele at either of these loci conferred a 3.98 (SNP2253) and 3.74 (SNP3195) -fold increase in risk of converting to bTB positive in this population of buffalo (Figure 3.3). Due to low frequency of the risk allele at both loci (0.099 and 0.142), homozygotes for each risk allele were rare, making the magnitude of risk equal across genotype and allele models for each SNP (i.e., heterozygotes and risk allele homozygotes are at equal risk of conversion). Additionally, due to a small number of risk allele homozygotes ($n_{2253}=1$ and $n_{3195}=3$), we lacked the power to assess an additive effect of the risk allele at either locus. As these loci were not in linkage disequilibrium and did not have a multiplicative effect on disease risk when included in the same model, we conclude that the association of each locus is likely driven by a unique resistance mechanism. We determined the average linkage block size for markers in high LD ($r^2>=0.9$) to be 29kb and therefore used this distance to guide our search for genes near each SNP that represent potential underlying infection resistance mechanisms. Within 29kb of SNP2253, we found one gene: peroxisomal membrane protein *PEX14* (18kb downstream). We found no annotated genes within 29kb of SNP3195.

Inflammatory Phenotypes

PEX14 plays an integral part in peroxisome generation, degradation, migration, and modulation of oxidative stress within the cell through the production and scavenging of reactive oxygen and nitrogen species (ROS and RNS; Fransen et al., 2012). Within macrophages, peroxisomes play a role in acidification of the phagosome and inflammasome activation through the production of ROS/RNS, therefore affecting the cell's inflammatory state directly (Demarquoy and Borgne, 2015; Eguchi et al., 1979). Because macrophages (specifically alveolar macrophages) are an important cell lineage for both innate respiratory pathogen recognition and the persistence of bTB infection (discussed below), we compared cytokine production across all captures among animals with and without the risk allele at SNP2253. Furthermore, by testing levels of IFNγ, IL-4, and IL-12 cytokines, we could roughly test at what point T-cell and phagocyte activation differs among SNP2253 genotypes and how this corresponds to inflammatory phenotype. We found that animals heterozygous or homozygous for the risk allele (G) at SNP2253 produced 34% less IL-12 than animals with the CC genotype following whole blood stimulation, while allelic variation at SNP2253 did not predict differences in IFNγ or IL-4 production, nor was IL-12 production affected by bTB status (Table 3.2 and Figure 3.4). These results suggested no difference in activation of type 1 and type 2 T-helper (T_H1 & T_H2) cells among SNP2253 genotypes, as seen in unchanged IFNγ and IL-4 production respectively, but that the expression of IL-12 from activated phagocytes in the blood (monocytes/macrophages) is reduced in animals possessing the risk allele. In contrast, we observed no significant patterns in cytokine production relative to SNP3195 genotype.

DISCUSSION

Here we identify two genomic regions highly associated with time to onset of bTB infection in African buffalo. One locus, which increases risk of bTB conversion by nearly 4 fold, is near *PEX14*, a gene with putative roles in intracellular respiratory burst, pathogen degradation, and inflammasome-mediated apoptosis (Eguchi et al., 1979; Fransen et al., 2012). Furthermore, production of IL-12 during whole blood stimulation is reduced in animals possessing the risk allele at this locus. Taken together, these putative candidate mechanisms and a reduction in IL-12 relative to genotype suggests appreciable differences in inflammatory phenotype among buffalo of different genotypes at SNP2253 which directly relates to time to onset of bTB infection.

Interleukin-12 (IL-12) is a pro-inflammatory cytokine released by classically activated monocytes, macrophages, neutrophils, or dendritic cells which causes activation and proliferation of $CD8^+$ T cells and is considered an important signaling cytokine for the T_H1 response against intracellular pathogens (Trinchieri, 2003). Most commonly, innate effector cells (mononuclear phagocytes) undergo activation through pathogen recognition via membrane-bound toll-like receptors (TLRs), or by cytokine stimulation from T_H1 cells producing IFNγ (Goerdt et al., 1999). Since pokeweed mitogens are used to test the efficacy of T-dependent immunity, we conclude that phagocytes classically activated by IFNγ are producing the IL-12 present in stimulated blood. We observed no difference in IFNγ production among SNP2253 genotypes, and thus conclude that the activation of T_H1 T cells is not directly affected by this locus. However, the observed reduction in IL-12 suggests differences in phagocyte activation among SNP2253 genotypes, and may be a putative mechanism for differences in bTB susceptibility in this population.

PEX14 is an integral part of the translocon pore (i.e., allows protein transport across the peroxisomal membrane) and represents a limiting reactant in the importation machinery of the peroxisome (Galiani et al., 2016; Komori et al., 1997). PEX14 also plays a critical role in both peroxisome formation and degradation, processes paramount to the control of cellular oxidative stress and respiratory burst (van Zutphen et al., 2008). Through the creation and scavenging of ROS and RNS, peroxisomes are able to modulate respiratory burst in several ways to either promote or block inflammatory responses. In plants, peroxisomal ROS production has been directly linked to inducible pre-invasion resistance mechanisms (Lipka et al., 2005). In animals, peroxisomes can modify cellular ROS concentrations leading to the activation of the inflammasome system, resulting in apoptosis in macrophages and increased local inflammation (Demarquoy and Borgne, 2015). Additionally, peroxisomes have been shown to co-localize with the macrophage phagosome during acidification, confirming their role in pathogen degradation (Eguchi et al., 1979; Fransen et al., 2012). Peroxisomes are also important organelles for the maintenance of anti-inflammatory state in non-activated immune cells. Expression of a related nuclear family receptor and transcription factor, peroxisomal proliferation-activation receptor gamma (*PPARγ*), acts to suppress the NF-kappa-B pro-inflammatory apoptotic cascade. In alveolar macrophages, *PPARγ* expression levels co-vary with CD36 expression, increasing uptake of apoptotic neutrophils and promoting an anti-inflammatory state in surrounding lung tissue (Asada et al., 2004). When *PPARγ* is down-regulated following TLR4 activation of these macrophages during infection, this leads to a pro-inflammatory state and apoptosis in infected cells (Chawla et al., 2001; Necela et al., 2008). Interestingly, another nearby gene, DNA fragmentation factor subunit alpha (*DFFA*; 35kb upstream of SNP2253), serves as the substrate for caspase-3 during TLR2 or TLR4-mediated apoptosis (Li et al., 2005). Though not within the linkage block distance of SNP2253, this gene adds evidence that differences in average time to onset of bTB may reflect underlying differences in phagocyte-mediated pathogen degradation, programmed cell death due to infection, and resulting IL-12 inflammatory phenotype.

Mycobacterial pathogens have evolved many mechanisms through which they evade immune recognition including the reduction of antigen presentation in phagocytes, active scavenging of ROS to circumvent the effects of cellular oxidative burst, the production of suppressive cytokines, and escape from the phago-lysosome during degradation (Raja, 2012). High variation in mycobacterial clearance in alveolar macrophages has been observed, as highly virulent strains of *Mycobacterium tuberculosis* often escape the phagosome, block host cell apoptosis, and are able to replicate in the cytosolic space, effectively avoiding a humeral immune response (Kaufmann, 1991; Kornfeld et al., 1999). The balance between host immune recognition and mycobacterial evasion determines disease outcome at the animal level, leading to successful immune clearance or localized tissue necrosis and morbidity (Welin and Lerm, 2012). Commonly, *M. tuberculosis* persists by evading macrophage apoptosis through the disruption of the TLR2 or TLR4 mediated apoptotic pathway (Sanchez et al., 2010). Since macrophages are often classically activated through these TLR pathways, we would expect differences in inflammatory phenotype among hosts with different disease outcomes, particularly pertaining to IL-12 production. In fact, IL-12 has been repeatedly identified as an important aspect of anti-mycobacterial immunity (Cooper et al., 2007; Jouanguy et al., 1999). IL-12 is highly up-regulated locally in humans with active pulmonary tuberculosis (Eruslanov et al., 2004; Taha et al., 1997; Taha et al., 1999; Zhang et al., 1994), and high variability has been demonstrated in IL-12 expression across murine families of varying *M. tuberculosis* susceptibility (Eruslanov et al., 2004). Additionally, mutations in IL-12 and IL-12 receptor genes have been clearly linked to mycobacterial susceptibility (Abhimanyu et al., 2012; Ottenhoff et al., 1998).

Here we demonstrate the first instance, to our knowledge, of differences in IL-12 production directly relating to bTB infection susceptibility and resistance in a wild mammal. Furthermore, genomic regions associating with time to onset of bTB contain genes directly related to inducible anti-bacterial immunity and apoptosis of infected phagocytes. Though we cannot provide an absolute mechanism of bTB resistance, some breakdown in phagocyte activation and resulting IL-12 production is a likely candidate for variation in susceptibly and inflammatory phenotype in this system.

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TABLES

Table 3.1: Cox proportional hazards models for time to onset of bTB infection including SNP genotype and allelic effect for the two significantly associating SNPs (n=160). All estimates are back-transformed and represent a multiplicative increase in risk of bTB conversion. For genotype models only significant pairwise comparisons are shown.

