CHARACTERIZATION OF GENETIC VARIATION AND BASIS OF INFLAMMATORY BOWEL DISEASE IN THE TOLL-LIKE RECEPTOR 5 GENE OF THE RED WOLF AND MANED WOLF

by

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ABSTRACT

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Characterizing Toll-like receptors across taxa can lead to an increasingly accurate documentation of the evolutionary processes active within this receptor class, as well as a greater understanding of the diseases associated with these receptors. This study examines two sequenced portions of the Toll-like receptor 5 protein coding gene in two imperiled canid species: the near threatened maned wolf (*Chrysocyon brachyurus*) and the critically endangered red wolf (*Canis rufus*), to characterize genetic variation and investigate the presence of single nucleotide polymorphisms (SNPs) previously associated with canine inflammatory bowel disease (IBD). Both maned and red wolves suffer from inflammatory bowel disease, threatening the sustainability of their crucial *ex situ* populations. In this thesis I report novel polymorphic positions found in maned and red wolf TLR5 gene and differences in variation with regard to nucleotide

polymorphisms and resulting amino acid variation between maned wolves, red wolves, gray wolves and domestic dogs. Although domestic dog SNPs associated with IBD were not found to be polymorphic in maned wolves and red wolves, all sampled individuals of both focal species and gray wolves lack the protective alleles present in many dog breeds, suggesting a genetic predisposition for IBD in these two wild canid species. This potential predisposition informs *ex situ* management practices and treatment for IBD.

INTRODUCTION

Project Justification

Habitat reduction, fragmentation, hunting and disease collectively pose a considerable threat to many wildlife species, including those in the family *Canidae*. Of the 35 extant non-domestic canid species, six are listed as vulnerable, endangered or critically endangered[1]. This study is interested in two canid species: the South American maned wolf (*Chrysocyon brachyurus*), and the North American red wolf (*Canis rufus*).

The red wolf, listed as critically endangered by the International Union for the Conservation of Nature (IUCN)[2], was once endemic to the southeastern United States but was considered extinct in the wild in 1980 due to habitat loss, hunting and the introgression of coyote genes[2,3]. The *in situ* population of red wolves was recovered with an intensive reintroduction program in North Carolina beginning in 1987, that has resulted in an approximately 100 individual population that exists within the current 6,000 km^2 reintroduction area[2]. The population has recently seen an increase in gunshot mortalities and is facing further threats from increased applications for take permits and recent legislation that threatens to end the reintroduction program[4].

Considering these threats, the *ex situ* population of red wolves is becoming increasingly important to the survival of the species and serves as a reservoir population

for a future reintroduction program. From 12 founders, the *ex situ* population consists of 191 wolves housed in 42 U. S. institutions[5]. Currently, the level of genetic diversity of this population is 89.32% which is lower than the ideal goal of 90%. [6]. A healthy, reproductively successful population is needed to reach this high level of genetic diversity.

The maned wolf, considered near threatened by the IUCN, currently faces many of its own challenges[7]. As of 2008 the *in situ* maned wolf population was estimated at 13,000 and is suspected to decline by at least 10% in the next ten years[8]. Habitat fragmentation poses the greatest threat to the survival of the species[9] and produces the compounding effects of road mortality[10] and exposure to disease from and competition with domestic dogs[11].

With habitable land in their native Cerrado rapidly decreasing, the future of the maned wolf may rely on the success of the *ex situ* population. Currently there are 88 individuals in the North American population derived from 31 founders. With a relatively large number of founding animals the breeding program has been able to maintain 92% genetic diversity[12]. The importance of the viability of *ex situ* populations for both maned wolves and red wolves is becoming increasingly apparent as threats facing both species mount. Unfortunately for these efforts, both maned wolves and red wolves suffer from suboptimal health[13,14] and reproductive difficulties[15,16,17,18]. Gastrointestinal disease has proven to be a major factor in mortalities in both red wolves and maned wolves and has a high prevalence in both populations[19,10,20].

This study will focus on inflammatory bowel disease (IBD), a common diagnosis in both species, that refers to any general inflammation of the gastrointestinal tract[21]. IBD is a multifaceted disorder that has been shown to have microbial[22] and genetic[23,24] bases in other species. In the domestic dog (*Canis lupus familiaris*) the disease is linked to single nucleotide polymorphisms (SNPs) in the Toll-like receptor 4 (TLR4) and Toll-like receptor 5 (TLR5) genes[25], with two protective alleles against IBD identified in TLR5 across 38 different breeds[26]. I hypothesized that genetic analysis could identify a similar causative genetic element for IBD in the maned wolf and red wolf, linked with the TLR5 gene.

The investigation of the genetic basis for inflammatory bowel disease in TLR5 also contributes to the current knowledge of variation in mammalian Toll-like receptors with regard to the evolutionary processes that act on these receptors, their potential species specific adaptation and the changes in innate immunity associated with domestication. Toll-like receptors have been found to be under positive selection in some species[27,28] and relatively conserved in others[29] This study contributes previously unsampled canid species to this current debate regarding the model of evolution for Toll-like receptors. I hypothesized that patterns of variation differ between these two wild canids most strikingly with those of domestic dog, and that this variation suggests species-specific adaptation based on differential microbial environments.

The aim of the present study was two-fold: to characterize polymorphisms and genetic variation within two selected regions of the TLR5 gene in maned wolves and red wolves and to determine the role of previously identified genetic markers for IBD in

these two disease prone species. This study reports novel polymorphisms in these previously unsampled threatened species, differing variability in TLR5 between maned wolves, red wolves, gray wolves and domestic dogs and a potential role for IBD SNPs in the pathogenesis of IBD within maned wolves and red wolves.

The Red Wolf

The red wolf is a native American canid belonging to the order *Carnivora*, the family *Canidae* and the subfamily *Caninae*. Originally identified as a variation of the gray wolf (*Canis lupus*)[30] it was subsequently defined as a separate species[31]. The red wolf belongs to one of the two North American canid clades that also includes coyotes (*Canis latrans*) and eastern wolves (*Canis lycaon*)[32]. At the time of its original identification in 1851 there were three subspecies associated with *Canis rufus*: *Canis rufus floridanus*, originating from Florida, *Canis rufus gregoryi* from Louisiana, and *Canis rufus rufus*, encompassing the Texas population[33]. The red wolf's original range extended from central Texas to Florida and Georgia and spread along the Mississippi to Indiana and Illinois. Increased agricultural development and shootings due to lengthened hunting seasons led to human induced declines in these regions, while parasites like heart worm, hookworm, tape worm and sarcoptic mange mites further reduced the population[34].

Due to the mounting threats facing the species, the red wolf was listed as endangered in 1967 and a recovery program initiative passed with the Endangered Species Act[35]. The fragmented nature of red wolf populations and the increased presence of coyotes in red wolf territory motivated the decision to use captive management as the

primary strategy for the recovery of the species. The founding captive breeding program consisted of 12 individuals identified from four hundred animals captured from Louisiana and Texas and brought to the Point Defiance Zoo in 1973. Reintroductions began in five counties in North Carolina and currently the population is estimated at 100 individuals[36].

Red wolves are identified by their intermediate size between gray wolves and coyotes. Red wolves collected prior to 1930 weighed between 21 and 41kg for males and 16 to 29kg for females[33] and red wolves captured in Texas in 1970 weighed an average of 23.7kg for males and 21.19kg for females[34]. The red wolf also has a distinctive narrow and elongated skull and earns its name from its reddish and sparsely haired pelt which can also have a black color morph[33]. An early description of red wolf coloration describes the wolf as having cinnamon patches on its upper back, top of the head, and outer surfaces of its limbs. Its face is a mottled black and grey, and the legs and feet are paler than the remainder of the red coat with a distinctive black line on the external forearm[31]. Another distinguishing characteristic for the red wolf is the size and angle of its ears. The ears are large in proportion to the narrow face and positioned at an angle that gives the wolf's head a triangular appearance that is absent in gray wolves and coyotes[34].

Red wolves travel in pairs or small family groups similar to gray wolves[36] but do not hunt in packs due to the small size of their prey species[34]. Prior to the near extinction of the species, common prey items included, rabbits (*Sylivagus sp.*), nutria (*Myocastor coypus*), and rats (*Rattus sp.*)[34], and following reintroduction prey shifted to raccoons (*Procyon lotor*), rabbits and white tailed deer (*Odocoileus virginianus*)[36]. In captivity, red wolves are fed dog chow with the wolves in this study all fed a commercial dog food

brand as the primary component of their diet [37]. The official red wolf husbandry manual states that it is, "not the responsibility of the RWSSP to feed red wolves a diet that they would find in the wild, " and that wolves should be fed commercial dry dog food with 22-28% protein" [38]. The husbandry manual continues to suggest that meat may be added as an encouragement to eat the dry chow but should not be a regular dietary component [38].

Territory sizes for reintroduced wolves vary based on the size of the pack with a range from 88.5 km^2 for individuals to 123.4 km^2 for packs. Red wolves are monoestrus and breed in January and February, yielding litter sizes of 3-4 pups which both parents raise[34]. Red wolves are monogamous and within a family group often retain nonbreeding offspring to serve as helpers for future pups causing delayed dispersal in these offspring and leading to direct fitness benefits including reduced pup and male adult mortality, and increased lifetime reproductive success in females[39,40]. Despite the delayed dispersal, inbreeding avoidance mechanisms are built into red wolf breeding behavior and the rates of philopatric reproduction are low[41].

