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RHINOCEROUS CONSERVATION IN KENYA – THE IMPLICATION OF TRANSLOCATION IN DISEASE TRANSMISSION DYNAMICS

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Rhinocerous Conservation in Kenya –The implication of Translocation in disease transmission dynamics

La conservación de los rinocerontes en Kenia- La implicación de la translocación en las dinámicas de transmisión de la enfermedad

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Chapter 1: Introduction, justification and research approach

Rhinoceros were so abundant in Kenya in the early 20th Century (Barclay, 1932, Brett, 1993, Hunter and Watson, 1952, Lloyd-Jones, 1925, Neumann, 1898, Patterson, 1909) that there were around 20,000 individuals in 1970's (AfRSG., 2008) widely distributed. However, the population declined catastrophically due to poaching and human settlement during the following 20 years, to less than 400 animals by 1990 (Brett, 1993, Emslie and Brooks, 1999, Gakahu, 1993, Okita-Ouma et al., 2007). This collapse resulted in small, isolated, demographically unviable populations scattered across fragmented regions in Kenya, with many facing local extinction. An ambitious translocation program for isolated rhinoceros populations that focused on moving rhinoceros into high security breeding nucleus sanctuaries enhanced their security and breeding prospects (Merz, 1994). Gradually new sanctuaries were established by translocating offspring from the nucleus sanctuaries and by 2008, the black rhinoceros population in Kenya has grown to over 740 animals (Emslie et al., 2009) in 16 subpopulations. In addition, white rhinoceros (*Ceratotherium simum. simum*) were imported from South Africa in order to enhance tourist value of sanctuaries that where hosting black rhinoceros and there are currently 400 white rhinoceros in Kenya.

Besides poaching, infectious diseases are potential threats of population viability as they can cause acute or chronic infections that may reduce fitness or cause death. Wild animals occupying areas endemic to certain parasites usually develop endemic stability and rarely develop clinical disease (Penzhorn et al., 1994, Penzhorn, 2006). However, when stressed by natural conditions or human induced factors, such as capture and translocation which is an important management tool, wild animals develop clinical disease (Penzhorn, 2006). Moreover, translocation is known to spread pathogens and vectors to other areas, thereby risking infection to immunologically naive populations (Woodford and Rossiter, 1993). Tick-borne piroplasms, *Babesia* and *Theileria* as well as filarial fly transmitted nematode, *Stephanofilaria* spp affecting a number of large herbivore mammals including rhinoceros have known to increase rhinoceros mortality rates of infected rhinoceros during translocation. Nevertheless, the occurrence, distribution and prevalence of these parasites in rhinos is not well described in Kenya. Therefore it was imperative to have knowledge of their epidemiology and spatial distribution to help in prevention and control during and after rhino translocation. The findings of the study will give information which will be directly beneficial to the conservation of the small and dwindling population of rhinos in Kenya.

Our study focused on some parasites such as *Theileria*, *Babesia* and filariasis affecting both black and white rhinos as they are the major causes of mortality associated with translocation stress. This is because there are hardly any studies of these parasites done in the rhinos of Kenya with few international reports regarding the parasites in other populations in Africa.

Theileria and *Babesia* are parasitic apicomplexan protozoa which inhabit erythrocytes and sometimes other cells of vertebrates. They are members of suborder piroplasmorina. Species of ticks are the usual vectors for these blood-borne parasites which mostly appear to be non-pathogenic. Their infections have been recorded worldwide, and are known to infect a wide range of native and non-native mammals. No tick species has yet been confirmed as a vector for any of the piroplasms in the native mammals so far described. *Theileria* is a parasitic hemoprotozoan of many ungulates (Bishop et al., 2004). transmitted by ticks specifically *Rhiphicephalus*, *Hyalomma*, and *Amblyomma* tick spp (Norval et al., 1992). Theileria has been reported to infect cattle, sheep and goats, buffalos (*Syncerus caffer*), water buffalo (*Bubalus bubalis*), Eland and waterbucks (*Kobus* spp.), Eland, and in many wild Bovidae (Burridge, 1975). They are known to be non-pathogenic in most cases and for the pathogenic spp the virulence varies depending on the stock of the parasite, the dose of parasite and the type of mammalian host (Norval et al., 1992). *Babesia* is a tick borne intra erythrocyte and generally host specific protozoan parasite that causes hemolytic disease referred to as Babesiosis or piroplasmosis (Brown *et al*., 2008). To date there are over 70 *Babesia* spp which have been recognized based on their morphology, serologic tests, and molecular characteristics. They are parasitic on fish, amphibians, birds, and mammals and are spread through the saliva of tick when it bites to feed (Gerrit, 2006) with longer periods of feeding associated with a higher probability of acquiring the parasite. Although it is possible for a single *Babesia* species to infect more than one vertebrate host, most *Babesia* spp are host specific. Ticks of the genus, *Boophilus*, *Rhipicephalus*, *ixodes*, *Haemaphysalis*, *Dermacentor*, and *Hyalomma* are the major vectors of the *Babesia* parasites (Bock et al., 2004). The parasite can survive in the tick as it molts through its various developmental stages resulting in all stages being infectious.

Stephanofilaria is a very important genus of filarial nematodes affecting rhinoceros, Asian elephant, hippopotamus, domestic and wild suid, buffalo and cattle. This genus belongs to the family **Filariidae, Weinland 1858**, of the order Spirurida, Railliet 1914 in the phylum Nematoda. Aside from being of economic importance to the livestock industry, *Stephanofilaria* is also important to

the conservation of endangered species such as rhinoceros where outbreaks have been reported (Mutinda et al., 2012, Kock and Kock, 1990). *Stephanofiliaria* spp cause dermal ulcerative lesions in specific regions of the body of their mammalian hosts (Agrawal and Shah, 1984, Bain et al., 1996, Kock and Kock, 1990, Schulz and Kluge, 1960, Tremlett, 1964). In rhinos, infection with *Stephanofiliaria dinniki* causes, erosive, ulcerating lesions 2-3cms deeper than the surrounding skin with crusting, and edges raised above the normal skin (Mutinda et al., 2012, Kock and Kock, 1990, Schulz and Kluge, 1960, Tremlett, 1964). In the white rhinoceros these lesions can be on average 23 cm in diameter and located behind the shoulder, in the axillary region or on the rump, whereas in the black rhinoceros the wounds were smaller, being on average 15 cm and located behind the shoulder or on the ribs (Mutinda et al., 2012). A more detailed description of the pathology of the lesions in black rhino was first described by Tremlett (1964) and Kock and Kock (1990). Such ulcerative lesions are known to exacerbate mortality due to animal translocation; an increasing important tool in the genetic management of small isolated populations.

This doctoral thesis provides knowledge that will enable better management of different parasites affecting wild animals and the better understanding of host- parasite interactions intearctions particularly this knowledge will be pivotal to improve the conservation of rhinocerous in Kenya.

Research goals

The main aim of the present thesis was to advance our understanding of rhinocerous parasitic diseases. The specific objectives of this study are to:

1. Identify *Theileria* parasites and their genetic diversity in black and white rhinoceros population in Kenya (chapter 1)

2. Examine the epidemiology of *Theileria* infections in black and white rhinoceros. Specically we aims to study the association of *Theileria* infection with host species, age, sex, location, season and population mix (black rhinoceros vs black and white rhinoceros populations) (Chapter 2)

3. Study the emerging filariosis in black and white rhinoceros and how to control the spread of the diseas (Chapter 3)

4. Study the re-emerging filariosis in black and white rhinoceros, and its relation with rhinoceros host species, sex and age (Chapter 4)

Capitulo 1: Introducción, justificación y enfoque de la investigación

Los rinocerontes fueron tan abundantes en Kenia a principios del siglo XX (Barclay, 1932, Brett, 1993, Hunter y Watson, 1952, Lloyd-Jones, 1925, Neumann, 1898, Patterson, 1909) que había alrededor de 20.000 ejemplares en 1970 (AfRSG., 2008) ampliamente distribuidos. Sin embargo, la población disminuyó catastróficamente debido al asentamiento humano y la caza furtiva durante los siguientes 20 años, observándose una reducción de 400 animales en 1990 (Brett, 1993, Emslie y Brooks, 1999, Gakahu, 1993, Okita Ouma et al., 2007). Este colapso se produjo en poblaciones pequeñas, aisladas, demográficamente inviables y dispersas en regiones fragmentadas en Kenia, con miras a la extinción local. Con el objetivo de mejorar la seguridad y las perspectivas de cría, se creó un ambicioso programa de translocación para las poblaciones de rinocerontes aislados (Merz, 1994). Poco a poco nuevos refugios fueron establecidos por translocación de crías procedentes de los refugios del núcleo y en 2008 la población de rinoceronte negro en Kenia ha crecido a más de 740 animales (Emslie et al., 2009) en 16 subpoblaciones. Además, los rinocerontes blancos (*Ceratotherium simum. simum*) fueron transladados de Sudáfrica con el fin de aumentar el valor turístico de los refugios donde se hospedan los rinocerontes negros y actualmente hay 400 rinocerontes blancos en Kenia.

Además de la caza furtiva, las enfermedades infecciosas son amenazas potenciales de la viabilidad de la población ya que pueden causar infecciones agudas o crónicas que pueden reducir la aptitud o causar la muerte. Ocupando áreas endémicas de ciertos parásitos de animales silvestres que generalmente desarrollan estabilidad endémica y rara vez desarrollan la enfermedad clínica (Penzhorn et al., 1994, Penzhorn, 2006). Sin embargo, los animales silvestres pueden desarrollar enfermedad clínica por condiciones naturales o por factores inducidos por humanos, como la captura y translocación, que es una herramienta de gestión importante (Penzhorn, 2006). Además, es conocido que la translocación supone una difusión de patógenos y vectores a otras áreas, arriesgando así la infección de poblaciones de individuos inmunológicamente comprometidos (Woodford y Rossiter, 1993). La transmisión por garrapatas de piroplasmas, *Babesia* y *Theileria* así como filaria transmitida por la mosca, *Stephanofilaria* spp, que afecta a un gran número de mamíferos herbívoros como rinocerontes han aumentado las tasas de mortalidad de los mismos, afectando sobre todo a poblaciones de rinoceronte durante la translocación. Sin embargo, la presencia, distribución y prevalencia de estos parásitos no se ha descrito detalladamente en Kenia.

Por lo tanto es imprescindible tener conocimiento de su epidemiología y distribución espacial para ayudar en la prevención y control durante y después de la translocación del rinoceronte. Los resultados del estudio aportarán información que beneficiará directamente a la conservación de las poblaciones pequeñas y reducidas de rinocerontes en Kenia.

Nuestro estudio se centró en algunos parásitos como la *Babesia*, *Theileria* y filariasis que afectan a ambos rinocerontes blanco y negro ya que son las principales causas de mortalidad asociada al estrés por translocación. Esto es porque hay pocos estudios de estos parásitos en el rinoceronte de Kenia y pocos datos en los informes internacionales de parásitos en otras poblaciones de África.
Theileria y *Babesia* son protozoos parásitos apicomplejos que habitan en los eritrocitos y a veces otras células de vertebrados. Son miembros del suborden piroplasmorina. Las especies de garrapatas son los vectores habituales para estos parásitos transmitidos por la sangre que en su mayoría parecen ser no patógenos. Sus infecciones se han registrado en todo el mundo y son conocidos por infectar a una amplia gama de mamíferos nativos y no nativos. Ninguna especie de garrapata ha sido confirmada todavía como un vector de piroplasmas en los mamíferos nativos descritos hasta ahora. *Theileria* es un hemoprotozoo parásito de muchos ungulados (obispo et al., 2004), transmitida por garrapatas específicamente *Rhiphicephalus*, *Hyalomma* y *Amblyomma* spp. (Norval et al., 1992). *Theileria* se ha citado como el responsable de la infección de especies de bovinos, ovinos y caprinos, búfalos (*Syncerus caffer*), búfalos (*Bubalus bubalis*), Eland y antílopes acuáticos (*Kobus* spp.) y en muchos bóvidos salvajes (Burridge, 1975). Son conocidos por ser no patógenos en la mayoría de los casos, y para los patógenos la virulencia varía dependiendo de la acción del parásito, la presencia en cuanto a cantidad del mismo y el tipo de hospedador (Norval et al., 1992). *Babesia* es un protozoo parásito transmitido por garrapatas en el interior de los eritrocitos y generalmente específico para el hospedador, causante de la enfermedad hemolítica denominada Babesiosis o piroplasmosis (Brown et al., 2008). Hasta la fecha hay más de 70 especies de *Babesia*, que han sido descritas basándose en su morfología, pruebas serológicas y características moleculares. Son parásitos de peces, anfibios, aves y mamíferos y se transmiten a través de la saliva de la garrapata cuando pica para alimentarse (Gerrit, 2006) con períodos de alimentación asociado a una mayor probabilidad de contraer el parásito. Aunque es posible que una sola especie de *Babesia* pueda infectar a más de un hospedador vertebrado, la mayoría de las especies de *Babesia* son específicas de un hospedador. Las garrapatas de los géneros, *Boophilus*, *Rhipicephalus*, *ixodes*, *Haemaphysalis*, *Dermacentor* y *Hyalomma* son los principales vectores de los parásitos de *Babesia* (Bock et al.,

2004). El parásito puede sobrevivir en la garrapata como muda durante diversas etapas del desarrollo, resultando en todas infecciosas.

Stephanofilaria es un género muy importante de nematodos que afectan al rinoceronte, elefante asiático, hipopótamo, cerdo doméstico y salvaje, búfalos y bovinos. Este género pertenece a la familia *Filariidae*, Weinland 1858, del Orden Spirurida, 1914 Railliet en el Filo Nematodos. Aparte de ser de importancia económica para la industria ganadera, *Stephanofilaria* también es importante para la conservación de especies en peligro de extinción, como el rinoceronte, de donde han sido los brotes registrados (Mutinda et al., 2012, Kock y Kock, 1990). *Stephanofiliaria* spp. causa lesiones ulcerosas cutáneas en regiones específicas del cuerpo de sus hospedadores mamíferos (Agrawal y Shah, 1984, Bain et al., 1996, Kock y Kock, 1990, Schulz y Kluge, 1960, Tremlett, 1964). En rinocerontes, la infección con *Stephanofiliaria dinniki* causa lesiones erosivas, úlceras con profundidad superior a 2-3cms de la piel circundante con formación de costras, y bordes que sobresalen por encima de la piel normal (Mutinda et al., 2012, Kock y Kock, 1990, Schulz y Kluge, 1960, Tremlett, 1964). En el rinoceronte blanco, estas lesiones pueden ser en promedio 23 cm de diámetro y se encuentran situadas detrás del hombro, en la región axilar o en la grupa, mientras que en el rinoceronte negro las heridas fueron menores, siendo en promedio 15 cm y situadas detrás del hombro o en las costillas (Mutinda et al., 2012). Una descripción más detallada de la patología de las lesiones en el rinoceronte negro fue descrita por primera vez por Tremlett (1964) y Kock y Kock (1990). Tales lesiones ulcerosas son conocidas por exacerbar la mortalidad debido a la translocación de animales; una herramienta cada vez más importante en la gestión genética de pequeñas poblaciones aisladas.