Model	Estimate	P-value
A. Time to onset of bTB x SNP2253 Genotype		
PC1	0.989	0.743
PC2	0.994	0.812
PC3	1.000	0.989
PC4	1.010	0.781
PC5	1.048	0.159
Treatment- Control	0.922	0.793
Herd- Lower Sabie	1.551	0.156
Initial Age (years)	0.609	<0.0001
SNP2253 genotype- CC vs CG	3.98	<0.0001
R^2=0.246		

Model	Estimate	P-value
B. Time to onset of bTB x SNP2253 Allele		
PC1	0.989	0.743
PC2	0.994	0.812
PC3	1.000	0.989
PC4	1.010	0.781
PC5	1.048	0.159
Treatment- Control	0.922	0.793
Herd- Lower Sabie	1.551	0.156
Initial Age (years)	0.609	<0.0001
SNP2253 Allele C	1.068	0.952
SNP2253 Allele G	3.98	<0.0001
R^2=0.246		

Model	Estimate	P-value
C. Time to onset of bTB x SNP3195 Genotype		
PC1	1.001	0.972
PC2	1.019	0.484
PC3	0.966	0.289
PC4	0.970	0.351
PC5	1.084	0.018
Treatment- Control	0.961	0.897
Herd- Lower Sabie	1.956	0.037
Initial Age (years)	0.605	<0.0001
SNP3195 CC vs CT	3.74	<0.0001
R^2=0.295		

Table 3.1 (continued)

Model	Estimate	P-value
D. Time to onset of bTB x SNP3195 Allele		
PC1	1.001	0.972
PC2	1.019	0.484
PC3	0.966	0.289
PC4	0.970	0.351
PC5	1.084	0.018
Treatment- Control	0.961	0.897
Herd- Lower Sabie	1.956	0.037
Initial Age (years)	0.605	<0.0001
SNP3195 Allele C	0.069	0.585
SNP3195 Allele T	3.743	<0.0001
R^2=0.295		

Table 3.2: Mixed effect maximum likelihood models for longitudinal production of (A) IL-12, (B) IFNγ, and (3) IL-4 by presence-absence of the SNP2253 risk allele (G; n= 370 blood samples from 129 animals over a 3 year period). Production of each cytokine was measured in ng/ml following incubation of whole blood with a pokeweed mitogen. Estimates in the IL-12 and IFNγ models are back-transformed and represent a multiplicative increase in cytokine production. Only significant pairwise comparisons among capture periods are shown (Bonferroni corrected alpha=0.005). Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. ln(IL-12 production)	248.7	<0.0001
Capture 1 vs 5	0.509	0.002
Herd- Lower Sabie	1.950	<0.0001
SNP2253 Allele G	0.660	0.006
B. ln(IFNγ production)	0.622	<0.0001
Treatment- Control	0.862	0.123
SNP2253 Allele G	0.910	0.449
C. IL-4 production	495.7	<0.0001
Herd- Lower Sabie	-59.66	0.061
TB Status	36.44	0.363
Age (years)	-11.73	0.112
SNP2253 Allele G	17.57	0.633

<u>FIGURES</u>

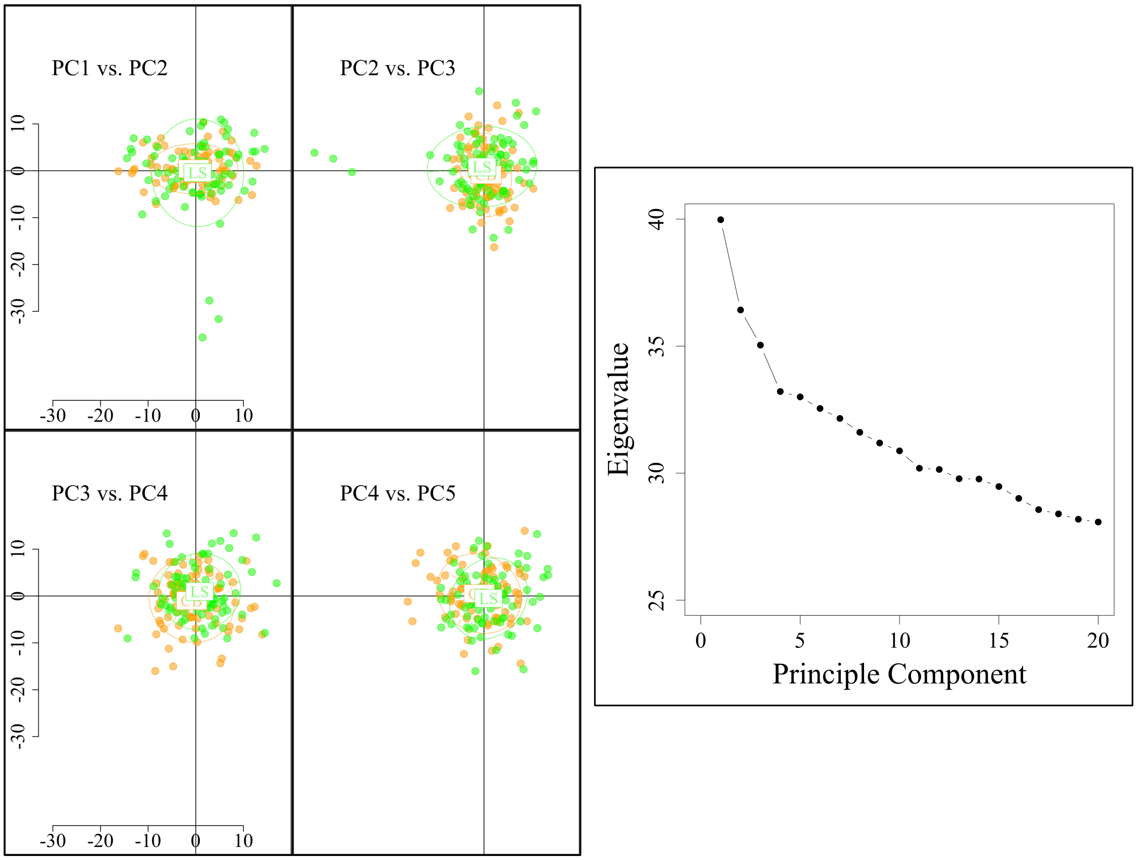

Figure 3.1: Principal components analysis of the genome-wide relationship matrix based on 1480 SNPs. The first four panels (left) show scatterplots of sequential combinations of the first five principle components in the analysis, color-coded by sample herd (“LS”= Lower Sabie, “CB”= Crocodile Bridge). Here we see no obvious clustering based on herd membership. The right panel plots the eigenvalues for the first twenty principle components. We see the decrease in eigenvalue levels off after principle component 4.

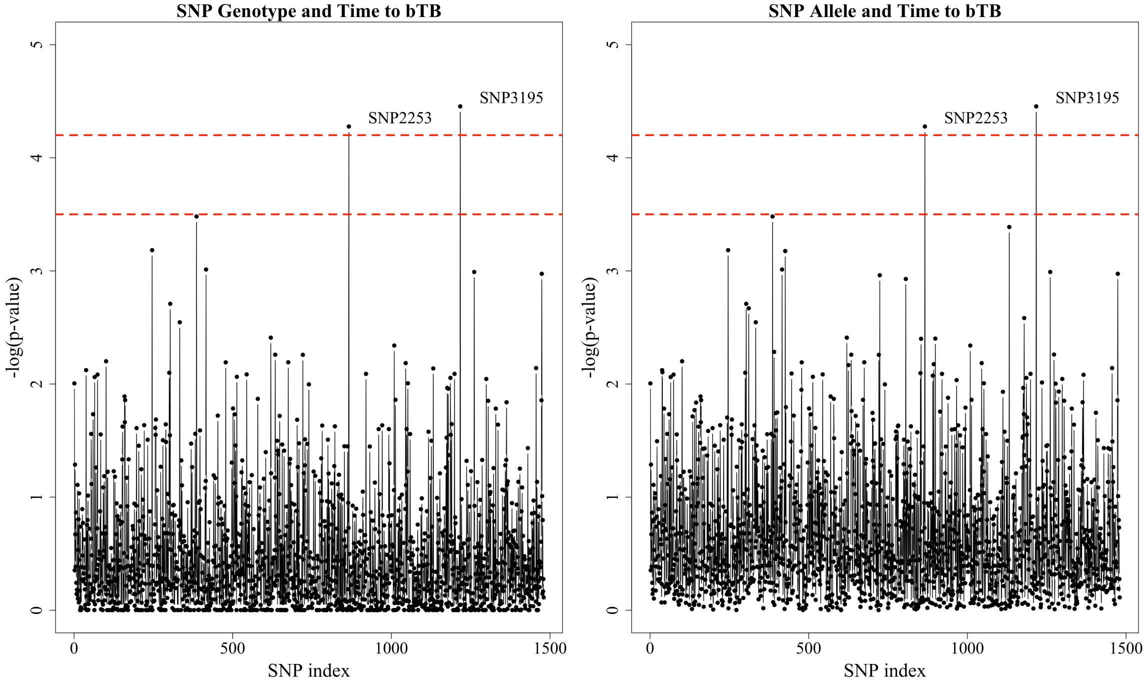

Figure 3.2: Manhattan plots depicting association of SNPs in both genotype (left) and allele (right) Cox proportional hazards models for time to onset of bTB. Significance is presented on the y-axis as negative log-transformed p-values and significant SNPs are labeled. Red lines denote significance thresholds for FDR-corrected q-values of 0.10 (lower) and 0.05 (upper). SNP2253 and SNP3915 are significantly associated with time to onset of bTB conversion in both models.