Today the reintroduced red wolf population faces many of the same challenges that led to the decline of the original *in situ* population. During the first 25 years of reintroduction illegal activities (e.g. poisoning, illegal take, and gunshot) accounted for 30% of red wolf mortality, while vehicle collisions (20%), health-related causes (16%) and intraspecific competition (6.5%) also were responsible for deaths. However, 1988-2003 gunshot related mortalities increased 375%[2]. In fact, anthropogenic related mortality has an additive effect with natural mortality in low density red wolf populations, which negatively impacts the survival and growth rate of these

populations[42]. In addition to threats directly related to anthropogenic disturbance, 67% of the free ranging red wolves have intestinal parasites including hookworms, ascarids, whipworms and tapeworms[13]. Due to the presence of domestic dogs in portions of the red wolf reintroduction area, red wolves also suffer from common domestic dog diseases including canine distemper, parvovirus, leptospirosis, hemobartonellosis, borrellosis, mange and rabies[2]. In a potential secondary reintroduction area in the Great Smoky Mountains National Park, the high prevalence of parvovirus in pups greatly influenced pup survival and was one of the main contributing factors to the demise of the reintroduction at that site[2].

One of the most contentious issues currently surrounding red wolf conservation is the question of the hybrid nature of the species. There are many studies that indicate that red wolves are a hybrid between gray wolves and coyotes[43,44,45,46] and still many others that insist the red wolf is a distinct species[47,48,49]. Hybrid or not, one of the greatest threats facing the species is the introgression of coyote genes[50,51,3] further exacerbated by the increasing presence of coyotes in the reintroduction area[52].

The extirpation of the wild red wolf in the 1980s motivated the creation of an *ex situ* breeding population. Originating from 12 founders, there are now 191 wolves at 42 institutions in the red wolf species survival plan[5]. The captive population has been managed to maintain 89.32% genetic diversity, which is lower than the ideal of 90%[6]. The *ex situ* population of wolves also shows no evidence of an inbreeding depression, and inbreeding has had no effect upon juvenile viability or litter size[53]. The captive population still suffers from high pup mortality[16], decreased reproductive success[15], and a

variety of health issues including pervasive lymphosarcoma[13,20,19]. This present study will focus on the deleterious effects of highly prevalent gastrointestinal disease on the population. This prevalence is demonstrated by a survey of 62 red wolf mortalities, finding that 8 individuals died of gastrointestinal causes, making it the single deadliest category of maladies for the *ex situ* population[19]. Research investigating gastrointestinal inflammation in the red wolf could help to improve the health of this species and the success of the captive breeding program overall.

The Maned Wolf

The maned wolf is a distinctive South American canid, classified in the order *Carnivora*, and the family *Canidae*[7]. The maned wolf is the only species in the genus *Chrysocyon* and diverged from other canid groups with its most closely related living relative, the bush dog (*Speothos venaticus*), approximately 3 million years ago[54]. The maned wolf has also been identified as the closest extant relative to the extinct Falklands Island Wolf (*Dusicyon australis*) by nuclear and mitochondrial DNA analysis[55] and comparisons of the external brain anatomy within the family *Canidae* [56]. The preferred habitats for this species are the Cerrado, pampas, and Chaco regions of South America. These areas are primarily grasslands, open save for sparse outcroppings of trees and scrubs, and traversed by small bodies of water. Historically, the maned wolf inhabited the entirety of the Cerrado in Brazil, down to Argentina, through the majority of Uruguay, into Bolivia, and potentially into a region of Peru because of its pampas habitat[57]. Currently, the range of the maned wolf has been reduced to central Brazil, as well as some lowland areas of Brazil, and Paraguay. Several individual wolves have been

captured in Uruguay and Argentina, but the populations in these areas are assumed to be small[57].

Maned wolves gain their name from the dorsal erectile black mane that contrasts with the remainder of their long red coat[58]. They also display black coloration on the muzzle, below the elbow on the forelimb and below the talus on the hind limb. White coloration appears on the throat, the inside of the ears, end of tail, and near the mandible [58]. Colloquially called the "fox on stilts," the maned wolf is tall and slight, its stature presumably improving vision in its grassland habitat[58,59]. Adult male maned wolves weigh an average of 23.76 kg with females weighing 22.7 kg[58].

The maned wolf has a unique diet in comparison to other canids. Plant material, including its fruit of choice, the wolf apple (*Solanum lyocarpum),* makes up 43.5% of the maned wolf diet with armadillos (*Dasypus sp*.), rodents, opossum *(Didelphis sp*.) and other small prey comprising the remaining 56.5%[60]. There are also noticeable seasonal differences in diet marked by the consumption of birds in the wet season and more small mammals and the wolf apple in the dry season[61]. In captivity maned wolves are currently primarily fed a species specific chow produced by Mazuri and supplemented with prey items and fruit to mimic the wolf apple. The type and quantity of supplemental prey and fruit varies by institution [62]. The Mazuri maned wolf diet was developed to reduce protein intake in an effort to ameliorate the effects and reduce the chance of development of cystinuria especially in male maned wolves[63]. The composition of the maned wolf Mazuri diet was formulated with the expectation that maned wolves and domestic dogs had similar protein requirements for growth and maintenance [64].

Socially, maned wolves are solitary canids, but will form monogamous pairs that share an average territory size of 27 km^2 [,58]. Females are monoestrus and have a gestation period of 60 to 65 days[65]. Maned wolves are also induced ovulators, making them distinct from other canid species[66].

The current population of maned wolves is confronting many of the threats that come with the exponential increase in the development of the agro-industrial complex in South America. The primary threat impacting the *in situ* population is habitat fragmentation and loss with 80% of the wolf's Brazilian Cerrado habitat converted for agricultural purposes[9] and only 1.5% of the region currently under protection[67]. Habitat fragmentation also has increasingly put wolves in contact with human settlements, exposing them to the dangers of road mortalities and domestic animal pathogens. Road mortalities contribute to approximately 10% of wild wolf mortalities[10]. Maned wolves in the *in situ* population have been shown to be susceptible to canine distemper, parvovirus, adenovirus, coronavirus, rabies, *Leptospira interrogans*, *Dirofilaria immitis* and *Toxoplasma gondii* [68,69,70]. While maned wolves are of great cultural significance in the areas they inhabit, some traditional practices threaten dwindling populations by incorporating ingredients composed of maned wolf parts. For example, consumption of pieces of maned wolf heart are thought to cure snake bite; a tooth necklace is believed to protect against dental problems; and the hide is used to alleviate back and kidney issues[58].

Because the *in situ* population is facing these threats the *ex situ* breeding population has been created to act as a hedge against extinction. The current captive population consists of 88 individuals derived from 31 founders. The breeding program

has maintained 92% genetic diversity and a mean inbreeding coefficient of 0.0280[12]. Like the red wolf, maned wolves suffer from suboptimal health [20,10,71,72,14] low reproductive success and high neonatal mortality[17,10]. In a mortality summary for the 2013-2014 year, out of 12 total adult mortalities, 3 were due to gastritis, 2 had elements of colitis and gastrointestinal mucosal friability, and 1 had intestinal mucosal hyperplasia[73]. In an 18 year study of maned wolf mortality, disorders of the digestive system were causal in 8% of captive adults[10]. The prevalence of gastrointestinal disease in captive maned wolves and its nature as a contributing factor in mortalities, make IBD an important focus of study for this population.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), most simply, refers to inflammation along any portion of the gastrointestinal tract[21]. In humans and murine models, IBD encompasses two primary diseases affecting the GI tract: ulcerative colitis and Crohn's disease[74]. Studies in murine and canine models have aided in the discovery of key aspects of the disease, suggesting that inflammation may be linked to the presence or absence of pathogens, a particular genetic background, and an inappropriate T cell response that can be perpetuated by interactions with dendritic cells[74].

To begin investigating IBD in wild canids, it is important to look at one key model for IBD, the domestic dog. Canine IBD can be classified into four forms based on the types of inflammatory cells that are predominantly present in the affected area: lymphocytic, plasmacytic, eosinophilic and granulomatous. Of the four major forms of inflammation the first three present most often and eosinophilic has the highest rate of

recurrence[21,75]. These cell types infiltrate the gastrointestinal mucosal barrier and can result in lesions anywhere along the gastrointestinal tract with plasmacytic and lymphocytic infiltrates found solely in the intestinal lamina propria[76,77].

Inflammatory Bowel Disease is a complex and multifactorial disease whose causative factors have not been fully elucidated. Studies in domestic dog suggest that IBD can result from disturbance in critical areas of the mucosal barrier, the gut microflora, and the mucosal immune system[75]. Current research has linked IBD to environmental, microbial and genetic factors in human and canine models[75].

Environmental factors linked to IBD include the presence of dietary, pathogenic, and microbial antigens that contribute to inflammation[75]. Much of the research regarding these factors has been conducted in humans; it has been shown that appendectomy and smoking have the greatest effect on the development and persistence of IBD[78]. Associations with IBD have also been made with early exposure to pathogens, the use of oral contraceptives, and infection with measles or *Mycobacterium avium paratuberculosis*[79]. In comparing environmental factors to genetic factors, a study of Swedish twins found that only 17% of identical twins were concordant for IBD, indicating the importance of environmental factors over genetics in contributing to disease prevalence[80]. Environmental factors described in animal models, including domestic dog, typically refer to infectious agents. Non-pylori *Heliobacter* is shown to cause colitis in cotton top tamarins (*Saguinus oedipus*), while *Mycobacterium avium paratuberculosis* causes Johne's disease, an analog of Crohn's disease in cattle *(Bos taurus)*[81]. Boxer dogs with granulomatous colitis were shown to have aggressive,

invasive, and adherent *Escherichia coli* colonizing their intestinal mucosal and contributing to the cycle of inflammation[82].

