Disease and Ethiopian wolves (*Canis simensis*): Trialling the feasibility of oral vaccination against rabies

A thesis submitted for the degree

Master of Science (by Research)

Christopher H. Gordon

Lady Margaret Hall College, University of Oxford

Hilary Term 2013

Christopher H. Gordon M.Sc. (by Research) in Zoology

Disease and Ethiopian wolves (*Canis simensis*): Trialling the feasibility of oral vaccination against rabies

ABSTRACT

Disease often plays a large role in the population dynamics of wild canids, and is the most immediate threat to the Endangered Ethiopian wolf (*Canis simensis*). Well-documented rabies epizootics have devastated the Bale wolf population three times since 1991, with mortality of 65-75% in affected areas. Intensive monitoring permitted the diagnoses of wolf carcasses found in 2008/09, which confirmed two separate introductions of the same rabies strain from the sympatric dog reservoir, with severe consequences for the affected populations. During emergency interventions, 98 wolves were vaccinated in 20 packs adjacent to affected packs, to contain the spread of rabies, as in 2003.

The impact of rabies upon Ethiopian wolves may be compounded by canine distemper virus (CDV). The first recorded CDV mortalities showed high mortality rates of 43-68% during two separate epizootics in 2005/06 and 2010. Mortality was higher in sub-adults (83-87%) than adults (34-39%), and breeding success immediately after an epizootic was unusually low. In the Web Valley, CDV in 2010 followed the rabies outbreak two years prior, affecting a wolf population that had not fully recovered, and four of seven packs disappeared. The combined impact of rabies and CDV at low inter-epizootic intervals markedly affected Ethiopian wolf pack persistence and population recovery, as was predicted by previous models.

The severe effect of recurrent epizootics on wolf population dynamics, combined with the fragility of small population sizes, highlight the need to improve current disease control policies. To date, efforts to protect wolves have focused on vaccination of the dog reservoir, and the emergency vaccination of wolves in response to rabies outbreaks. Oral vaccination potentially offers an efficient and economical method to inoculate both dogs and wolves against rabies, and was identified as a key tool for wolf conservation. To test its suitability, field trials of the oral vaccine *Rabigen® SAG2Dog* were conducted on dogs. In total, 142 vaccines were delivered to 116 dogs using a variety of baits. Intestine baits had highest uptake rates but meat baits were chewed the longest, maximising the potential for vaccine absorption. Rabies antibody titres in pre- and post-vaccination samples were assessed using the FAVN test at a 0.5IU/ml protection threshold. The number of dogs to seroconvert following vaccination was low (11%), even at a reduced threshold of 0.2IU/ml (37%). There were limited anamnestic responses to the vaccine (an enhanced reaction in previously exposed dogs) in baseline positive dogs (25%). Low sero-conversion and logistical difficulties of maintaining a cold chain for this vaccine, offer little evidence that *Rabigen® SAG2Dog* would provide effective rabies protection for dogs.

Bait uptake and the effectiveness of *Rabigen® SAG2Dog* were also tested on wolves. Meat baits had highest uptake (50-53%), and targeted delivery had better uptake than random delivery. Subsequently, *Rabigen® SAG2Dog* was delivered using the favoured meat bait and targeted delivery to one wolf pack. Oral vaccine baits were consumed by five of eight wolves, and blood samples were drawn from four of these wolves and one control two weeks later. Two of the four wolves that ate baits had positive rabies titres (>0.5 IU/ml), while a third had raised levels. All wolves from the pack are alive 18 months after delivery. This small, controlled trial confirms the potential for *Rabigen® SAG2Dog* to provide rabies protection to Ethiopian wolves. Further extended trials will be required before oral rabies vaccination can be implemented as a proactive disease management approach.

ACKNOWLEDGEMENTS

There are so many people that spring to mind when I think of who has helped me to complete this Masters. First are Leta Edea and Antenna Girma, who both assisted me through all the field trials. It will be difficult to forget all the many hours spent sitting out in Sanetti or Web Valley, huddled behind a rock to escape the incessant wind, while watching baits distributed in the landscape, eagerly scanning the horizon for signs of a wolf. And I certainly won't forget the lightning storm rolling in, knowing that we were the tallest object on Sanetti, wondering at what point to stop the experiment. I must also thank Anne-Marie Gordon, Claudio Sillero-Zubiri and Brian Barbre for conducting a few trials to help boost sample sizes. Leta and Antenna were ever-present during the targeted delivery trials on horseback – I thoroughly enjoyed the hours we spent together on horseback looking for wolves, trying to deliver baits to them. I must also thank my steed, Guracha, for bearing my weight through all the trials, and for forgiving my naivety as a horseman.

For the vaccine delivery to the wolves, I'd like to thank Leta once again and Alo Hussein, for his invaluable knowledge of every wolf, as well as Addisu Tsegaye and Geremew Mebratu from the Bale Mountains National Park. I must also thank Leta for all his hard work in meticulously preparing the baits for these trials. I would like to express my sincere gratitude to Virbac in France, and particularly Dr. Philippe Mahl, for kindly donating the Rabigen® SAG2Dog oral rabies vaccines and the placebo sachets for these trials. Many thanks to Philippe, Dr. Michel Aubert and Dr. Florence Cliquet for all their advice on Rabigen® SAG2Dog and previous oral vaccine trials. Valuable comments for the vaccine trial methodologies were kindly provided by Prof. Richard Kock, Prof. Rosie Woodroffe, Dr. Karen Laurenson, Prof. Claudio Sillero, Dr. Darryn Knobel and Dr. Paul Johnson. Thank you also to Richard, Rosie and Karen for writing letters of support for the trial proposal. Thank you to Dr. Alemayehu Lemma at the Faculty of Veterinary Medicine of Addis Ababa University and Ato Alemu of the Ethio-Belgium Project for storing these vaccines in their -30°C freezer. I am grateful to the Ethiopian Wildlife Conservation Authority for providing permissions for the oral vaccine trials on Ethiopian wolves. In particular, I would like to thank Dr. Kifle Argaw, Ato Ewnetu Bilata, Ato Yeneneh Teka, and Dr. Fekede Regassa.

The EWCP capture team were phenomenal as usual, all the guys are the epitome of professionalism. It has been a great honour to be a part of the team that has handled 53 wolf captures during my time in Ethiopia – to have physically held 10% of the world's population of this species is truly humbling. I must give special thanks throughout the captures to Edris Ebu, Alo, Claudio, Leta, Anne-Marie, Muktar Abute, Mustafa Dule and Burka Kediro. Everybody's dedication to checking traps day and night, and processing wolves in all conditions was fantastic, and allowed for the completion of this exciting vaccine trial. Thanks also to Dr. Fekede Regassa for his assistance in the field during the wolf captures.

The vaccine trial on the wolves didn't finish with these captures, and I would like to thank Leta, Anne-Marie and Muktar for all their help processing and coding the blood samples after hours in our "mobile lab" in the Web Valley. Permission to export these samples was kindly and efficiently (as always) provided by the ever-smiling Ato Yeneneh Teka. Thank you to Prof.

Tony Fooks for the continued support of the laboratory at the Animal Health and Veterinary Laboratories Agency (AHVLA) for the rabies FAVN tests. Dr. Dan Horton kindly analysed all the samples, while IPA analyses were undertaken by Janine Gielbert. I sincerely appreciate that the methodology for IPA was started almost from scratch.

Many thanks to Dr. Bewket Siraw at the Ministry of Agriculture in Addis Ababa for providing the necessary permissions for the oral vaccine trials on domestic dogs. Thanks also go to Dr. Melaku Assefa for writing all the necessary import and export permits, but particularly for his assistance in the field for the dog captures. The vaccine delivery to dogs, and the baseline and post-vaccination captures were a combined effort by our well-oiled team, including Leta, Muktar, Kebede Wolde, Abubakkir Hussein, Habtamu Mulugeta and Ashebir Anbessa. Thank you to each and every one of you guys for all your hard work during those two weeks. Not only did we work tirelessly from dawn to dusk, but Leta, Muktar, Kebede and Abubakkir never complained when helping to process all the samples and prepare the next day's baits. Dan Horton's compliments on the serum quality are a fitting tribute to all our hard work during those two weeks. Thanks again to Tony and Dan at AHVLA for the rabies FAVN tests of all these samples. Once again, I would like to express my gratitude to Virbac and Philippe for kindly donating the Rabigen® SAG2Dog oral rabies vaccines and the placebo sachets for these trials.

We were fed and watered throughout both bait and vaccine trials by Yasin Adishu and Awol Sultan – thank you both so much for all your efforts in the kitchen. Thanks also to the camp guards in Sanetti and Web Valley, namely Hussein Wakayo, Adishu Sheyemo, Ibrahim Nure, Gobe Ahhamed, and Hussein Abdulmanan. A hot fire in the wood-burning stoves was always appreciated after a long day sitting out watching baits.

I cannot thank enough the various individuals and organisations who have financially supported EWCP, and the research in particular. Funding for these trials has been generously provided by the Born Free Foundation, the Wildlife Conservation Network, the US Fish and Wildlife Service, the Oppenheimers, the Saint Louis Zoo and the National Geographic Society. Thank you to all of you. I would like to extend grateful thanks to EWCP, and particularly to Claudio, for supporting the costs of the Masters, and for allowing me to conduct this research while also continuing my day-to-day role as Technical Coordinator for EWCP. I have been extremely fortunate.

The information for Chapters Three and Four are a result of many people's efforts, dating back to before my arrival, and during my time, at EWCP. I would like to thank Alo, Mustafa, Sultan Washo, Ibrahim Muhammed, Ibrahim Abduke, and Leta for all the post-mortem samples they collected which enabled us to gather such detailed information on disease in Ethiopian wolves. The hours that these guys spend in the field under tough conditions in the Bale Mountains are phenomenal. A huge thank you to Edris for the vital role he plays behind the scenes organising the team, and his dedication in ensuring a rapid response during these epizootics. You have been a great friend and help throughout our time in Ethiopia. Many thanks to Dr. James Malcolm, Dr. Graham Hemson and Anne-Marie for all their hard work in keeping EWCP ticking, and for all their organisation of disease monitoring, carcass recovery and the vaccination interventions – I am well placed to appreciate all their efforts and to extend a huge thank you to each of you for all the work that has gone in to keeping EWCP running as the first-class conservation project that it is. Yeneneh Teka kindly organised the

export of all these earlier samples, and many thanks to Tony, Dan and Dr. Ashley Banyard for running FAVN and Elisa tests on the samples.

I would like to extend a big thank you to Jorgelina Marino for her efforts in preparing the fantastic maps for this thesis, and for helping me to calculate the densities, home ranges and time series graphs for Sanetti and Web Valley. I know that all these tasks were very time-consuming, and I am extremely grateful for the help. The statistical analyses of my results remained a worry to me right to the end of this write-up. Thank you so much to Dr. Anouska Kinahan for guiding me down the right path in Bale. Without the knowledge that is Paul Johnson, I would have got nowhere. I am eternally grateful.

The various chapters of this thesis have morphed through various guises throughout the write up. Many people have uncomplainingly given invaluable input and comments to improving these chapters, while some have commented on multiple chapters, or even multiple versions of chapters. I only hope I have interpreted all your comments how you intended them. I really appreciate the time taken to read and comment on what has often needed much improvement. Thank you so much to each of you: Claudio, Anne-Marie, Karen, Jorgelina, Graham, James, Tony, Dan, Fekede, and Michel.