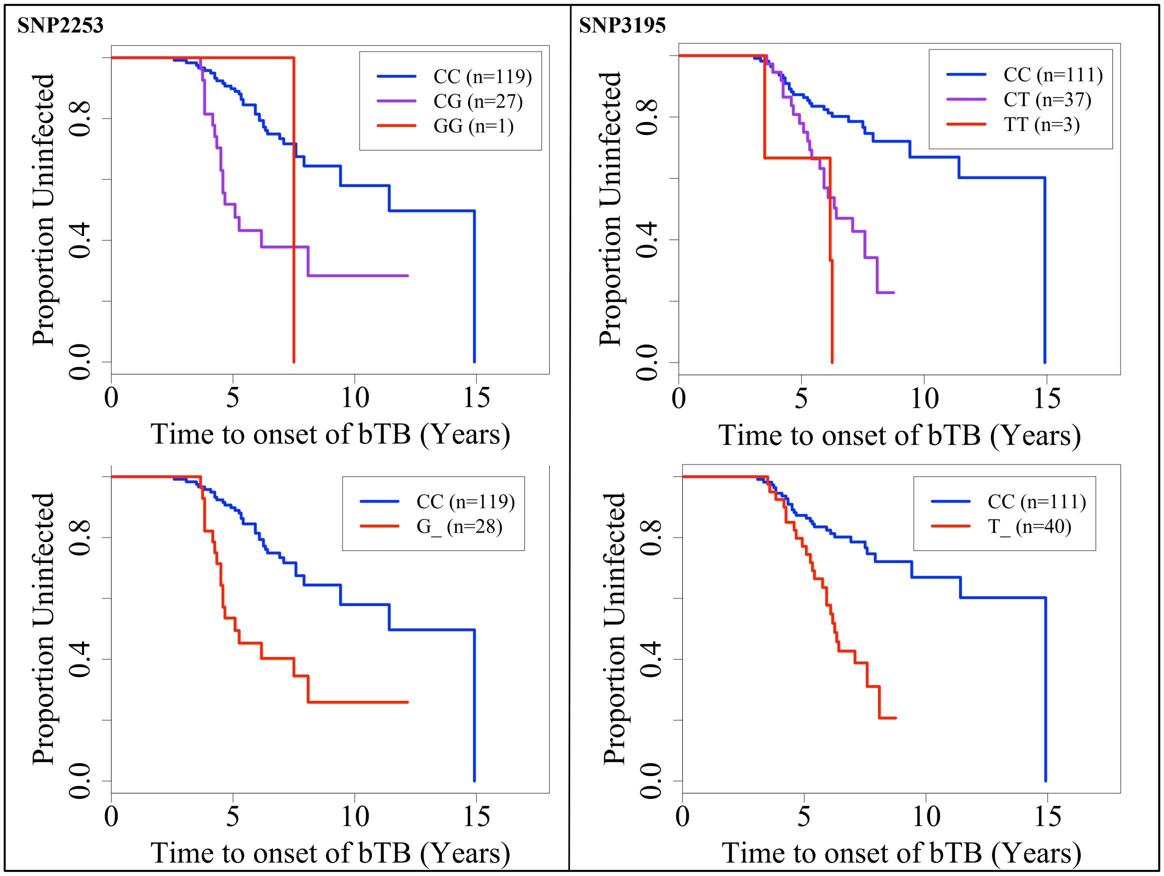

Figure 3.3: Kaplan-Meyer time-to-event curves by SNP genotype (top) and alleles (bottom) for SNP2253 (left) and SNP3195 (right). At both loci, the less common allele confers a greater risk of bTB infection in both the genotype and allele models.

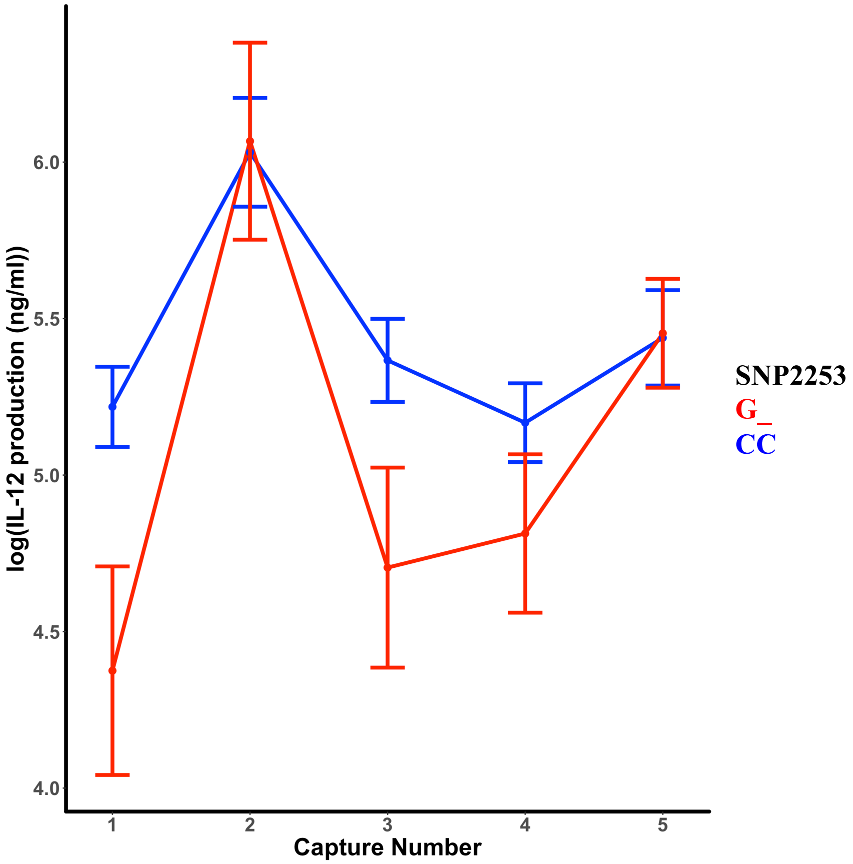

Figure 3.4: Interleukin 12 (IL-12) production by SNP2253 genotype. Animals heterozygous or homozygous for the 'G' risk allele (red) produce 34% less IL-12 than CC animals following pokeweed mitogen stimulation of whole blood.

CHAPTER 4

Cream always rises to the top: all African buffalo calves get fatty milk, but those with fat moms get passive immunity

Hannah F. Tavalire, Brianna R. Beechler, and Anna E. Jolles

ABSTRACT

Energetic trade-offs are an important driver of reproductive investment in wild systems. Trans-generational effects of limited resources can manifest as differences in maternal nutritional provisioning during lactation in mammals. Here we characterize differences in milk composition across lactation in African buffalo relative to maternal traits. We observed high variability in immune-active milk components relative to maternal age, size, and condition, but fat content remained relatively consistent across cows. Furthermore, we found that higher fat content in milk was predictive of higher survival to weaning and associated with increased immune function during early life. In contrast, we observed that immune-active components of milk – IgG and lactoferrin- generally associated with reduced immune function in claves, but higher growth (lactoferrin only). These findings are in agreement with general laboratory findings that maternally-derived immune components 'free' up energetic resources for growth in the calf by providing passive immunity during early life. In this system where resources are dramatically seasonally variable, we expect that selective pressures favor increased milk fat content because of its impact on survival to weaning, and that immune-active components are provided relative to maternal resource availability.

INTRODUCTION

A central question in ecology is how organisms regulate trade-offs between multiple physiological functions competing for limited resources. Balancing investment in both health and reproduction is critical, but can prove challenging. This delicate energy balance is further complicated in natural populations by seasonal and inter-annual fluctuations in resource availability (Stearns, 1976), making environmental context a main driver of trade-offs between maintenance, immunity, and reproduction (French et al., 2009).

Many studies examining energetic trade-offs are limited to offspring number or size as a measure of maternal investment. In mammals, lactation prolongs the period when resource allocation decisions must balance maternal and offspring needs, therefore representing a large portion of maternal cost (Rogowitz, 1996). Energetic trade-offs in wild mammal populations may result in context-dependent variation in milk composition: When resources are limited, we might expect to see a milk composition which ensures offspring survival, but does not provide excess nutrition to the neonate, as it is in a mother's best interest to minimize the energetic cost per unit offspring growth (Bernhart, 1961). Despite generalized expectations such as this, in fluctuating environments, there is still striking variation in resource partitioning among individuals (e.g., reindeer: Gjostein et al., 2004; rhesus macaques: Hinde et al., 2009; Iberian red deer: Landete-Castillejos et al., 2009; harbor seals: Lang et al., 2005). This inter-individual variation in the quality and composition of milk available to the offspring is often quantified as a 'maternal effect' (Rossiter, 1996). However, maternal effects are not limited to nutritional inputs alone, but can include transfer of antibodies and other immune components, as well as non-adaptive features of milk, such as bacterial load (Bernardo, 1996).