Microbial factors can disrupt the delicate balance of commensal and pathogenic microbes inhabiting the mucosal barrier of the intestine, thus, contributing to IBD[75]. Commensal bacteria aid the body in metabolism, GI development and immune homeostasis in the gastrointestinal tract[83]. In humans, patients with ulcerative colitis and Crohn's disease can be differentiated from each other and from healthy patients using abnormalities in their microbial communities. Individuals with IBD show lower levels of *Lachnospiraceae, Bacteroidetes*, and butyrate-producing bacteria[83]. These organisms are involved in the maintenance of GI health with butyrate specifically acting as a source of energy for epithelial cells, thus allowing them to bolster the strength of the epithelial barrier[83]. In domestic cat (*Felis catus*), *Bifidobacterium* and *Bacteroides* levels are significantly lower, while *Desulfovibrio* levels are higher in IBD positive individuals than non-affected cohorts [84]. Likewise, in the small intestine of dogs with IBD, *Bacteroidetes* are lacking in comparison to unaffected individuals and members of the *Enterobacteriaceae* family are more prevalent[85]. Abnormalities in the microbial community are a potential causative factor for the development of IBD in wild canids. Genetic factors have been found to play a role in the pathogenesis of IBD and many genes and proteins have been suggested as potential players. Over 30 genes have been identified that are associated with IBD in the human and mouse[86]. One of the earliest genes to be confirmed and one of the most studied is the nucleotide binding oligomerzation domain 2 (NOD2). NOD2 produces proteins that are involved with

recognizing pathogens within the cell and, thus, play an important role in maintaining the integrity of the intestinal mucosal barrier[87]. Other genes associated with IBD fall into clusters according to location and function. The two most important clusters are the cytokine cluster and the major histocompatibility complex cluster. Genes within these clusters produce pro-inflammatory molecules and regulate the immune system's response to foreign antigens[86]. Mutations in NOD2 or many of the genes within these clusters can produce deleterious effects that result in the disruption of homeostasis at the intestinal mucosal border. Toll-like receptors (TLRs) recognize pathogen associated molecular patterns on the surface of bacterial cells and help to initiate an appropriate immune response through the production of cytokines[88]. In domestic dogs and multiple other species, a group of genes that code for toll-like receptors are associated with IBD[25]. For instance, the expression of TLR3 and TLR4 is significantly different between human patients with and without IBD[89] and polymorphisms in theTLR4 gene have been associated with both ulcerative colitis and Crohn's disease[90]. In mice, individuals that lack Toll-like receptors 2, 4, 5 or 9 are more likely to develop colitis and have a higher mortality risk[91]. Toll-like receptors recognize pathogen associated molecular patterns on the surface of bacterial cells and help to initiate an appropriate immune response through the production of cytokines [88]. Two non-synonymous single nucleotide polymorphisms in TLR4 and three in TLR5 are associated with IBD in domestic dogs, and serve as genetic markers for the disease[25,26].

The present study will use methods developed in domestic dogs to look for the presence or absence of these SNPs and similar diagnostic markers within maned wolf and

red wolf TLR5. TLR5 will be the focus of this research because SNPs within this gene have been shown to be diagnostic for IBD across many different dog breeds[26].

Toll-like receptors

Toll-like receptors, pattern recognition receptors essential to the functioning of the innate immune system, belong to a large family of interleukin 1 receptors [92]. These transmembrane receptors consist of a cytoplasmic Toll-interleukin 1 receptor (TIR) domain, responsible for downstream signal transduction, transmembrane domains and leucine rich (LRR) extracellular domains [93]. These extracellular LRR motifs form a ligand-binding horseshoe shaped solenoid that recognizes pathogen associated molecular patterns (PAMP) [94,95] on the surface of bacterial cells and helps to initiate an appropriate immune response through the production of cytokines [96]. The pathogen recognition capability of TLRs make them an important component of the innate immune system and indicates a greater specificity for this system [93]. Six TLR families have been identified in vertebrates, with each family recognizing a specific class of PAMP [29]. Receptors within the TLR5 family are responsible for detecting bacterial flagellin and mammalian TLR5 recognizes flagellin from both Gram-negative and gram-positive bacteria [97].

Phylogenetic analysis places the origin of TLRs at 700 mya [94]. Studies attempting to identify the applicable model of evolution for these immune genes have oscillated between claiming that vertebrate TLRs are highly conserved because of the functional constraint of PAMPs [29] or that they are experiencing positive selection as a result of their constant interaction with rapidly evolving pathogens [28]. Signatures of positive selection have been found in all mammalian TLRs in primates, while other vertebrates have higher

rates of positive selection in non-viral TLRs than in viral TLRs [28]. In all species where adaptive selection has been identified, selective pressure is focused on the LRR extracellular domain because of its interaction with PAMPs [28] while a large portion of the TIR domain remains relatively conserved [92]. In TLR5, evidence of adaptive evolution has been found in the LRR pattern recognition extracellular domain [27], [98] and a specific signature of adaptively evolving codons within this domain identified in domesticated mammals [98].

Because of their important role in the innate immune system, TLRs have been associated with many maladies. Mutations in TLRs or their associated signaling pathways have been linked to pneumococcal disease, systemic lupus erythematosus, chagas cardiomyopathy, malaria and tuberculosis in humans [99].

Of particular interest to this study, TLRs have also been implicated in the pathogenesis of many gastrointestinal disorders [99]. A healthy gut is characterized by its ability to regulate its immune response to food antigens and commensal bacteria while remaining able to respond to pathogens. When this balance is disrupted, it can lead to inflammation and IBD. TLRs play an important role in maintaining this balance [100]. In humans, polymorphisms in the TLR2 gene as well as the TLR4 gene are more likely to be present in patients with colorectal cancer, and a TLR9 polymorphism has been associated with Crohn's disease. SNPs in TLR1, 2 and 6 are shown to associate with both ulcerative colitis and Crohn's disease [100] and genomic methods implicate TLR7 and 8 in celiac susceptibility in humans [99]. In mice, individuals that lack TLR 2, 4, 5 or 9 are more likely to develop colitis and have a higher mortality risk [91,101]. Polymorphisms in

the TLR5 gene are significantly associated with IBD in domestic dogs [25], with two protective SNPs identified across many different dog breeds [26].

HYPOTHESES AND OBJECTIVES

The overall aims of this study were to characterize polymorphisms within two selected regions of TLR5 and to determine the role of previously identified genetic markers for IBD in maned wolves and red wolves. My central questions were as follows: (1) Are methods developed in the domestic dog for extracting, amplifying and sequencing TLR5 applicable in the maned wolf and red wolf, (2) Are there any mutations or diagnostic regions of TLR5, including the SNPs identified in domestic dog, that are significantly associated with disease activity in maned wolves or red wolves, and (3) Are there significant differences in TLR5 DNA sequence variation and resulting amino acid variation between red wolves, maned wolves and other canid species. My first objective was to test the hypothesis that there is sufficient homology between the maned wolf, red wolf and domestic dog genomes to successfully extract, amplify, and sequence red and maned wolf TLR5 using methods developed in the domestic dog. Objective 2 was to test the hypothesis that mutations or variable regions of TLR5, including SNPs identified in domestic dog, can be associated with disease activity in maned wolves and red wolves by correlating a disease activity scoring index with genetic evidence of mutations or variable regions . Objective 3 was to test the hypothesis that there will be identifiable and quantifiable differences in variation between maned wolves and red wolves and other

canid species due to differential evolutionary processes acting on this gene. This study is the first to attempt to characterize TLR5 and apply genetic methods to the question of IBD in two threatened canid species. Inflammatory Bowel Disease is a devastating disease for the *ex situ* populations and a better understanding of the pathogenesis of this disease could potentially lead to new avenues for disease prevention and treatment.

MATERIALS AND METHODS

Sample Collection

Thirty one maned wolves (24 *ex situ* and 7 *in situ)* and fifteen red wolves were sampled for this study. Due to the opportunistic collection of samples, an IACUC was not required by either the Smithsonian Conservation Biology Institute's IACUC committee or George Mason's IACUC committee. *Ex situ* maned wolf samples were collected during routine physical examinations from individuals housed at the Smithsonian Conservation Biology Institute in Front Royal, VA and at three other Species Survival Plan (SSP) participating Association of Zoos and Aquariums (AZA) accredited institutions, including: Louisville Zoo in Louisville, KY, White Oak in Yulee, FL and Fossil Rim Wildlife Center in Glen Rose, TX . Maned wolf *in situ* samples represent populations situated in Bolivia (N=5), Argentina (N=1) and Brazil (N=1). Red wolf *ex situ* samples were collected from individuals at the Point Defiance Zoo and Aquarium in Tacoma, WA. For extant *ex situ* individuals of both species blood was collected opportunistically into one 3ml EDTA coated tube by the veterinary staff during routine examinations. For deceased *ex situ* individuals, necropsy samples were requested with a biomaterials request form. The primary tissue for collection was liver. A $4mm^2$ cube of tissue was collected in a 1.5 ml cyrovial and frozen at -20°C or -80°C (if available) and

subsequently shipped frozen to SCBI. *In situ* maned wolf samples were obtained from DNA extracted for previous studies that investigated genetic variability of maned wolves throughout their range [102]. Bolivian samples were from populations in Noel Kempff Mercado National Park[103] and the samples from Argentina and Brazil were from samples stored in the frozen tissue collection at the Conservation Genetics Laboratory at Departamento de Biodiversidad y Genética-IIBCE-Uruguay.