I have thoroughly enjoyed being a fleeting, and rarely seen, member of WildCRU. Thank you so much to Claudio and Prof. David Macdonald for allowing me the opportunity of joining the team. So many faces have been seen throughout my time there, but special thanks to the following for sharing offices, and/or discussing various conservation and research topics: Jorgelina, Mark Stanley-Price, Paul, Sandra Baker, Amy Dickman, Lucy Tallents, Christos Astaras, Kirsten Bilham, and Carly Easby. A massive thank you to Dawn Burnham for all your help.

There are many people who have helped me through this period in various ways. Thank you to Thadaigh, Anouska, Harenna, and Brian, my Bale neighbours, for many fun Saturday evenings. Come Dine with Me Bale was an inspired idea, apart from when it came to my turn! The EWCP team have been superb throughout my time – you guys are amazing and will be thoroughly missed. A special thanks in particular to Edris, Zegeye, Musti, Alo and Weggy. A massive thank you to my Addis family: Stephen – your generosity in opening your house to Anne-Marie and I will never be forgotten, and neither will (most of) our whiskey drinking sessions. You are a true friend, to whom I will always be in debt. You can keep the lion statue though!

I am eternally grateful to my Mum and Dad for giving me the opportunities to follow my career dreams, and being supportive throughout and no matter what. As if that weren't enough, there has always been a warm bed, a hot meal and much love on my way through home to/from Oxford/Ethiopia. Family time has always been, and will always be, hugely important to us, and that applies to all the Gordon/Bagnall/Cochrane clan – a huge thank you for all your love and support throughout – all the fantastic family meals, the long dog walks and the gin and tonics around the kitchen counter – you are all very special to me. Thank you also to my South African family for always offering me a fabulous place to relax, unwind, and forget about the Masters, while being spoilt with great food and family in Cape Town and at the farm.

There are two people left to thank who deserve special praise. I never appreciated when I started how time-consuming it would be to juggle a full-time job with a Masters. Claudio has been a fantastic boss and supervisor throughout, allowing me to manage both of these tasks, particularly during my write up, while always offering invaluable guidance. I am very grateful for the time taken to improve me as a scientist, helping me to develop my critical thinking and scientific writing. Thank you for reading countless drafts of manuscripts. Thank you also for your unwavering support throughout - I have felt it since day one, and I only hope that I have lived up to your expectations for this job. It has been a huge honour to be entrusted with the care of these magnificent wolves.

Last, but really first, is my amazing wife. There are so many things to thank you for. You have always given me the support and belief that I can achieve this, even during my lowest points. You have always challenged me intellectually, helping me to improve where needed. You have tolerantly edited countless manuscripts, generously giving your time even when there was none. You have always made sure I was fed and watered, things that would have been likely overlooked without you. You have never complained that your husband has not been around or has been working late. I could thank you endlessly for all your brilliance and love, but instead, I will just declare that *Ek is baie lief vir jou*.

CONTENTS

CHAPTER I

General Introduction

Introduction

The resilience and adaptability of wild canids (Order Carnivora: Family Canidae) has allowed them to occupy many different niches, with members of this family ranging in size, habits and diet (Macdonald & Sillero-Zubiri, 2004). This flexibility has led wild canids to spread globally, where in many areas the rapidly expanding human populations have resulted in increased contact between wild canids and sympatric domestic dogs (Butler *et al*., 2004). Due to their shared receptivity of numerous pathogens with domestic dogs (Woodroffe *et al*., 2004), and despite their adaptability, some wild canid species are under threat from canid-related diseases.

Infectious viruses are a significant cause of population declines in wildlife (Dobson & Foufopoulos, 2001), particularly in carnivores. For instance, rabies caused dramatic population declines in Endangered African wild dogs (Gascoyne *et al*., 1993; Hofmeyr *et al*., 2004) and Blanford's foxes (*Vulpes cana*) (Geffen, 1994), while a canine distemper virus outbreak killed 90% of the Endangered island fox (*Urocyon littoralis*) on Santa Catalina Island in California (Timm *et al*., 2009).

Both these viruses represent major threats to the continued existence of the Endangered Ethiopian wolf (*Canis simensis*), considered the rarest canid in the world, and Africa's most threatened carnivore (Marino *et al*., 2011; Sillero-Zubiri & Macdonald, 1997). Less than 450 adult and sub-adult wolves remain, restricted to just six enclaves above 3,000m in the Ethiopian highlands (Marino, 2003; Marino *et al*., 2011). Well-documented rabies outbreaks caused the death of 65-75% of wolves in affected areas of the Bale Mountains in 1991/92 (Sillero-Zubiri *et al*., 1996b) and 2003 (Randall *et al*., 2004).

In addition to rabies, canine distemper virus (CDV) has emerged as a further significant threat to the Ethiopian wolf. Serosurveys in the early 1990s had already suggested that CDV had the ability to impact wolf populations (Laurenson *et al*., 1998) and CDV-related mortality was confirmed recently, with significant population declines observed in two separate CDV epizootics in 2005/06 and 2010 (Banyard *et al*. 2012; Chapter 4).

The long-term monitoring of Ethiopian wolf populations and constant field presence of the Ethiopian Wolf Conservation Programme (EWCP) in the Bale Mountains allows the rapid detection of carcasses and subsequent diagnoses of samples where feasible. Using detailed wolf demographic data combined with extensive knowledge of disease impacts and transmission, modellers have investigated the impact of different disease scenarios on Ethiopian wolves in relation to other factors. More specifically, these models have provided simulations of differing inter-epidemic intervals on wolf populations of varying sizes, while investigating the effect of rabies and CDV both on their own, and in combination with one another (Haydon *et al*., 2002; Mace & Sillero-Zubiri, 1997). These models reveal the relevance of population size in relation to the probability of local extinctions. The likelihood of extinction is greater when these disease pressures are applied to the smaller wolf populations in North Ethiopia, some of which number under 30 individuals (Haydon *et al*., 2002).

Disease monitoring and access to baseline information is critical in reacting promptly to disease epizootics in wild canids. Ethiopian wolves present a valuable example of the impact of disease on an endangered species, and long term data from the Bale Mountains offer an insight into the role of mitigation measures in disease control. Population declines from disease epizootics highlight the need to integrate veterinary epidemiology into carnivore conservation (Cleaveland *et al*., 2007; Haydon *et al*., 2006), particularly when infectious viruses affect endangered species. Throughout Africa, domestic dogs act as the principal reservoir for rabies (Talbi *et al*., 2009), and consequently, since 1996, the dog population in and around Bale has been regularly vaccinated against rabies by EWCP, in an attempt to remove the main rabies vector around wolf range, and thus protect wolves. However, despite vaccinating over 68,000 dogs in Bale since 1996 (EWCP, 2012), there have been two major rabies epizootics in the wolf population since the dog vaccination campaign started. During both the 2003 and 2008/09 epizootics, the timely intervention of capturing and vaccinating wolves parenterally (Knobel *et al*., 2008; Randall *et al*., 2006) may have prevented the severity experienced during the 1991/92 rabies outbreak, but not before significant wolf losses were sustained prior to these reactive vaccinations.

While population fluctuation tends to be a trait of most wild canid species, disease-induced declines in small population sizes might increase the possibility of local extinction. Thus, when trying to protect small populations of an extremely rare and threatened canid, losses from disease that jeopardize population survival are unacceptable, emphasising the limitations of reactive emergency vaccination as a management option. While a proactive approach to capture and inject a proportion of the wolf population would offer a more efficient alternative in terms of preventing large outbreaks, this invasive method is deemed unsuitable since the manipulation of individuals in such rare species should ideally be kept to a minimum. The inadequacy of the dog vaccination campaign to eradicate the risk of large rabies outbreaks that affect the wolves, and the limitations of reactively capturing and vaccinating wolves, highlight the need for novel disease control methods in this species.

Modelling data from previous outbreaks (Haydon *et al*., 2002; 2006) suggest that targeted, low-coverage and less-invasive vaccination strategies could be effective in curtailing disease outbreaks and enhancing the long-term persistence of Ethiopian wolf populations. The models predict that a combined vaccination campaign in both wolves and dogs would be the most effective method for protecting this endangered species (Haydon *et al*., 2002). The development and success of oral rabies vaccines in other wild canids (e.g. Aubert & Mutinelli, 2002; Bingham *et al*., 1999; Follman *et al*., 1996) presents the opportunity to deliver vaccines non-invasively, directly and pro-actively to the wolves. Indeed, oral vaccination was identified as a key conservation tool for Ethiopian wolf conservation in the 10-year National Action Plan for this species (EWCA, 2011). Oral vaccines could offer a more efficient and feasible option to reach and maintain the required 70% inoculation level for domestic dogs, in order to reduce rabies prevalence in target areas (Coleman & Dye, 1996). Delivering the vaccine orally could also speed up inoculation campaigns for semi-feral and hard-to-handle dogs in rural settings, while reducing costs in terms of financial and human resources.

The main aim of this thesis is to investigate the feasibility of the live attenuated oral rabies vaccine Rabigen® SAG2Dog on Ethiopian wolves and domestic dogs, with a view to designing a rabies control policy for orally vaccinating both the dog reservoir and the wolves. First and foremost, this vaccine had to meet rigorous safety standards to be delivered to an endangered species, with all trials to date confirming the safety and suitability of SAG2 for

this purpose. Furthermore, a suitable delivery vehicle and protocol needed to be tested to maximise bait uptake by wolves and dogs and reduce consumption by non-target species. The hypotheses are that local baits will provide a more effective delivery vehicle for the vaccine sachets than the commercial bait matrix, and that targeted delivery of baits, rather than their random distribution, will maximise bait uptake and limit uptake by non-target species. While oral vaccines have been used extensively on some wild canid species, trials have not been conducted on endangered canids. Thus, the expected lessons to be learnt from these trials will have a far greater impact beyond their applicability on Ethiopian wolves.

Structure of the thesis

This thesis consists of a review of disease in canids, four data chapters, and a general discussion.

Chapter II: A history of disease in Ethiopian wolves

A literature review presents the current state of knowledge of disease in canids in general, and in Ethiopian wolves in particular, to provide background to the subsequent chapters of this thesis. All viral diseases known to affect wild canids are examined, before focusing discussions on rabies and CDV. Monitoring of Ethiopian wolves in the Bale Mountains spanning four decades has provided detailed information on the impact of disease on wolf numbers, through the episodic recovery of wolf carcasses and the disappearance of known wolves. All major disease epizootics recorded since 1988 are discussed in detail, reviewing all resulting scientific publications describing wolf mortality, emergency wolf vaccinations, and the models that predict both the impact of these disease epizootics on different sized wolf populations and the effect of wolf vaccination campaigns of varying intensity.

The Bale wolf population was heavily impacted by rabies in 1991/92, 2003, and 2008/09 (Johnson *et al*., 2010; Randall *et al*., 2004; Sillero-Zubiri *et al*., 1996b). Emergency vaccinations of wolves in 2003 and 2008/09 most likely prevented a similar spread of the disease through the Bale Mountains as was experienced in 1991/92 (Haydon *et al*., 2006; Knobel *et al*., 2008; Randall *et al*., 2006). Additionally, CDV impacted upon Bale's wolves in

2005/06 and in 2010, with high mortality rates. Disease control to date has focused on the reservoir dog population, in addition to the emergency vaccination interventions on the wolves. These disease control and prevention options are evaluated, with the chapter reviewing new disease management options that have been successfully tested on other species.