Esta tesis doctoral aporta conocimientos que permiten una mejor gestión de los diferentes parásitos que afectan a los animales salvajes y la mejor comprensión de las interacciones huésped-parásito, particularmente este conocimiento será fundamental para mejorar la conservación de rinocerontes en Kenia.

Objetivos de la investigación.

El principal objetivo de la presente tesis fue avanzar en nuestra comprensión de las enfermedades parasitarias en rinocerontes. Los objetivos específicos de este estudio son:

1. Identificar los parásitos de *Theileria* y su diversidad genética en la población de rinoceronte blanco y negro en Kenia (Capítulo 1).

2. Examinar la epidemiología de infecciones por *Theileria* en rinoceronte blanco y negro. Especialmente se pretende estudiar la asociación entre la infección por *Theileria* con la edad, sexo, ubicación, temporada y mexcla de poblaciónes (poblaciones de rinoceronte negro y blanco vs rinoceronte negro) de los hospedadores
(Capítulo 2).

3. Estudio de la filariosis emergente en el rinoceronte blanco y negro y cómo controlar la propagación de la enfermedad (Capítulo 3).

4. Estudio de la filariosis reemergente en el rinoceronte blanco y negro y su relación con el sexo y la edad en las especies de rinoceronte (Capítulo 4).

Chapter 2: Three novel haplotypes of *Theileria bicornis* in black and white rhinoceros in Kenya

Transboundary and Emerging Diseases

SHORT COMMUNICATION

Three Novel Haplotypes of Theileria bicornis in Black and White Rhinoceros in Kenya

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Summary

Piroplasms, especially those in the genera Babesia and Theileria, have been found to naturally infect rhinoceros. Due to natural or human-induced stress factors such as capture and translocations, animals often develop fatal clinical piroplasmosis, which causes death if not treated. This study examines the genetic diversity and occurrence of novel Theileria species infecting both black and white rhinoceros in Kenya. Samples collected opportunistically during routine translocations and clinical interventions from 15 rhinoceros were analysed by polymerase chain reaction (PCR) using a nested amplification of the small subunit ribosomal RNA (18S rRNA) gene fragments of Babesia and Theileria. Our study revealed for the first time in Kenya the presence of Theileria bicornis in white (Ceratotherium simum simum) and black (Diceros bicornis michaeli) rhinoceros and the existence of three new haplotypes: haplotypes H1 and H3 were present in white rhinoceros, while H2 was present in black rhinoceros. No specific haplotype was correlated to any specific geographical location. The Bayesian inference 50% consensus phylogram recovered the three haplotypes monophyletically, and Theileria bicornis had very high support (BPP: 0.98). Furthermore, the genetic p-uncorrected distances and substitutions between T. bicornis and the three haplotypes were the same in all three haplotypes, indicating a very close genetic affinity. This is the first report of the occurrence of Theileria species in white and black rhinoceros from Kenya. The three new haplotypes reported here for the first time have important ecological and conservational implications, especially for population management and translocation programs and as a means of avoiding the transport of infected animals into non-affected areas.

Introduction

The eastern black rhinoceros (Diceros bicornis michaeli) is indigenous to East Africa, but is currently extant only in Kenya and Tanzania. Black rhinoceros were so abundant in Kenya in the early twentieth century (Brett, 1990) that they were treated as agricultural pests and hampered human settlement in eastern Kenya (Hunter, 1952). There were around 20 000 individuals in the 1970s (Emslie and Knight, 2012) when the species was still widely distributed throughout Kenya. The population then declined catastrophically over the following 20 years as a result of authorized hunting, poaching and human settlements and bottomed out at fewer than 400 animals by 1990 (Okita-Ouma et al., 2007). This decline created a situation of small isolated, demographically unviable populations scattered across fragmented regions of Kenya, many of which now face local extinction. An ambitious translocation programme for isolated rhinoceros populations begun in 1993 (KWS, unpublished data) and focuses on moving

rhinoceros into high-security breeding sanctuaries, thereby enhancing their security and breeding prospects (Merz, 1994). New sanctuaries have been gradually established by translocating offspring from these breeding nuclei and by 2008 the black rhinoceros population in Kenya had risen to over 630 animals in 16 subpopulations (Emslie et al., 2009).

In Kenya today, in addition to the eastern black rhinoceros, there are also populations of both the southern white (Ceratotherium simum simum) and northern white (Ceratotherium simum cottoni) rhinoceros. The southern white, indigenous to southern Africa, was introduced into Kenya and Uganda in the early 1980s (KWS, unpublished data); on the other hand, the northern white is extinct from its natural range in central African countries (Rookmaaker and Pierre-Olivier, 2012), although four of these rhinoceros were introduced into the Ol Pejeta Conservancy in Kenya in 2009.

Although small in size, the population of the Eastern black rhinoceros in Kenya represents about 90% of this subspecies' global population (Emslie, 2011) and has an annual population growth of about 4%. In Kenya, the translocation of individual rhinoceros from various subpopulations is frequently undertaken to establish, re-establish or augment local populations and is an important conservation tool used to manage the species in situ (Woodford and Rossiter, 1993). Translocation has associated disadvantages such as the risk of introducing destructive pathogens into naive wildlife populations and the exposure of translocated animals to pathogens in the new release site (Woodford and Rossiter, 1993; Chipman et al., 2008). The process of capture and translocation is an inherently stressful event for game animals and often compromises animals' immune defences (Woodford and Rossiter, 1993). In natural systems, parasites co-evolve with their hosts and develop equilibrium or endemic stability in which infected hosts do not develop the disease (Penzhorn et al., 1994; Penzhorn, 2006). However, when the host–parasite equilibrium is altered by various stressor conditions, latent infections can develop into disease (Penzhorn et al., 1994; Cindy, 2002; Penzhorn, 2006; Mutinda et al., 2012).

A group of blood-borne protozoans of the order Piroplasmida and generically referred to as piroplasms have been linked to rhinoceros morbidity and mortality in Tanzania, South Africa and Kenya (Nijhof et al., 2003; Penzhorn, 2006; Obanda et al., 2011). This suggests that infectious diseases (and not only poaching) are an emerging threat to the conservation of rhinoceros (Ramsey and Zainuddin, 1993; Penzhorn et al., 1994). The connection between translocation and the onset of diseases such as piroplasmosis is of interest because translocation is a tool that is frequently employed in the management of thisrhinoceros metapopulation in situ (Emslie et al.,2009). Piroplasm parasites associated with mortalities in black rhinoceros in South Africa and Tanzania were recognized as the novel species Theileria bicornis and Babesia bicornis (Nijhof et al., 2003). It is unclear whether or not these lethal species occur and circulate in the Kenyan rhinoceros metapopulation. Moreover, it is not known whether other known or unknown species of piroplasms may latently infect rhinoceros in their various Kenyan subpopulations.

With the advance of current molecular and genetic techniques, more novel species of Theileria and Babesia are being discovered and their phylogenetic relationships are becoming increasingly well understood. Analyses of 18S rRNA gene fragments have been successfully applied in the identification and classification of several previously unknown Theileria and Babesia species (Quick et al., 1993, 1993; Persing et al., 1995; Schnittger et al., 2000; Bhoora et al., 2009; Chaisi et al., 2013). Furthermore, the phylogenetic classification of cattle-infecting piroplasms via the analysis, and comparison of 18S rRNA gene fragments has been shown to match traditional taxonomy and provides additional information on their evolutionary relationships (Quick et al., 1993). In the present study, we investigated the genetic diversity and occurrence of piroplasm species infecting both black and white rhinoceros populations in Kenya using a nested amplification of the 18S rRNA genes of Babesia and Theileria.

Materials and Methods

Study population

In this study, blood samples from 15 rhinoceros (Diceros bicornis michaeli n = 12 and Ceratotherium simum simum n = 3) were collected from various national parks and rhinoceros sanctuaries in Kenya (Table 1). Samples were taken from animals from different subpopulations located in different geographical and ecological habitats.

Sampling was carried out opportunistically during scheduled immobilizations for population and health management or during the ear-notching activity that is routinely performed for identification purposes. Rhinoceros were immobilized using 4 **1**g/kg (body weight) of etorphine hydrochloride (Novartis, Johannesburg, South Africa) combined with 5000 IU hyaluronidase (Kyron Laboratories, Benrose, South Africa) and 80 **1**g/kg (body weight) total dose of xylazine hydrochloride (Kyron Laboratories). The animals were darted, and upon recumbency blood was drawn from the radial vein of the foreleg and preserved in ethylenediamine tetra-acetic acid tubes. The sex and age of the animals were identified using morphological criteria (Mutinda et al., 2012). Samples were conserved in frozen liquid nitrogen and transported to the laboratory. Animals were revived by the injection of 18 **1**g/kg (body weight) diprenorphine (M5050 ; Kyron Laboratories) and 6 **1**g/kg (body weight) atipamezole hydrochloride (ANTISEDAN ;

Table 1. Theileria bicornis haplotypes identified in the sampled white (Ceratotherium simum simum) and black (Dicerous biconis michaeli) rhinoceros populations

Species	Age	Sex	Geographical location	Theileria bicornis haplotype
D. bicornis michaeli	Subadult	Female	Lake Nakuru National Park	HI
C. simum simum	Adult	Female	Lake Nakuru National Park	H2
C. simum simum	Adult	Male	Meru National Park	H2
C. simum simum	Subadult	Male	Meru National Park	H2
D. bicornis michaeli	Juvenile	Male	Ngulia	H3
D. bicornis michaeli	Adult	Female	Mugie	H3
D. bicornis michaeli	Adult	Male	Mugie	H3
D. bicornis michaeli	Subadult	Male	Meru National Park	H3
D. bicornis michaeli	Subadult	Male	Nairobi National Park	H3
D. bicornis michaeli	Juvenile	Female	Nairobi National Park	H3
D. bicornis michaeli	Juvenile	Female	Nairobi National Park	H3
D. bicornis michaeli	Subadult	Male	Lake Nakuru National Park	H3
D. bicornis michaeli	Subadult	Female	Solio	H3
D. bicornis michaeli	Subadult	Male	Mugie	H3
D. bicornis michaeli	Adult	Male	Mugie	H3

Kyron Laboratories) into an ear vein. The white rhinos were also injected with 25 **1**g/kg (body weight) naltrexone hydrochloride (Naltrexone; Kyron Laboratories) intramuscularly to prevent re-narcotization. All rhinos were back on their feet in approximately 3 min.

DNA isolation and PCR amplification

Genomic DNA was extracted from blood using a genomic DNA extraction kit (DNeasy blood and Tissue Kit; Qiagen, Southern Cross Biotechnologies, South Africa) following the manufacturer's protocol. A nested PCR amplification specific for the 18S rRNA gene of Babesia and Theileria was performed (Maamun et al., 2011). A primary amplification was carried out in a 50 **1**l reaction volume containing 3 **1**l of the genomic DNA, 45 **1**l of Platinum blue supermix (Applied Biosystems, Johannesburg, South Africa) and 0.25 **1**M each of the forward and reverse primers. The forward primer was ILO-9029, (5^{0}-CGGTAATTCCAGCTC CAATAGCGT-3^{0}) and the reverse primer, ILO-9030 (5^{0}-18 TTTCTCTCAAAGGTGCTGAAGGAGT-3^{0}). The amplification (Thermocycler, Veriti; Applied Biosystems, Johannesburg, South Africa) was preceded by a 30-s polymerase activation step at 95°C followed by 30 cycles each of 1 min of denaturing at 94°C, 1 min of annealing at 53°C for 30 s and extension for 1 min at 72°C. Amplification was terminated by a final extension step of 72°C for 9 min. The secondary amplification was performed in a 50-**1**l reaction volume containing 2 **1**l of the primary amplification product, 45 **1**l of platinum blue supermix and 0.3 **1**M each of the forward and reverse primers. The forward primer was MWG4/70, (5^{0}-AGCTCGTAGTTGAATTTCTGCTGC-3^{0}) and the reverse primer, ILO-7782 (5^{0}-AACTGACGACCTC CAATCTCTAGTC-3^{0}). The secondary PCR (Thermocycler, Veriti; ABI) was begun with an initial denaturation at 95°C for 30 s, followed by 30 cycles of 1 min each at 94°C, annealing at 55°C for 30 s and extension at 72°C for 1 min. The PCR was completed with a final extension step of 72°C for 9 min.

Polymerase chain reaction products showing successful amplification from agarose gel analysis were directly sequenced for both strands. PCR products were purified for direct sequencing by enzymatic treatment using exonuclease I and shrimp alkaline phosphatase (PCR Product Presequencing Kit; Amersham Biosciences, Buckinghamshire, UK). All DNA sequencing was carried out by direct cycle sequencing on both strands of purified PCR DNA products from PCR amplification using MWG4/70 and ILO-7782 primers (0.1 **1**M from each). Sequencing reactions were carried out with the ABI PRISM DigDye Terminator v3.1 cycle sequencing kit and analysed on an ABI310 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Molecular analyses

The complementary reads were used to resolve rare ambiguous base-calls in Sequencher v.4.9. Sequences were aligned in Seaview v.4.2.12 (Gouy et al., 2010) under ClustalW (Larkin et al., 2007) default settings. Incomplete terminal sequences were removed from the alignment. Nucleotide substitutions and p-uncorrected distances were performed in MEGA v5 (Tamura et al., 2011), and phylogenetic analyses were performed with Mr. Bayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). Sequence BLAST searches were conducted in GenBank to identify homologous sequences (Altschul et al., 1990). Sequences with

98% or greater similarity to our target sequences and others belonging to the same genus (as suggested from the BLAST searches) of African origin were also included in the alignment. The designated out-group was T. gondii following Nijhof et al. (2003).

The most appropriate substitution model for the Bayesian inference was determined by the Bayesian information criterion (BIC) in Model test v.0.1.1 (Posada, 2008). Mr. Bayes was used with default parameters and Markov chain settings and with random starting trees. The gamma shape parameter and proportion of invariant sites were estimated from the data. Each run consisted of four chains of 10 000 000 generations, sampled every 10 000 generations for a total of 1000 trees. A plateau was reached after few generations with 25% (250 trees) of the trees resulting from the analyses discarded as 'burn-in'.