Mammals not only provide invaluable nutritional resources to the neonate through lactation, but many immune-active constituents as well (Carlier and Truyens, 1995; Grindstaff, 2008; Hasselquist and Nilsson, 2009). Discussions of passive transfer in bovids often credit antibodies received through colostrum as the primary means of neonatal immune protection (for example, Bolin and Ridpath, 1995; Hurley and Theil, 2011). However, in recent years, many studies have more closely examined milk, finding it to be full of immunomodulatory components that are not only present in colostrum, but fluctuate throughout lactation, including lactoferrin, cytokines, and live immune cells (Cross and Gill, 2000; Ewaschuk et al., 2011; Takahashi et al., 1992). Though these immune components have been well characterized in domesticated bovids (Stelwagen et al., 2009), few studies follow their effects on the growth and development of the neonate, especially in wild species.

Milk composition varies predictably over time in many species, optimizing protein concentrations for maximum growth of the neonate and minimum maternal cost (Bernhart, 1961; Ontsouka et al., 2003). African buffalo (*Syncerus caffer*) undergo parturition and conception around the time when grasses are at their highest protein content, showing plasticity in calving date depending on inter-annual variation in timing of the first green flush (Ryan et al., 2007). There is also a great deal of variation in calving date among individuals, with calving beginning in October and finishing as late as April (encompassing most of the wet season). Here we quantify maternal effects of rearing environment as variable milk composition in African buffalo. First, we characterize variation in energetic and immune components of milk, then determine the impacts of milk composition on calf survival, growth, and early immune function. To our knowledge, this is the first longitudinal study of lactational maternal effects in a wild mammalian population.

METHODS

Study System and Field Sample Collection

This work was part of a larger study monitoring the spread of foot and mouth disease virus in a herd of African buffalo (*Syncerus caffer*) in Kruger National Park, South Africa. This herd consists of ~70 animals in a 900 hectare enclosure near the Satara Rest Camp (central region of the park). Electrified perimeter fences exclude predators, and supplemental food and water are provided during the dry season, but otherwise this enclosure mimics natural conditions of buffalo in the area. The main stock of this herd was isolated in 2001 as part of a disease-free breeding program within the park. Additionally, after a poor calf crop in 2014, ten new adult female buffalo and their calves were captured within Kruger and added to the herd in June of 2015. In subsequent analyses, we account for differences in survival, immunity, and milk composition between habituated "resident" buffalo and wild "new" buffalo with the 'animal status' factor.

As part of the aforementioned study, all buffalo were captured every 2-3 months (five times per year) by passive enclosure near their water source, after which they were driven into a system of corrals, or captured by helicopter. At each capture, animals were immobilized by dart using etorphine hydrochloride (M99, Captivon, Karino, South Africa). Following data collection, immobilization was reversed using diprenorphine (M5050) or naltrexone (40 mg/ml, Kyron). Animals were kept under observation until fully recovered and all immobilizations were conducted by a veterinarian according to the

South African National Parks Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. All animal work for this study was approved by the institutional animal care and use committee at Oregon State University (ACUP #4478 & 4861).

Age at each capture was determined in young animals by tooth emergence and in older animals by wear pattern per established methods in this species (Jolles, 2007). Calf age was estimated at first capture from body size and horn emergence, and estimates were improved by incorporating pregnancy data from each calf's cow. Pregnancy status and stage were determined by rectal palpation done by a veterinarian. This method shows 100% sensitivity in Egyptian buffalo after 51 days of gestation (*Bos bubalis*; Karen et al., 2011). Body length was measured from poll to base of tail and horn length was measured from base to tip of the horn in young animals. Horn width was measured in older animals at the widest horizontal point. Body condition was assessed on a five-point scale through palpation and visual inspection of four areas where buffalo deposit fat: spine, hips, ribs, and base of tail. Condition ranged from 1 (very poor) to 5 (excellent) at each area, and was then averaged across these areas for an overall estimate of condition (Ezenwa et al., 2009). This method has been shown to correlate with fat deposits in the liver (Ezenwa et al., 2009).

Peripheral blood was collected from the jugular vein of immobilized buffalo and stored in heparinized tubes on ice in the field until processing that day. All blood samples for immune assays were collected within fifteen minutes of sedation. All reproductively mature female buffalo were visually inspected for signs of lactation and manually milked on all four quarters (Jolles et al., 2005). Milk was harvested into a 15mL Falcon tube and stored on ice, then frozen for later processing. Useable harvested volumes ranged from 200uL to 3mL. Some samples were obtained during very late pregnancy or from cows with calves that were less than a week old and are considered colostrum in subsequent analyses. Calves were assigned to cows based on behavioral patterns and verified by genetic parentage analyses using 1999 single nucleotide polymorphism (SNP) markers and the program COLONY (see previous chapters for genomic methods; Jones and Wang, 2010).

We harvested 60 total milk samples (four of which were colostrum) within a three-year period over 14 captures. In total, we had milk samples from 21 cows corresponding to 23 calves, 5 of which we "new" animals in 2015. On average, we obtained 2.6 milk samples per cow-calf pair before weaning.

Milk Component Quantification and Milk Bacterial Killing Assay (BKA)

Milk samples were processed in batches, and we observed no effect of storage time on lactoferrin, immunoglobulin G (IgG) or free fatty acid (FFA) concentrations. We chose FFA as a measure of nutrition in milk because this is the main energy source in buffalo milk, due to their adaptation to an arid environment (Osthoff et al., 2009). Lactoferrin and IgG were quantified using commercially available kits optimized for milk (Bethyl Laboratories Inc.) using a 1:1000 and 1:50,000 dilution, respectively. FFA was quantified using a commercially available kit (Zen Bio) and a 1:10 dilution.

We adapted the milk bacterial killing assay (BKA) methods from well-established methods used for whole blood and plasma in African buffalo (Beechler et al., 2012). Briefly, each milk sample was diluted 1:10 and filtered to remove dead cells (bacterial and host) using a 20 μm filter. To control for background differences in milk composition that may affect intrinsic bacterial growth rate, we pasteurized a replicate of each milk sample for 1 hour at 68 degrees Celsius as a nutrient control. We then inoculated each sample and nutrient control in a 96 well plate with 10^5 cells/ml of either *Staphylococcus aureus* or *Escherichia coli* and incubated for one hour at 37 degrees Celsius, during which time bacterial killing occurred. After incubation, bacteria were 'rescued' with the addition of nutrient broth and allowed to grow for 12 hours at 37 degrees Celsius. We assessed the total reduction in bacterial growth in untreated samples relative to nutrient controls using absorbance readings before and after the 12-hour incubation step.

Immune Assays

We assessed calf immune function over time with whole blood and plasma BKAs and cytokine production of whole blood. Whole blood and plasma BKA methods are similar to those describes above for the milk BKA, but with no nutrient control, no filtering step, and different dilution schemes. In place of a nutrient control, bacterial growth in blood and plasma samples was compared to control wells containing only bacteria and broth. See Beechler et al. (2012) for further detail. We used cytokine-specific ELISAs (Abd Serotec) to quantify levels of interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) following stimulation with pokeweed mitogen (*Phytolacca americana*; Sigma). Pokeweed is well established as a mitogen to elicit a T-dependent immune response in peripheral blood samples (Keightley et al., 1976; Moretta et al., 1979; Stanilova et al., 2005). Heparinized whole blood was incubated with 0.1mg/ml of pokeweed mitogen for seventy-two hours at 37 degrees Celsius, after which samples were spun, and the plasma supernatant was removed and stored at 4 degrees Celsius until ELISA testing. All cytokine ELISAs were run within four days of sample storage and we observed no effect of storage time.

Statistical Approach

We determined relationships among milk components and their impact on milk bacterial killing using Pearson's product moment correlations. IgG was log transformed to meet assumptions of normality for all analyses. When applicable, normality was assessed using data visualization and Shapiro-Wilks testing. All model selection was done using Akaike's Information Criterion (AIC) and residual plot visualization, unless otherwise noted. All statistics were run in R version 3.2.4 (R Core Team, 2016).

We used linear mixed effect models fit by maximum likelihood to determine drivers of variation in milk components over time with cow ID as a random effect. Full models for each milk component included maternal age, condition, age-length residuals, age-horn residuals, animal status (see above), pregnancy status, as well as calf age, calf sex, whether the sample was colostrum, and month and year in the study.

We assessed the effects of milk composition on overall calf survival using a Cox mixed-effect model fit by maximum likelihood. We then determined calf survival to weaning (15 months) using a Cox proportional hazards model with milk composition averages for each calf. Calf sex and animal status were included as covariates in each full survival model.

We assessed calf growth relative to milk composition using both total growth and longitudinal growth and condition data. We chose length as a measure of growth because it highly correlates with weight in young buffalo ($r^2=0.780$, $p<0.0001$) and it offered a more complete dataset. We used linear mixed effect models to determine the effects of concurrent milk composition on calf growth as age-length residuals, age-horn length residuals, and condition. Condition during lactation and total length gained by weaning were assessed using linear models and averages of each milk component. Colostrum samples were excluded from these analyses due to inconsistencies in composition relative to milk.

In a similar statistical approach, we compared milk composition to measures of immunity in the calf (BKAs and cytokine production) both longitudinally across lactation and averaged before and after weaning. We used linear mixed effect models to determine the effect of milk composition on concurrent immune activity. We then averaged each milk component over the course of lactation and compared these averages to immune measures averaged before and after weaning. By comparing concurrent and previous milk components to immunity, we can determine both long and short-term effects of milk composition on immune function.