Chapter III: Recurrent rabies epizootics in Ethiopian wolves highlight the need for new control approaches

The threat of rabies to Ethiopian wolf populations has always been of concern. The discovery of carcasses, combined with intensive monitoring of focal wolf areas, allowed for the detailed documentation of major rabies outbreaks in 1991/92 and 2003 (Randall *et al*., 2004; Sillero-Zubiri *et al*., 1996b). Chapter Three reports on more recent rabies outbreaks in the Bale wolf population in 2008 and 2009. Mortality rates during the latest epizootics were consistent with mortality in previous outbreaks. Timely emergency interventions, involving the capture and parenteral vaccination of wolves adjacent to affected packs, halted the spread of rabies during both outbreaks. This latest report of rabies epizootics in Bale suggests that significant disease events may be more frequent than previously thought, affecting population survival and subsequent recovery. Modelling predicts that a large wolf population such as Bale's can recover from five-year intervals between major epizootics, but that the probability of extinction would increase as intervals reduce (Haydon *et al*., 2006). The 2008 rabies outbreak in Web Valley was followed by a 2010 outbreak of CDV (reported in Chapter Four), with four of seven packs consequently going extinct. Repeated population crashes, such as the one described, have the potential to result in local extinctions. The observed and potential impact of rabies and CDV outbreaks highlight the need to assess and improve disease control policies in the context of Ethiopian wolf conservation.

Chapter IV: The emergence of canine distemper virus as a threat to Ethiopian wolves

Chapter Four discusses two recent CDV epizootics in 2005/06 and 2010, which resulted in extensive mortality in wolves in Bale, with higher rates seen in sub-adults than adults. The 2010 epizootic spread extensively through all sub-populations of the Bale Mountains, with total mortality likely exceeding all previously recorded disease episodes. Previous modelling showed that the impact of epizootics would be increased by reducing inter-epizootic

intervals (Haydon *et al*., 2006), but that extinction probabilities for rabies alone were higher than for rabies and CDV (Haydon *et al*., 2002), although estimated CDV mortality rates used in these models were lower than observed rates in both these epizootics. In Web Valley in 2010, a CDV outbreak occurred 20 months after a rabies outbreak, before the population had fully recovered. The effect was devastating, with high mortality rates, and four of seven packs completely disappearing. This combined impact of both diseases seriously reduces the chance of pack survival, exacerbating the slow recovery of wolf populations. Increased disease incidence at low intervals may even lead to local extirpation in smaller populations, as predicted by models (Haydon *et al*., 2002). The conservation implications of this emerging threat are discussed and the potential relevance of a CDV control programme, alongside current efforts to reduce incidences of rabies, is considered.

Chapter V: Trialling an oral live attenuated rabies vaccine on domestic dogs in Ethiopia

Parenteral vaccination of domestic dogs in and around wolf populations is challenging, as dogs in rural Ethiopia are notoriously difficult to handle, making injectable hand vaccinations time-consuming and impractical. In Ethiopia, vaccination of dogs remains important for reducing disease incidence and transmission risks to wolves. Oral rabies vaccination offers a more efficient and economical method to inoculate dogs against rabies, and was identified as a key tool for effective Ethiopian wolf conservation (EWCA, 2011). This chapter describes a trial of the oral vaccine Rabigen® SAG2Dog on domestic dogs in Ethiopia under field conditions. The trial constitutes a first step towards implementing new disease control methods to reduce disease threats to Ethiopian wolves. A trial of this oral vaccine investigated the suitability of various baits to successfully deliver the vaccine to dogs, and the efficacy of the vaccine under field conditions, tested by taking blood samples from each dog before and after vaccination. The results of the trial are analysed, with conclusions discussed in the context of the disease control strategy for Ethiopian wolf populations.

Chapter VI: Testing the suitability of the oral live attenuated rabies vaccine Rabigen® SAG2Dog to protect endangered Ethiopian wolves

To date, efforts to protect Ethiopian wolves from canid-related pathogens have involved the parenteral vaccination of the domestic dog reservoir. Emergency interventions in response to severe rabies outbreaks have concentrated on the parenteral vaccination of wolves, but

only once significant wolf losses had occurred. Oral rabies vaccination offers a more cost-efficient and proactive approach to protecting wolves against rabies. Chapter Six reports on the first in-situ trial to deliver an oral rabies vaccine to Ethiopian wolves, in order to test its effectiveness. The chapter investigates the ideal bait for delivering the vaccine to the wolves, and evaluates different delivery methods to maximise uptake while reducing delivery to non-target species. The chapter describes the subsequent delivery of the live attenuated oral vaccine Rabigen® SAG2Dog to one wolf pack in the Bale Mountains during November 2011, using the favoured bait. Blood samples drawn two weeks after vaccination allowed analysis of the efficacy and safety of the vaccine, and confirmed bait ingestion in individual wolves. The results are discussed here in the context of SAG2's suitability to protect Ethiopian wolf populations from high mortality events and possible disease-induced local extinctions.

Chapter VII: General Discussion

This chapter summarizes and discusses the results of all four data chapters in the wider context of Ethiopian wolf and canid conservation. The effects of rabies and CDV outbreaks on Ethiopian wolf population dynamics are discussed, highlighting the extinction risks posed by reduced inter-epizootic intervals. The chapter argues that active disease management is necessary for the protection of Ethiopian wolf populations, and the first trials of oral rabies vaccines on wolves and dogs are a positive step towards achieving this. Recommendations are made for future steps in the development of oral vaccination as a practical tool to manage disease risk, with the ultimate goal of minimising, and eventually eradicating rabies and CDV as significant threats to Ethiopian wolf survival.

CHAPTER II

A history of disease in Ethiopian wolves

Abstract

Infectious diseases are a major cause of population declines in wildlife. Rabies is recognized as the most common cause for disease-related mortality in wild canids, which are also affected by other viral diseases such as canine distemper virus (CDV), canine parvovirus (CPV), canine adenovirus 1 (CAV) and canine coronavirus. Intensive monitoring of the Bale Mountains' Ethiopian wolf (*Canis simensis*) population since the early 1980s has led to these wolves being considered one of the best known carnivore populations in the world. During this 30-year period, disease has had a major impact on wolf numbers in Bale, as indicated by episodic recovery of wolf carcasses and mass disappearance of individuals from known wolf packs. Analyses of samples collected from carcasses, determined disease epizootics and their origins, and informed reactive disease control measures. The Bale wolf population was heavily impacted by rabies in 1991/92, 2003, and 2008/09, with mortality rates between 65-75% in affected sub-populations. Emergency interventions in 2003 and 2008/09 prevented a similar spread of the disease through the Bale Mountains, as was experienced in 1991/92. Additionally, CDV impacted upon Bale's wolves in 2005/06 and 2010, with mortality rates of 43-68%. This chapter presents a literature review on disease in canids and the current state of knowledge of disease dynamics in Ethiopian wolves. I evaluate the disease control and prevention options implemented thus far, and suggest future disease management options.

Introduction

While wild canids (Order Carnivora: Family Canidae) tend to be widespread and relatively common, six of the 35 species are formally under threat, whilst the Falkland wolf (*Dusicyon australis*) is extinct (IUCN, 2012). Growing human populations across the globe, and the resultant encroachment of wilderness areas, have increased the potential for contact between wild canids and domestic dogs, which are often kept to protect livestock, aid hunting efforts, clean waste or as companions (Atickem *et al*., 2010; Green *et al*., 1984b; Sillero-Zubiri & Switzer, 2004). The most immediate threat to wild canids resulting from this contact with sympatric domestic dogs is the transmission of infectious diseases. Infectious diseases are a major cause of population declines in all wildlife (Dobson & Foufopoulos, 2001), not just in wild canids. Diseases are readily transmitted between wild and domestic canid species due to their shared receptivity of numerous pathogens, with both often acting as the reservoir for viruses (Woodroffe *et al*., 2004). An *epizootic* is defined by Merriam Webster Dictionary as an outbreak of disease affecting many animals of one kind at the same time, while a *disease outbreak* is defined as a sudden rise in the incidence of a disease. In the context of this literature review, a disease outbreak is more specifically used to describe the sudden rise in the incidence of a disease within a short time frame.

Viral Disease in Wild Canids

Viral diseases known to affect wild canids include rabies, canine distemper virus (CDV), canine parvovirus (CPV), canine adenovirus 1 (CAV), and canine coronavirus. Rabies is recognised as the most common cause of disease-related mortality in wild canids (Funk *et al*., 2003; Macdonald, 1980; Woodroffe *et al*., 2004). Rabies (Order Mononegavirales: Family Rhabdoviridae) is classified within the *Lyssavirus* genus (Banyard *et al*., 2012), which in turn is divided into seven genotypes. Only genotype 1 (rabies or RABV) is spread in terrestrial vectors, including carnivores, while all other genotypes are almost exclusively limited to bats (WHO, 2004). Rabies can infect all species of mammal, although some are more susceptible than others, particularly carnivores (Macdonald, 1995). The virus is transmitted by a bite, transferring from the saliva of the infected aggressor into the wound of the naïve individual. Infected animals demonstrate behavioural changes including aggression, hydrophobia and excessive salivation, with the virus eventually causing fatal encephalitis (Banyard *et al*.,

2012). However, recent studies have challenged the classic description of rabies infection, demonstrating rabies seropositivity in a proportion of healthy, unvaccinated domestic dogs in rural Africa (Cleaveland *et al*., 1999), suggesting that seropositive dogs were unlikely to have current rabies infections, but that seropositivity was, more likely consistent with an aborted infection (Fekadu, 1991).

The social structure of many canid species and their ability to deliver a damaging bite wound, make them an ideal reservoir host for transmitting rabies. In Africa, the domestic dog acts as the principal reservoir of the rabies virus (Talbi *et al*., 2009), due in part to the large numbers of potential host animals. Rabies is endemic in Ethiopia and is widespread in domestic dog populations throughout the country (Fekadu, 1982; Mebatsion *et al*., 1992; Tefera *et al*., 2002). In recent times, rabies has caused dramatic population declines, and even the local extinction, of African wild dogs (*Lycaon pictus*) (Gascoyne *et al*., 1993; Hofmeyr *et al*., 2004) and Blanford's foxes (*Vulpes cana*) (Geffen, 1994). Rabies-related mortalities have also been reported in other wild canids, including bat-eared foxes (*Otocoyon megalotis*) (Thomson & Meredith, 1993), and black-backed jackals (*Canis mesomelas*) (Cleaveland, 1998; Cleaveland & Dye, 1995).