Ethics

The Committee of the Department of Veterinary and Capture Services of the Kenya Wildlife Service (KWS) approved the study and the animal capture, translocation and sample collection. KWS guidelines on Wildlife Veterinary Practice-2006 were followed. All KWS veterinarians complied with the Veterinary Surgeons Act, Cap. 366, Laws of Kenya, that regulates veterinary practice in Kenya.

Results and Discussion

Amplification products of 385 bp were generated from the samples taken from Kenyan black and white rhinoceros. The PCR products were sequenced, and BLAST analyses indicated that the sequences were most similar to T. bicornis (Nijhof et al., 2003). Three haplotypes were recovered (H1, H2 and H3) from the sampled animals and are shown in Table 1. Their sequences are deposited in GenBank with accession numbers KC771140 (H1), KC771141 (H2) and KC771142 (H3). Haplotypes H1 and H3 were present in white rhinoceros, while H2 was presented in black rhinoceros. No specific haplotype was correlated with any specific geographical location. The best-fitting model for the BML tree was TIM3+I+G (lnL = 1584.9442, BIC = 3396.1116). The Bayesian inference 50% consensus phylogram recovered all the haplotypes monophyletic with T. bicornis with very high support (BPP: 0.98) (Fig. 1). Furthermore, the genetic p-uncorrected distances and substitutions between T. bicornis and the three haplotypes were the same in all three haplotypes, indicating very close genetic affinity (Table 2).

Members of the Order Piroplasmidae include an assemblage of intra-erythrocytic protozoans that are vectored by diverse ticks and cause disease in humans, livestock and wildlife. Infections with piroplasms range from severe acute to mild subclinical forms. Piroplasms, especially T. parva, cause major economic losses in the livestock industry. Although wild animals are just as susceptible to piroplasms, most parasites occur latently due to co-evolution with their hosts. However, under stressful conditions, latent infections can flare up and cause clinical piroplasmosis. Capture and translocation is a management strategy that is widely used to manage populations in situ, and, in many cases, the stress of the translocation can be linked to the onset of

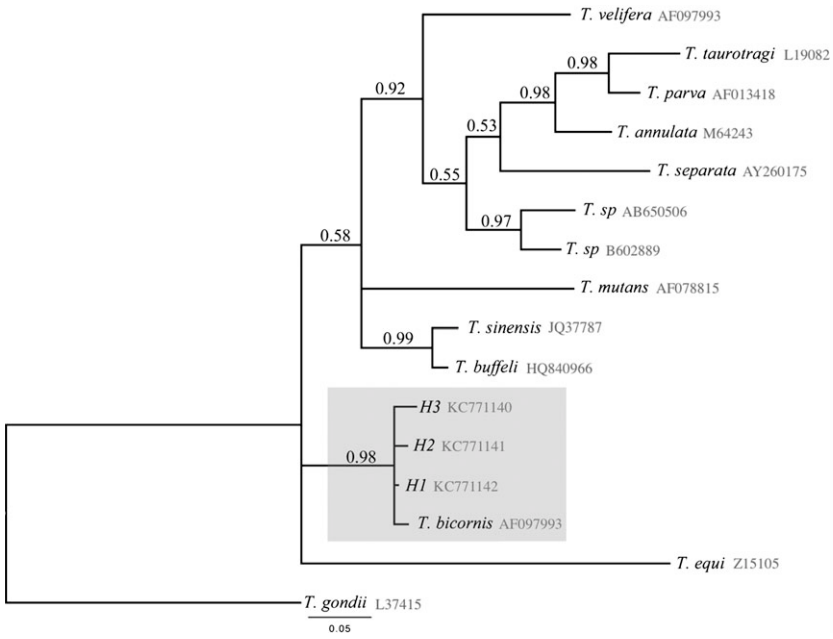

Fig. 1. Bayesian inference 50% consensus phylogram of Theileria species (18S rRNA partial sequence). Values by nodes are the posterior probabilities recovered from the Bayesian analysis. All three recovered haplotypes (H1, 2, 3) and T. bicornis are shown in the shaded area. GenBank accession numbers are in grey colour.

Table 2. Nucleotide substitutions (above diagonal) and p-uncorrected distances (below diagonal) for each pairwise comparison between Theileria species

	H1	H2	H3	T.buf	T.sin	T.bic	T.sp(el)	T.sp(bu)	T.equ	T.mut
H1					6	4	6	31	525	3022
H2					–	2	4	30	508	3023
H3					0.005	–	2	29	506	3020
					0.011	0.005	–	31	508	3023
					0.085	0.082	0.087	–	539	3039
					0.089	0.086	0.091	0.088	547	3025
					0.076	0.071	0.076	0.085	504	3027
					0.079	0.073	0.079	0.082	502	3027
					0.105	0.103	0.108	0.107	560	3031
					0.093	0.087	0.093	0.085	590	3026
					0.065	0.059	0.065	0.074	458	3025
					0.062	0.056	0.062	0.082	476	3026
					0.072	0.078	0.078	0.073	549	3012
					0.081	0.081	0.087	0.065	567	3028
					0.115	0.109	0.115	0.124	638	3109
					0.139	0.139	0.139	0.151	749	3141

All three recovered haplotypes (H1, 2, 3) and T. bicornis are shown in the shaded area. Abbreviations on the top of the table correspond to the three first letters of the specific name. For GenBank accession numbers, see Fig. 1.

clinical piroplasmosis. Various Babesia and Theileria spp. have been found incidentally to occur in wildlife. Nijhof et al. (2003) showed that Babesia bicornis could be linked to fatal disease, although these authors presented no evidence to suggest that T. bicornis is pathogenic for the black rhinoceros.

Findings from this study confirm for the first time the presence of new T. bicornis haplotypes circulating among both white and black rhinoceros in Kenya; however, these haplotypes were not shared between these two rhinoceros species. Theileria bicornis was first isolated from black rhinoceros but since then has been detected in nyala, white rhinoceros and cattle (Muhanguzi et al., 2010; Pfitzer et al., 2011), thereby suggesting that it has a wide host range. Although this parasite has never been associated with fatal clinical infection in black rhinoceros in South Africa, it is closely related to T. equi (Nijhof et al., 2003), which is pathogenic in equids and causes stress-induced piroplasmosis in, for instance, recently captured Grevy's zebra and plains zebra in Kenya and Uganda (Dennig, 1966).

Our results indicate that the new T. bicornis haplotypes are widespread among the major rhinoceros subpopulations in Kenya. Such a wide distribution may be advantageous as many individuals are not immunologically naive to this pathogen, and hence, there is less risk of clinical disease in cases where individuals are released into endemic areas.

Despite the positive impact of rhinoceros population management in Kenya, translocations have key shortcomings associated with the risk of introducing destructive pathogens into naive wildlife population and the exposure of translocated animals to pathogens in the new release sites (Woodford and Rossiter, 1993; Chipman et al., 2008). The findings of our study suggest that these translocations could have exposed the naive rhinoceros population to endemic areas where there are other piroplasm hosts such as buffalo (Syncerus caffer), hartebeest (Alcelaphus buselaphus), eland (Taurotragus oryx) (Burridge, 1975) and bushbuck Tragelaphus scriptus, among others (Benson et al., 2006). This type of management may therefore increase the possibility of successfully transmitting piroplasms from the alternative host to naive rhinoceros and is likely to account for the maintenance of these three new haplotypes. These parasites have co-evolved with their hosts and have developed an equilibrium or endemic stability in which infected hosts do not develop the disease (Penzhorn et al., 1994; Penzhorn, 2006). However, when the host–parasite equilibrium is altered by various stressor conditions, latent infections may develop into disease (Penzhorn et al., 1994; Cindy, 2002; Penzhorn, 2006).

Nevertheless, the transmission dynamics and the specific vectors of T. bicornis are still unknown, although ixotid ticks of the species Amblyomma rhinoceroscerotis and Dermacentor rhinoceros, known to specifically feed on both

black and white rhinoceros, are suspected to play a role in parasite transmission between individuals (Horak and Penzhorn, 1997). Tick distribution is dynamic and it is possible that the tick species that are vectors for T. bicornis are widely spread in all the parks sampled and thus help to ensure transmission.

Conclusions

This is the first record of T. bicornis and its three new haplotypes in both black and white rhinoceros in Kenya. The parasite is well established in this country's rhinoceros population because several major subpopulations were found to be positive, thereby suggesting that the majority of the rhinoceros are immunologically challenged and exist in a state of endemic stability with this parasite. This may explain the lack of clinical theileriosis, even during capture. However, as this parasite could be multihost, it is likely to be maintained in a habitat even in the absence of rhinoceros. Further epidemiological studies that include the identification of other hosts within rhinoceros sanctuaries still need to be carried out. Although we did not detect B. bicornis, further investigations in other subpopulations are required in the future.

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Declaration of Interest

The authors report no conflict of interests. The authors alone are responsible for the research and the writing of this paper.

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Chapter 3: Epidemiology of *Theileria bicornis* among black and white rhinoceros metapopulation in Kenya

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RESEARCH ARTICLE Open Access

Epidemiology of Theileria bicornis among black and white rhinoceros metapopulation in Kenya

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Abstract

Background: A huge effort in rhinoceros conservation has focused on poaching and habitat loss as factors leading to the dramatic declines in the endangered eastern black rhinoceros (Diceros bicornis michaeli) and the southern white rhinoceros (Ceratotherium simum simum). Nevertheless, the role disease and parasite infections play in the mortality of protected populations has largely received limited attention. Infections with piroplasmosis caused by Babesia bicornis and Theileria bicornis has been shown to be fatal especially in small and isolated populations in Tanzania and South Africa. However, the occurrence and epidemiology of these parasites in Kenyan rhinoceros is not known.

Results: Utilizing 18S rRNA gene as genetic marker to detect rhinoceros infection with Babesia and Theileria, we examined blood samples collected from seven rhinoceros populations consisting of 114 individuals of black and white rhinoceros. The goal was to determine the prevalence in Kenyan populations, and to assess the association of Babesia and Theileria infection with host species, age, sex, location, season and population mix (only black rhinoceros comparing to black and white rhinoceros populations). We did not detect any infection with Babesia in the sequenced samples, while the prevalence of T. bicornis in the Kenyan rhinoceros population was 49.12% (56/114). White rhinoceros had significantly higher prevalence of infection (66%) compared to black rhinoceros (43%). The infection of rhinoceros with Theileria was not associated with animal age, sex or location. The risk of infection with Theileria was not higher in mixed species populations compared to populations of pure black rhinoceros.

Conclusion: In the rhinoceros studied, we did not detect the presence of Babesia bicornis, while Theileria bicornis was found to have a 49.12% prevalence with white rhinoceros showing a higher prevalence (66%) comparing with black rhinoceros (43%). Other factors such as age, sex, location, and population mix were not found to play a significant role.

Keywords: Ixodid, Ticks, Piroplasms, Diceros bicornis michaeli, Ceratotherium simum simum

Background

The populations and distribution ranges of the black rhinoceros (Diceros bicornis) and the white rhinoceros (Ceratotherium simum) have declined in the whole of Africa. The rate of their population decline is faster than any other large terrestrial mammal in recent times [1], a fact that supports their endangered status and calls for robust international efforts towards their recovery. These rhinoceros have been exterminated in the majority of African countries, while their range among the remaining principal countries; Kenya, Tanzania, Namibia, Zimbabwe and South Africa [1] is greatly reduced and currently restricted in artificially created sanctuaries. Habitat loss and vicious poaching are the leading twin drivers of population decline of the rhinoceros [2]. However infectious diseases are also an incipient threat to endangered species [3] having been classified among the top five causes of species extinctions [4].

Piroplasms, which are blood-borne protozoan parasites in the genera Babesia and Theileria (Order Piroplasmidae), are globally distributed and transmitted by a diverse species of Ixodid ticks. These parasites infect a wide range of domesticated and wild mammals as well as humans. Infections may lead to severe disease and death or it may

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remain latent depending on virulence of the species and host immune status. Piroplasms have historically been known to infect rhinoceros with some infections associated with fatalities [5-7]. However the causal species were unknown until 10 years ago when Babesia bicornis and Theileria bicornis were independently associated with stress-induced mortality [8]. The first genetic work on piroplasms by Otiende et al., [9] has shown the existence of infection by piroplasm and the occurrence of three new haplotypes of Theileria bicornis circulating in both black and white rhinoceros in Kenya. The factors influencing piroplasms prevalence among Kenyan populations were previously unknown. Piroplasms have coevolved with rhinoceros and they coexist with the host without signs of clinical disease. However, stress induced by translocation has been linked to immune suppression and is a major cause of post translocation morbidity and/or fatality. Translocation is at the core of in situ management of rhinoceros metapopulation and yet it is incriminated as a disease inducer besides its inherent role in the spread of pathogens. The link between translocation and piroplasmosis is intricate because it is based on the modulatory effects of stress hormones on the immune system. Translocation elicits stress hormones, which allow uninhibited proliferation of piroplasms in the host resulting in disease and death. However, effects of stress hormones are not predictable or homogenous in the population as underlying individuals' conditions, such as injury, pregnancy, co-infection, vary and may elicit different immune response [10].

The goal of this study is to determine the epidemiology of Theileria biconis in Kenya. Specifically, we intended to (a) determine their prevalence in both species of rhinoceros and among sub-populations then (b) test the association of infection prevalence with host species, age, sex, location, season and population mix (black rhinoceros vs black and white rhinoceros populations). Information generated will be useful in guiding management and veterinary options such as translocation, differential diagnosis and chemotherapy.

Methods

Study area

Lake Nakuru National Park (LNNP) central coordinates are 0°22′S 36°05′E and is 4 km from Nakuru town center. The park covers an area of 188 km^2 completely fenced, of which 44 km^2 lies in the shallow alkaline soda lake, thereby leaving 144 km^2 for wildlife use. The area around the lake is flat bare lowland of 1200 m altitude surrounded by hills and gentle cliffs that rise to 1750 m above sea level. The park receives mean annual rainfall of 850 mm with rainfall in the months of April to May and again in October – November. The park consists of open grassland with elevated areas occupied by dry forests of Acacia xanthophloea, Olea capensis sub sp. Macrocarpa and Croton dichogamus. Marshland along the river inflows and springs are covered by Cyperus laevigatus and Typha spp. Other striking plant species include the invasive Tarchonanthus spp. bush land, the deciduous (Teclea & Olive) forest and the Euphorbia candelabrum forest. The park has 33 white and 69 black rhinoceros that freely interact with other diverse species.