DNA extraction

DNA was extracted from whole blood and tissue using a Qiagen DNeasy blood and tissue kit (#69581, Qiagen, MD). Spin column purification was used to purify 100 ul of anticoagulated blood. Qiagen tissue lysis buffer was substituted for phosphate buffered saline (PBS) and DNA eluted in 100 µl buffer AE with no repeat elution.

The Qiagen DNeasy blood and tissue kit spin column purification was used to extract DNA from tissues. A sliver of tissue weighing approximately 1 g was cut from the collected 4 mm^2 cube with a thin scalpel and used for extraction. The sample was incubated at 56º C overnight for thorough lysis and eluted in 100 µl buffer AE with no repeat elution.

DNA Amplification

DNA concentration and quality was measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, DE). Two fragments surrounding the three IBD associated SNPs [25] were selected for amplification (Fig. 1).

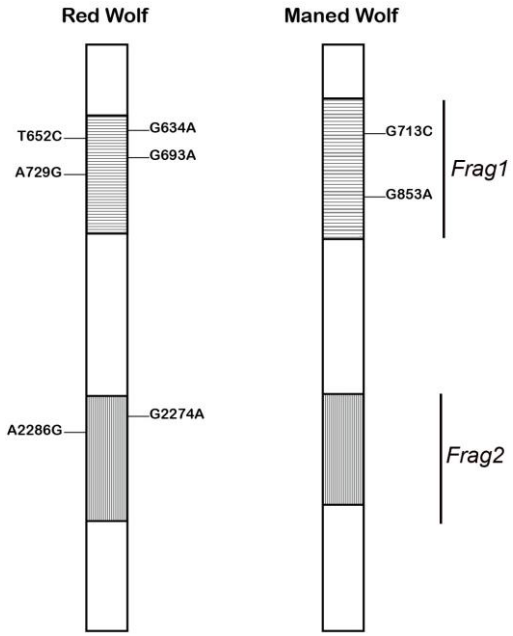

Figure 1: Single nucleotide polymorphisms identified in TLR5 in 31 maned wolves and 15 red wolves within the indicated Fragment 1 and 2 regions

The first fragment (Frag1) is 423 bp long and contains SNPs G22A (G727A in this study) and C100T (C805T); and the second fragment (Frag2), contains SNP T1844C (C2549T) and is 413 bp in length. Primers (Supp Fig. 1) were designed to amplify these fragments using the Primer3 software[104] against domestic dog TLR5 Genbank accession NW_0119176 and Ensembl accession ENSCAFT00000018059 . AmpliTaq Gold Taq and buffer (#4398813, Applied Biosystems, NY) were used for all polymerase chain reactions (PCR) but cycling conditions varied between fragments (Supp Fig. 2). All reactions were run on a Biorad DNA engine Peltier thermal cycler tetrad (Bio-Rad, CA). To inspect products for specific binding, and for the quality and quantity of amplified DNA, PCR products were run on a 1.5% agarose gel using GelRed dye (Biotium), a BioRad

PowerPac Basic gel box (Bio-Rad, CA) and Tris-Acetate (TAE) buffer. Gels were visualized using a MultiDoc-it Digital Imaging System (UVP, CA).

DNA Purification

Effective purification methods varied based on fragment and species due to the variation in size of nonspecific bands appearing in PCR products. For maned wolf samples nonspecific bands were typically less than 100 bp, TLR5 fragments 1 and 2 were purified using 2 µl of EXOsapIT (#78250, Affymetrix, CA) per 7 µl of PCR product and incubated first at 37ºC for 25 min followed by 80ºC for 15 min. In red wolves, where contaminating products are typically larger than 100 bp, products were purified with solid phase reversible immobilization using carboxyl coated magnetic beads (SPRI beads) (#B23317, Beckman Coulter, MD). Samples were incubated for 10 min at room temp, then 5 min on a magnetic separation plate, subsequently washed with 100% ethanol and eluted with Qiagen Elution Buffer (#19086, Qiagen) and 20% Tween (EBT) [105].

Sequencing

Purified products were sequenced using Big Dye Terminator v3.1 (#4337455, Applied Biosystems). Samples were heated to 96º C for 2 min, followed by 24 cycles of 96º C for 10 sec, 50º C for 10 sec and 60º C for 4 min. Sequenced fragments were cleaned using a Sephadex G50 (#17-0041-01, GE Healthcare, VA) column. After the application of water to dry Sephadex powder and the subsequent solidification of the powder, sequencing products were applied to the column, and centrifuged at 2500 RPM for 5 min in an Allegra X-15R plate centrifuge (VWR, PA). Ten microliters of Hi-Di Formamide (#4311320, Life Technologies, NY) were added to each well of sample and

the plate was sequenced on an ABIPRISM3100 genetic analyzer (Life Technologies). All fragments were sequenced on both the forward and reverse strands to confirm polymorphic positions.

Clinical Analysis

Disease status in maned wolves was assessed using a questionnaire developed for veterinary staff (Supp. Maned Wolf IBD Questionnaire). The questionnaire asked for general information about each wolf, including: identification number, sex, age and date of record review, as well as information regarding preexisting conditions, prior illness, and treatment regimen. The second portion of the questionnaire is a modified version of the canine inflammatory bowel disease activity index, developed for domestic dogs[76]. The index provides a scoring based on the severity of six diagnostic symptoms to evaluate disease activity within individuals during an episode of IBD. The symptoms include attitude/activity, appetite, vomiting, stool consistency, stool frequency, and weight loss. Cautionary notes were provided for potential species specific irregularities for these measurements. For example, for veterinary staff reviewing clinical reports for stool consistency in maned wolves, the questionnaire advises that maned wolf stool consistency is usually poor and the scoring scale has been adjusted to reflect this difference. Users of the questionnaire were asked to choose three gastrointestinal episodes within the clinical record for the lifetime of the animal using the criteria of: severity of episode (with preference for the most severe); how many of the six symptoms were presented; and, the amount of detail present in clinical notes. Each symptom within each episode was scored on a scale of 0-3 (3 being the most severe symptoms). The

average of the symptom scores for all three episodes was used to assess the overall disease activity of the individual. An overall score of 0-3 indicates a wolf with clinically insignificant disease and scores above 3 indicate the definitive presence of IBD, the severity of which increases accordingly with the overall score. If a wolf was continuously presenting signs of gastrointestinal distress users were asked to score their overall gastrointestinal health. Wolves with an overall score of 0-1 were considered control individuals and IBD positive was defined as individuals with an overall score above 3. Wolves under two years of age were excluded from scoring due to the increased presentation of disease associated symptoms in middle aged dogs. For both maned wolf SNPs the minor allele was present in a heterozygous condition, so individuals were classified as either heterozygous or homozygous for each SNP to correlate with CIBDAI scores.

Red wolves with intestinal inflammation do not present characteristic clinical signs[106] that can be scored, and thus CIBDAI was not used to assess disease activity in sampled red wolves. All sampled red wolves had varying degrees of intestinal inflammation clinically characteristic of inflammatory bowel disease as assessed by intestinal biopsies performed at Pt Defiance Zoo and Aquarium[106].

Data Analysis

Sequenced fragments were aligned using the software program Sequencher 5.3 (Gene Codes, MI) and inspected manually for the presence of polymorphic positions. Subsequent contigs were aligned with available published domestic dog sequences for TLR5 (Genbank accession NW_0119176 and Ensembl accession

ENSCAFT00000018059). SNP position was reported in reference to ENSCAFT00000018059. The number of SNPs in the two amplified regions of red wolf and maned wolf TLR5 were counted and compared to the number of SNPs in the same two regions in domestic dog [107]. Heterozygous positions were identified in Sequencher and corroborated by manual inspection. For heterozygous loci, the gametic phase was determined using the software PHASE [108]. Mean heterozygosity was calculated and compared between *ex situ* and *in situ* maned wolf samples using a Mann-Whitney U test and between maned wolves, red wolves and previously published values for domestic dog and gray wolf [107] using a one way ANOVA with a Bonferroni post hoc. Nucleotide diversity (Θ) was calculated using a Tajima's test of neutrality in MEGA 5.22[109] . To investigate patterns of selection rates of non-synonymous (dN) and synonymous (dS) substitutions were calculated using both the codon based HyPhy selection model and the Nei and Gojobori (1986) method, with Jukes-Cantor correction using MEGA software version 5.2.2 [109] .

Translation of fragment sequences into amino acids was performed using Sequencher (Gene Codes, MI). Amino acid change ratio was calculated by dividing the length of the resulting translation for each fragment by the number of amino acid changes created by nonsynonymous SNPs and compared to the amino acid ratio for each fragment calculated using published data for domestic dog TLR5 [107]. Protein domain predictions were made in SMART [110] and used to identify domains encompassed in Frag1 and Frag2 as well as the domain type for identified SNPs. PROVEAN [111] was used to predict the functional impact of SNPs resulting in non-synonymous mutations by taking into

consideration the amino acid sequence surrounding the residue of interest and classifying the mutation as either deleterious or neutral.The relationship between CIBDAI scores and alleles present in the two maned wolf SNPs (G713C and G853A) was assessed using an independent T test.