Similarly, canine distemper virus (CDV) presents a significant threat to wild canids. Despite its name, CDV (Order Mononegavirales: Family Paramyxoviridae) has possibly the widest host range of all morbilliviruses, affecting most carnivore families (e.g. Canidae, Ursidae, Mustelidae, Viverridae, Felidae, Hyaenidae) (Barrett, 1999; Deem *et al*., 2000; Harder & Osterhaus, 1997). CDV is an acute febrile disease showing high mortality rates, second only to rabies (Deem *et al*., 2000). CDV is transmitted via the aerosolization of virus particles, which are highly contagious (Greene & Appel, 1990). Infected animals show common respiratory symptoms such as pneumonia (Deem *et al*., 2000), and ocular symptoms such as optic neuritis (Gellatt *et al*., 1985). There are also often signs of jaundice, ulceration, anorexia, diarrhoea and neurological abnormalities (Banyard *et al*., 2012; Deem *et al*., 2000). CDV may be lymphotropic, and as such, infection may induce a profound immunosuppression that often leads to secondary opportunistic infections, that in turn lead to the death of the host (von Messling *et al*., 2004). A CDV outbreak in lions (*Panthera leo*),

other wildlife species and domestic dogs in Tanzania and Kenya showed that virus isolates were serologically indistinguishable between infected wildlife and dogs (Kock *et al*., 1998; Roelke-Parker *et al*., 1996). Further molecular testing of separate virus isolates showed genetic clustering within geographic areas, rather than host species, suggesting that a single virus strain caused mortality in a range of species and that the virus was freely transmissible between domestic dogs and wild carnivore populations (Cleaveland *et al*., 2000).

Despite the high mortality rates and the increasingly close contact between domestic dogs and wild canids, confirmed mortality of wild canids from CDV has been reported in just five canid species. The first evidence of CDV in wild canids affected black-backed jackal populations in Kenya in 1978 (Alexander *et al*., 1994). More significantly, CDV killed 90% of the Endangered island fox (*Urocyon littoralis*) on Santa Catalina Island, California (Timm *et al*., 2009). Three separate CDV outbreaks in populations of African wild dogs in Botswana and Tanzania caused significant mortality in 61-94% of individuals (Alexander *et al*., 1996; Goller *et al*., 2010; van de Bildt *et al*., 2002). CDV has also affected populations of bat-eared foxes (Roelke-Parker *et al*., 1996). There is a single report of one grey wolf (*C. lupus*) dying from CDV in Canada (Stronen *et al.,* 2011) but in the Yellowstone National Park in the USA, peaks in CDV seroprevalence correlated with high wolf pup mortality, suggesting that CDV might have contributed to the observed mortality (Almberg *et al*., 2009). Stephenson *et al*. (1982) speculated that the virus was introduced into the wolf populations sporadically from domestic dogs, as CDV seropositivity within packs showed only occasionally over long time periods (Almberg *et al*., 2009). However, Banyard *et al*. (2012) have recently suggested that CDV requires either large spatial scales or multi-host transmission for persistence.

Canine parvovirus (CPV) emerged relatively recently as a disease affecting domestic dogs, with the first reports in the USA in 1978 (Appel *et al*., 1978). However, retrospective serological evidence suggests that the virus was present in Europe between 1974 and 1976 (Banyard *et al*., 2012). Its origins remain unknown but may have resulted from a spillover of the closely related feline panleucopoenia virus infecting wild canids (Hoelzer & Parrish, 2010). The virus causes severe haemorrhaging enteritis and associated leucopoenia, with infection characterised by severe diarrhoea and vomiting, which in turn, can lead to death

from dehydration (Banyard *et al*., 2012). The first occurrence of CPV in a wild canid was reported in maned wolves (*Chrysocyon brachyurus*) (Fletcher *et al*., 1979). Subsequently, CPV was detected in serology studies of wild grey wolves in Minnesota (Mech *et al*., 1986); of 77 wolves sampled between 1980 and 1983, 57% were seropositive for CPV. A more dramatic demonstration of the impact of CPV on wolves was the death of nine pups and two yearlings from captive wolf packs (Mech *et al*., 1986). The first documented description of CPV infection in a free roaming wolf did not come until 1997 in Minnesota, USA (Mech *et al*., 1997), when CPV was confirmed from a faecal sample. Long-term studies on wild wolf populations in the USA, mainly using serosurveys, suggest that when endemic, CPV-induced mortality occurs predominantly among pups and does not necessarily lead to a population decrease (Mech & Goyal, 1995). However, CPV may limit the ability of a wolf population to increase and may have a greater impact on small isolated populations (Banyard *et al*., 2012). There is little information on the mechanisms of CPV transmission to wild canids.

Canine adenovirus 1 (CAV) is an infectious canine hepatitis that can cause lethal hepatic infection in young Canidae and Ursidae (Kelly, 1993; Whetstone, 1988). Disease symptoms vary from coughing and fever to ataxia and convulsions (Banyard *et al*., 2012). The virus invades the liver, causing swelling, cell damage, and often a rapid progression to acute death due to shock (Whetstone, 1988). In addition to acute hepatocellular necrosis, severe acute haemorrhages are observed on the serosal surfaces of the lymph nodes and the liver (Kelly, 1993). An infected animal sheds the virus in the faeces and urine, with other animals contaminated via the mouth or nose as the virus lodges in the tonsils. The virus is not airborne. During serosurveys, 95% and 13% of gray wolves in Alaska and Canada respectively, were seropositive for CAV antibodies (Choquette & Kuyt, 1974; Stephenson *et al*., 1982), while 16% of San Jaoquin kit foxes (*V. macrotis mutica*) in California were seropositive (McCue & O'Farrell, 1988). CAV antibodies were also recorded in 72-97% of island foxes on four of the six Channel Islands, California (Garcelon *et al*., 1992). CAV-related mortality has been observed in captive red fox (*V. vulpes*) cubs (<6 months) (Cabasso, 1981).

Canine Coronavirus (CCV) was initially reported as the causal agent of acute enteritis in domestic dogs in 1974 (Keenan *et al*., 1976). Over the next 10 years, the epidemiology of

CCV was investigated further, with the virus generally regarded as a mild, usually non-lethal, but highly contagious infection of young dogs, especially puppies less than 12 weeks of age (Ford, 2003; Keenan *et al*., 1976). CCV-infected individuals show symptoms of diarrhoea, vomiting and anorexia (Banyard *et al*., 2012). New reports since the late 1990s indicate that CCV appears to be more virulent than previously thought (Bandai *et al*., 1999; Naylor *et al*., 2001). CCV was always previously seen in conjunction with CPV (Green *et al*., 1984a), but there is recent evidence that CCV can cause death in dog puppies in the absence of CPV (Evermann *et al*., 2005). CCV-only infections have not been reported in wild carnivores (Bartz & Montali, 1987), although CCV antibodies have been observed in both wild and captive coyotes (*C. latrans*) (Green *et al*., 1984a; Foreyt & Evermann, 1985), and in island foxes (Garcelon *et al*., 1992). It is unlikely that CCV is an important cause of mortality in adult coyotes (Green *et al*., 1984a), but the virus may cause enteric diseases in coyote pups, either directly or in conjunction with other intestinal pathogens (Foreyt & Evermann, 1985).

Disease Management for Wild Canids

Disease outbreaks have raised awareness of the need to integrate veterinary epidemiology into carnivore conservation and management (Breed *et al*., 2009; Cleaveland *et al*., 2007; Delahay *et al*., 2009; Haydon *et al*., 2006), particularly when infectious viruses have affected endangered species. Disease management for prevalent and severe viruses, such as rabies, have been particularly well considered. The World Health Organisation (WHO) and the World Organisation for Animal Health (OIE) both recommend using parenteral vaccination of domestic dog reservoir populations as a disease control method where possible (WHO, 2004). Models suggest that 70% of domestic dogs have to be vaccinated against rabies to prevent 95% of rabies epizootics (Coleman & Dye, 1996). Alternative approaches to managing disease have been considered where vaccination of such levels of dogs is not possible. Studies have confirmed the effectiveness of parenteral vaccination of certain wild canids to control rabies. For example, Hofmeyr *et al*. (2004) showed that vaccinated African wild dogs in South Africa survived a rabies challenge, whereas 10 out of 12 unvaccinated pups died with the challenge.

The case of the endangered Ethiopian wolf (*Canis simensis*) represents a valuable example of disease impact on an endangered species, and the role of mitigation measures in disease control. This literature review describes the complexity of disease-related issues faced by the Ethiopian wolf, investigating the current knowledge of diseases affecting this species, and the disease control methods that have been used to date to alleviate these threats. While habitat loss represents the principal long-term threat to the continued survival of this species, it is disease that is the greatest short-term issue (Laurenson *et al*., 1997; Marino *et al*., 2011) particularly in the smaller isolated populations (Haydon *et al*., 2002). Throughout 30 years of monitoring, disease had a major impact on wolf numbers in the largest wolf population, found in the Bale Mountains, as reported by episodic recovery of wolf carcasses and the unexplained disappearance of known individuals from monitored packs.

The Natural History of Ethiopian Wolves

The Ethiopian wolf is a relatively recent addition to the fauna of the Horn of Africa, first appearing during the last glacial period, circa 100,000 years ago (Gottelli *et al*., 2004), when it colonised the then widespread Afroalpine environment. With the onset of the present interglacial period, approximately 18,000 years ago, Afroalpine habitats started to shrink, replaced by montane forests at lower altitudes. As a result, Ethiopian wolves were stranded in mountain refuges, a process that has left its mark on the current genetic structure of extant populations (Gottelli *et al*., 1994, 2012; Kennedy *et al*., 2011). By approximately 10,000 years ago, the wolves were likely restricted to fewer than a dozen Afroalpine enclaves (Gottelli *et al*., 1994). Today, they survive in six montane habitats above 3,000m in the Ethiopian highlands, with approximately 450 adult and sub-adult individuals remaining (Marino *et al*., 2011). Approximately half of the global population are found in the Bale Mountains National Park (BMNP) (Marino *et al*., 2011).

Ethiopian wolves were poorly known until recently (Marino, 2003; Sillero-Zubiri, 1994). The first systematic studies of their ecology and behaviour were started in the Bale Mountains (Fig. 2.1) of Ethiopia in the early 1980s by the Bale Mountains Research Project (BMRP), followed by a detailed four-year doctoral field study from 1987 to 1992 (Sillero-Zubiri, 1994). Population data has been collected since, with an interruption during 1993-95 due to

political instability in Ethiopia. The early studies alluded to the disease threats faced by wolves, following a rabies outbreak in 1991/92 that removed up to 75% of known individuals in affected areas (Sillero-Zubiri *et al*., 1996). As a result of these losses, the Ethiopian wolf was reclassified by the IUCN Red List from Endangered (Ginsberg & Macdonald, 1990) to Critically Endangered in 1996, with the listing later downgraded back to Endangered in 2004 (Sillero-Zubiri & Marino, 2011). This sharp decline prompted the establishment of the Ethiopian Wolf Conservation Programme (EWCP), which has held a full-time conservation and monitoring presence in the Bale Mountains since 1995.