Nairobi National Park (NNP) central coordinates are 1°16′S, 36°49′E and is about 10 km from the city of Nairobi and covers an area of 117 km^2. A large section of the park is fenced with only 20 km left open for wildlife dispersal. Average annual rainfall is 800 mm with rainy season between April-May and October-November. The vegetation consists of mosaic grassland, thickets and Acacia and deciduous forests as well as woodlands especially along River Mbagathi that crosses it. The park has 77 black and 13 white rhinoceros besides many other wildlife species.

Ngulia rhino Sanctuary (NgRS) is within Tsavo West National Park occupying a fenced area of 90 km^2 at 3^0 01′S to 3^0 06′S and 38^0 06E to 38^0 10′E. Altitude ranges from 600 m of lowlands to 1800 m of craggy hills with average annual rainfall of 600 mm. Dry period is between December to March, while rains occur in the months of April to June and again in October and November. The vegetation is thickly wooded by Commiphora-Acacia woodland, dotted with baobab trees. This sanctuary contains 77 black rhinoceros without white rhinoceros, though other small-medium sized wildlife occurs in small density.

Meru Rhino Sanctuary (MRS) is 48Km^2 (central coordinates, N 00^0 15.125, E $038^0$06.481) located within the Meru National Park has 44 black and 45 white rhinoceros. Altitude ranges from 1000 to 3400 m above the sea level. Average rainfall is 635-762 mm with the wet season occurring in late March to April, while the dry season begins from October. Major rivers such as Makutano, Kanjoo, Kathithi, Rujuwero and Kindani traverse MRS, which contributes to mosaic vegetation types that include thickets, bushland and grassland as well as a thick forest on its southern edge.

Solio and Mugie Rhino Sanctuaries are in the Laikipia-Samburu ecosystem, which is characterized by savannah-type grassland dominated by Euclea divinorum, Acacia spp and Euphorbia woodland while annual rainfall averages 300 to 700 mm. Rainfall level varies annually with intermittent patterns that peaks in April-May, July–August and October–November. The rest of the months are dry. Solio Sanctuary, 0°16′S 37°00′E / 0.27°S 37°E/ -0.27; 37, is 68.9 km^2 and has altitude of 1932 m, located at the base of the Aberdare ranges. The sanctuary holds 50 black and 110 white rhinoceros. Mugie rhino sanctuary (ceased to be a rhino sanctuary at the time of this study, as the entire

population was translocated) was 90 km^2 with an altitude 1990 m.

Sampling design

This study was carried out between 2011 and 2012 in various sub populations of black and white rhinoceros in Kenya. Sampling was cross-sectional whereby samples were collected from apparently healthy rhinos immobilized for translocation or tagging. The biodata of each rhinoceros was obtained from the KWS rhinoceros database. Rhinoceros < 2 years of age were not immobilized for translocation. Juvenile rhinoceros are <3.5 years, sub-adults are (3.6 – 7 years) and adults are >7 years. Rhinoceros were immobilized using a combination of etorphine hydrochloride (M99®), Hyaluronidase (Kyron Laboratories, Benrose 2011, South Africa) and xylazine (Norvatis, [PTY] Ltd, South Africa). Venous blood was drawn from the front limb of the rhinoceros and then collected in EDTA tubes. Blood in EDTA tubes were gently mixed by turning the tubes up and down and then transferring aliquots in labeled cryovials followed by quick freezing in liquid nitrogen. The samples were transported in liquid nitrogen to Forensic and Genetics Laboratory of Kenya Wildlife Service in Nairobi for analysis.

DNA isolation and PCR amplification

Genomic DNA was extracted from blood using a genomic DNA extraction kit (DNeasy blood and Tissue Kit, QIAGEN, Southern Cross Biotechnologies, South Africa) following the manufacturers' protocol. A nested PCR amplification specific for the 18S rRNA gene of Babesia and Theileria was performed. A primary amplification was carried out in 50 µl reaction containing 3 µl of the genomic DNA, 45 µl of Platinum blue supermix, 1 µl (10 mM) each forward and reverse primers. The forward primer was ILO-9029, (5′-CGGTAATTCCAGCTCCAA TAGCGT-3′) and reverse, ILO-9030 (5′-TTTCTCTC AAAGGTGCTGAAGGAGT-3′) primer [11]. The amplification (Thermocycler, Veriti, ABI) was preceded by a 30 sec polymerase activation step at 95°C followed by 30 cycles of 1 min each at 94°C, annealing at 53°C for 30 sec, extension for 1 min at 72°C. Amplification was terminated by a final extension step 72°C for 9 min. The secondary amplification was in a 50 µl reaction containing 2 µl of the primary amplification product, 45 µl of Platinum blue supermix, 1.5 µl (10 mM) each of forward and reverse primers. The forward primer was MWG4/70, (5′-AGCTCGTAGTTGAATTTCTGCTGC-3′) and the reverse was ILO-7782 (5′-AACTGACGACCTCCAAT CTCTAGTC-3′) [11]. The secondary PCR (Thermocycler, Veriti, ABI) was initiated with an initial denaturation at 95°C for 30 sec, followed by 30 cycles of 1 min each at 94°C, annealing at 55°C for 30 s and extension at 72°C for 1 min. The PCR was completed with a final extension step of 72°C for 9 min. PCR products showing successful amplification on agarose gel analysis were directly sequenced for both strands. PCR products were purified for direct sequencing by enzymatic treatment using exonuclease I and shrimp alkaline phosphatase (PCR Product Presequencing Kit, Amersham). All DNA sequencing was carried out by direct cycle sequencing on both strands of purified PCR DNA products from PCR amplification. Sequencing reactions were carried out with the ABI PRISM DigDye Terminator v3.1 cycle sequencing kit and analyzed on an ABI310 DNA sequencer (Applied Biosystems, CA).

Statistical analysis

Statistical analyses were performed using Fisher Exact test for count data and Chi Square test to determine the relationship between infection of rhinoceros with Theileria and the following variables: rhinoceros age, location, sex, and rhinoceros species. To confirm our results, we also used a Generalized Linear Model with a binomial error, and a complementary log-log link function. All possible interactions were included in the first model. As rhinoceros species are essentially clustered with locality (game park or sanctuary) a generalized linear mixed effect model was also applied, considering locality (game park or sanctuary) as random effect: Theileria Infection ~ Rhinos Species + (1 | Locality). Statistical significance was assessed at $p < 0.05$.

Ethic

The Committee of the Department of Veterinary and Capture Services of the Kenya Wildlife Service (KWS) approved the study including animal capture, translocation and sample collection. KWS guidelines on Wildlife Veterinary Practice-2006 were followed. All KWS veterinarians were guided by the Veterinary Surgeons Act Cap 366 Laws of Kenya that regulates veterinary practice in Kenya.

Results

A total of 114 blood samples of black (n = 82) and white rhinoceros (n = 32) were sampled from seven rhinoceros populations and molecularly examined for infection with Babesia and Theileria. We did not detect any infection with Babesia in the obtained sequences, while the overall Theileria prevalence was 49.1% (56/114). All Theileria sequences belonged to the three haplotypes already described by Otiende et al., [9]. The prevalence of Theileria infection was higher in white rhinoceros (66%) than in black rhinoceros (43%) (Table 1). We confirmed this result using a generalized linear model ($b = -0.652$, $p = 0.023$). The simplified glm model was Theileria Infection ~ Rhinos Species, family = binomial (cloglog):

Table 1 Proportion of rhinoceros infected as a function of age, location, sex and species evaluated using Fisher's exact test

Variable	Variable categories	% Negative	% Positive	N	p-value
AGE	Juvenile	58.8	41.2	17	0.764
	Sub-Adult	48.2	51.8	56	
	Adult	51.2	48.8	41	
LOCATION	LNNP	46.7	53.3	35	0.559
	Meru N. P.	50.0	50.0	12	
	Mugie	57.9	42.1	20	
	Ngulia	55.2	44.8	29	
	NNP	55.6	44.4	10	
	Solio	87.5	12.5	8	
SEX	Female	41.7	58.3	59	0.7026
	Male	30.0	70.0	55	
SPECIES	C. simum	34.4	65.6	32	0.037
	D. bicornis	57.3	42.7	82	

Theileria Infection ~ 0.0656 ± 0.2287 Rhinos Species - 0.6516 ± 0.2857. Since prevalence was higher in white rhinoceros, we tested whether presence of white rhinoceros together with black rhinoceros in the same locality was a risk factor for infection with Theileria in black rhinoceros. We found no significant association between infection of black rhinoceros with Theileria and the presence or absence of white rhinoceros in the same locality ($\chi^2 = 0321$; $p = 0.571$). The results were confirmed by applying a generalized lineal mixed effect model, considering locality (game park or sanctuary) as random effect. The last model in this case was Theileria Infection ~ Rhinos Species, family = binomial (logit): Theileria Infection ~ 0.0647 ± 0.3722 - 0.9414 ± 0.434 (Rhinos Species). Prevalence seemed to increase with age, but the infection by age-groups (juveniles, sub-adults and adults) was not statistically significant (Fisher test, $p = 0.764$, Table 1). Females of both species had higher prevalence 54% (29/54) than males 45% (27/60), this difference however was not statistically significant (Fisher test, $p = 0.702$, Table 1). Inter-population variations in prevalence (Figure 1) were not statistically different (Fisher test, $p = 0.681$, Table 1).

Discussion

The six sampled rhinoceros sub-populations in Kenya were infected with piroplasms but we molecularly detected only Theileria and not Babesia in all studied samples from black and white rhinoceros species.

Ticks and wildlife are the maintenance hosts of piroplasms but efficient transmission fundamentally requires presence of the protozoan, a competent tick species and the host.

Even with B. bicornis and T. bicornis originally identified from black rhinoceros [8] T. bicornis has now been detected in white rhinoceros, Nyala (Tragelaphus angasii) and Cattle [12-14]. This means that T. bicornis is a multi-host pathogen with possibility of having diverse tick species as vectors. Since D. rhinocerinus have been found in other animals such as cattle, sheep, donkey, elephant (Loxodonta africana), buffalo (Syncerus caffer) and eland (Taurotragus oryx) [15,16] this tick could be important in the cross-transmission of rhinocerotid piroplams. Pathogenicity of T. bicornis remains unresolved but T. equi, which is its close relative [8,17] and recently seen in white rhinoceros [13], has been reported to cause clinical piroplasmosis in translocated equids [18].

Translocation is intimately associated with flare up of latent infections that result in clinical state [18-20]. This is because translocation leads to elevation of glucocorticoids, whose effects are viewed to be obligatorily immunodepressant [10], yet in most cases, especially in transient acute stress, they prepare an animal to survive [10,21,22]. In the present study, sampling was carried out on asymptomatic individuals and despite underlying infection with T. bicornis, some of them were subjected to a longer period of stressor condition; during >1000 km road transportation to a new sanctuary in Ruma National Park. Nevertheless, for six months post-release monitoring of this population, they remained asymptomatic. This outcome may support the notion that T. bicornis is apathogenic or it may suggest that translocation-stress did not suppress immunity to induce clinical state. Theories behind disease induction by translocation-stress often focus on single parasite infections. However, in nature, wild animals are infected and infested simultaneously with a

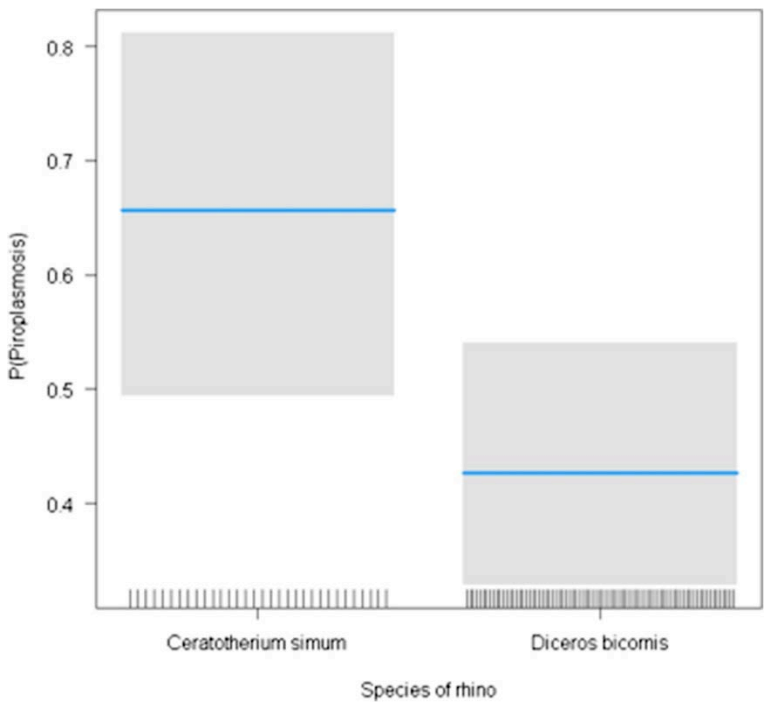

Figure 1 Variation of infection intensity of rhinoceros by species.

plethora of parasites that elicits complex immune response that may promote one parasite over the other. For instance, in concomitant infection involving African trypanosome superimposed with piroplasm leads to inhibition of the piroplasm in spite of the trypanosome immunodepressant effect [23]. In reference to fatal piroplasmosis [8], the deceased rhinoceros were subject to diverse and combined stressors; two black rhinoceros in Tanzania did not undergo prior capture and translocation event; the third fatal case involved high parasitemia, severe cold and injury while the fourth case was pregnant and developed translocation myopathy. This suggests that stressor factors that trigger clinical disease are many with maximum effect attained under synergistic state.

In the present study, the prevalence of T. bicornis was relatively high (49.1%) but clinical disease was absent in the metapopulation, a state that could mimics endemic stability [24]. This state, which was initially coined for bovine babesiosis and now widely applied in many diseases and hosts, is based on the premise that (1) severity of clinical disease increases with age and (2) that after one infection, the probability that subsequent infections result in disease is reduced [25].

We noted that higher prevalence of T. bicornis (odds ratio, 2.502) being detected in white rhinoceros than in black species (Table 1) indicating a species effect. However, we did not find significant effect associating species with prevalence, suggesting that white rhinoceros, even though more susceptible, is not a risk factor to black rhinoceros prevalence. Our result show that the Kenyan white rhino has higher prevalence of T. bicornis (66%) compared to 32.1% - 46.6% in the South African populations [13,26]. The high prevalence of T. bicornis in white rhinoceros suggests they are important hosts in the epidemiology of this piroplasm. On the contrary, according to a theory postulated by Schmidt & Ostfeld, [27] we suggest that white rhinoceros could benefit black rhinoceros by acting as 'sinks' for rhinocerotid piroplasms.