RESULTS

I detected two polymorphic positions between the two selected fragments in maned wolves, both within Frag1 and six polymorphic positions in red wolves with four in Frag1 and two in Frag2 (Table 1) (Fig. 1). In contrast, inspection of previously published domestic dog and gray wolf SNP data[107] revealed that dogs and gray wolves have more SNPs within these two TLR5 regions, with seven SNPs in Frag1 and three SNPs within Frag2 in domestic dogs and 5 SNPs in Frag1 and 6 SNPs in Frag2 in gray wolves (Table 2). No polymorphic positions were shared between maned wolves and red wolves. Neither of the SNPs identified in maned wolves were found to be polymorphic in domestic dog and only one red wolf SNP was common to domestic dogs (A729G). A729G was also polymorphic within the published gray wolf SNP data set in addition to G2274A (Table 1).

Table 1: Polymorphic sites in TLR5 in 31 maned wolves and 15 red wolves

Position	SNP ID	Codon[a]		Aa Subst[b]	Protein Domain[c]	Provean Output[d]	Allele Frequency			
		Allele 1	Allele 2				Maned Wolf	Red Wolf	Gray Wolf[e]	Domestic Dog[e]
634	G634A	**G**TC	**A**TC	val/ile*	ncp[1]	neutral	G(1)	G(.86)		G(1)
652	T652C	**T**GG	**C**GG	trp/arg*	ncp	neutral	T(0)	T(.73)		T(1)
693	G693A	CC**G**	CC**A**	pro/pro	ncp		G(1)	G(.91)		G(1)
713	G713C	C**G**C	C**C**C	arg/pro*	ncp	neutral	G(.75)	G(1)		G(1)
729	A729G	GC**A**	GC**G**	ala/ala	ncp		A(0)	A(.82)	A(.4)	A(.39)
853	G853A	**G**TC	**A**TC	ile/val*	ncp	neutral	G(.54)	G(0)		G(0)
2274	G2274A	CG**G**	CG**A**	arg/arg	LRR[2]		G(1)	G(.92)	G(.76)	G(1)
2286	A2286G	GC**A**	GC**G**	ala/ala	LRR		A(0)	A(.92)		A(0)

SNP position is in reference to ENSCAFT00000018059 and SNP ID includes the most frequent allele first, followed by the position and the least frequent allele.

[a] Bold letter is variable allele

[b] Amino acid substitution * non-synonymous aa change

[c] Protein domain predicted by SMART [1] ncp, no confident prediction [2] LRR, leucine rich repeat region

[d] Provean function prediction of non-synonymous SNPs only

[e] Gray wolf and red wolf allele frequencies provided by Francino et al. 2014

In contrast to the finding of more SNPs in domestic dog and gray wolf TLR5, dogs and gray wolves did not significantly differ from maned and red wolves in mean heterozygosity at these SNPs ($P \geq 0.1$) (Table 2). Tajima's D nucleotide diversity measures for maned and red wolves found a greater average variability in maned wolves ($\Theta = 0.002599$) than in red wolves ($\Theta = 0.0013765$) echoing the trend seen in heterozygosity with more variability in maned wolves than red wolves. Within maned wolves there was no significant difference in mean heterozygosity between *ex situ* and *in situ* samples ($P \geq 0.1$)**.**

Table 2: Comparison of number of SNPs, synonynmous and non-synonymous substitutions, and SNP heterozygosity in maned wolves, red wolves, gray wolves and domestic dogs

Species[a]	Number of SNPs			Mean Heterozygosity[d]
	Syn[b]	Non-Syn[c]	Σ	
Maned Wolf	0	2	2	0.44 ± 0.09*
Red Wolf	4	2	6	0.23 ± 0.10*
Gray Wolf	6	5	11	0.27 ± 0.17*
Domestic Dog	6	4	10	0.26 ± 0.15*

Differences in mean heterozygosity between species were tested for significance by a one way ANOVA with a Bonferroni post hoc
* P≥0.10
[a] Mean heterozygosity for gray wolf and domestic dog extrapolated from data published in Francino et al. 2014
[b] Synonymous
[c] Non-synonymous
[d] Mean ± SD

Tests of selection for each species by fragment revealed no evidence of non-neutral selection using a Z-test for Frag1 in maned wolves and Frag2 in red wolves ($P \geq 0.05$). The ratio of dN/dS could not be calculated for these fragments because of the lack of synonymous mutations in each. In red wolf Frag1, though the codon based Z test of selection showed only neutral selection ($P \geq 0.05$), HyPhy calculated dN/dS at 0.146, indicating a slight evidence of purifying selection (Table 3). Tests of selection were not performed for maned wolf Frag2 due to the lack of synonymous or non-synonymous mutations.

Tests of selection between species found evidence for both purifying and positive selection. The dN/dS ratio for Frag1 between maned and red wolves was 0.3362 indicating purifying selection. The Nei Gojobori method with a Jukes Cantor correction for purifying selection also found purifying selection between these species in Frag1 ($P \leq 0.05$) with the probability at 0.05. For maned wolf and red wolf Frag2 strong evidence was found for positive selection ($P \leq 0.05$) with a codon based Z test of selection using a Nei Gojobori model with Jukes Cantor correction yielding an overall probability of 0.03 (Table 3).

Table 3: Tests for Neutral, Purifying and Positive Selection and dN/dS for maned wolves (MW) and red wolves (RW)

Species/Frag[a]	dN/dS[b]	Selection Test[c]					
		Neutral Selection		Purifying Selection		Positive Selection	
		Probability[d]	Test Statistic[e]	Probability[f]	Test Statistic	Probability[g]	Test Statistic
MW[1] Frag1		0.17	1.39	1.00	-1.35	0.08	1.43
RW[2] Frag1	0.146	0.27	1.11	0.13	1.15	1.00	-1.14
RW Frag2		0.14	1.48	1.00	-1.40	0.07	1.45
MW and RW Frag1	0.3362	0.09	1.73	**0.05**	-1.67	1.00	-1.69
MW and RW Frag2		0.07	-1.81	1.00	-1.76	**0.03**	1.88

MW Frag2 not included due to lack of variable sites, significant values in bold
[a]Species abbreviations [1] Maned Wolf [2] Red Wolf
[b] dN/dS for fragments with both non-synonymous and synonymous changes only
[c] Codon-based Z test of Selection, Nei-Gojobori method with Jukes-Cantor correction
[d] Probability of rejecting the null hypothesis of dN=dS
[e] Test statistic= dN-dS
[f] Probability of rejecting the null hypothesis of dN=dS for dN<dS
[g] Probability of rejecting the null hypothesis of dN=dS for dN>dS

We utilized an amino acid change ratio to compare the effect of these described polymorphic sites on resulting proteins. Domestic dogs and gray wolves had a higher amino acid change ratio than either maned or red wolves, suggesting a higher level of conservation in the latter species (Table 4).

Table 4: Comparison of amino acid change ratio in maned wolves, red wolves, gray wolves and domestic dogs

Species[a]	Protein length (aa)	AA change ratio[b]
Maned Wolf	288	1/144
Red Wolf	279	1/139.5
Gray Wolf	288	1/57.6
Domestic Dog	288	1/72

[a] Amino acid change ratio for gray wolf and domestic dog extrapolated from data published in Francino et al. 2014
[b] Amino acid ratio: amino acid changes caused by nsSNPs divided by the protein length

Protein domain structure was predicted for the selected fragments with Frag1 for both maned wolves and red wolves consisted of three unknown domains and two low complexity regions while Frag2 consisted of three leucine rich repeat (LRR) regions, one leucine rich repeat C-terminal (LRR-CT) region and one unknown region. All SNPs in Frag1 in both species were found to be in areas with unknown SMART predictions while both SNPs in red wolf Frag2 were found to be in the LRR region (Table 1).

Both maned wolf SNPs were found to be nonsynonymous in comparison with two out of six red wolf SNPs, five out of eleven gray wolf polymorphisms and four out of ten domestic dog polymorphisms. All nonsynonymous maned wolf and red wolf SNPs were found in Frag1 while domestic dog and gray wolf nonsynonymous SNPs were more evenly distributed between the two fragments.

The functional impact of these nonsynonymous SNPs was tested using PROVEAN and all identified red wolf and maned wolf nonsynonymous SNPs were shown to have a neutral effect on protein function (Table 1). Comparatively, three nonsynonymous domestic dog SNPs and four gray wolf nonsynonymous SNPs present within Frag1 and Frag2 were reported to have a probably damaging or possibly damaging impact on protein function [107]. One of these SNPs is T1844C, a SNP previously associated with domestic dog IBD [25], which was shown to be deleterious [107]. All identified domestic dog and gray wolf SNPs [107] with a potential functional impact are not present as polymorphic positions in maned or red wolves.

The SNPs identified as associated with domestic dog IBD (G727A, C805T and C2549T) were not polymorphic in maned or red wolves. Red wolves and maned wolves

did however show a complete lack of the protective T allele in C805T and C2549T and the risk allele A in G727A (Fig. 2). Gray wolves also lack these protective alleles [107] indicating that the non-protective C is potentially ancestral. Provean predictions show that the deleterious impact of the leucine to serine amino acid change in the C2549T SNP is retained in maned wolves and red wolves (Table 5).