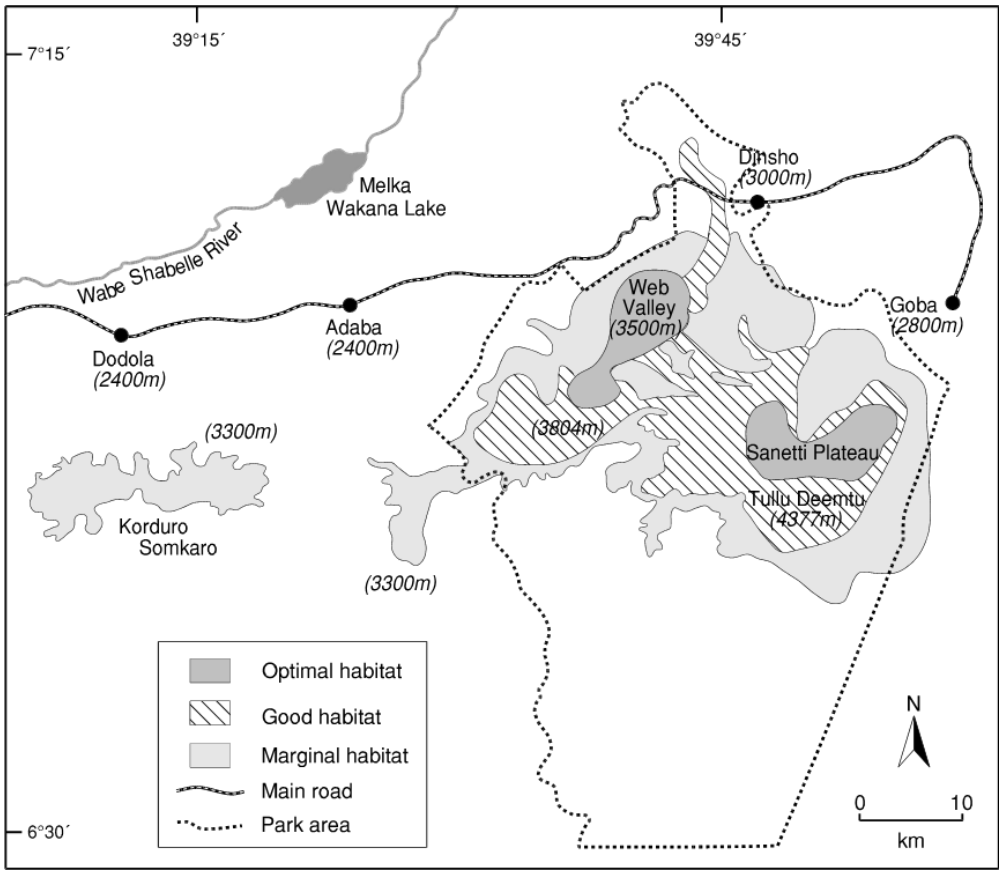

Figure 2.1: *The Bale Mountains National Park, showing optimal, good and marginal wolf habitats, including the optimal habitats of Sanetti, Web Valley and East Morebawa (from Malcolm & Sillero-Zubiri, 1997).*

In 1983, the BMRP established a road count along 31km of the Sanetti Plateau, which recorded all wolf observations. These Sanetti counts were complemented by horse-back counts started in the Web Valley in 1987, and both have continued to present, constituting a 30-year time-series of wolf abundance. Since 1988, close observations of selected focal packs in the high density wolf sub-populations of Sanetti and Web have provided robust

population demography over time. These efforts were extended to East Morebawa in more recent times. At present, 18 packs are intensively monitored in three focal areas, with up to 41 packs monitored at lower intensity elsewhere in Bale (Gordon *et al*., 2012). The social nature, diurnal habits and small home ranges of Ethiopian wolf packs make for easy observations. Focal pack observations, in particular, supply reliable information on population change, individual survival rates, breeding success, and the extinction and formation of new packs. All these factors make the Ethiopian wolf population in the Bale Mountains one of the best known carnivore populations in the world.

Ethiopian Wolf Ecology and Behaviour

Ethiopian wolves became specialist predators of Afroalpine rodents, with adaptations such as a slim body, and long legs and muzzles, thereby improving their hunting efficiency. Their mean mass is 16.2kg (14.2-19.3kg) for adult males and 12.8kg (11.2-14.15kg) for adult females (Sillero-Zubiri & Gotelli, 1994). This reduced body size reflects the difficulty in maintaining condition with a diet composed almost exclusively of small diurnal rodents, mainly grass rats (*e.g., Arvicanthis spp, Lophuromys spp, Otomys spp*), and molerats (*Tachyoryctes macrocephalus*, *T. splendens*) (Sillero-Zubiri & Gottelli, 1995a). In the Bale Mountains, wolves are consequently most active during the day, the peaks in their foraging activity associated with the activity of rodents above the ground (Sillero-Zubiri *et al*., 1995). In the largest population in the Bale Mountains, wolves are present at unusually high densities, often reaching above 1.4 adults and sub-adults/km² (Marino *et al*., 2006). These wolf densities correlate closely with the abundance and biomass of their rodent prey, which in Bale includes the giant molerat (*T. macrocephalus*) (Sillero-Zubiri *et al*., 1995).

Ethiopian wolves combine solitary foraging with intense sociality. As rodent prey is not easily shared, it maximises energy expenditure to hunt alone. However, the wolves live in territorial, cooperatively-breeding packs (Sillero-Zubiri *et al*., 2004), meeting in early mornings and evenings to patrol and maintain their territory boundaries. Family groups can contain up to 20 adult and sub-adult wolves, but the average pack consists of six adult and sub-adult wolves protecting a home range of approximately 6km² (Sillero-Zubiri & Gottelli, 1994). A dominant pair produces one litter of up to seven pups each year during a breeding

season that runs from October to December (Sillero-Zubiri *et al*., 2004). In periods of high density, 75% of packs breed, but this figure can increase to 83% when the population is in a recovery period (Marino *et al*., 2013). All pack members guard the den, chasing potential predators, and regurgitate, or later carry, rodent prey to feed the pups. By ten weeks old, the pups live on solid foods supplied by helpers, and are essentially independent by six months. The independent period from six months to one year is one of the hardest, as the young wolves attempt to hone their hunting skills while maintaining body condition. On average, mortality in juveniles (<1 year) is 37% when the population is in a high density period, although this mortality rate falls to 29% when the population is in a recovery phase (Marino *et al.*, 2013). Full adult appearance is attained at two years and both sexes become sexually mature during their second year.

Ethiopian Wolves as a Model to Describe Disease Impact

Disease was first identified as a concern to Ethiopian wolf survival during a month-long survey in 1987 (Malcolm, 1988), the report warning about the dangers of the large numbers of domestic dogs closely interacting with wolves, and the potential for disease transmission. Less than four years after this warning, the first confirmed rabies outbreak had killed wolves (Sillero-Zubiri *et al*., 1996). Since those losses in 1990, disease has played a major and repeated role in impacting on wolf numbers in the Bale Mountains, as reported by recovered wolf carcasses and missing individuals from known wolf packs. Monitoring protocols ensured that samples were collected where possible, allowing analysis of disease epizootics and their origins, and informing reactive disease control measures. This thesis highlights the increasing threat disease is playing on this species. This chapter describes the chronological history of known rabies and CDV epizootics in wolves in Bale. The literature review further discusses the disease control methods that were implemented at various stages, citing their successes and failures, and providing evidence to necessitate the trial of new control methods (Chapter Five; Chapter Six).

Accounts of Disease Epizootics in Ethiopian Wolves

Canine Pathogen Serosurveys

Mebatsion *et al*. (1992) conducted rabies serosurveys on wolves and domestic dogs in and around BMNP, using both Enzyme Linked Immunosorbent Assay (ELISA) and Rapid Fluorescent Focus Inhibition Test (RFFIT) tests. Rabies antibodies were detected in 80% of dog (n=10) and 13.3% of wolf (n=15) samples (Mebatsion *et al*., 1992). Serie and Andral (1963) earlier noted the occurrence of rabies-neutralizing antibodies in apparently healthy stray dogs in Ethiopia. Afroalpine rodents (n=28) were also surveyed as possible reservoir hosts for rabies, but all tests were negative (Mebatsion *et al*., 1992).

CDV-specific seropositivity was detected in 30% of 30 Ethiopian wolf samples collected from seemingly healthy animals captured between 1989 and early 1992 (Laurenson *et al*., 1998), indicating that they had been exposed to CDV and that individuals can survive infection from this virus. In addition, 67% of the wolves sampled had been exposed to canine adenovirus (CAV), and 13% to CPV. These results suggested that CDV and CPV were not endemic or persistent in the BMNP wolf population, but that CAV may have been endemic in this population (Laurenson *et al*., 1998). Despite evidence of CAV infection in wolves, none of the dogs sampled in the BMNP during this period were CAV seropositive, although this virus appeared highly sero-prevalent and endemic in sampled urban dogs. All dogs tested for CPV antibodies in both BMNP and nearby villages were seropositive.

CDV was prevalent in domestic dogs in Bale as well. Thirty one dogs were tested in the Web Valley in 1995/96, with all dogs less than two years old seronegative, and all dogs over two years seropositive (Laurenson *et al*., 1998). Furthermore, in Dinsho, a large village near the park boundary, 60-80% of 146 dogs sampled over three years were seropositive. These results, combined with anecdotal evidence of a non-rabies disease outbreak in dogs, strongly suggest a CDV epizootic in domestic dogs in 1993/94, although it was not possible to assess whether there was mortality in the wolf population and whether it accounted for its slow recovery following the 1991/92 rabies epizootic (Laurenson *et al*., 1998).

Rabies Epizootic 1990-92

Sillero-Zubiri *et al*. (1996) reported 12 of 23 known wolves from three packs in Sanetti dying or disappearing between April and June 1990, a 52% mortality rate. A second outbreak followed with 25 wolves dying and 16 wolves disappearing from five packs in the Web Valley between November 1991 and February 1992, a 77% mortality rate. Sillero-Zubiri *et al*. (1996) described clinical symptoms in sick wolves consistent with a rabies infection such as complete ataxia and convulsions. Three of five packs in the Web Valley went extinct at this time, negatively affecting population recovery chances in this area. As wolves follow an expansionist strategy, there is a time delay before new packs are formed (Marino *et al*., 2006; 2013), usually when large packs split (Sillero-Zubiri, 1994). Additionally, because dominant females monopolise breeding in a pack (Sillero-Zubiri, 1994), reduced numbers of breeding units limit the number of new pups born into the population each breeding season (Marino *et al*., 2006). The number of pups born each year during the recovery from this rabies epizootic correlated with the number of breeding units, not with the density of wolves (Marino *et al*., 2013).

Two samples collected in 1991/92 in the Web Valley confirmed rabies as the cause of this disease outbreak (Sillero-Zubiri *et al*., 1996). No samples were collected from the Sanetti carcasses, but clinical signs observed during the outbreak suggested that rabies was also the cause of these wolf losses. The virus isolated from Web Valley samples was of serotype 1 canid origin (Sillero-Zubiri *et al*., 1996), supporting the view that domestic dogs were the most likely source of infection. In all likelihood, the wolves' intense sociality aided the spread of rabies once transmitted from a domestic dog to an individual wolf.

Rabies Epizootic 2003

Rabies affected the BMNP wolf population again between August 2003 and January 2004, when a total of 38 wolf carcasses were found in the Web Valley, with two carcasses also found in Morebawa and one in Gaysay (Fig. 2.2). In total, 72 of 95 (76% mortality rate) known wolves died or disappeared in ten study packs in Web Valley, with the rabies virus detected in 13 of 15 brain samples (Randall *et al*., 2004). The nucleoprotein gene sequences were identical for all 13 samples, suggesting a single point source of infection (Randall *et al*.,

2004). All available evidence suggests that dogs are the reservoir for rabies both in the Bale Mountains and Ethiopia (Fekadu, 1982; Mebatsion et al., 1992; Tefera et al., 2002). Case traceback in this outbreak suggested that rabies may have been brought into wolf habitat by an unvaccinated immigrant dog accompanying people and their livestock searching for seasonal grazing (Randall *et al*., 2004).