RESULTS

Variation in Nutritional and Immune Components of Milk

Here we see high overall variability in milk composition relative to maternal traits and lactational stage. Free fatty acid (FFA) concentration marginally correlated with lactoferrin (r=0.282, p=0.071) and was weakly negatively correlated with immunoglobulin G (IgG; r=-0.368, p=0.016) in buffalo milk samples, but overall we observed no striking patterns in compositional correlations (Figure 4.1). Lactoferrin, but not IgG, was also marginally correlated with the *E. coli* killing ability of milk samples (r=0.402, p=0.058; Figure 4.2), likely due to its antimicrobial properties (Hettinga et al., 2011; Stelwagen et al., 2009).

We assessed maternal drivers of milk composition through 15 months of lactation, when weaning begins and milk is no longer a substantial component of calf diet (Smithers, 1983). Controlling for concurrent pregnancy and animal status (“new” versus “resident”, see methods), lactoferrin content was highly influenced by maternal condition and increased significantly with increasing maternal age, and over the course of lactation (as the calf aged; Table 4.1A; Figure 4.3). IgG concentration was 26.15 time higher in late pregnant cows, as this is when colostrum is being produced (Ontsouka et al., 2003). IgG also significantly varied with maternal age-horn length residuals, as cows with larger horns produced more IgG in their milk (Table 4.1B). Maternal condition was retained in the best-fit model but was not a significant driver of IgG concentration. FFA content of milk increased during lactation with calf age and varied marginally across years in the study, but was not predicted by any maternal traits (Table 4.1C).

Calf Survival and Growth

Overall calf survival was best predicted by status as a "new" animal in June 2015 and was not significantly influenced by concurrent milk composition as it was measured here (Table 4.2A). However, when components were averaged across lactation, higher FFA marginally reduced death risk before weaning (15 months; Table 4.2B). Though retained in the best-fit model, average IgG and lactoferrin concentrations did not significantly affect calf survival to weaning.

After controlling for sex-based differences in growth (males grow more slowly), we found that calves that received milk with higher lactoferrin concentrations tended to be larger for their age (Table 4.3A). Though IgG concentration was retained in the best-fit model, it did not significantly associate with patterns in age-length residuals. However, calves receiving milk with higher IgG concentrations tended to have smaller horns for their age (Table 4.3B). Overall length gain by weaning (15 months of age) was largely driven by age at first measurement, although average lactoferrin was retained in the best-fit model (Table 4.3C). Condition during lactation varied across years and months, while condition over the life of the calf (pre- and post-weaning) was negatively associated with increasing IgG concentration, after accounting for age (Table 4.3D/E).

Calf Immune Function

To assess the effects of milk composition on calf immune function, we compared lactoferrin, IgG, and FFA concentrations to calf whole blood bacterial killing ability, plasma bacterial killing ability, and cytokine production throughout early development. We observed no striking patterns in immune response measures relative to calf age (Figure 4.4), though calf age is retained as a covariate in some of the immune models.

E. coli killing ability of calf whole blood and plasma were marginally associated with differences in FFA concentrations at the same time point, while IgG concentration marginally associated with increased whole blood killing (TABLE 4.4A/B). *S. aureus* killing ability of calf whole blood was significantly associated with decreasing lactoferrin in concurrent milk samples (Table 4.4C), while year was the best predictor of plasma killing ability (Table 4.4D). TNFα production before weaning was negatively associated with concurrent IgG concentration and for every unit increase in IgG, TNFα production in whole blood stimulated with pokeweed decreased by 5.9% (Table 4.4E).

We compared pre- and post-weaning averages of immune measures to pre-weaning milk compositional averages to assess long and short-term effects of milk constituents on immune function. In models of immunity during lactation (pre-weaning), lower average IgG marginally associated with higher average IFNγ production (Table 4.5A). After weaning, FFA and lactoferrin were marginally associated with differences in average TNFα production (Table 4.5B). Aside from marginal associations with cytokine production, average milk composition was not predictive of average immune response before or after weaning.

DISCUSSION

Here we demonstrate high variability in milk immune components, both over the course of lactation and among cows of varying age, size, and condition. In contrast, we observed consistency among cows in nutritional provisioning in the form of free fatty acids. Furthermore, we provide some evidence that higher FFA reduces mortality risk in calves before weaning and may have positive impacts on calf immune function. The immune active constituents of milk, IgG and lactoferrin, appear to have distinct functions and effects on calf growth and immune function. We demonstrated that lactoferrin can kill bacteria in milk, and thus may act to sterilize the milk the calf is receiving. Accordingly, higher lactoferrin is associated with increased growth rates in calves, and reduced immune activation. By contrast, higher IgG levels were associated with improved bactericidal activity of calf blood, but reduced endogenous cytokine production.

Bovine calves are born agammaglobulinemic with an underdeveloped immune system due to cotyledonary placental attachment, which does not allow for transfer of antibodies from mother to calf (Goddeeris & Morrison, 1994). Therefore, the neonate relies heavily of colostrum for antibody transfer and protection during the first few weeks of life (Husband et al., 1972; Ploegaert et al., 2011). IgG is the most abundant antibody in bovine colostrum and milk, making up 81% and 73% of total antibodies, respectively, with transfer dropping off precipitously 24-48 hours after parturition (Butler, 1973; Hurley and Theil, 2011). Although here we did not directly assess the effects of milk IgG concentration on calf infections, we did find a general negative association between higher IgG levels in milk and immune activation- measured as cytokine production, after accounting for calf age. We also observed that milk concentrations of IgG and FFA were significantly negatively correlated, which may explain opposing associations with calf horn growth in this study. Furthermore, maternal antibodies have been shown to have conflicting stimulatory and suppressive effect on the calf's immune system (Lemke et al., 2003). Several vaccination studies have demonstrated that calves who received maternal antibodies mount a quicker, more effective immune response against pathogens to which the mother was vaccinated before parturition (for example, Hurley and Theil, 2011; Shahid et al., 2002). However, maternal antibodies can also have suppressive effects by protecting the neonate from a costly innate immune response through instructive priming and direct protection (Grindstaff, 2008). Due to the specificity of antibody-driven immune priming, associated suppressive effects can have detrimental effects on more general immune responses involving novel pathogens (Carlier and Truyens, 1995; Hasselquist and Nilsson, 2009). Therefore, it is not surprising that we observed a negative association between increasing IgG and stimulated cytokine production and a positive association between IgG and whole blood bacterial killing ability. Passive immunity would likely dampen the response to a novel pathogen in calves (measured here as decreased cytokine production), but is predicted to have positive effects on more general, circulating immune components (resulting in higher bacterial killing ability).

Along with antibodies, lactoferrin can make up 30% of milk protein in some species (Hanson et al., 2003). Lactoferrin and lactoperoxidase have antibacterial properties and are common constituents of bovine milk (Piccinini et al., 2007). Levels of these proteins have been shown to fluctuate during lactation and do not drop off after colostrum production ceases (Stelwagen et al., 2009). In agreement with these findings, we observed a lactoferrin increase over the course of lactation. Additionally, we demonstrated that lactoferrin has strong antibacterial properties and associates with higher growth in buffalo calves. We observed a strong correlation between lactoferrin concentrations and bacterial killing ability of milk. Lactoferrin is a key component of 'nutritional immunity,' acting to reduce bacterial growth by sequestering iron, a limiting nutrient (Gil and Rueda, 2002; Lonnerdal, 2009). In agreement with our growth findings, Robblee et al. (2003) showed that pre-weaning average daily gain and gain-to-feed ratios increased, while fecal scores decreased (less diarrhea), in beef production calves supplemented with lactoferrin-rich milk replacer.

Fat content appears to be the main driver of calf success, and has been previously identified as the primary energy source to the neonate in this system (Osthoff et al., 2009). Furthermore, FFA's consistency across cows and lactational stage, and its strong influence on calf survival to weaning, suggest that fat content is likely under strong stabilizing selection in this arid environment.

With relatively consistent FFA content among cows, energetic trade-offs, and resulting maternal effects, seem to play out as differences in immune-active milk components relative to maternal age, size, and condition. Indeed, it has been demonstrated in many laboratory studies that energetic trade-offs are common between parental care and maternal health across taxa (e.g., Moreno et al., 2001; Saino et al., 1997; Steiger et al., 2011), especially when maternal costs are maximized during lactation (Festa-Bianchet, 1989). Though FFA provides the main signal of survival and increased growth in this system, higher lactoferrin and IgG levels may result in passive immunity, allowing for more energetic investment in growth, while maternally derived compounds regulate immune response. Further studies will focus on the duration of maternal protection in this system, and how these immune active components affect long-term immune development.

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TABLES

Table 4.1: Linear mixed effect models for drivers of milk concentrations of (A) lactoferrin, (B) IgG, and (C) FFA up to 15 months since the start of lactation. Comparisons among years are considered significant at a Bonferroni corrected alpha of 0.017. Only significant comparisons are shown for factors with multiple levels (e.g., year). Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. Lactoferrin (ng/ml)	100000	0.007
Maternal Condition	46070	0.0003
Maternal Age (yrs)	3822	0.020
Calf Age (mos)	5844	0.0007
Pregnant	27480	0.083
Animal Status (New)	47560	0.024
B. Immunoglobulin G (ng/ml)*	66.43	<0.0001
Maternal Condition	5.386	0.575
Pregnant	26.15	0.013
Maternal Age-Horn Length Residuals	1.219	0.014
C. Free Fatty Acids (μM)	2305.5	0.002
Calf Age (mos)	92.73	0.050
Year	NA	NS

*Estimates were natural log back-transformed and represent a multiplicative effect on the response median.