Further, our results show that prevalence among the age-groups of rhinoceros did not differ significantly (Table 1) contrary to the infection pattern in white rhinoceros population in South Africa in which female sub-adults had significantly higher prevalence [13]. Nevertheless, the age-associated inclination in our result is comparable with that of Govender et al., [13] in that peak infections were observed among sub-adults (Table 1). It is postulated that sub-adult rhinoceros of both sexes are subject to numerous stress-related changes such as, reproductive maturity, courtship, mating and territorial fights [13,28] that may suppress immunity and enhance susceptibility [29,30].

Sex-biased prevalence is observed in many parasitic infections with males having higher prevalence and intensity of infections than their conspecific females [31]. In our results, there was no significant sex-biased difference in prevalence (Table 1) though females (56%) seemed to have higher prevalence than males (45%) an observation

that concurs with that of Govender et al., [13]. Factors predisposing females to piroplasm are likely to be comparable with those affecting sub-adult rhinoceros.

The occurrence of T. bicornis in all the sampled rhinoceros sub-populations could have been facilitated by the regular translocations of individuals. Translocation assists spread of both tick vector and haemoparasites among habitat patches/populations. We observed apparent variations in prevalence among the sub-populations (Figure 2) but there was no significant difference (Table 1). This means that local factors in these habitats, such as ecological and weather differences, mammalian diversity, sanctuary size, were not sufficient to cause significant disparity in prevalence. According to Lopez et al., [32], frequent introduction of parasites in to a patch/habitat via host migration contributes to local patch prevalence. This implies that rhino sanctuaries' that frequently receive new individuals are likely to harbor higher parasite prevalence. Okita-Ouma et al., [33] points out that LNNP is more of a source population that has supported 41

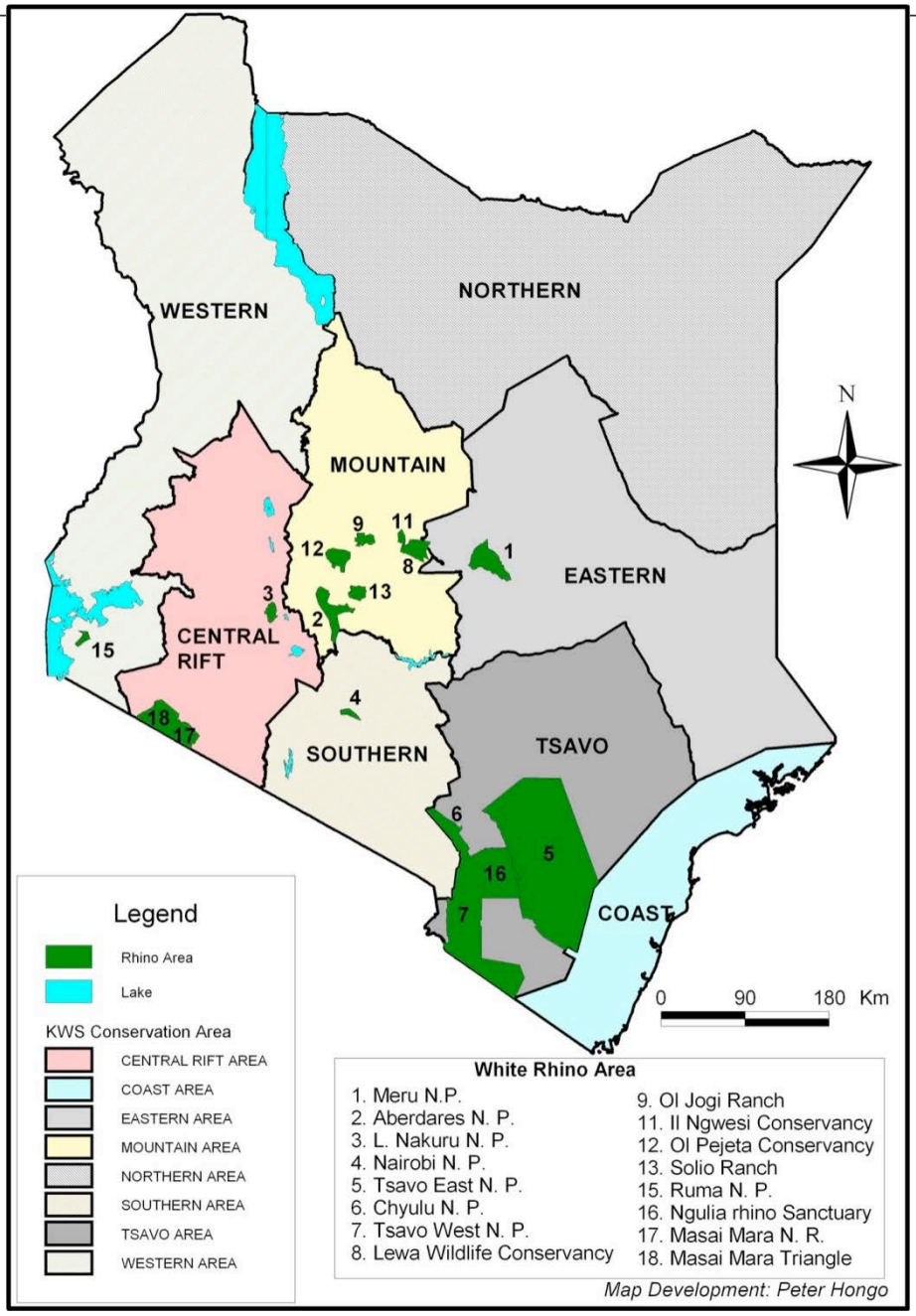

Figure 2 Locations of black rhinoceros conservation areas in Kenya.

outward translocations and received one inward translocation, whereas Ngulia RS is a recipient population having received 16 inward translocations and only one outward translocation. However, lack of significant associ- ation between location and prevalence (Table 1) does not concur with the postulation of Lopez et al., [32].

Conclusion

In the analyzed samples we did not detect the presence of Babesia bicornis, while Theileria bicornis was found to have 49.12% prevalence with white rhinoceros showing a higher prevalence than black rhinoceros. Other factors such as age, sex, location, and population mix were not found to play a significant role.

Competing interests
The authors declare no conflict of interest in this work.

Authors' contributions
Conceived and designed the experiments: MYO SA VO. Performed the experiments: MWK MNM JNM. Analyzed the data DO LK FG DM RCS. Contributed reagents/materials/analysis tools: RCS SA. Wrote the paper: MYO SA. All authors read and approved the final manuscript.

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Chapter 4: Putative filariosis outbreak in white and black rhinoceros at Meru National Park in Kenya

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Parasites & Vectors

SHORT REPORT Open Access

Putative filariosis outbreak in white and black rhinoceros at Meru National Park in Kenya

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Abstract

Background: Habitat and food supply loss and disruption, together with man's pursuit of the animal's unique horn pose significant threats to the charismatic rhinoceros. Filarial worms have been thought to cause cutaneous lesions in black rhinoceros (Diceros bicornis) in Kenya and South Africa, but never in white rhinoceros (Ceratotherium simum) in the wild, despite the fact that the two species live often in close proximity. Stephanofilaria dinniki has been implicated in the past as the causal agents for such lesions.

Findings: In this paper we report a putative filariosis outbreak in both black and white rhinos at Meru National Park in Kenya. Four black and five white rhinos were affected by various degrees of filarioid-like lesions, while apparently all sympatric wild and domestic animals were filarial worm-free. Affected rhinos were captured and successfully treated. Comparison between the epidemiological aspects of white and black rhinoceros filariosis, and the possible relations between this outbreak and annual seasons, the presence of oxpeckers and other host species are discussed.

Conclusions: Our study highlights (i) that filarial infection is not restricted to black rhinos, but it affects both rhinoceros species, and (ii) the importance of the earlier detection and immediate treatment (capture-treat and release) of filarioid infections, which is of pivotal interest for wildlife conservation, and especially the endangered and isolated white and black rhinoceros populations.

Keywords: Filariosis, Stephanofilaria dinniki, Diceros bicornis, Ceratotherium simum, Treatment, Threatened species

Findings

The world rhinoceroses' population has been reduced by more than 90% only in the past 30 years. From the 30 known rhinoceros species once inhabiting our planet, only five threatened species persist today. Only the black (Diceros bicornis) and white rhinoceros (Ceratotherium simum) still roam in Africa. The dreadful plight of this charismatic species is due to habitat and food supply loss and disruption, and mainly because of man's pursuit of the animal's unique horn, which poses the single most dangerous threat [1-3].

The causal agent of the frequent occurrence of ulcerative wounds behind the shoulder of black rhinoceros in Kenya and South Africa has been the subject of speculation for many years. The seasonal appearance of the lesions led to the belief that they were associated with secondary sex skin glands, which became active during the breeding season [4,5]. Stephanofilaria dinniki has been implicated in the past as the causal agent for such lesions [6,7]. To the best of our knowledge, in all reports only black rhinos have been shown to be affected and not the white rhino species [8], despite the fact that the two species live often in close proximity [6]. Even so, filarial lesions in white rhinos at Meru National Park (Kenya) have been reported in captivity [9].

The aims of the present study are: (i) to describe for the first time filariosis simultaneous outbreaks in white and black rhinos from wild populations, (ii) to report the efficacy of filarid infection treatment in this wild animal.

Meru National Park and Rhino Sanctuary, Kenya

This National Park is situated in northern Kenya, covers an area of 870 square kilometres. It has abundant rainfall, 635–762 mm in the west of the park and 305–

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356 mm in the east. The rainfall results in tall grass and lush swamps, which make it difficult to spot wild animals. It has a wide range of wild animals like elephant, hippopotamus, lion, leopard, cheetah, black rhinoceros and some rare antelopes, with incursions from cattle, camel, goats and sheep. The Meru National Park is a home for 22 black rhinos and 48 white rhinos. During the dry season there are constant incursions of livestock that include goats, camels and sheep in the park area.

Case report

In May 2011 during our routine monitoring of the health status of wild and domestic animal populations in Meru National Park, we identified five white and four black (Figure 1) rhinos with filariosis-like lesions, while apparently all other sympatric wild and domestic animals were filarioid-free. The affected white rhinos were 2 males and 3 females, and the infected black rhinos were 3 males and 1 female. The affected rhinos were between 3.5 and 27 years old (age estimation was based on known morphometric criteria; [10]).

The spatial distribution of the infected white and black rhinos within Meru National Park was scattered and isolated points with an average distance of 3 km ranging between 2 and 4 km (No map or GPS references were giving in this paper for security reasons, and to protect these rhinoceroses of being poached).

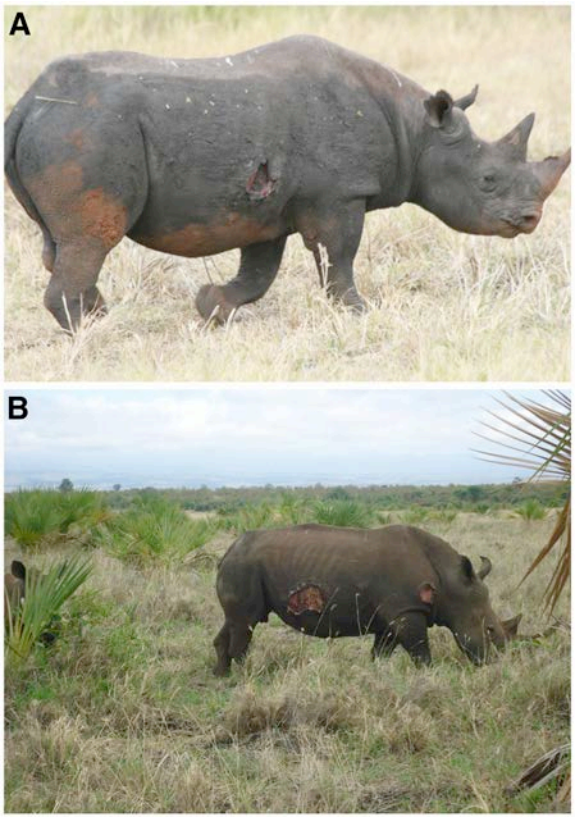

Figure 1 Extensive filarial like lesion in (A) a black rhino, Toyo, and (B) a white rhino, Stella.

Macroscopic examination of the filariosis-like lesions and parasite collection

Attempts were made to collect worms surgically from the subcutis of the immobilized rhino, and small pieces of the affected skin were collected for histopathological examination. The lesions were characterized by erosive ulcerations and crust formation. The affected skin area appeared to peel off towards the healthy side with dry and crusty edges falling off leaving a massive reddish area with neither smell nor maggots. Wounds apparently healed up well but left a big scar. The central area of the lesion was depressed and the peripheral edge of the lesion rose above the normal edge of the skin. There was a difference in thickness between the normal area of the skin and the lesion area of about 2–3 cm [11], (Figure 2).

The wounds were quite big; the mean size was 23 ± 8 cm in diameter in the white rhinos, while the black rhinos had much smaller wounds of 15 ± 5 cm. The location of the wounds was varied; white rhinos had massive wounds on the rump, behind the shoulder and the axillary regions, while the black rhinos had wounds on the ribs and behind the shoulder. Red-billed oxpeckers were observed in both rhino species.

Filariae-infected rhinos capture and treatment

Infected rhinos were darted with Dan-inject^{W} dart gun from a helicopter. The dart contained 5 mg Etorphine Hydrochloride (M99^{W}) and 30 mg Xylazine (ILIUM, Troy Laboratories, Australia.) for adult black rhinos and 6 mg Etorphine Hydrochloride and 50 mg Xylazine for adult white rhinos. The animals went down in

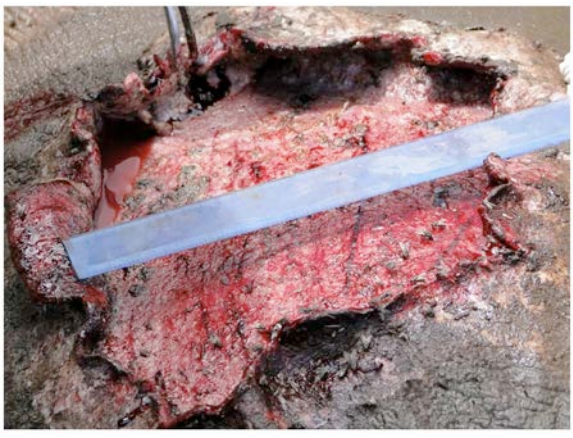

Figure 2 A white rhino, Rosie, wound at time of treatment showing macroscopic appearance and size of filarial like lesion.

approximately 6 minutes. After getting down, anaesthetic plan was improved by injecting 5 mg of Nalorphine into an ear vein. In case of the presence of accompanying calves, they were driven away by the help of a vehicle. The animals were then placed on sternal or lateral recumbency to be sampled and treated.