Rabies Epizootic 2008/09

Only five years later, another rabies outbreak affected the Web Valley and Gaysay, with 39 wolf carcasses recovered between August 2008 and January 2009 (Johnson *et al*., 2010). A further 13 known wolves went missing during this period, most likely having died of rabies with their carcasses not discovered. The mortality rate of adult and sub-adult wolves during this outbreak was 63%. In May 2009, a total of 11 wolf carcasses were discovered in West Morebawa, following a second outbreak of this 2008/09 epizootic. This second rabies outbreak affected a non-focal area, where lower monitoring efforts reduced the accuracy of information on wolf losses and mortality rates. It is possible that mortality rates in this area were lower than in the Web Valley, due to lower wolf densities. Models predict that lower population densities reduce the probability of disease transmission (see Sterner & Smith, 2006), while Hochachka and Dhont (2000) showed there to be a density-dependent decline in the abundance of the host species during a *Mycoplasma* epizootic.

Rabies was confirmed from four carcasses that were recovered in the first outbreak, while samples were taken from just one wolf during the second outbreak. Phylogenetic comparison of the virus nucleoprotein-coding sequences showed that all five sequences were identical (Johnson *et al*., 2010), suggesting a single point source of infection. The likely source of the first outbreak was the dog reservoir living in and around BMNP, although it was unclear whether the second outbreak occurred due to wolf-to-wolf transmission from the earlier outbreak in Web Valley or was a repeated transmission from another dog (Johnson *et al*., 2010). These results are described in more detail in Chapter Three.

CDV Epizootic 2005/06

In July 2005, 49% (65/132) of dogs died in Ayida, a village close to Ethiopian wolf habitat in the Worgona Valley, situated in the Central Peaks area of BMNP between Web Valley and Sanetti. Serum was collected from 16 dogs that had been reported to show clinical symptoms and consequently survive the disease outbreak: 56% (9/16) of samples were positive for CDV antibodies. From September 2005, 47% of adult and sub-adult wolves (9/19) disappeared from Worgona Valley in a four-month period. Between January and April 2006, 14 wolf carcasses were recovered in Sanetti. In addition, 17 known wolves disappeared from this area, bringing wolf mortality in Sanetti to approximately 53.5% (31/58). Sub-adults showed higher mortality rates than adults (83% cf. 34%). Samples were collected from three of 14 carcasses, with analysis at the Animal Health and Veterinary Laboratories Agency (AHVLA) returning two CDV positive results (AHVLA, Unpublished Data). The P gene analysed from one sample was most closely associated with previous CDV outbreaks in the United States and Germany. While a CDV outbreak was suspected in the wolf population in 1992/93 (Laurenson *et al*., 1998), the 2005/06 outbreak confirmed CDV as a serious threat to the Ethiopian wolf, with the first confirmed wolf mortalities. Ayida village was the most likely source of the infection in wolves, validating the assertion that CDV is transmitted to wild canids by domestic dogs (Cleaveland *et al*., 2000). These results are discussed in more detail in Chapter Four.

CDV Epizootic 2010

A second confirmed CDV epizootic affected BMNP wolves during 2010, spreading through all sub-populations. A total of 31 wolf carcasses were recovered from all areas, in addition to three dog carcasses. Five of seven wolf samples returned positive CDV results. H and P gene data from two samples suggested this outbreak was the result of the introduction of a CDV virus that originated in Asia. Mortality rates, as calculated by discovered carcasses and known wolves that disappeared during the epizootic, were high: 43% (27/63) in Sanetti, 68% (21/31) in Web Valley, and 47% (16/34) in East Morebawa. As was observed with the 2005/06 epizootic, sub-adults had a higher mortality rate (87%) than adults (39%). It seems that juveniles are significantly affected by CDV as only two of 29 juveniles survived to sub-adult status after the 2010 epizootic, a 93% mortality rate.

In the Web Valley, the inter-epidemic interval between the 2008 rabies outbreak and the 2010 CDV outbreak was just 20 months. The brevity of this interval meant that wolf numbers had only just started to recover following the rabies epizootic. The CDV epizootic was consequently severe for the Web Valley wolf population, eradicating four out of seven packs from the area, including 86% of sub-adult wolves that were born during the 2008/09 breeding season. These consecutive epizootics are the likely reason for mortality rates in Web Valley being greater than those seen in the other high density wolf areas. These results are discussed in more detail in Chapter Four.

Table 2.1: An outline of five disease epizootics in the Bale Mountains' Ethiopian wolf population, summarising the affected areas and mortality rates.

Virus / Outbreak	**Areas Affected**	**Mortality Rates**	**Positive Samples**	
Rabies 1990-92	Sanetti (1990) Web Valley (1991/92)	12/23 (52%) – Sanetti 41/53 (77%) – Web Valley	2/2	Sillero-Zubiri *et al*., 1996
Rabies 2003	Web Valley & Gaysay	72/95 (76%) – Web Valley	13/15	Randall *et al*., 2004, 2006
CDV 2005/06	Worgona Valley (2005) Sanetti (2006)	9/19 (47%) – Worgona Valley 31/58 (53%) – Sanetti	2/3	Chapter Four
Rabies 2008/09	Web Valley & Gaysay (2008) West Morebawa (2009)	52/83 (63%) – Web Valley 33/67 (49%) – West Morebawa	5/5	Johnson *et al*., 2010; Chapter Three
CDV 2010	Morebawa, Web Valley, Central Peaks, Sanetti, Chafadalacha	27/63 (43%) – Sanetti 21/31 (68%) – Web Valley 16/34 (47%) – East Morebawa	5/7	Chapter Four

Disease in other Ethiopian Wolf Populations

Disease outbreaks have been reported periodically in domestic dog populations surrounding the Simien Mountains National Park in northern Ethiopia, but there has never been a confirmed outbreak of disease in the wolf population there (Marino *et al*., 2011). In 2010, several wolf carcasses were recovered in the Guassa-Menz wolf population in central Ethiopia. Samples taken from one wolf returned negative results for both rabies and CDV, although this carcass was in an advanced state of decomposition, preventing reliable screening (Marino *et al*., 2011). Local villagers reported CDV-like symptoms in the surrounding domestic dog populations at that time.

Disease Control Efforts

Domestic Dog Vaccination

Following the 1991/92 rabies epizootic in wolves, EWCP began parenteral rabies vaccination campaigns in 1996 to mitigate this threat, targeting domestic dogs living in and around wolf habitat in the BMNP. The WHO and the OIE both recommend using parenteral vaccines on the domestic dog reservoir as a disease control method where possible, with the aim to reduce the incidence of rabies in the dog reservoir. From 1996 to 2000, the campaign also vaccinated dogs against CDV and CPV, but these vaccines were stopped due to financial limitations. The majority of vaccinations took place in the Bale Mountains, with sporadic efforts in and around other smaller Ethiopian wolf populations such as Simien Mountains, Arsi Mountains, South Wollo and Guassa-Menz.

Domestic Dog Confinement

Ethiopian wolves are sympatric with domestic dogs both outside and within protected areas due to expanding human settlements into suitable Afroalpine habitats. Controlling free-ranging dogs by keeping them confined to households, by fencing or tethering them, would reduce the chance of dog-to-wolf transmission (Woodroffe *et al*., 2004). The EWCP carried out an education campaign from 1996 for several years that encouraged dog owners living in wolf habitat to tie up their dogs (in compliance with Ethiopian law) or to keep fewer dogs, as well as providing owners with collars and chains (Randall *et al*., 2006). This strategy met with limited success in Bale where cultural beliefs preclude the handling of dogs (Randall *et al*., 2006) and where restraint limited the dogs' primary roles of guarding livestock (Sillero-Zubiri & Switzer, 2004) and removing human waste (Atickem *et al*., 2010). The chains that were provided were used by dog owners for other purposes.

Domestic Dog Sterilisation

Between 2000 and 2002, EWCP carried out a domestic dog sterilisation campaign in the Bale Mountains to try to reduce future dog numbers (EWCP, 2004). In total, 496 dogs in target areas were sterilised, including 69% of the male dog population and 22% of females (EWCP, 2004). However, to limit future dog numbers, an estimated 95% of the male dog population would have to be sterilised, as well as a very high percentage of females (EWCP, 2004). The

campaign encountered several problems that contributed to the lower number of dogs sterilised, including dogs not being home, dogs not being caught, owners not being home, owners being afraid that sterilisation would reduce their dog's ability to guard, owners wanting their female dogs to be able to breed, and time and financial restrictions. The campaign was consequently discontinued.

Rabies Intervention 2003

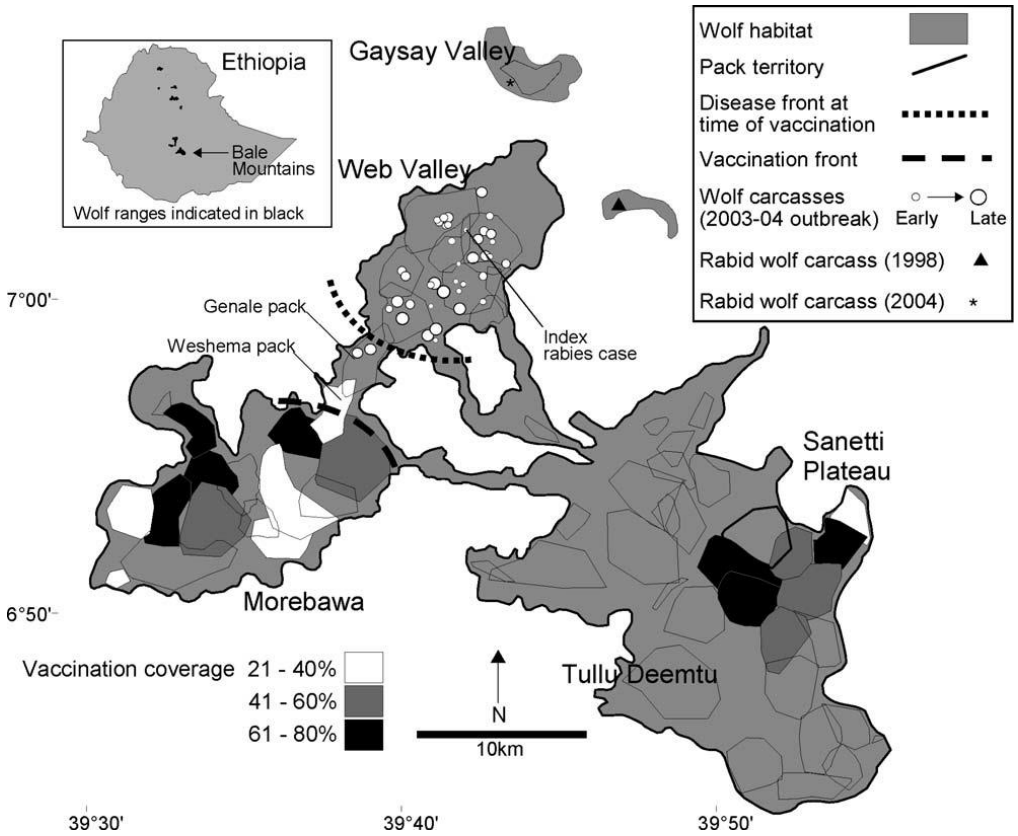

Figure 2.2: *A map showing carcass discovery (indicated by white circles) in the Web Valley and Gaysay during the 2003 rabies epizootic. Known wolf pack territories are designated by polygons. Within pack vaccination coverage during the 2003–2004 emergency vaccination campaign is shown in the lower legend. Unvaccinated packs are designated by unfilled polygons (from Randall et al., 2006).*

In response to the 2003 rabies epizootic, emergency parenteral vaccinations of the wolves prevented the same rabies spread that had been seen in 1991/92 (Haydon *et al*., 2006; Knobel *et al*., 2008; Randall *et al*., 2006). Experts were consulted as to the best course of action, and permission was obtained from the Ethiopian Wildlife Conservation Authority to undertake reactive parenteral rabies vaccination of Ethiopian wolves. Planning of the emergency interventions was assisted by extensive empirical data provided by the EWCP monitoring teams (Randall *et al*., 2006), existing theoretical models (Haydon *et al*., 2002)

and action plans (Laurenson *et al*., 1997). Knobel *et al*. (2008) captured and vaccinated a total of 77 wolves against rabies using *Nobivac Rabies* vaccine. Vaccination coverage was 37% from 16 packs in Morebawa and 48% from nine packs in Sanetti (Fig. 2.2).