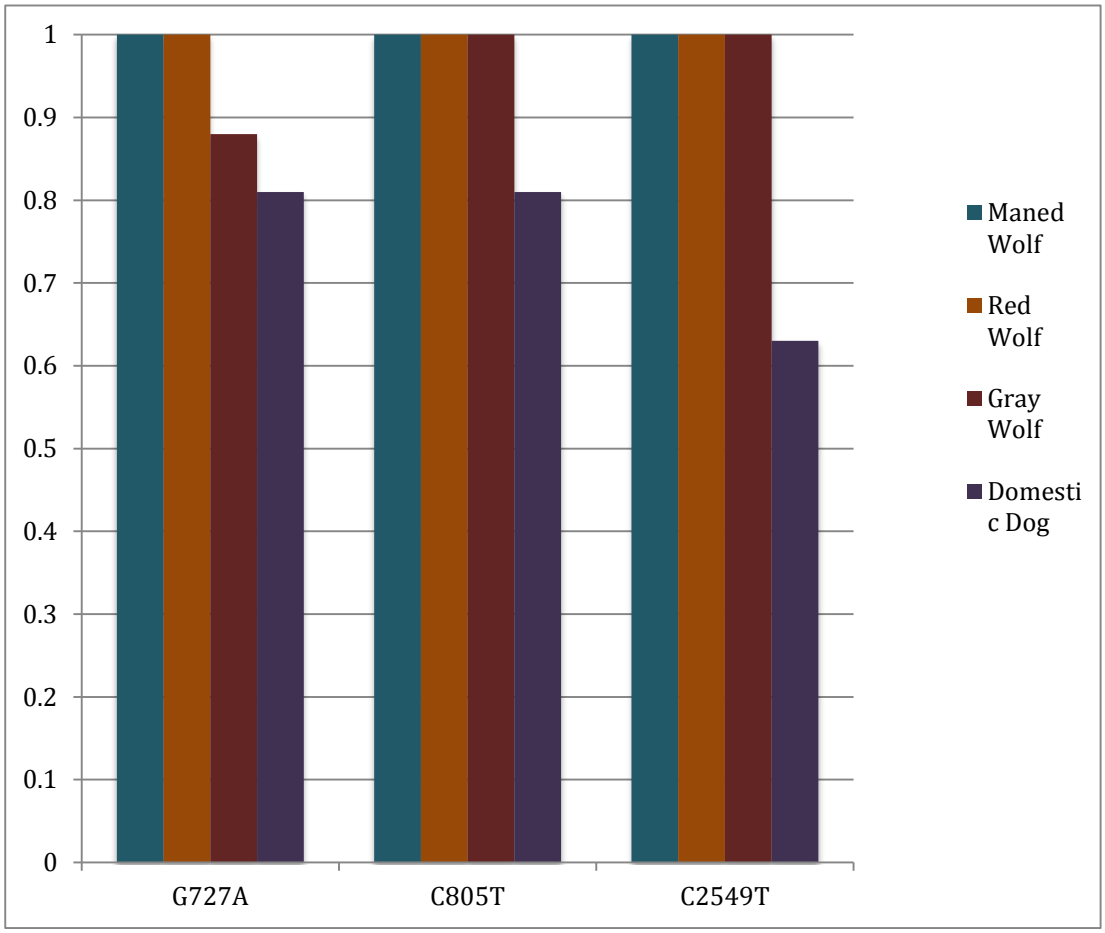

Figure 2: Observed allele frequency for IBD associated SNPs A in G727A, C in C805T and C2549T) in maned wolves and red wolves. Allele frequencies for IBD SNPs provided by Francino et al. 2014.

Table 5: Polymorphic sites associated with Inflammatory Bowel Disease in maned wolves, red wolves, gray wolves and domestic dogs

Position	SNP ID	Codona		AA Subst[b]	Protein Domain[c]	Provean Output[d]	Allele Freq			
		Allele 1	Allele 2				Maned Wolf	Red Wolf	Gray Wolf[e]	Domestic Dog[e]
727	G727A	**G**CG	**A**CG	ala/thr*	ncp[1]	neutral	G(1)	G(1)	G(.88)	G(.81)
805	C805T	**C**GC	**T**GC	arg/cys*	ncp	neutral	C(1)	C(1)	C(.99)	C(.81)
2549	C2549T	T**C**G	T**T**G	ser/leu*	LRR CT[2]	deleterious	C(1)	C(1)	C(.98)	C(.63)

SNP position is in reference to ENSCAFT00000018059 and SNP ID includes the most frequent allele first, followed by the position and the least frequent allele.

[a] Bold letter indicaties a variabe allele

[b] Amino acid substitution * non-synonymous aa change

[c] Protein domain predicted by SMART [1] ncp, no confident prediction [2] LRR CT, leucine rich repeat C-terminal region

[d] Provean function prediction of non-synonymous SNPs only

[e] Gray wolf and red wolf allele frequencies provided by Francino et al. 2014

Of the eight wolves scored with CIBDAI, four were in the control range (CIBDAI 0-1), indicating no disease activity and four were in the range suggestive of active disease presence (CIBDAI >3) (Table 6). CIBDAI scored individuals were either heterozygous or homozygous for SNPs G713C and G853A. No significant correlation was found between homozygous or heterozygous position and mean CIBDAI score (Table 6) indicating that these SNPs are not accurate predictors of disease activity as scored by CIBDAI.

Table 6: Canine Inflammatory Bowel Disease Activity Index scoring results for two SNPs in 8 maned wolves

SNP	G713C[a]	CIBDAI[b]	G713C/G[c]	CIBDAI
G713C	Flint	4.5	Hope	0
	Blue	4		
	Ibera	5		
	Rocko	0		
	Echo	0		
	Uno	0		
	Calysta	4		
Mean CIBDAI		2.5*		0*
SNP	G853A[d]	CIBDAI	G853A/G[e]	CIBDAI
G853A	Blue	4	Calysta	4
	Uno	0	Flint	4.5
			Ibera	5

		Rocko	0
		Hope	0
		Echo	0
Mean CIBDAI	2*		2.25*

The relationship between homozygous or heterozygous state and CIBDAI score were tested with an independent T test

* P≥0.10

[a] below individuals are homozygous at the G713C position for allele G

[b] Canine inflammatory bowel disease activity index

[c] below individuals are heterozygous at the G713C position for alleles C and G

[d] below individuals are homozygous at the G853A position for allele G

[e] below individuals are heterozygous at the G853A position for alleles A and G

DISCUSSION

Toll-like receptors are increasingly becoming a target of research due to their crucial role as sentinels of the innate immune system and their association with many common and debilitating diseases in human and animal models [99]. This study contributes to the existing body of research by characterizing the TLR5 locus in two threatened canid species. It additionally provided a greater understanding of the complexity of this immune gene in nonIn this study we contribute to the body of research of the TLR5 locus with regard to two threatened canid species for which a greater understanding of this immune gene will be an important factor in maintaining healthy *ex situ* populations.

The first hypothesis of this study, that there would be sufficient homology between the maned wolf, red wolf and domestic dog genomes to extract, amplify and sequence red and maned wolf TLR5 with domestic dog methods was rejected due to nonspecific nature of domestic dog primers for either species, the need for different PCR cycling conditions and different purification methods. The second hypothesis, regarding the ability to correlate a disease activity score with the presence of a genetic mutation or with the presence of domestic dog IBD associated SNPs can be partially accepted. While I was unable to find mutations that correlate with disease activity score in maned wolves and the disease activity score proved inappropriate for red wolves, domestic dog IBD SNPs were found to be potentially informative for IBD in these two wolf species. Lastly, the third hypothesis postulated that there were identifiable and quantifiable differences in

variation between maned wolves and red wolves and other canid species. I was able to both identify and quantify variation in TLR5 between maned wolves, red wolves, gray wolves and domestic dog and to tie this variation to evolutionary processes acting on this gene.

The larger number of SNPs in domestic dog and gray wolf and the lack of significant difference between mean heterozygosity in all four species implies that these regions may be more variable in gray wolf and domestic dog but that heterozygosity has been maintained across canid lineages over evolutionary time. This suggests a role for balancing selection in canid TLR5 which has been implicated in the evolution of innate immunity in humans [112]. The higher amino acid change ratio in domestic dogs and gray wolves indicates that the observed genetic variation results in changes in amino acid composition within these two TLR5 regions and suggests that there is a higher level of sequence conservation between two highly divergent canids. Future studies should screen for variation across a larger number of canids to confirm levels of variation across this family.

Consistent with published research identifying the leucine rich repeat region of TLR5 as a site under adaptive selection due to its direct interaction with evolving pathogens [28]. Signatures of adaptive selection were detected within the LRR sequenced here between maned and red wolves, indicating that this ligand binding pocket is adapting to compete with evolving microbes. The higher number of SNPs in both gray wolf and domestic dog and their higher propensity to be nonsynonymous and damaging suggests signs of deleterious allele accumulation in this region of TLR5. Deleterious

allele accumulation has been shown in domestic dogs and could be a result of a previously documented bottleneck in domestic dogs and in the European population of wolves referenced in this study [107, 113] . The complete lack of overlap in variable sites between maned wolf, red wolf, gray wolf and domestic dog and the conservation of just one polymorphic position between red wolves, gray wolves and domestic dog suggests a potential species-specific function for TLR5 in maned wolves and red wolves as seen in other species [116].