Each rhino was treated with 15,000 mg intramuscularly, long acting Amoxicillin Trihydrate (Betamox^{W}), Ivermectin 200 mg (dosage of 1 ml per 50 kg body weight) and local wound treatment, cutting dead tissue debridement followed by spraying with povidone iodine and fly repellent in some wounds (Figure 3).

Animals were revived by the injection of 18 mg Diprenorphine (M5050^{W}) and 6 mgs Atipamezole Hydrochloride (ANTISEDAN^{W}) into an ear vein. The white rhinos were also injected with 25 mg Naltrexone Hydrochloride (Naltrexone; Kyron Laboratories) intramuscularly to prevent re-narcotisation. All rhinos got up in approximately three minutes.

Results and Discussion

In the present study we report for the first time a putative filarid outbreak in both black and white rhinos from Meru National Park in Kenya. We failed to collect worms surgically from the subcutis of the immobilized rhinos, and the histopathological examination was negative for the collected samples. Only Stephanofilaria dinniki has been implicated in the past as the causal agent for such filarioid-lesions, and hence we expect this parasite species to be the causative agent of the outbreak of this disease.

The life cycle of S dinniki parasite is unknown. Species of the order to which it belongs require blood-sucking arthropods to complete the cycle [12]. Members of the phylum Arthropoda found associated with rhino are mosquitos, flies and ticks, and hence they can be considered in the transmission of this parasite. Oxpeckers have been seen to peck at the lesions but they are probably not involved in the life cycle but may be attracted by ticks and loose skin. Further studies are needed to understand the life cycle and the vectors of this parasite.

When living conditions deteriorate for hosts, during periods of overcrowding or food shortage, animal become stressed. This stress has often been linked to epidemics, which are attributed to the immunocompromised status of the stressed hosts [13-15]. In addition, the prevailing environmental conditions could have contributed to lesions and possible filariosis outbreak in both rhino species in Meru National Park. The Rhino Sanctuary at the time of the outbreak was wet, bushy and thick, compounded by dense under growth, while the open areas were most likely swampy patches. Kock & Kock [16] reported that poor body condition and heavy rainfall were thought to predispose to development of recrudescence of lesions.

The relationship between oxpeckers and the large mammalian species in Africa is, controversially, usually classified as "cleaning symbiosis" as it is thought to be equally beneficial to both species [17,18]. Keet et al. [19] reported that red-billed (Buphagus erythrorynchus) oxpeckers could play an important role in the pathogenesis of filarioid epidemiology and lesions. The carnivorous behaviour of these birds, ingesting the blood containing embryonated eggs, could reduce the likelihood of parasite spread, but at the same time they feed on the superficial necrotic skin causing the development of larger ulcers. In the white and black rhinos we observed with lesions we also observed the presence of yellow- and red-billed oxpeckers, which could possibly play an important epidemiological role in filarioid control (Figure 4).

The treated rhinos recovered completely within three months, and no new cases presented in the last

Figure 3 Photo showing treatment and sampling of an immobilized white rhino.

Figure 4 A black rhino, Doreen, showing signs of filarid-like lesions, together with the presence of an oxpecker.

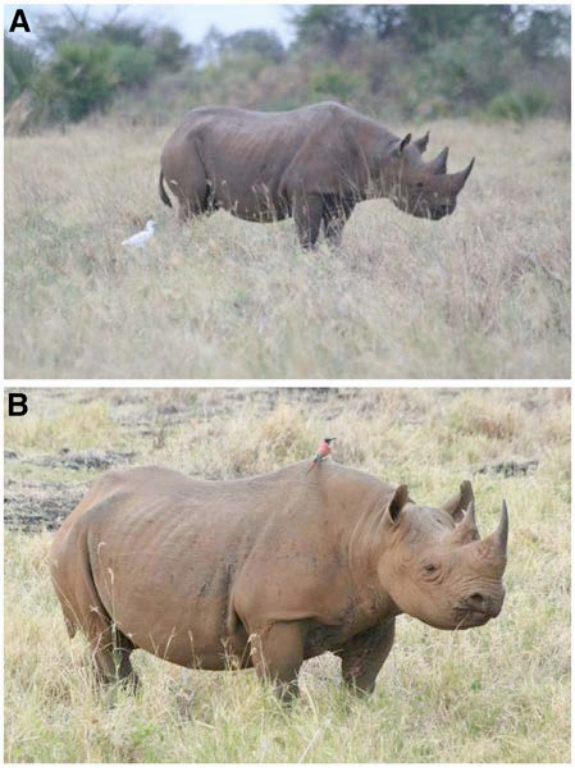

Figure 5 A black rhino, Kelele, (A) before and (B) after treatment.

monitoring in May 2012. Rhinoceros recovery was evaluated by the reductions in the mean number and surface area of lesions (Figure 5). This shows that the disease can be controlled in an ecosystem via therapeutic treatment of positive cases "capture-treat and release", which is in concordance with other studies showing the high efficacy of ivermectin against other filarioid infections, but it has not previously been tested in rhinoceros species [20-22].

One explanation of the high frequency of filarioid outbreaks in wildlife, during the last decades, could be the intrusion of humans and their livestock into wildlife habitat ranges, resulting in great changes in the interface between wildlife and people/livestock, and wildlife habitat losses, and hence the interaction between human/livestock and wildlife is changing from often sporadic and fragile to more permanent and substantial, providing significant opportunities for parasite transmission [23]. The permanent loss of wildlife genetic diversity could have contributed negatively, making wild animals more susceptible to parasite infection [24,25].

The presence of parasites in any ecosystem generates complex parasite webs within the system, and it is through these webs that zoonotic parasites move from one host to other [26,27]. Hence, more studies are needed to understand the filarioid-navigating web including potential molecular analyses [28,29], and the range of underlying causal factors of its unexplicable emergence and re-emergence [24,30], which poses a substantial threat to the conservation of the global biodiversity [23].

Ethics

The Committee of the Department of Veterinary and Capture Services of the Kenya Wildlife Service (KWS) approved the study including animal capturing and treatment protocols. KWS guidelines on Wildlife Veterinary Practice-2006 were followed. All KWS veterinaries were guided by the Veterinary Surgeons Act Cap 366 Laws of Kenya that regulates veterinary practices in Kenya.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MM, MO, FG, LK and VO conceived and designed the experiments. MM, MO, FG, LK, VO, DN, EN, EK, IL, RCS, LR and SA performed the fieldwork experiments. Manuscript was analysed, discussed and written by all co-authors. All authors read and approved the final manuscript.

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Chapter 5: Emerging and re-emerging filariosis infection threatens the conservation of the white and black rhinoceros in Kenya

Emerging and re-emerging filariosis infection threatens the conservation of the white and black rhinoceros in Kenya

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Abstract

Stephanofilaria is a genus of filarial nematodes affecting a number of large herbivore mammals, causing filariosis disease. In 2011-2012, we reported the first case of filariosis outbreak in white and black rhinoceros in Kenya with nine cases, all successfully treated. In the present paper we highlight how filariosis disease has been converted in to a deadly emerging and re-emerging disease threatening the conservation of white and black rhinoceros. After our first report, the rhinoceros population was affected by 21 more cases between 2012 and 2015. White rhinoceros where affected (86%) more than black rhinoceros (14%). Males (71%) were more vulnerable than females (29%), and many adults (86%) where infected compared to sub-adults and juveniles (14%). There were more cases occurring in the dry season (67%), compared to cases occurring in the wet season (33%). All affected animals were treated; nonetheless we had a high mortality rate of 14%. Our study reveals that filariosis emergence in black and white rhinoceros in Kenya was not just a simple outbreak, but it has been converted in to a deadly emerging and re-emerging disease threatening the conservation of these endangered species with high mortality rate. More studies are needed to build the missing links in the epidemiology of the causal parasite, including the intermediate hosts, environmental effects, possible rhinoceros-domestic animal co-infection if it exists, and the role of oxpeckers, which may aid the spread of this deadly emerging and re-emerging disease.

Keywords: *Stephanofilaria dinniki*; *Diceros bicornis*; *Ceratotherium simum*; Treatment; Threatened species; Intermediate Host; Lewa, Co-infection; Oxpeckers; Endangered

Finding

Rhinos are one of the big five game species that attract tourist to protected areas and conservancies where these animals occur and are therefore an important source of tourism revenue in many African economies. Rhinos also play an important ecological role in African ecosystems. Specifically rhinos like many mega-herbivores can greatly accelerate the nutrient cycle in ecosystems through grazing or browsing and subsequent excretion through defecation and urination, returning nutrients to the soil at rates that are orders of magnitude faster than processes of leaf loss and decay through excreta (Ripple et al., 2015). Rhinoceros were abundant in Kenya in the early 20th Century (Barclay, 1932, Brett, 1993, Hunter and Watson, 1952, Lloyd-Jones, 1925, Neumann, 1898, Patterson, 1909). However, the population declined catastrophically due to poaching and human settlement during the following 20 years, to less than 400 animals by 1990 (Brett, 1993, Emslie and Brooks, 1999, Gakahu, 1993, Okita-Ouma et al., 2007). This collapse resulted in small, isolated, demographically unviable populations scattered across fragmented regions in Kenya, with many facing local extinction.

Stephanofilaria is a very important genus of filarial nematodes affecting rhinoceros, Asian elephant, hippopotamus, domestic and wild suid, buffalo and cattle. This genus belongs to the family Filariidae, Weinland 1858, of the order Spirurida, Railliet 1914 in the phylum Nematoda. There are nearly a dozen species in this genus infecting specific hosts; *Stephanofilaria dinniki.* infects the black and white rhinos (Round, 1964), *Stephanofilaria thelazioides* infects the hippopotamuses (Boomker et al., 1995), *Stephanofilaria boomkeri* infects domestic and wild African suids (Bain et al., 1996), *Stephanofilaria stilesi* infects cattle in Australia, *Stephanofilaria zaheeri* (syn *Stephanofilaria assamensis)* infects cattle and Buffalo in Europe and Asia while an unknown species of *Stephanofilaria* infects Indian or Asian elephants (Agrawal and Shah, 1984, Shaw and Sutherland, 2006).

Stephanofilaria causes significant economic loses to the cattle industry in northern Australia and in parts of Asia (Johnson and Toleman, 1988, Shaw and Sutherland, 2006). Dermatitis from *Stephanofilaria* infection has also been reported in Africa, and North America, suggesting that it is a globally widespread problem (Oduye, 1971, Dies and Pritchard, 1985, Guglielmone et al., 1999). Johnson and Toleman (1988) reported a prevalence of between 61.2-95% of

Stephanofilarial lesions in *Bos indicus* cattle in northern Australia whereas in Bengal, India, Sigh et al (2011) report the prevalence of *Stephanofilarial* lesions among cattle to be 18% in the south and 38.34 % the north.

Stephanofilaria is also important to the conservation of endangered species such as rhinoceros where outbreaks have been reported (Mutinda et al., 2012, Kock and Kock, 1990). *Stephanofiliaria* spp cause dermal ulcerative lesions in specific regions of the body of their mammalian hosts (Agrawal and Shah, 1984, Bain et al., 1996, Kock and Kock, 1990, Schulz and Kluge, 1960, Tremlett, 1964). In rhinos, infection with *Stephanofiliaria dinniki* causes, erosive, ulcerating lesions 2-3cms deeper than the surrounding skin with crusting, and edges raised above the normal skin (Mutinda et al., 2012, Kock and Kock, 1990, Schulz and Kluge, 1960, Tremlett, 1964). In the white rhinoceros these lesions can be on average 23 cm in diameter and located behind the shoulder, in the axillary region or on the rump, whereas in the black rhinoceros the wounds were smaller, being on average 15 cm and located behind the shoulder or on the ribs (Mutinda et al., 2012). A more detailed description of the pathology of the lesions in black rhino was first described by Tremlett (1964) and Kock and Kock (1990). Secondary infection of *Stephanofilarial* caused wounds is another major cause of mortality in captive rhino populations (Clausen and Ashford, 1980).

Meru National Park and Rhino Sanctuary, Kenya

This National Park is situated in northern Kenya and covers an area of 870 Km^2. It has abundant rainfall, 635–762 mm in the west of the park and 305–356 mm in the east. The habitat of Meru National Park varies from woodland to open grasslands intersected by permanent rivers and associated riverine vegetation. Within the National park lies the rhino sanctuary which is surrounded by a perimeter electric fence. The southern portion of the rhino sanctuary is dominated by forests while the rest of the sanctuary is dominated by thickets and grassland interspersed with several rivers such as Mukutano, Kanjoo and Rujuwero. Meru National Park has a rich diversity of wild animals including elephant, hippopotamus, lion, leopard, cheetah, black rhinoceros and some rare antelopes, with incursions from cattle, camel, goats and sheep. The Meru National Park is a home for 22 black rhinos and 48 white rhinos. During the dry

season there are constant incursions of livestock that include goats, camels and sheep in the park area but not into the rhino sanctuary.

Filariosis emerging and re-emerging

In 2011-2012, we reported the first case of filariosis outbreak in white and black rhinoceros in Kenya with nine cases all successfully treated. After our first report, the rhinoceros population was affected with 21 more cases between 2012 and 2015 (Fig 1-3). White rhinoceros where more affected (18/21 cases; 86%) than black rhinoceros (3/21 cases; 14%) (χ^2=18.667, P<0.0001). More male (15/21; 71%) were affected than females (6/21; 29%) (χ^2=7.714, P=0.005) More cases were reported in the dry season (14/21; 67%), comparing with the wet season (7/21; 33%) (χ^2=4.667, P=0.031). Adults were more affected (18/21; 86%) comparing with sub-adults and juvenile (3/21; 14%) (χ^2=21.429, P<0.0001). All affected animals were treated, but even so we had a high mortality rate of 14% (3/21) affecting only the white rhinoceros.