During capture, blood samples were drawn, with the serum analysed later. Of the 77 wolves captured, 74 were regarded as seronegative (rabies-neutralizing antibody titres < 0.5 IU/ml) (Knobel *et al*., 2008). All 19 wolves recaptured one month after vaccination exhibited titres above this protection threshold. Of the five wolves sampled one year after vaccination, the only individual with a high titre had received a booster dose of 1ml at one month post-vaccination, suggesting that the ideal parenteral vaccination strategy for Ethiopian wolves was a 1ml dose of rabies vaccine with a booster 1–6 months later (Knobel *et al*., 2008).

Of the seven animals captured in the Web Valley post-outbreak, three tested seropositive. The presence of these three unvaccinated, seropositive wolves in the outbreak area raises the possibility that these animals may have survived exposure to the virus (Knobel *et al*., 2008). It was speculated that this exposure was due to a low dose of virus by non-bite transmission, most probably through the mucous membranes following oral–oral contact with an infected animal (Dutta, 1998; Fooks *et al*., 2006).

Knobel *et al*. (2008) proved the effectiveness of parenteral rabies vaccines on Ethiopian wolves. They also identified the importance of vaccinating at least one adult female in each wolf pack to ensure persistence of that pack. The spread of this 2003 rabies epizootic was limited by the strategic vaccination of adjacent packs to detain the virus within the Web Valley. Empirical and theoretical evidence suggested that this strategy was effective in halting the spread of the outbreak (Haydon *et al*., 2006).

Rabies Intervention 2008/09

As had occurred following the rabies outbreak of 2003, an emergency vaccination campaign began in the Web Valley on 20th October 2008. Morebawa was the most immediately threatened sub-population at the time of the first rabies outbreak in 2008. Consequently, trapping efforts focused in Web Valley, East Morebawa and the Web Isthmus, with a total of

50 wolves vaccinated from 11 packs, creating a barrier of vaccinated wolves to prevent the spread of the disease. The overall trapping coverage was 63-68% of wolves, attaining or exceeding the target of 40% (Haydon *et al*., 2002) in all but one pack. Female wolves were caught from eight of 11 packs, ensuring maintenance of a breeding pair in those packs. After excluding vaccinated packs from the analysis, an estimated 67% of wolves were lost from six other packs in Web Valley. In vaccinated packs, 29% of wolves disappeared, but of these, only one had been vaccinated.

By the time the second rabies outbreak was discovered in May 2009, this outbreak was considered too far advanced to protect the West Morebawa population. Emergency interventions therefore focused on the high density Sanetti sub-population. A total of 48 individuals from nine packs were vaccinated. The overall vaccination coverage was 58% of wolves. Vaccination attained or exceeded the target of 40% (Haydon *et al*., 2002) in all packs. Female wolves were trapped in seven of nine packs, ensuring maintenance of breeding potential for those seven packs. All these results are discussed in more detail in Chapter Three.

Disease Modelling

Haydon *et al.* (2002) modelled the effects of rabies and CDV on the persistence of Ethiopian wolf populations through population viability analyses (PVA). In the absence of disease, simulation models showed that wolf populations of all sizes were remarkably stable and persistent. These results were complemented by empirical data. However, when rabies epizootics were introduced into the PVA model, extinction probabilities rose linearly with the regularity of infection from the dog reservoir and particularly quickly in smaller, more vulnerable wolf populations, such as those found in North Ethiopia. Simulations of the effect of CDV on the long-term persistence of Ethiopian wolf populations suggested that CDV may not be significant (Haydon *et al*., 2002), although estimated CDV mortality rates when building the PVA were lower (15-20%) than observed in 2005/06 or 2010. The model suggested that direct vaccination of as few as 20-40% of wolves in each pack against rabies, including an adult female, would be enough to ensure pack persistence, and therefore protect the wolf population from reducing to very low densities that would make recovery

unlikely (Haydon *et al*., 2002). Furthermore, a PVA model predicted that if the rabies virus was introduced once every five years on average into a sub-population, vaccination of individuals in the corridors between sub-populations would reduce the probability of catastrophic meta-population reduction almost fourfold from 0.38 to 0.10 (Haydon *et al*., 2006). These models were crucial when planning the rabies interventions that were implemented in 2003 and 2008/09.

Discussion

Rabies is endemic in Ethiopia and is widespread in dog populations throughout the country (Fekadu, 1982; Mebatsion et al., 1992; Tefera et al., 2002). Despite vaccinating over 68,000 dogs against rabies in the Bale Mountains from 1996 to present (EWCP, 2012), rabies remains a major threat to the Ethiopian wolf, having caused two large-scale outbreaks in a seven-year period. The failure of the dog vaccination campaigns to prevent the 2003 and 2008/09 rabies outbreaks was probably in part due to low vaccination coverage because of growing dog populations and due to an insufficiently wide vaccination zone to cover the movements of seasonal herders and their dogs (Randall *et al*., 2006). Rural dog population growth in Bale was estimated at 7.5% per annum before vaccinations started in 1996 (Laurenson *et al*., 1997), with dog population turnover estimated at around 25% per annum in Bale (Laurenson *et al*., 1997). These two factors would reduce vaccination coverage over time without the necessary increase in vaccination effort, making it harder to maintain the 70% required vaccination coverage (Cleaveland *et al*., 2003; Coleman & Dye, 1996).

Population viability models indicated that disease-induced population fluctuations and extinction risks could be markedly reduced with the vaccination of a relatively small proportion of wolves (Haydon *et al*., 2002), the required percentage dependent on the size of the wolf population. The disease control strategy adopted for emergency interventions on Ethiopian wolves in 2003 and 2008/09 was both multi-disciplinary and adaptive over time (Randall *et al*., 2006). Detailed demographic and epidemiological data coupled with quantitative analyses provided practical guidance for successful control interventions, which

showed that even low levels of vaccination were effective in controlling the spread of the rabies outbreak (Knobel *et al*., 2008; Haydon *et al*., 2006).

CDV has now been confirmed as a major threat to the Ethiopian wolf. The 2010 CDV outbreak affected all three core sub-populations of BMNP with 21 wolves dying and a further 41 wolves disappearing from these three areas. Ten more carcasses were recovered in lower density non-focal areas. These losses, combined with an unknown number of dead wolves from lower density areas, suggest that this was the single most catastrophic disease event recorded for Ethiopian wolves, to date. The 2008 rabies outbreak, followed by the 2010 CDV outbreak in the Web Valley, compounded the loss of wolves in this area. Four out of seven packs were eradicated in the Web Valley, including the pack that had lost its adult female during the rabies outbreak. The time taken for total wolf numbers to recover from epizootics indicated low population growth, and that individuals clearly did not always benefit from access to extra food resources (Marino *et al*., 2013). Because reproduction is limited to the dominant female in the pack (Sillero-Zubiri, 1994), any delay in the formation of new breeding units after a severe outbreak can slow population growth and jeopardize recovery (Marino *et al*., 2006). Such devastation from consecutive or concurrent disease outbreaks was identified in Haydon *et al*.'s (2002) models. However, the size and structure of the Bale Mountains' wolf population lends robustness to total wolf numbers, allowing the population as a whole to recover in the face of such continuous and sustained disease threats. For example, two new packs were formed in the Web Valley following the CDV 2010 outbreak, several of the founder members coming through the corridor from East Morebawa, confirming that corridors facilitate migration and recovery after epizootics (Hess, 1996). The disease scenario seen in the Bale Mountains in the past 30 years would likely have caused local extirpation in smaller, less adaptable, wolf populations.

A long-term disease management plan has been developed for tackling both rabies and CDV as threats to the Ethiopian wolf (EWCA, 2011; Randall *et al*., 2006), with vaccination of both host and target populations continuing to be important strategies for disease management (Randall *et al*., 2006). Rabies vaccinations in domestic dog populations are continuing, while

parenteral vaccinations of domestic dogs against CDV have restarted following the 2010 CDV outbreak.

The parenteral vaccination of endangered wild canids can control spillover infections and lessen the severity of outbreaks, as has been proved in African wild dogs and Ethiopian wolves (Haydon *et al*., 2006; Hofmeyr *et al*., 2004; Knobel *et al*., 2008). However, CDV vaccines for wild species are not currently at the same stage of development as rabies vaccines. Inactivated oil-emulsion CDV vaccines have been successfully trialled in captive African wild dogs, with no adverse reactions observed, and elevated CDV antibodies seen in 89% (n=9) of wild dogs (Cirone *et al*., 2004). However, inactivated vaccines may not protect against overwhelming CDV exposure (Wimsatt *et al*., 2006). Trials of the Nobivac D and P antigens, contained within the modified live virus vaccine, *Nobivac Puppy DP™,* have also been conducted on wildlife, including lions (Kock *et al*., 1998), red foxes (*Vulpes vulpes*), ferrets (*Mustela furo*), mink (*Mustela vison*), and raccoons (*Procyon lotor*) (D. Sutton, pers. comm.), with no adverse effects recorded during any trials. Other modified live virus vaccines have been trialled with good antibody responses and no adverse reactions in fennec foxes (*V. zerda*), maned wolves (*Chrysocyon brachyurus*) and bush dogs (*Speothos venaticus*) (Montali *et al*., 1983), and in African wild dogs (van Heerden *et al*., 2002) when combined with prior vaccination with inactivated vaccines. Monovalent canarypox-vectored CDV recombinant vaccines hold the greatest promise for protection of wild canids against CDV (Deem *et al*., 2000). A significant serological response was observed following oral administration of the canarypox-vectored CDV recombinant vaccine, *PureVax*, in island foxes (Vickers *et al*., 2004). This vaccine also elicited antibody responses and no adverse reactions in fennec foxes (Coke *et al*., 2005). A small-scale trial on a small number of Ethiopian wolves would allow controlled testing of parenteral CDV vaccines on this species, should a vaccine be required for future emergency interventions against CDV.