In reference to the role of TLR5 in maned and red wolf inflammatory bowel disease, when correlated with CIBDAI scores, no alleles were found to be significantly associated with a positive score of disease activity in maned wolves. This lack of significance indicates that the newly identified polymorphic positions in maned wolves are not appropriate markers for inflammatory bowel disease in this species. Genetic predisposition for IBD in maned wolves and red wolves may be more related to the SNPs implicated in domestic dog IBD, G727A, C805T and C2549[25]. The non-polymorphic nature of these SNPs within maned wolves and red wolves makes them also unsuitable as diagnostic markers for inflammatory bowel disease in these species. However, all sampled *ex situ* and *in situ* maned wolves and red wolves lacked the protective T in the alleles validated across all dog breeds, C805T and C2549T[26] suggesting that wolves may harbor a genetic predisposition to IBD. The high prevalence of IBD in the maned wolf captive population, the ubiquitous presence of intestinal inflammation in the red wolf population and the retention of the deleterious effect of the C2549T SNP are further evidence for this predisposition. A large population of gray wolves also lacks the

protective allele T in both C805T and C2549T, indicating that the non-protective allele C is ancestral and that the T allele emerged in domestic dogs[107].

Taken together, the low SNP number in maned wolves and red wolves in comparison to domestic dog and gray wolf, the neutral functional impact of observed nonsynonymous SNPs and the low amino acid change ratio suggest that these fragments in maned wolves and red wolves are less variable than in domestic dogs and gray wolves. This conservation within species and the purifying selection present in Frag1 is in agreement with previous studies that identify TLRs as a conserved class of protein[29], while the evidence of positive selection in Frag2 agrees with studies that find a role for adaptive selection in mammalian Toll-like receptors[98]. The observation of distinct variation between species, even between maned and red wolves in nucleotide diversity, especially in the ligand binding site, points to a potential specificity of function of TLR5 influenced by differential microbial environments[117]. The functional import of these characterized fragments lies in their relationship to IBD. The lack of the protective T in the maned wolf, red wolf and gray wolf populations and the emergence of this allele in the domestic dog population, suggests a role for this allele in domestication and that the non-protective allele is ancestral. The retention of the damaging impact of this SNP implies that all maned and red wolves may potentially be genetically susceptible to IBD.

Recent work on dog domestication has identified a host of genes containing a signature of domestication, typically in mutations that allow the species to better adapt to an environment in close proximity to humans[118]. Changing diet including adaptation to a starch based diet plays an essential role in the domestication process and provides a

relevant link to IBD[119]. Since TLR5 recognizes bacterial flagellin, a shift in gut microbiome composition as a result of diet change[120] can result in an inappropriate hypo or hyper activation of the TLR pathway and lead to inflammation[89]. It is possible that the protective T allele in domestic dog C805T and C2549T developed as a protection against this type of inflammation.

Ex situ maned wolves and red wolves in the United States are primarily fed a starch based diet [12,5] in contrast to their *in situ* omnivorous and carnivorous diets respectively [61,36]. The resulting changes in the gastrointestinal microbial community can result in dysregulation of TLR5 and the development of IBD. This relationship between putative genetic predisposition, inappropriate diet and a resulting foreign microbial community could explain the high prevalence of IBD in *ex situ* maned wolves and red wolves.

Future studies should focus on documenting the gastrointestinal microbiome compositions of *ex situ* and *in situ* maned and red wolves and correlating these microbial results with clinical, histopathological and serum markers of IBD. Further characterization of the full sequence of TLR5 for these sampled populations of maned and red wolves could additionally inform the evolutionary history of extracellular Toll-like receptors within the *Canis* genus.

RESEARCH IMPACT AND SIGNIFICANCE

The environmental, social, and biological challenges that have led to the decline of both the now critically endangered red wolf and near threatened maned wolf are not unique to these species. Habitat fragmentation and degradation that threatens to destroy the maned wolf's native Cerrado, is the leading cause of anthropogenically related extinction [9]. Roads and associated infrastructure that account for a large proportion of red and maned wolf mortality [2,10] have been shown to cause a decline of between 25 and 38% in species abundance within 17 km of a road in 33 mammal species [121]. Human wildlife conflict, created by livestock predation in the case of the maned wolf and by mere carnivore presence in the case of the red wolf has already led to the extinction of another canid species, the Falkland Island wolf, hunted due to its suspected depredation of sheep flocks [122]. Of importance to this study, disease has negatively affected dwindling wild canid populations that are often exposed to pathogens like canine distemper and parvovirus from neighboring feral domestic dog populations[123].

These threats emphasize the growing importance and necessity of healthy and viable *ex situ* populations that can serve as current sources for the *in situ* population, like the red wolf or, like the maned wolf, as an ark population in reserve for anticipated future declines. Twenty five species have been saved from extinction in the wild through

captive breeding programs and currently *ex situ* populations have served as reservoirs for reintroduction for 121 bird and mammal species[124]. The model of reintroduction to replenish diminishing populations or to reinstate species to a depopulated landscape has been largely successful for canid species. The archetype of carnivore recovery, the return of the gray wolf to Yellowstone National Park, has faced opposition but overall is regarded as a success. The return of the wolf had profound and far reaching ecological impacts, with a reduction in coyote and elk density and change in behavior in both species leading to a transformation of the surrounding forests[125,126,127]. The red wolf, reintroduced into a different landscape, an amalgamation of reserve and private property on a peninsula, has faced many of the same challenges as the gray wolf but the intensity of public pressure surrounding the red wolf encroachment on private lands has hindered the success of the population[37].The current review of the red wolf reintroduction project [128] threatens to end the program altogether and emphasizes the essential nature of the red wolf *ex situ* population for the survival of the species.

The viability of *ex situ* efforts that directly result in reintroduction, as in the case of the red wolf and may in the future for the maned wolf depend heavily on maintaining populations of healthy, breeding and genetically viable individuals. The impact of this study with regard to species conservation is in its implications for improving the health of the *ex situ* population. Having a greater understanding of the genetic basis for inflammatory bowel disease can assist in narrowing the list of causative elements for this complex and multifactorial disease.

In addition to the significance of this study for the pathogenesis of inflammatory bowel disease, describing the partial sequence of Toll-like receptor 5 from two threatened wild canid species is informative for the study of variation within Toll-like receptor genes and the evolutionary processes that act on these genes in mammals.

Toll-like receptors are an important part of the innate immune system and though initially thought to be less specific than the adaptive immune system, are now known to specifically recognize different pathogen types[93]. Wildlife studies of immune system variation have traditionally focused on the major histocompatibility complex (MHC), a set of genes involved in regulating functions associated with immunity and kin recognition [129]. This almost singular focus on MHC has recently been questioned, considering that non-MHC genes account for more than half of genetic variability associated with infection risk. Recent studies have focused on other gene families including, chemokine receptors, immunoglobulin receptors, interferon genes, natural macrophage proteins and Toll-like receptors[129]. These investigations into immune associated genes have greatly informed the understanding of immune response and variation in wildlife species.

This immune variation is the result of a co-evolutionary arms race between host and pathogen, the host attempting to evolve beyond a debilitating infection and the pathogen attempting to maintain its ability to infect[130]. The participation of Toll-like receptors in this race is debatable with many studies indicating that Toll-like receptors experience positive selection[27], while others insist on the conservation of this class of protein due to conserved nature of its target antigen, the pathogen associated molecular

pattern[29]. This variable or conserved nature of TLR5 based on species is informative to the reconstruction of the evolutionary history of environmental pathogen exposure for a particular species. Differences between species may reflect not only differential microbial environments but also, in the case of conserved Toll-like receptor regions, a potential inability to respond to novel antigens.

The differential patterns in Toll-like receptor variation observed here between maned wolves, red wolves, gray wolves and domestic dog not only emphasize a species-specific function for Toll-likereceptor 5 but may be informative for the process of domestication. The protective alleles associated with domestic dog inflammatory bowel disease appear only in this species, indicating a potential functional adaption to domestication similar to that seen in their adaptation to a starch based diet[119]. The study of the process of dog domestication is essentially the study of the ability of a species to adapt to life in close proximity to humans and human settlement. This proximity is increasingly becoming the reality for many non-domestic species as human settlements and agricultural lands infringe on previously wild spaces. Understanding domestic adaptations to these conditions may help us to predict how wild species will adapt to this changing landscape. Additionally understanding the genetic differences between domestic and wild canids can inform conservation research regarding current zones of admixture between gray wolves and domestic dog and help to address the potential threat of hybridization and introgression for dwindling gray wolf populations[131].

Though this study addresses the process of domestication, its most important result lies in its implications for the health of non-domestic *ex situ* populations and often

their treatment as domestics with regard to food and handling. The differential pressures of captivity have even led some *ex situ* populations to develop genetic adaptations to captivity, which, over many generations make the population unsuitable for reintroduction[124] due to the inherently inappropriate nature of these adaptations to *in situ* survival. In the short-term captivity can lead to negative behavioral patterns like pacing[132] and can diminish natural behaviors like predator avoidance within a few generations[133]. Wide ranging species like the red and maned wolf are more vulnerable to these captive behavioral aberrations and health issues[132]. To preserve the short term clinical health and the long term genetic health of *ex situ* populations it is therefore important to guard against aspects of captivity that diminish these measures of health and are detrimental to reintroduction success.