The missing links in *Stephanofilaria* epidemiology

1-Intermediate hosts and life cycle

Although much is known regarding the life cycle and intermediate hosts of most *Stephanofiliria* infecting cattle and buffalo (Hibler, 1966, Agrawal and Shah, 1984), there is a dearth of information regarding the intermediate host of the species of *Stephanofilaria* infecting rhinos. The life cycle of *S. stilesi* is more documented than most species of *Stephanofilaria*. In Northern Australia *Stephanofilaria stilesi* infections are transmitted by the buffalo fly *Haematobia irritans exigua* (Allingham et al., 1998, Johnson and Toleman, 1988). Whereas in parts of Europe, North and South America and the Caribbean, Middle East and central Asia, the horn flies *Haematobia irritans irritans* and *Haematobia tillilans* are important vectors of *Stephanofilaria stilesi* (Guglielmone et al., 1999, Saparov et al., 2014, Singh et al., 2011). Horn flies develop in cattle manure. These flies become infected by an average of two microfilariae by feeding on infected ulcerated skin lesions. Ingested microfilaria larvae develop in the abdominal haemocoele and it takes eighteen to twenty-one days to grow from L1 to L3, with the first moult at eight to ten days, and the second at fourteen to sixteen days. After this is migrates to the head and proboscis, in order to enter the definitive host the next time the fly feeds (Hibler, 1966). The cycle repeats again when these flies bite on cattle creating lesions that enable infective *Stephanofilaria* larvae

to invade the skin (Fig 4). In India, Sigh et al (2011) found two of three species of hematophagus flies, *Musca conducens* and *Haematobia* sp but not *Stomoxys calcitrans* to be vectors of infective larvae even though all these species were associated with animals infected with *Stephanofilaria assamensis*. In Uzbekistan, Saparov et al (2014) established *Haematobia atripalpis*, *Haematobia (Lyperosia) titillans* and *Haematobia (Lyperosia) irritans* as vectors of *Stephanofilaria* infecting cattle. Flies from the family *Muscidae* are involved in the transmission of many filarioid nematodes and related species relevant to this study. The two main genera that seem to be involved here are *Haematobia* (biting flies) and *Musca* (nuisance flies) (Holdsworth et al., 2006). In muscid flies, for example the Buffalo fly *Haematobia irritans exigua*, both sexes are involved are carriers of *Stephanofilaria* (Anderson, 2000). *H. irritans* transmits *S. stilesi* in North America (Holdsworth et al., 2006, Fallis, 1980), as well as in India (Mohan, 1973). *Musca conducens* transmits Stephanofilarial dermatitis of cattle in West Bengal, India (Singh et al., 2011).

Studies on biting flies of black rhinos in east Africa revealed that some of the species of flies known to be vectors of *Stephanofilaria* elsewhere are associated with rhino in East Africa and include *Haematobia* sp, other species associated with ulcerative skin lesions in Rhinos include *Rhinomusca dutoiti*, *R. brucei* (Mihok et al., 1996, Parsons and Sheldrick, 1964). Studies on *Stephanofilaria* vectors elsewhere suggests that a Muscid fly, most likely *Haemotobia*, or *Musca,* are involved in the transmission of *S. dinniki* in rhino. In Fig 5 the life-cycle of *S. dinniki* in the rhino (black and white) and fly vector respectively are proposed. More work needs to be done to confirm the identity of the filarial species infecting rhinos in Kenya, whether is actually *Stephanofilaria dinniki* or another similar species.

2-Environmental influences on epidemiology of *Stephanofilaria* infections

The occurrence of ulcerative filarial lesions in rhinos caused by *Stephanofilaria dinniki* is strongly seasonal (Kock and Kock, 1990, Mutinda et al., 2012) and suggests that either vector arthropod abundance or nutritional stress of hosts are key factors driving the incidence of infection. A high incidence of Stephanofilarial lesions occur during the end of the rainy season in both Kenya and in Zimbabwe (Kock and Kock, 1990, Mutinda et al., 2012) and during the monsoon rains and during summer in India and South Africa (Agrawal and Shah, 1984, Schulz and Kluge, 1960). However, the peak in disease incidence occurs at the end of the rains when

food is likely abundant suggesting that the incidence of disease is driven primarily by vector abundance. However, in the south of the United States, Hibler (1966) found that the highest incidence of infection of horn flies with *S. stilesi* microfilariae were in the spring and autumn and concluded that the summer was too hot for development of the larval stages in the fly.

3-Putative rhinoceros-domestic animal co-infection

Although domestic ruminants specifically cattle can be infected with *Stephanofilaria* and has been demonstrated elsewhere, the incidence of *Stephanofilaria* infections in domestic ruminants is not known in Kenya. There is an urgent need to establish the presence of *Stephanofilaria* disease in Kenya through surveillance of cattle from areas where cattle overlap or share habitat with rhinoceros.

Another area for future research is identifying whether the same vectors transmitting Stephanofialrial worms of cattle elsewhere are implicated in the transmission of *Stephanofilaria dinniki* in rhinos. The life cycle of *Stephanofilaria dinniki* in both the rhino and the arthropod vectors once identified should be examined further in order to identify which stages of larvae are found in the mammalian hosts and in the arthropod host.

4. The putative role of oxpeckers

Oxpeckers feed exclusively on the on ectoparasites on rhinos, particularly ticks, as well as insects infesting wounds and the flesh and blood of some wounds as well. They are sometimes classified as parasites, due to the fact that they open wounds on the animals' backs (Craig, 2009). Oxpecker/rhino interactions are the subject of some debate and ongoing research. They were originally thought to be an example of mutualism, but recent evidence suggests that oxpeckers may be parasites instead (Weeks, 2000). Oxpeckers do eat ticks, but often the ticks have already fed on the ungulate host and no statistically significant link has been shown between oxpecker presence and reduced ectoparasite load (Weeks, 2000). Oxpeckers have been observed to open new wounds and enhance existing ones in order to drink the blood of their perches (McElligott et al., 2004). Our observation of lesions that are generally characterised by superficial redness, erosive ulceration and crust formation with serrated edges is due to the oxpecker activity;

Oxpeckers also feed on the earwax and dandruff of rhinos; less is known about the possible benefits of this to the mammal, but it is suspected that this is also a parasitic behaviour.

Conclusions

Our studies revealed that filariosis outbreak in black and white rhinoceros in Kenya was not just a simple outbreak but it has been converted into a deadly emerging and re-emerging disease threatening the conservation of these endangered species. More studies are needed to build the missing links in the epidemiology of the causal parasite, which will help for the effective control of the new emerging and re-emerging disease.

Figure 1 Macroscopic appearance of a filarial-like lesion in a white rhinoceros

Figure 2 Filarial lesion in a white rhinoceros

Figure 3 The epidemiology of filariosis infection between 2012 and 2015

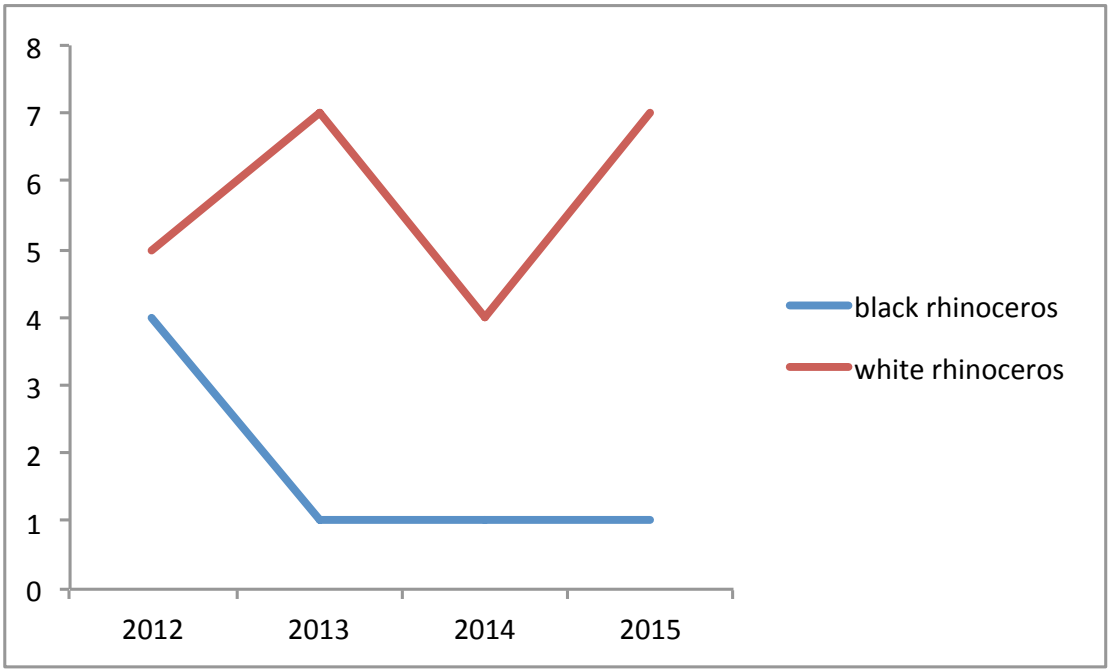
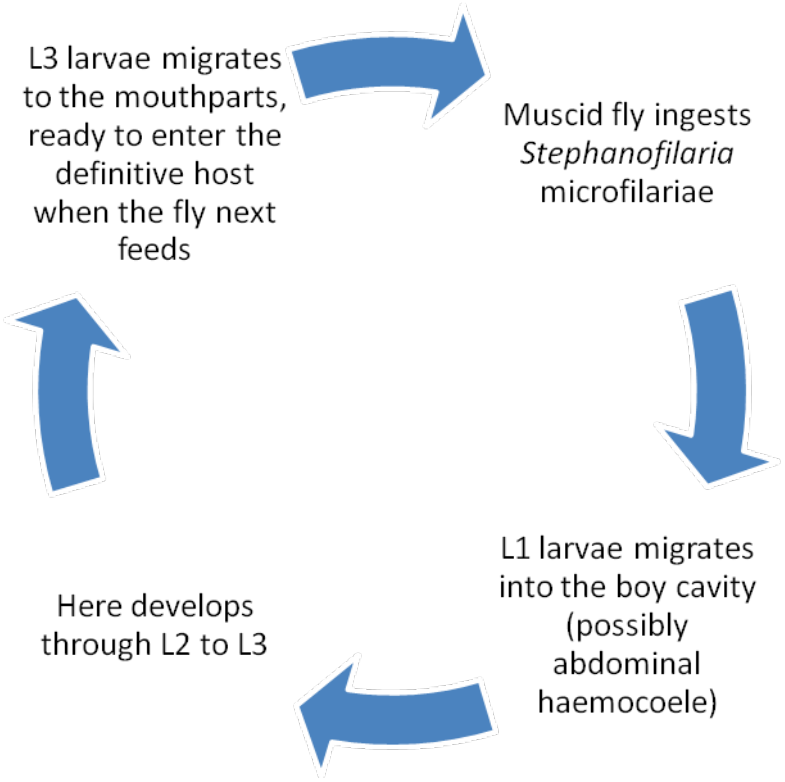


Figure 4 Filarial lesion in a juvenile black rhino showing possible dipterid vectors

Figure 5 Putative Life cycle of *Stephanofilaria*



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Chapter 6: Discussion

In our first chapter of this thesis, sampling was carried out on asymptomatic individuals and despite underlying infection with *T. bicornis*, some of them were subjected to a longer period of stressor condition; during >1000 km road transportation to a new sanctuary in Ruma National Park. Nevertheless, for six months post-release monitoring of this population, they remained asymptomatic. This outcome may support the notion that *T. bicornis* is apathogenic or it may suggest that translocation-stress did not suppress immunity to induce clinical state. Theories behind disease induction by translocation-stress often focus on single parasite infections. However, in nature, wild animals are infected and infested simultaneously with a plethora of parasites that elicits complex immune response that may promote one parasite over the other. For instance, in concomitant infection involving African trypanosome superimposed with piroplasm leads to inhibition of the piroplasm in spite of the trypanosome immunodepressant effect (Millott and Cox, 1985). In reference to fatal piroplasmosis (Nijhof et al., 2003), the deceased rhinoceros were subject to diverse and combined stressors; two black rhinoceros in Tanzania did not undergo prior capture and translocation event; the third fatal case involved high parasitemia, severe cold and injury while the fourth case was pregnant and developed translocation myopathy. This suggests that stressor factors that trigger clinical disease are many with maximum effect attained under synergistic state.

In the present study, the prevalence of *T. bicornis* was relatively high (49.1%) but clinical disease was absent in the metapopulation, suggesting a state of endemic stability (Penzhorn et al., 2008). We noted that higher prevalence of *T. bicornis* being detected in white rhinoceros than in black species indicating a species effect. However, we did not find significant effect associating species with prevalence, suggesting that white rhinoceros, even though more susceptible, is not a risk factor to black rhinoceros prevalence. Our result show that the Kenyan white rhino has higher prevalence of *T. bicornis* (66%) compared to 32.1% - 46.6% in the South African populations (Bigalke et al., 1970, Govender et al., 2011). The high prevalence of *T. bicornis* in white rhinoceros suggests they are important hosts in the epidemiology of this piroplasm. On the contrary, according to a theory postulated by Schmidt & Ostfeld, (2001) we suggest that white rhinoceros could benefit black rhinoceros by acting as ‘sinks’ for rhinocerotid

piroplasms. Further, our results show that prevalence among the age-groups of rhinoceros did not differ significantly contrary to the infection pattern in white rhinoceros population in South Africa in which female sub-adults had significantly higher prevalence (Govender et al., 2011). It is postulated that sub-adult rhinoceros of both sexes are subject to numerous stress-related changes such as, reproductive maturity, courtship, mating and territorial fights that may suppress immunity and enhance susceptibility (Glaser and Kiecolt-Glaser, 2005, Muehlenbein, 2006). Sex-biased prevalence is observed in many parasitic infections with males having higher prevalence and intensity of infections than their conspecific females (Klein, 2004). In our results, there was no significant sex-biased difference in prevalence though females (56%) seemed to have higher prevalence than males (45%).

The occurrence of *T. bicornis* in all the sampled rhinoceros sub-populations could have been facilitated by the regular translocations of individuals. Translocation assists spread of both tick vector and haemoparasites among habitat patches/populations. According to Lopez et al., (2005), frequent introduction of parasites in to a patch/habitat via host migration contributes to local patch prevalence. This implies that rhino sanctuaries' that frequently receive new individuals are likely to harbor higher parasite prevalence. Okita-Ouma et al., (2010) points out that LNNP is more of a source population that has supported 41 outward translocations and received one inward translocation, whereas Ngulia RS is a recipient population having received 16 inward translocations and only one outward translocation. However, lack of significant association between location and prevalence does not concur with the postulation of Lopez et al., (2005).

In our study that focused on the genetic diversity, three haplotypes were recovered (H1, H2, and H3) among sampled animals. The Bayesian Inference 50% consensus phylogram recovered all the haplotypes monophyletic with *Theileria bicornis* with very high support (BPP: 0.98). Furthermore, the genetic *p*-uncorrected distances and substitutions between *T. bicornis* and the three haplotypes were the same as within all the three haplotypes, indicating very a close genetic affinity (Chapter 2). Our results indicate that the new *T. bicornis* haplotypes are widespread among the major rhinoceros sub populations in Kenya. This wide distribution may be beneficial as many individuals are not immunologically naïve to this pathogen hence there is a low risk of clinical disease in cases where individuals are released into endemic areas.