Table 4.2: Cox proportional hazards models for (A) overall calf survival relative to concurrent milk composition across all time points, and (B) calf survival to weaning relative to values of each immune or nutritional milk component averaged across lactation. All estimates were natural log back-transformed and represent a multiplicative increase in risk of death per unit increase in the predictor. Best-fit models were selected using AIC and residual plots.

Model	Estimate	P-value
A. Overall calf survival		
Lactoferrin (ng/ml)	1.000	0.95
Free Fatty Acids (μM)	0.996	0.44
Animal Status (New)	1658	0.035
Sex (Male)	0.266	0.67
47 observations, 10 events		
B. Calf survival to weaning		
Average Lactoferrin (ng/ml)	1.000	0.43
ln(Average Immunoglobulin G (ng/ml))	8.629	0.25
Average Free Fatty Acids (μM)	0.999	0.09
Animal Status (New)	2.396	0.006
21 observations, 6 events		
Adj. R^2=0.59		

Table 4.3: Linear mixed effect and linear models for drivers of calf growth during different stages. Models (A) and (B) describe milk components and animal traits affecting calf length and horn length scaled to age (residuals), model (C) describes factors influencing total length gain by weaning (15 months), and models (D) and (E) describe factors affecting condition of the calf up to 15 months and up to 2.5 years of age. Comparisons among months and years are considered significant at a Bonferroni corrected alpha of 0.003 or 0.017, respectively. Only significant comparisons are shown for factors with multiple levels (e.g., month). Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. Age-Length Residuals	2.550	0.633
Lactoferrin (ng/ml)	0.00007	0.029
Immunoglobulin G (ng/ml)*	0.524	0.146
Sex (Male)	-9.150	0.010
B. Age-Horn Length Residuals	2.830	0.011
Immunoglobulin G (ng/ml)*	0.767	0.012
C. Total Length Gained by Weaning	67.85	<0.0001
Average lactoferrin (ng/ml)	-0.00012	0.105
Calf Age (mos)	-6.047	0.006
D. Calf Condition During Lactation	3.113	<0.0001
Lactoferrin (ng/ml)	-0.000003	0.173
Month (Aug vs Feb)	-1.440	0.0005
Month (Oct vs Feb)	-1.044	0.002
Month (Aug vs June)	-1.461	0.0001
Month (Oct vs June)	-1.065	0.0002
Month (Dec vs June)	-0.856	0.0029
Year (2015 vs 2014)	0.924	0.003
E. Overall Calf Condition	3.262	<0.0001
Average Immunoglobulin G*	0.943	0.004
Calf Age (mos)	-0.017	0.015
Month (Oct vs Feb)	-0.600	0.0002
Month (Dec vs Oct)	0.475	0.002
Year (2015 vs 2014)	0.783	<0.0001
Year (2016 vs 2014)	0.891	<0.0001

*Estimates were natural log back-transformed and represent a multiplicative effect on the response median.

Table 4.4: Linear mixed effect models for immune response parameters significantly predicted by concurrent milk component concentration. Models below include factors affecting *E. coli* killing ability of calf whole blood (A) and plasma (B), *S. aureus* killing ability of calf whole blood (C) and plasma (D), and TNFα production in response to whole blood stimulation with pokeweed (E). Comparisons among months and years are considered significant below a Bonferroni corrected alpha of 0.003 or 0.017, respectively. Only significant comparisons are shown for factors with multiple levels (e.g., month). Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. Proportion *E. coli* Killed (Whole blood)	0.042	0.894
Immunoglobulin G (ng/ml)*	1.035	0.057
Free Fatty Acids (μM)	0.000009	0.102
Calf Age (mos)	-0.023	0.193
Month	NA	NS
Year	NA	NS
B. Proportion *E. coli* Killed (Plasma)	-0.159	0.394
Free Fatty Acids (μM)	0.00006	0.085
Sex (Male)	0.080	0.219
Animal Status (New)	-0.158	0.114
Month (June vs Feb)	0.701	0.001
Month (Dec vs Feb)	0.641	0.001
Year (2016 vs 2014)	0.837	0.001
Year (2016 vs 2015)	0.774	0.0006
C. Proportion *S. aureus* Killed (Whole blood)	0.574	0.001
Lactoferrin (ng/ml)	-0.000003	0.026
D. Proportion *S. aureus* Killed (Plasma)	0.400	0.005
Lactoferrin (ng/ml)	-0.000001	0.180
Animal Status (New)	0.149	0.102
Year (2015 vs 2014)	-0.389	0.003
E. TNFα Production	0.485	0.132
Immunoglobulin G (ng/ml)*	0.941	0.017
Year	NA	NS

*Estimates were natural log back-transformed and represent a multiplicative effect on the response median.

Table 4.5: Linear regression models for average values of IFNγ production before weaning and TNFα production after weaning relative to average concentration of milk components during lactation. No other immune response parameters were predicted by average milk components at either time point. Best-fit models were selected using AIC, conditional R^2, and residual plots.

Model	Estimate	P-value
A. Average IFNγ Before Weaning (ng/ml)	127.5	0.0009
Average Immunoglobulin G (ng/ml)*	0.026	0.061
Calf Age (mos)	-2.895	0.093
Animal Status (New)	22.25	0.128
Sex (Male)	-15.01	0.200
B. Average TNFα After Weaning (ng/ml)	-0.226	0.243
Average Lactoferrin (ng/ml)	-0.00002	0.085
Average Free Fatty Acids (μM)	0.00009	0.068

*Estimates were natural log back-transformed and represent a multiplicative effect on the response median.

FIGURES

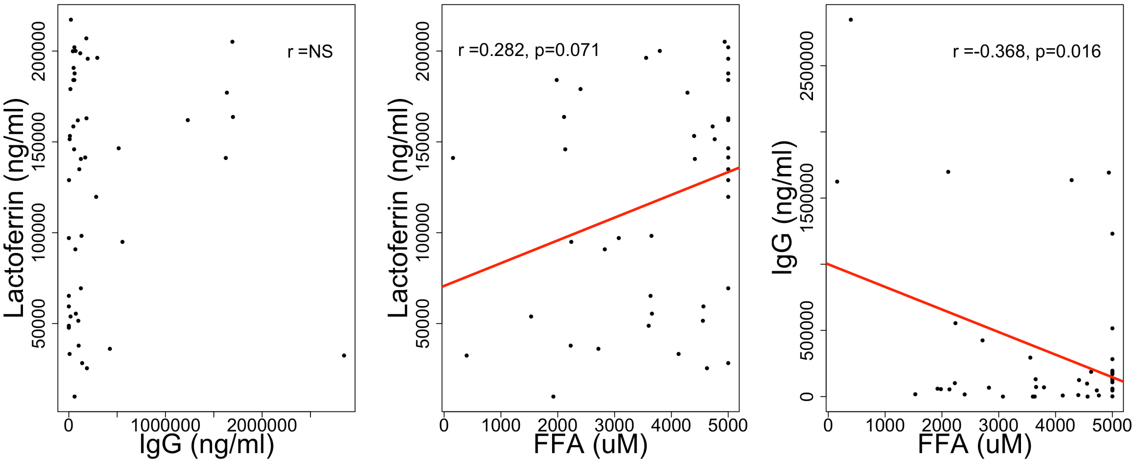

Figure 4.1: Correlations of pairwise milk components.

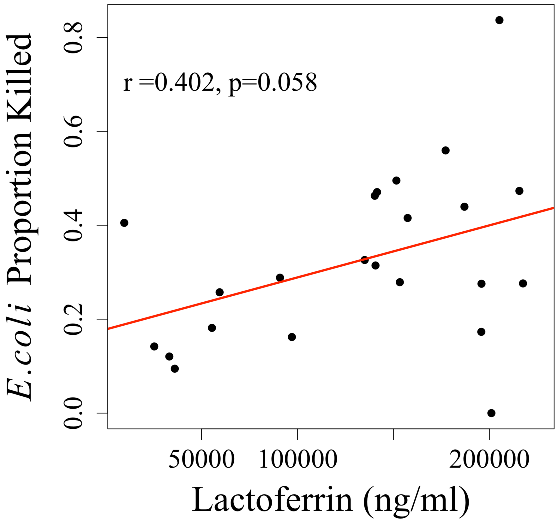

Figure 4.2: Positive correlation of milk *E. coli* killing ability and lactoferrin concentration.