Considering that Toll-like receptor 5 acts in the gastrointestinal region, diet is the aspect of captivity that is most relevant to the pathogenesis of inflammatory bowel disease. In captivity maned and red wolves are often fed unnatural diets. *Solanum lycocarpum,* the fruit that comprises 43.5% of the maned wolf diet[60] is not able to be imported into the United States or Europe and is thus not available for much of the captive population of maned wolves, including the wolves sampled for this study. Maned wolves are instead offered a supplement of various fruits including apples, papayas, grapes, and coconut in addition to a primarily plant based commercial food produced by Mazuri. This food was developed specifically for maned wolves to address another contributor to poor health in captivity, cystinuria[63]. An overabundance of protein in the diet can lead to the development of cysteine crystals in the urine and subsequent urinary

tract issues especially in male wolves[72]. Red wolves are fed a variety of diets falling on a spectrum from dog food to a more varied carcass based diet. The red wolves sampled for this study reside at a facility that feeds primarily dog chow due to the need to sustain a large breeding population and the costs associated with feeding this population[37].

The importance of differences in diet between captive and wild individuals lies in the resulting changes these diet differences can have on an individual's microbial community[134]. Feeding a wild canid a diet developed for domestic dog may assist in developing a microbial community more similar to domestic animals than to their native congeners[135]. Since Toll-likereceptor 5 specifically recognizes bacterial flagellin, a dog like microbiome interacting with a maned or red wolf Toll-likereceptor evolutionarily adapted to the native microbiome of these species could result in a hypo or hyper activation of this portion of the innate immune system. While dogs have had approximately11,000-32,000 years[118,136] of domestication to adapt to a starch based diet, maned and red wolves have been managed by species survival plans in captivity for 30 and 35 years respectively. Adaptation to a starch based diet should not be a goal of captive breeding programs and should be avoided at all costs for any *ex situ* programs that may eventually result in reintroduction.

There is also room in the management of captive populations for the application of the precautionary principle with regard to potential reintroduction. It may behoove captive programs to always manage for reintroduction in an attempt to limit the affects of captivity as they have significant genetic and health implications for the managed population.

This study uses genetic methodology to contribute to the conservation of the near threatened maned wolf and the critically endangered red wolf. By characterizing a key Toll-likereceptor gene in both species this study not only addresses the potential genetic basis for a debilitating gastrointestinal disease in captivity but provides insight from two rare species to the scientific debate regarding the evolutionary nature of mammalian Toll-likereceptor genes. The comparison of maned and red wolf TLR5 with domestic dog and grey wolf additionally contributes to research focused on the process of domestication. The potential negative role management practices may play in the pathogenesis of inflammatory bowel disease in both of these imperiled canid species emphasizes the need for careful adaptive management of *ex situ* populations especially for species whose future in the wild is uncertain.

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SUPPLEMENT

Supp. Fig. 1

Primer	Manufacturer	Direction	Primer sequence
TLR5 Fragment 1	Eurofins MWG (Huntsville, USA)	Forward	5'-GTT TCT CAA GGA CCC AGC AC-3'
		Reverse	5'-TCC TGA AGG CTT CTC TGT CG-3'
TLR5 Fragment 2	Eurogins MWG (Huntsville, USA)	Forward	5'-GCT GCA CCT GAA CCA CAA C-3'
		Reverse	5'-TGA AGA GGG AGA ACG TGA GG-3'

Supp. Fig. 2

Frag1 cycling conditions		
Cycle Number	Settings:	
1	95°C	10 minutes
35	95°C 57°C 72°C	1 minute 1 minute 2 minutes
1	72°C	7 minutes
Frag2 cycling conditions		
Cycle Number	Settings:	
1	95°C	8 minutes
2	95°C 64°C 72°C	30 seconds 30 seconds 1 minute
2	95°C 62°C 72°C	30 seconds 30 seconds 1 minute

2	95°C 60°C 72°C	30 seconds 30 seconds 1 minute
2	95°C 58°C 72°C	30 seconds 30 seconds 1 minute
2	95°C 56°C 72°C	30 seconds 30 seconds 1 minute
1	72°C	15 minutes

Maned Wolf IBD Questionnaire:
Please complete a separate questionnaire for each wolf.

Section A: General Information

Name:_____
Accession #:_____
Sex: _____
Age:_____

Date of analysis:_____

Section B: Preexisting conditions and Prior illness
Does this individual have a history of (yes/no):

a. Gastrointestinal disease or inflammation _____
b. Gastrointestinal bacterial infection (e.g. salmonella, clostridium)_____
c. Hypoadrenocortiscm _____
d. Immune related disease _____
e. Any other Infectious disease _____
f. Any chronic health issues (please describe)_____

_____

_____

g. Is this wolf currently medicated for gastrointestinal issues?

_____

h. Is this wolf receiving any medication currently?, If so, what?

_____

Section C: CIBDAI, Inflammatory Bowel Disease scoring questionnaire (attached)

This scoring system, developed for domestic dog, will give us a standardized way to look at the symptomatic presentation of IBD in maned wolves. To complete this scoring index we ask you to:

1. Choose three gastrointestinal episodes that you have on record for each wolf.
    a. A gastrointestinal episode is a defined as a period of gastrointestinal distress defined by any to all of the following symptoms: vomiting, diarrhea, poor stool consistency, change in stool frequency, change in appetite/attitude/activity, and weight loss.
    b. Choose gastrointestinal episodes using these criteria listed in order of importance:
        i. Severity of the episode, preference for most severe
        ii. how many of the 6 symptoms are presented
        iii. detail available in clinical notes
    c. If you do not have three gastrointestinal events, score as many as possible

d. If the wolf has been continuously presenting signs of gastrointestinal distress, score their overall gastrointestinal state.

2. Answer the scoring questionnaire for each episode. For example, if you have two wolves and you have been able to find three gastrointestinal episodes for each wolf you should fill out 6 scoring questionnaires.

IBD Scoring Questionnaire

Please complete this questionnaire for each of the three gastrointestinal episodes for each wolf.

Gastrointestinal Episode 1:

1. When was this gastrointestinal episode? _____
2. How long did it last (in days?)?_____
3. Was this wolf undergoing any treatment for IBD prior to or during this episode? _____
4. Was this wolf put on any treatment in response to this episode? _____

1. Attitude/activity =_____

a. normal
b. Slightly decreased
c. moderately decreased
d. severely decreased

2. Appetite =_____

(Consider seasonal changes in consumption, typically more in winter, less in summer when answering this question)

a. Normal
b. Slightly decreased
c. moderately decreased
d. severely decreased

3. Vomiting=_____

a. None
b. Mild (1time/week)
c. moderate (2-3 times/week)
d. severe (>3 times/week)

4. Stool consistency=_____

(maned wolves have typically soft stools, the scoring has been altered to reflect this)

a. normal= slightly soft feces
b. soft feces or fecal blood mucus or both
c. very soft feces
d. watery diarrhea

5. Stool frequency=_____

a. normal
b. slightly increased (2-3 times/day)
c. moderately increased (2-5 times/day)
d. severely increased (>5 times/day)

6. Weight loss=_____

a. none
c. moderate (5-10% loss)

b. mild (<5% loss) d. severe (>10% loss)

Gastrointestinal Episode 2:

1. When was this gastrointestinal episode? _____
2. How long did it last? _____
3. Was this wolf undergoing any treatment for IBD prior to or during this episode? _____
4. Was this wolf put on any treatment in response to this episode? _____

1. attitude/activity =_____
   a. 0=normal
   b. 1=slightly decreased
   c. 2= moderately decreased
   d. 3=severely decreased

2. Appetite =_____
   (consider seasonal changes in consumption, typically more in winter, less in summer)
   a. 0=normal
   b. 1= slightly decreased
   c. 2= moderately decreased
   d. 3= severely decreased

3. Vomiting=_____
   a. 0=none
   b. 1=mild (1time/week)
   c. 2= moderate (2-3 times/week)
   d. 3=severe (>3 times/week)

4. Stool consistency=_____
   (maned wolves have typically soft stool, the scoring has been altered to reflect this)
   a. 0=normal= slightly soft feces
   b. 1=soft feces or fecal blood mucus or both
   c. 2=very soft feces
   d. 3=watery diarrhea

5. stool frequency=_____
   a. 0=normal
   b. 1=slightly increased (2-3 times/day)
   c. 2= moderately increased (2-5 times/day)
   d. 3= severely increased (>5 times/day)

6. weight loss=_____

a. 0=none
b. 1=mild (<5% loss)
c. 2= moderate (5-10% loss)
d. 3=severe (>10% loss)

Gastrointestinal Episode 3:

1. When was this gastrointestinal episode? _____
2. How long did it last? _____
3. Was this wolf undergoing any treatment for IBD prior to or during this episode? _____
4. Was this wolf put on any treatment in response to this episode? _____

1. attitude/activity =_____
   a. 0=normal
   b. 1=slightly decreased
   c. 2= moderately decreased
   d. 3=severely decreased

2. Appetite =_____
   (consider seasonal changes in consumption, typically more in winter, less in summer)
   a. 0=normal
   b. 1= slightly decreased
   c. 2= moderately decreased
   d. 3= severely decreased

3. Vomiting=_____
   a. 0=none
   b. 1=mild (1time/week)
   c. 2= moderate (2-3 times/week)
   d. 3=severe (>3 times/week)

4. Stool consistency=_____
   (maned wolves have typically soft stool, the scoring has been altered to reflect this)
   a. 0=normal= slightly soft feces
   b. 1=soft feces or fecal blood mucus or both
   c. 2=very soft feces
   d. 3=watery diarrhea

5. stool frequency=_____
   a. 0=normal
   b. 1=slightly increased (2-3 times/day)
   c. 2= moderately increased (2-5 times/day)

d. 3= severely increased (>5 times/day)

6. weight loss=_____
   a. 0=none
   b. 1=mild (<5% loss)
   c. 2= moderate (5-10% loss)
   d. 3=severe (>10% loss)

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