In the third part of this thesis we describe for the first time a putative filarid outbreak in both black and white rhinos from Meru National Park in Kenya and attempt to unravel the missing link in the epidemiology of *Stephanofilaria dinniki*. The Rhino Sanctuary at the time of the outbreak was wet, bushy and thick, compounded by dense under growth, while the open areas were most likely swampy patches. Kock & Kock (1990) reported that poor body condition and heavy rainfall were thought to predispose to development of recrudescence of lesions. We also collected worms surgically from the subcutis of the immobilized rhinos, and examined its histopathology. The histopathological examination was negative for the collected samples.

Stephanofilaria dinniki has been implicated in the past as the causal agent for such filarioid-lesions, and hence we expect this parasite species to be the causative agent of the outbreak of this disease. The life cycle of *S dinniki* parasite is unknown but studies of other related species in this genus suggest that it may require blood-sucking arthropods to complete the cycle (Hitchins and Keep, 1970). Many arthropods tend to be associated with rhino including mosquitos, flies and ticks, are likely to play a role as vectors of this parasite. Oxpeckers have been seen to peck at the lesions but they are probably not involved in the life cycle. They may be attracted by ticks and loose skin. Further studies are needed to understand the life cycle and the vectors of this parasite.

In the fourth part we studied the re-emergence of filariosis diseases between 2011 and 2015. We found that White rhinoceros where affected (86%) more than black rhinoceros (14%). Males (71%) were more vulnerable than females (29%), and many adults (86%) where infected compared to sub-adults and juveniles (14%). There were more cases occurring in the dry season (67%), compared to cases occurring in the wet season (33%). All affected animals were treated; nonetheless we had a high mortality rate of 14%.

Capitulo 6: Discusión

En nuestro primer capítulo de esta tesis, se realizaron muestreos en individuos asintomáticos y a pesar de la infección subyacente con *T. bicornis*, algunos de ellos fueron sometidos a un período más largo en la condición de estrés; durante >1000 km de camino a una nueva área en el Parque Nacional de Ruma. Sin embargo, durante los seis meses posteriores a la liberación que se estuvo monitoreando a esta población, se mantuvieron asintomáticos. Este resultado puede apoyar la idea de que *T. bicornis* no es patogénico o puede sugerir que ese estrés por la translocación no suprimió la inmunidad para inducir el estado clínico. Las teorías que apoyan la inducción de la enfermedad por el estrés de la translocación a menudo solo se centran en infecciones parasitarias. Sin embargo, en la naturaleza, los animales salvajes son infectados e infectados simultáneamente con varios parásitos provocando una compleja respuesta inmune que pueda facilitar la presencia de nuevas infecciones secundarias.

Por ejemplo, la infección simultánea con tripanosoma africano y piroplasma conduce a la inhibición del piroplasma a pesar del efecto inmunodepresor del tripanosoma (Millott y Cox, 1985). En referencia a la piroplasmosis letal (Nijhof et al., 2003), los rinocerontes fallecidos fueron sometidos a diversos y combinados factores de estrés; dos rinoceronte negros en Tanzania no experimentaron un proceso de captura ni translocación previa; el tercer caso letal fue provocado por factores tales como la elevada parasitemia, el frío severo y lesiones; mientras que el cuarto caso estaba preñada y desarrollo miopatía debida a la translocación. Esto sugiere que los factores estresantes que desencadenan la enfermedad clínica causan su máximo efecto bajo estado sinérgico.

En el presente estudio, la prevalencia de *T. bicornis* fue relativamente alta (49.1%) pero la enfermedad clínica estuvo ausente en la metapoblación, sugiriendo un estado de estabilidad endémica (Penzhorn et al., 2008). Observamos una mayor prevalencia de *T. bicornis* en ejemplares de rinoceronte blanco con respecto a ejemplares negros indicando un efecto de la especie. Sin embargo, no encontramos un efecto significativo al asociar especies con prevalencia, sugiriendo esto que el rinoceronte blanco, aun siendo más susceptible, no es un factor de riesgo para la prevalencia en el rinoceronte negro. Nuestros resultados muestran que el rinoceronte blanco de Kenia tiene mayor prevalencia de *T. bicornis* (66%) frente a 32,1% -

46,6% en las poblaciones de Sudáfrica (Bigalke et al., 1970, Govender et al., 2011). La alta prevalencia de *T. bicornis* de rinoceronte blanco indica que son hospedadores importantes en la epidemiología de este piroplasma. Por el contrario, según una teoría postulada por Schmidt & Ostfeld, (2001) se sugiere que el rinoceronte blanco podría beneficiar al rinoceronte negro al actuar como sumideros de piroplasma para Rhinocerotidae. Además, nuestros resultados muestran que la prevalencia entre los grupos de edad de rinoceronte no difirió significativamente con respecto al patrón de infección en la población de rinoceronte blanco en Sudáfrica, en las que hembras sub-adultas tenían una prevalencia significativamente mayor (Govender et al., 2011). Se postula que los rinocerontes subadultos de ambos sexos están sujetos a numerosos cambios relacionados con el estrés, como madurez reproductiva, cortejo, apareamiento y peleas territoriales que pueden suprimir la inmunidad y aumentar la susceptibilidad (Glaser y Kiecolt-Glaser, 2005, Muehlenbein, 2006). En cuanto a la sex-ratio se observa que los machos presentan una mayor prevalencia e intensidad de infección parasitaria con respecto a las hembras de la misma especie (Klein, 2004). En nuestros resultados, no se observaron diferencias significativas de sex-ratio respecto a la prevalencia aunque las hembras (56%) parecen tener mayor prevalencia que los machos (45%).

La presencia de *T. bicornis* en todas las subpoblaciones muestreadas de rinocerontes podría haber sido facilitada por los desplazamientos regulares de individuos. La translocación ayuda a la propagación de vectores de garrapatas y hemoparásitos entre zonas de hábitats/poblaciones. Según López et al., (2005), la frecuente introducción de parásitos en una zona/hábitat mediante la migración del hospedador contribuye a la prevalencia local en la zona. Esto implica que las poblaciones de los rinocerontes que reciben con frecuencia nuevos individuos son propensas a albergar una mayor prevalencia parasitaria. Okita Ouma et al., (2010) señalan que LNNP es más una población emisora, puesto que ha soportado 41 desplazamientos hacia el exterior y recibido un desplazamiento hacia el interior; mientras que Ngulia RS es una población receptora al haber recibido 16 translocaciones hacia el interior y solo una hacia el exterior. Sin embargo, la ausencia de asociación significativa entre la localización y la prevalencia no coincide con la hipótesis de López et al., (2005).

En nuestro estudio, que se centró en la diversidad genética, se encontrarón tres haplotipos (H1, H2 y H3) entre los animales muestreados. Se obtuvo un filograma consenso por inferencia bayesiana con un 50%, encontrando un alto apoyo en todos los haplotipos monofiléticos con *Theileria bicornis* (BPP: 0,98). Además, las distancias genéticas *p* sin correjir y las sustituciones entre *T. bicornis* y los tres haplotipos eran los mismos, dentro de los tres haplotipos, indicando una estrecha afinidad genética (capítulo 2). Nuestros resultados indican que los nuevos haplotipos *T. bicornis* están extendidos entre las mayores subpoblaciones de rinocerontes en Kenia. Esta amplia distribución puede ser beneficiosa ya que muchos individuos han tenido contacto inmunológico previo con este patógeno, por lo tanto hay un riesgo bajo de enfermedad clínica en los casos donde los individuos son liberados en áreas endémicas.

En la tercera parte de esta tesis se describe por primera vez un brote aparente de filaria en ambas especies de rinoceronte blanco y negro del Parque Nacional Meru en Kenia, el cual trata de desentrañar el eslabón perdido en la epidemiología de *Stephanofilaria dinniki*. El santuario de los de rinocerontes durante el momento del brote de esta enfermedad estaba húmedo, espeso y abundante, compuesta por un denso decrecimiento bajo, mientras que las áreas abiertas fueron probablemente zonas pantanosas. Kock & Kock (1990) describió que las bajas condiciones físicas y las lluvias ocasionaron una mayor predisposición al desarrollo del agravamiento de las lesiones. También recogimos quirúrgicamente gusanos del tejido subcutáneo de rinocerontes inmovilizados y examinamos su histopatología. Los resultados de las muestras examinadas resultaron negativos.

Stephanofilaria dinniki fue relacionado en el pasado como el agente causal de lesiones tales como la filaria y, por lo tanto, esperamos que esta especie de parásito sea el agente causal del brote de esta enfermedad. El ciclo vital del parásito de *S dinniki* es desconocido, pero estudios relacionados con otras especies de este género sugieren que pueden requerir hematófagos para completar el ciclo (Hitchins y mantenga, 1970). Muchos artrópodos tienden a asociarse con rinocerontes como mosquitos, moscas y garrapatas, los cuales son propensos a jugar un papel como vectores de este parásito. Se han observado Picabueyes en las lesiones, pero probablemente no están implicados en el ciclo vital. Pueden ser atraídos por las garrapatas y la

piel muerta. Se necesitan estudios adicionales para entender el ciclo vital y los vectores de este parásito.

En la cuarta parte se estudió la reaparición de enfermedades de filariosis entre 2011 y 2015. Encontramos que en el caso de la especie de rinoceronte blanco había una afección del 86%, en contraposición a la especie de rinoceronte negro (14%). Los machos (71%) eran más vulnerables que las hembras (29%), mientras que los adultos (86%) estaban más infectados que los jóvenes (14%). Hubo más casos en la estación seca (67%), comparado con casos que ocurren en la temporada lluviosa (33%). Todos los animales afectados fueron tratados; sin embargo, hemos tenido una alta tasa de mortalidad del 14%.

Chapter 7: Conclusions

1. Molecular genetic analyses of the 18S rRNA, revealed an absence of *Babesia bicornis*, but the presence of *Theileria bicornis.* Genetic diversity analyses revealed the first record of *T. bicornis* and its three new haplotypes in black and white rhinoceros in Kenya. Our results further indicate that the new *T. bicornis* haplotypes are widespread among the major rhinoceros sub populations in Kenya.
2. The prevalence of *T. bicornis* was 49.12% prevalence with white rhinoceros showing a higher prevelance than black rhino suggesting that black rhino can be used as a transmission sink for *Theileria* in white rhinoceros
3. There was variation in *T. Bicornis* prevalence among rhinoceros sub-populations but there was no significant difference suggesting that local factors in these habitats, such as ecological and weather differences, mammalian diversity, sanctuary size, were not sufficient to cause significant disparity in prevalence.
4. Our study suggested that the lack of clinical *theileriosis* even during capture could be due to the fact that majority of the rhinoceros are immunologically challenged and exist in a state of endemic stability with the parasite.
5. The causasive agent for filarioid-lesions outbreak in black and white rhinos in Meru National Park is likely to be by *Stephanofilaria dinniki* because this species has been implicated in the past as the causal agent for such filarioid-lesions.
6. The prevailing environmental conditions could have contributed to lesions and possible filariosis outbreak in both rhino species in Meru National Park. The Rhino Sanctuary at the time of the outbreak was wet, bushy and thick, compounded by dense under growth, while the open areas were most likely swampy patches. Kock & Kock reported that poor body condition and heavy rainfall were thought to predispose to development of recrudescence of lesions.
7. Our study reveals that filariosis emergence in black and white rhinoceros in Kenya was not just a simple outbreak, but it has been converted in to a deadly emerging and re-emerging disease threatening the conservation of these endangered species with high mortality rate.

Capitulo 7: Conclusiones

1) Los análisis moleculares genéticos del ARNr 18S, reveló una ausencia de *Babesia bicornis*, pero presencia de *Theileria bicornis*. Análisis de diversidad genética revelaron el primer registro de *T. bicornis* y sus tres haplotipos nuevos en el rinoceronte blanco y negro en Kenia. Nuestros resultados indican que los haplotipos nuevos de *T. bicornis* están extendidos entre las grandes subpoblaciones de rinocerontes en Kenia.
2) La prevalencia de *T. bicornis* fue de un 49,12% siendo mayor en el rinoceronte blanco que en el negro sugiriendo que éste puede ser utilizado como un sumidero para la transmisión de *Theileria* en rinocerontes blancos.

3) Se observó una variación en la prevalencia de *T. bicornis* en las subpoblaciones de rinoceronte pero no hubo diferencias significativas, lo que sugiere que los factores locales en estos hábitats, tales como las diferencias ecológicas y climáticas, diversidad de mamíferos, tamaño de la población, no fueron suficientes para causar disparidad significativa en cuanto a la prevalencia.

4) Nuestro estudio sugiere que la falta de teileriosis clínica incluso durante la captura podría ser debido al hecho de que la mayoría de los rinocerontes están inmunológicamente comprometidos y se encuentran en un estado de estabilidad endémica con el parásito.

5) Es probable que el agente causal del brote de lesiones de filaria en rinocerontes blancos y negros en el Parque Nacional de Meru sea debido a la especie *Stephanofilaria dinniki* porque ya ha sido implicada en el pasado como el agente causante.

6) Las condiciones ambientales podrían haber contribuido a las lesiones y al posible brote en ambas especies de rinocerontes en el Parque Nacional de Meru. El área de rinocerontes en el momento del brote fue húmeda, espesa y abundante, compuesta por un importante decrecimiento, mientras que las áreas abiertas fueron probablemente zonas pantanosas. Kock & Kock señaló que la baja condición corporal y las lluvias predispusieron al desarrollo del agravamiento de las lesiones.

7) Nuestro estudio revela que la emergente filariosis en especies de rinoceronte blanco y negro en Kenia no fue un simple brote, sino una enfermedad letal y reemergente que amenaza la conservación de estas especies en peligro de extinción con una alta tasa de mortalidad.

PERSPECTIVES

Further studies on its epidemiology need to be done including the identification of other hosts within rhinoceros sanctuaries. Although we did not detect *B. bicornis,* further investigations in other sub-populations we did not obtain samples from need to be conducted.

Another area for future research is identifying whether the same vectors transmitting Stephanofialrial worms of cattel elsewhere are implicated in the transmission of *Stephanofilaria dinniki* in rhinos. The life cycle of the *Stephanofilaria* in both the rhino and the arthropod vectors once identified should be identified by examining which stages of larvae are found in the mammalian hosts and in the arthropod host.

Future studies should focus of the genetics of disease resistance in black and white rhinos including resistance to tick infestation. The vulnerability of White rhinocerous to the deadly filarial disease needs a better understanding from a genetic and a pathological view point.

Chapter 8: Bibliography

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