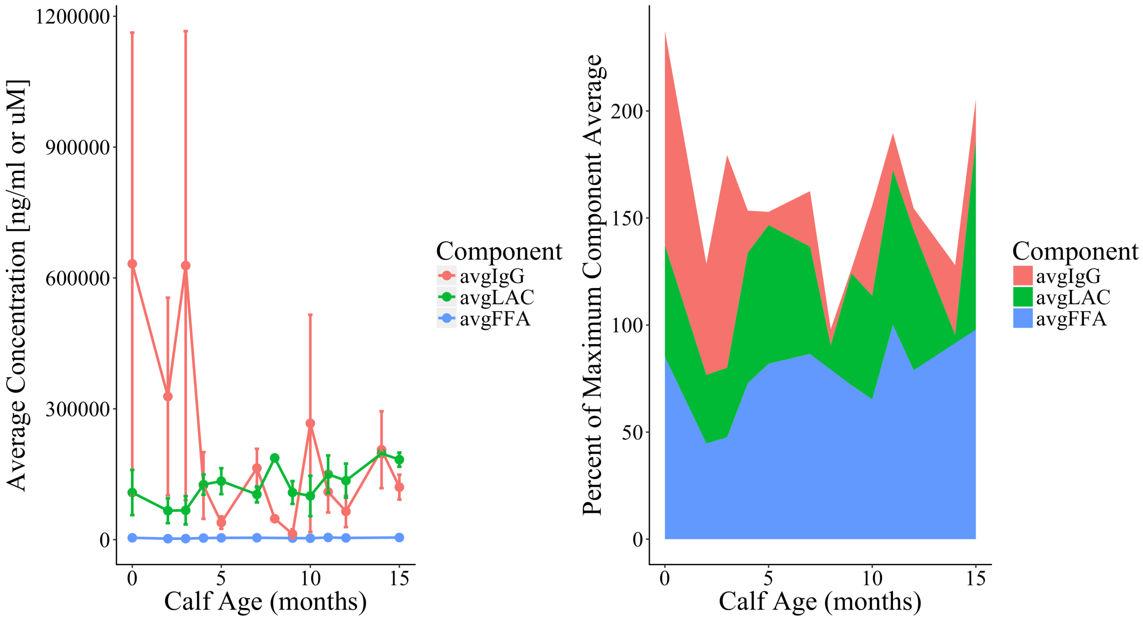

Figure 4.3: Average concentration (left) and relative abundance (right) of each nutritional and immune milk component over the course of lactation, relative to calf age. Concentration of IgG and lactoferrin are both shown in ng/ml while FFA concentration is measured in micromoles. Relative abundance is shown as the percent of each individual component's maximum value such that the width of each stacked area covaries with relative concentration of that component over time. Note that relative concentration can be directly compared among components in the line graph but not the stacked plot.

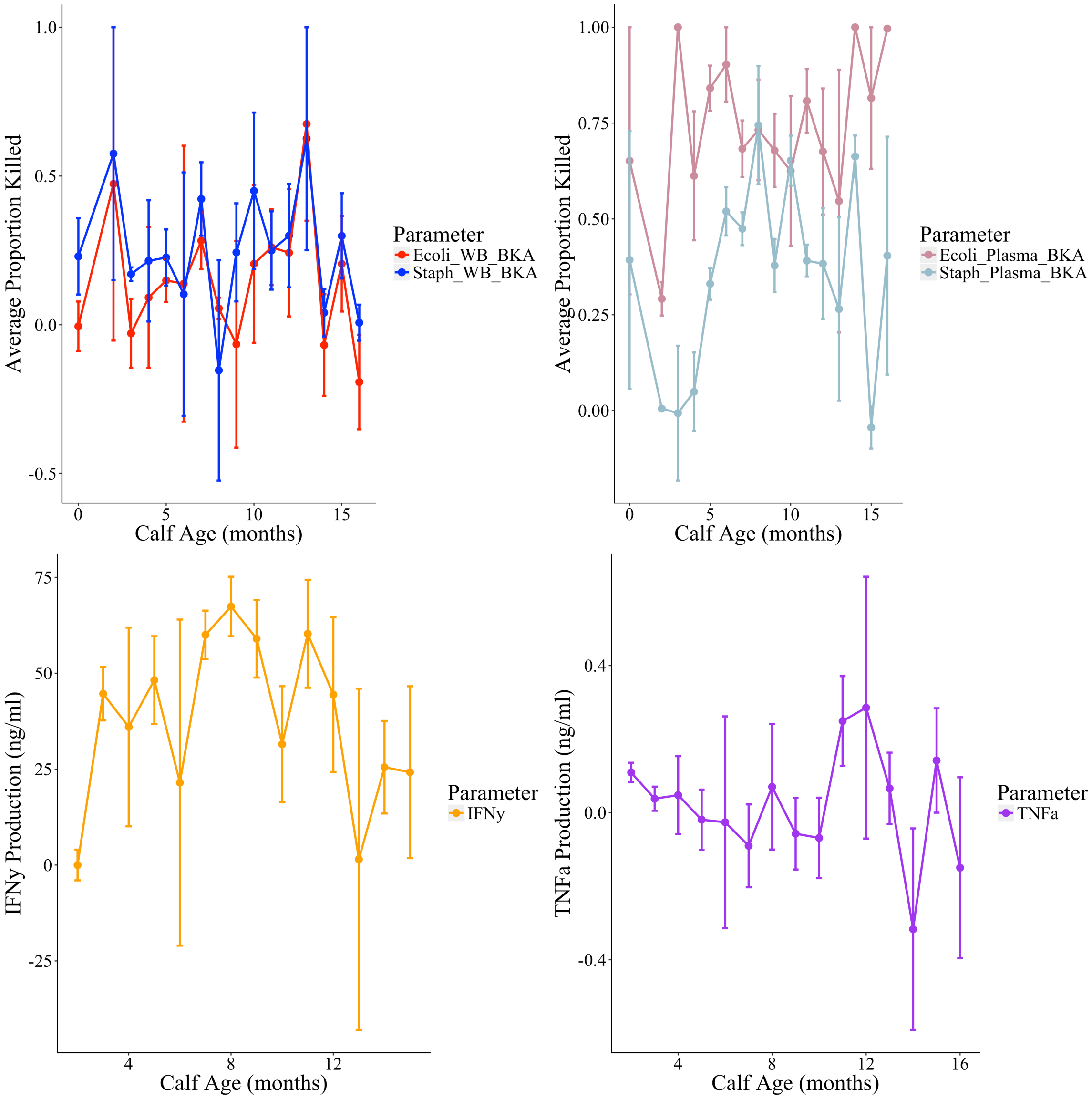

Figure 4.4: Measures of immunity relative to calf age up to 15 months (weaning).

CHAPTER 5

General Conclusion

Disease acts as a powerful driver of evolution and can shape patterns of resource allocation in wild systems where resource availability varies dramatically (Boots, 2011; French et al., 2009). In the face of ubiquitous pathogen challenges, host populations often evolve resistance mechanisms to prevent infection, and these mechanisms often come at a fitness cost (Antonovics and Thrall, 1994; Boots and Haraguchi, 1999). Though costly themselves, optimum resistance phenotypes evolve relative to the negative fitness effects of infection, such that the benefits of preventing infection outweigh the costs of the underlying defense mechanisms (Best et al., 2008; Boots and Haraguchi, 1999; Miller et al., 2005). Therefore, organisms must strike a balance between investment in immunity and investment in their ultimate fitness.

In mammals, the energetic balancing act does not end with parturition, but is prolonged by the energetic demands of lactation (Rogowitz, 1996). Mammalian mothers must meet their own maintenance needs, while maximizing the growth and development of the neonate. Therefore, maternal resources are predicted to determine the quality and quantity of milk received by the neonate (Hinde et al., 2009; Rogowitz, 1996). Differences in maternal provisioning can thus be quantified as 'maternal effects' of rearing environment, and are predicted to have dramatic impacts on neonatal health (Rossiter, 1996).

This dissertation defines the impact of genetic background and maternal effects on disease susceptibility and early immune function in African buffalo. I characterized the delicate balance of energy allocation in a resource-limited environment, by demonstrating that host investment in resistance mechanisms and maternal investment in immune provisioning is context- and resource- dependent. In chapter 2, I provided a novel example of highly heritable infection resistance to a zoonotic pathogen in a wild system, and identified multiple costs of resisting. Additionally, I discovered two distinct forms of resistance- infection and proliferation resistance- that do not appear to be correlated among individuals and confer distinct fitness advantages. In chapter 3, I proposed putative mechanisms of infection resistance, which corroborate clinical findings in humans and other animals (Cooper et al., 2007; Cross et al., 1999; Reeme et al., 2013). I demonstrated measureable variation in immune function among genotypes and conclude that variation in bTB susceptibility in this buffalo population is related to IL-12 production and macrophage activation. In my final data chapter, I determined that buffalo milk composition is dependent upon maternal resources and can affect calf immune function, growth, and survival. Specifically, increasing fat concentration positively associated with increased survival, while immune active components of milk generally associated with increased growth but decreased immune function early in life. To my knowledge, this body of work represents the first record of longitudinal milk composition and its effects on neonatal growth, survival, and immunity in a wild system.

The work presented here provides novel validation of past theoretical and clinical work in the fields of evolutionary ecology and eco-immunology. Using this incredibly unique dataset, I found heritable variation in response to disease, costs of resisting disease, and variable impacts of milk components on neonatal health. Taken together, these chapters highlight the intricate energetic trade-off between immunity and reproduction in a wild animal and provide a new model system for the evolution of disease resistance in wild populations.

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APPENDIX

Appendix Table 1: Drop-in-deviance (A) and log-rank test (B) results comparing time-to-event models for onset of bTB infection at each Most Recent Common Ancestor (MRCA) clade level. Treatment and final age were included as covariates in the best-fit Cox proportional hazards models, while log-rank tests were run on univariate models (clade only). Second MRCA clade was the lowest (most basal) clade level to be retained in the best-fit Cox PH model during AIC-based model selection.

A.

Clade Level	Δ log likelihood	$X_{(df)}$	P-value
Base MRCA Clade	14.7	$29.5_{(105)}$	1.000
Second MRCA Clade	51.9	$96.7_{(70)}$	0.018
Third MRCA Clade	29.1	$56.2_{39)}$	0.037
Fourth MRCA Clade	21.2	$44.8_{(27)}$	0.017

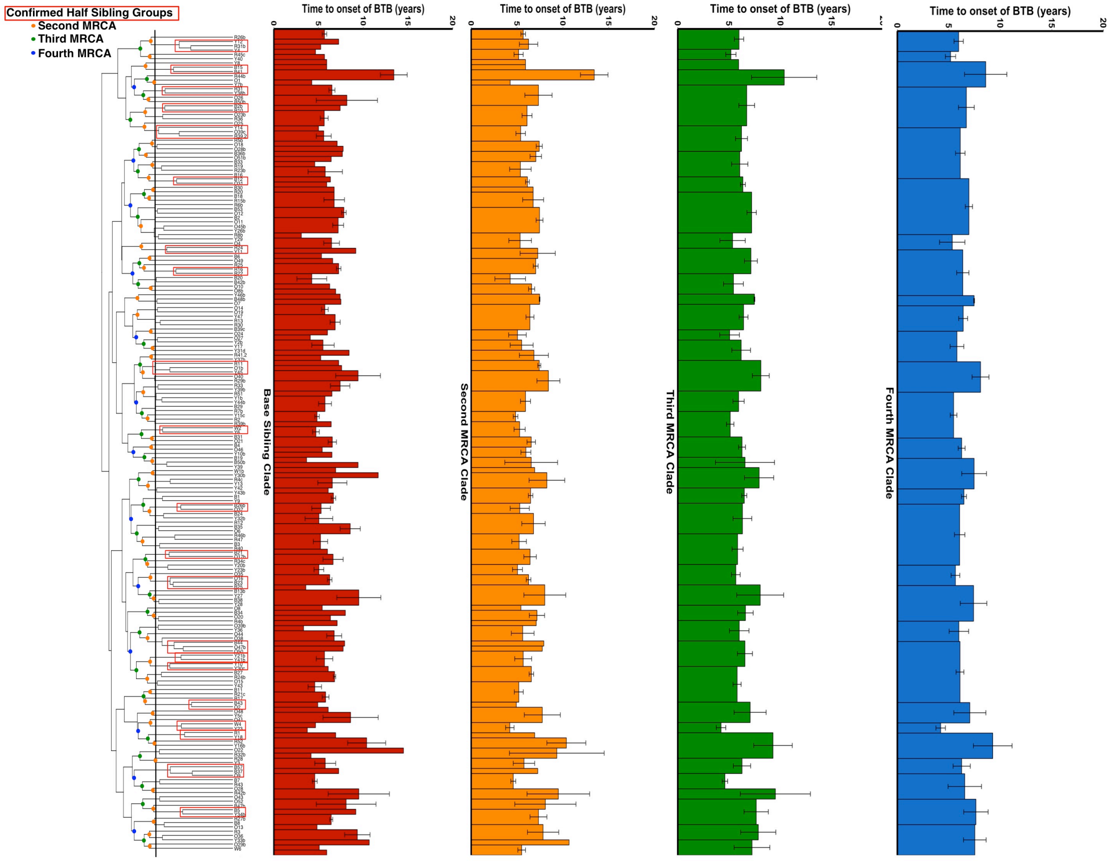
B.

Clade Level	$X_{(df)}$	P-value	Bonferroni Adj. P-value
Base MRCA Clade	$364_{(107)}$	0.0001	0.00009
Second MRCA Clade	$118_{(68)}$	0.00015	0.0002
Third MRCA Clade	$69.1_{(39)}$	0.0021	0.0006
Fourth MRCA Clade	$44.8_{(27)}$	0.0169	0.0013

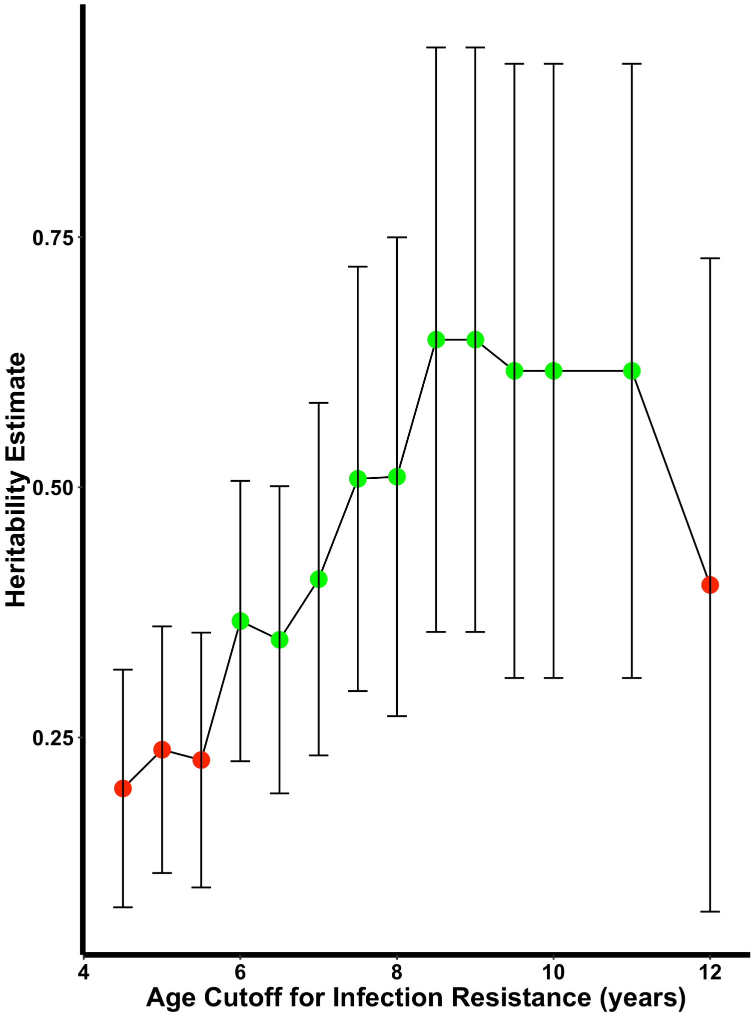
Appendix Table 2: Sample sizes, heritability estimates, and conditional R^2 for each infection resistance age cutoff model. Age cutoff values represent the age under which negative animals were truncated to account for animals that should be right censored (i.e., did not convert to bTB positive during the observation period). Herd and treatment were included in each model as covariates. Conditional R^2 ($R^2_{GLMM(c)}$) describes the proportion of variance explained by both the fixed and random factors in each model.

Age Cutoff	Sample Size (n)	Number of Clades (N)	(n/N)	h^2 Estimate (±SE)	$R^2_{GLMM(c)}$
4.5	99	39	2.54	0.199 (±0.119)	0.200
5	92	36	2.56	0.238 (±0.123)	0.254
5.5	88	35	2.51	0.228 (±0.127)	0.244
6	75	30	2.50	0.366 (±0.140)**	0.382
6.5	64	26	2.46	0.348 (±0.153)*	0.370
7	48	19	2.52	0.408 (±0.176)*	0.433
7.5	36	15	2.40	0.509 (±0.212)*	0.517
8	30	13	2.30	0.511 (±0.239)*	0.526
8.5	23	11	2.09	0.648 (±0.292)*	0.672
9	23	11	2.09	0.648 (±0.292)*	0.672
9.5	21	10	2.10	0.616 (±0.307)*	0.669
10	21	10	2.10	0.616 (±0.307)*	0.669
11	21	10	2.10	0.616 (±0.307)*	0.669
12	19	9	2.11	0.402 (±0.326)	0.457

* $p<0.05$
**$p<0.01$



Appendix Figure 1: Time to onset of bovine tuberculosis infection by most recent common ancestor (MRCA) clade hierarchical groupings. In the dendrogram on the left half sibling groups confirmed by the program COLONY have been outlined in red. We assigned base MRCA groups based on the least recent convergence of these confirmed groups, denoted here with the solid black vertical line. Individuals that share a common ancestor (node) to the right of this line (more recently) were considered to be part of one base MRCA clade. Continuing left (more distantly) from this black line, secondary nodes group animals by second MRCA clade, tertiary nodes group animals by third MRCA clade, and quaternary nodes group animals by fourth MRCA clade. Grouping nodes were only assigned at points where animals that are not related at the previous level converge (e.g., such that the same animals did not make up the whole of the second and third MRCA clade). Bar plots to the right display average values and standard error of time to onset of bTB by base, secondary, third, and fourth MRCA clades moving right. Bar widths are proportionate to group size and nested groups can be traced horizontally.



Appendix Figure 2: Heritability estimate by infection resistance age cutoff. Age cutoff values represent the age under which negative animals were truncated to account for animals that should be right censored (i.e., did not convert to bTB positive during the observation period). Herd and treatment were included in each model as covariates. Green points denote a heritability estimate significantly different from zero and red points are not significantly different from zero. Error bars represent the standard error of each heritability estimate. When bTB negative buffalo less than six years of age were excluded from the analysis, a significant heritability estimate was obtained.