The high prevalence of rabies in the surrounding domestic dog population and the recurrent incursion of rabies into the BMNP wolf population makes the development of an effective oral vaccination protocol all the more urgent, as suggested previously by EWCA (2011), Haydon *et al*. (2006), Knobel *et al*. (2008) and Randall *et al*. (2006). Trials have proved the

safety and efficacy of oral rabies vaccines in a number of wild canids, including coyotes, black-backed jackals, side-striped jackals (*C. adustus*) and red foxes (Artois *et al*., 1993; Aubert & Mutinelli, 2002; Bingham *et al*., 1995; Brochier *et al*., 1996; Fearneyhough *et al*., 1998) as well as domestic dogs (Cliquet *et al*., 2007; Fekadu *et al*., 1996). Oral vaccines present the opportunity to deliver vaccines non-invasively, directly and pro-actively to wolves, while increasing vaccination coverage in the "semi-feral" dogs surrounding wolf habitats. Furthermore, oral vaccination is cost-effective in terms of both human and financial resources. The first trials of a live attenuated oral rabies vaccine in both dogs in Ethiopia, and the wolves themselves, are reported and discussed in Chapters Five and Six. With these potential solutions, disease management may move from a crisis discipline to one in which proactive planning leads to effective disease control (Randall *et al*., 2006). This is critical in endangered populations such as the Ethiopian wolf, for which the ultimate consequence of severe infectious disease outbreaks is extinction.

CHAPTER III

Recurrent rabies epizootics in Ethiopian wolves highlight the need for new control approaches

Abstract

Disease is the most immediate threat to the endangered Ethiopian wolf. The largest population in the Bale Mountains experienced devastating declines due to well-documented rabies epizootics in 1991/92 and 2003. Subsequent rabies outbreaks affected this population in 2008/09, as reported here. During the first outbreak, 39 carcasses were discovered in Web Valley and Gaysay between 28th August 2008 and 15th January 2009, and a further five known wolves disappeared. The mortality rate in Web Valley was 61%, a rate consistent with mortality in 1991/92 and 2003. Eleven carcasses were discovered in West Morebawa in May 2009, approximately 12km South-West of the first outbreak, constituting the second outbreak. Due to reduced knowledge of the wolf population of this area, the estimated 45% mortality rate is likely an underestimation. Five of six sampled wolf carcasses from both outbreaks tested positive for the same rabies virus strain. Phylogenetic analysis of the strain pointed to domestic dogs as the likely source. Emergency interventions, involving the capture and parenteral vaccination of 98 wolves in 20 packs, adjacent to affected packs, apparently stopped the spread of rabies during both outbreaks.

The latest epizootic demonstrates that rabies remains a major threat despite ongoing disease control efforts, and that epizootics may be more frequent than previously thought. This is a cause for concern because wolf population models suggest that similarly-sized populations can recover from five year intervals between epizootics, but the probability of extinction increases as intervals reduce. The 2008 rabies outbreak in Web Valley was followed by a 2010 CDV outbreak, as reported in Chapter Four, with four packs going extinct. Repeated population crashes may reduce genetic diversity of a population that numbers only 250 wolves, but more seriously have the potential to cause extirpation of the smaller wolf populations. These latest rabies outbreaks highlight the need to assess and improve rabies control policies in the Ethiopian context.

Introduction

Encroachment of wilderness areas by a rapidly expanding human population has increased the potential for contact between wild canids and domestic dogs, which are often kept to protect livestock, aid hunting efforts, clean waste or as companions (Butler *et al*., 2004; Sillero-Zubiri & Switzer, 2004). Due to their shared receptivity of numerous pathogens, the most immediate threat to wild canids from this contact is disease. Both wild and domestic canid populations act as reservoirs for viruses (Woodroffe *et al*., 2004). Infectious diseases are a significant cause of population declines in wildlife (Dobson & Foufopoulos, 2001), with rabies recognized as the most common reason for epizootics in wild canids (Funk *et al*., 2003; Woodroffe *et al*., 2004). Rabies has caused dramatic population declines and even the local extinction of African wild dogs (*Lycaon pictus*) (Gascoyne *et al*., 1993; Hofmeyr *et al*., 2004) and Blanford's foxes (*Vulpes cana*) (Geffen, 1994).

In Africa, domestic dogs act as the principal reservoir for rabies (Talbi *et al*., 2009), and rabies remains endemic and widespread in dog populations throughout Ethiopia (Fekadu, 1982; Mebatsion *et al*., 1992; Tefera *et al*., 2002) with evidence suggesting that dogs act as the main reservoir in highland areas where Ethiopian wolves (*Canis simensis*) persist (Laurenson *et al*., 1998; Mebatsion *et al*., 1992; Randall *et al*., 2004). Mebatsion *et al*. (1992) found that rabies antibodies were present in 80% of dog (n=10) samples and 13% of wolf (n=15) samples. It is likely that the wolves' intense sociality aids the spread of disease once transmitted from a domestic dog to an individual wolf. The Ethiopian wolf in the Bale Mountains has seen dramatic population declines due to rabies in 1991/92 and 2003 (Randall *et al*., 2004; Sillero-Zubiri *et al*., 1996b). On both occasions, 52% and 76% of affected sub-populations died or disappeared during the epizootics (see Chapter Two). Today, Ethiopian wolves are restricted to half a dozen enclaves above 3,000m in the Ethiopian highlands, with approximately 450 adult and sub-adult individuals remaining (Marino *et al*., 2011). The largest wolf population is found in the Bale Mountains National Park (BMNP), constituting over half the global total, where wolves are present at high densities, reaching 1.4 adults and sub-adults/km² in ideal habitats (Marino *et al*., 2006).

Disease outbreaks that threaten endangered species have raised awareness of the need to integrate veterinary epidemiology into carnivore conservation and management (Breed *et al*., 2009; Cleaveland *et al*., 2007; Delahay *et al*., 2009; Haydon *et al*., 2006). In 1996, following the 1991/92 rabies epizootic, the Ethiopian Wolf Conservation Programme (EWCP) began vaccinating the reservoir population of "semi-feral" dogs in Bale against rabies, using injectable vaccines, in an attempt to create a *cordon sanitaire* of vaccinated dogs in and around wolf territories (Laurenson *et al*., 1998), as recommended by the World Health Organisation (WHO, 2004). Models predict that 70% of dogs have to be vaccinated against rabies to prevent 95% of rabies epizootics (Coleman & Dye, 1996). Despite vaccinating approximately 40,000 domestic dogs in the Bale Mountains between 1996 and 2003 (EWCP, 2004), rabies affected the BMNP wolf population in 2003, when 75 known wolves died or disappeared (Randall *et al*., 2004).

Alternative approaches to managing disease in wolves have been considered. Studies have proven the effectiveness of parenteral vaccination of wild canids to control rabies (Hofmeyr *et al*., 2004), reducing losses brought about by a rabies outbreak. It was just such emergency parenteral interventions in 2003 that limited the spread of rabies in Ethiopian wolves to less than the extent observed in 1991 (Knobel *et al*., 2008; Randall *et al*., 2006), but not before significant losses were sustained in one wolf sub-population prior to the reactive vaccinations. Analysis of the 2003 intervention provides evidence of the effectiveness of reactive vaccination in curtailing rabies outbreaks in wolves (Haydon *et al*., 2006; Randall *et al*., 2006). Population viability models indicate that disease-induced population fluctuations and extinction risks can be markedly reduced with the vaccination of a relatively small proportion of approximately 40% of wolves in a population size similar to the Bale Mountains (Haydon *et al*., 2006).

This chapter describes a rabies epizootic in the Bale Mountains' Ethiopian wolf population in 2008/09, and the emergency interventions that were implemented to control the spread of the outbreaks. Furthermore, it explores this epizootic in the context of recent rabies outbreaks to investigate the impact of epizootics on population dynamics. Finally, it assesses

the current rabies control methods utilised for Ethiopian wolf conservation and suggests future rabies management options.

Methodology

The BMNP is situated in south-central Ethiopia (6°54' N, 39°42' E) and contains the largest remaining contiguous Afroalpine ecosystem (Yalden, 1983), upon which Ethiopian wolves are reliant (Sillero-Zubiri, 1994). Wolves in BMNP are found at high densities in three major sub-populations linked by narrow valleys: Web Valley, Morebawa and Sanetti (Fig. 3.1), but wolves are found throughout the Afroalpine range, such as the Gaysay area to the North of the Web Valley. Ethiopian wolves forage alone but live in family groups that contain, on average, six (2-20) adults and sub-adults. The dominant female produces one litter of up to seven pups each year (Sillero-Zubiri *et al*., 2004). Wolves are classed as juveniles up to one year, when they become sub-adults, and at two years they attain full adult appearance and sexual maturity.

Since 2001, EWCP closely monitors focal packs in the three sub-populations, providing robust population demography over time. Currently, 18 focal packs are intensively monitored, with up to 41 packs monitored at lower intensity elsewhere in Bale (Gordon *et al*., 2012), such as in West Morebawa. Close observations of focal wolf packs supply reliable information on population change, survival and breeding success, and allow the detection of wolf carcasses during disease epizootics. In West Morebawa, the site of the second rabies outbreak in 2009, monitoring efforts were lower and thus mortality rates are less accurate.

Where wolf carcasses were found, and samples recovered, post-mortem sample collection followed extensive established protocols. Samples of lymph node, spleen and brain, were preserved in 96% ethanol, while brain samples were also stored in glycerol saline. Tissue samples were coded and shipped to the United Kingdom for analysis at the Animal Health and Veterinary Laboratories Agency (AHVLA). Brain samples were analysed for rabies following methods previously described by Johnson *et al*. (2004, 2010). In short, RNA was extracted using the Trizol® technique (InVitrogen). Reverse transcription and polymerase

chain reaction (PCR) amplification of a 606 bp fragment of the rabies nucleoprotein coding sequence was then performed.

Strategy for emergency interventions followed prior modelling recommendations (Haydon *et al*., 2006), with the aim of creating a *cordon sanitaire* between infected and naive areas. As Morebawa was the most immediately threatened sub-population following the 2008 outbreak, trapping efforts focused in the Web Valley, East Morebawa and the Web isthmus. Once the 2009 outbreak was discovered, the epidemic was considered too far advanced to protect the remainder of the West Morebawa sub-population, while the six East Morebawa focal packs had already been vaccinated in 2008. Therefore, the second emergency intervention focused in Sanetti. Wolf captures followed detailed standard trapping protocols established by Sillero-Zubiri (1996) and Knobel *et al*. (2008). Rubber-lined *Soft Catch*™ leg-hold traps (Woodstream Corporation, Pennsylvania, USA) were deployed in sets of three, and checked every 2–3 hours. Trapped animals were immediately covered with a blanket to induce passivity. Adults and sub-adults were vaccinated intra-muscularly with a dose of 1ml vaccine (*Nobivac Rabies*; Intervet, UK) in both quadriceps, while juveniles received two doses of 0.5ml. All trapped individuals were ear-tagged (*Rototag*; Dalton ID Systems, UK) to facilitate monitoring of the outbreak and the wolves post-vaccination.

Wald chi-squared values were calculated to test hypotheses concerning effect of age and sex on trapability and re-captures (a binary response) using logistic regression. Statistical analyses were run in SPSS 14.0.

Results

Rabies Outbreak 2008

A wolf carcass was discovered in Tarura pack territory in the Web Valley on the 28th August 2008 (index case). A further 38 wolf carcasses were recovered from one pack in Gaysay and eight packs in Web Valley between 5th October 2008 and 15th January 2009 (Fig. 3.1), while five known wolves disappeared. The population of adult and sub-adult wolves in Web Valley

declined from 66 individuals in April 2008 to 26 in April 2009, a 61% mortality rate (Table 3.1). Of 39 discovered carcasses, four of five sampled wolves were rabies positive.

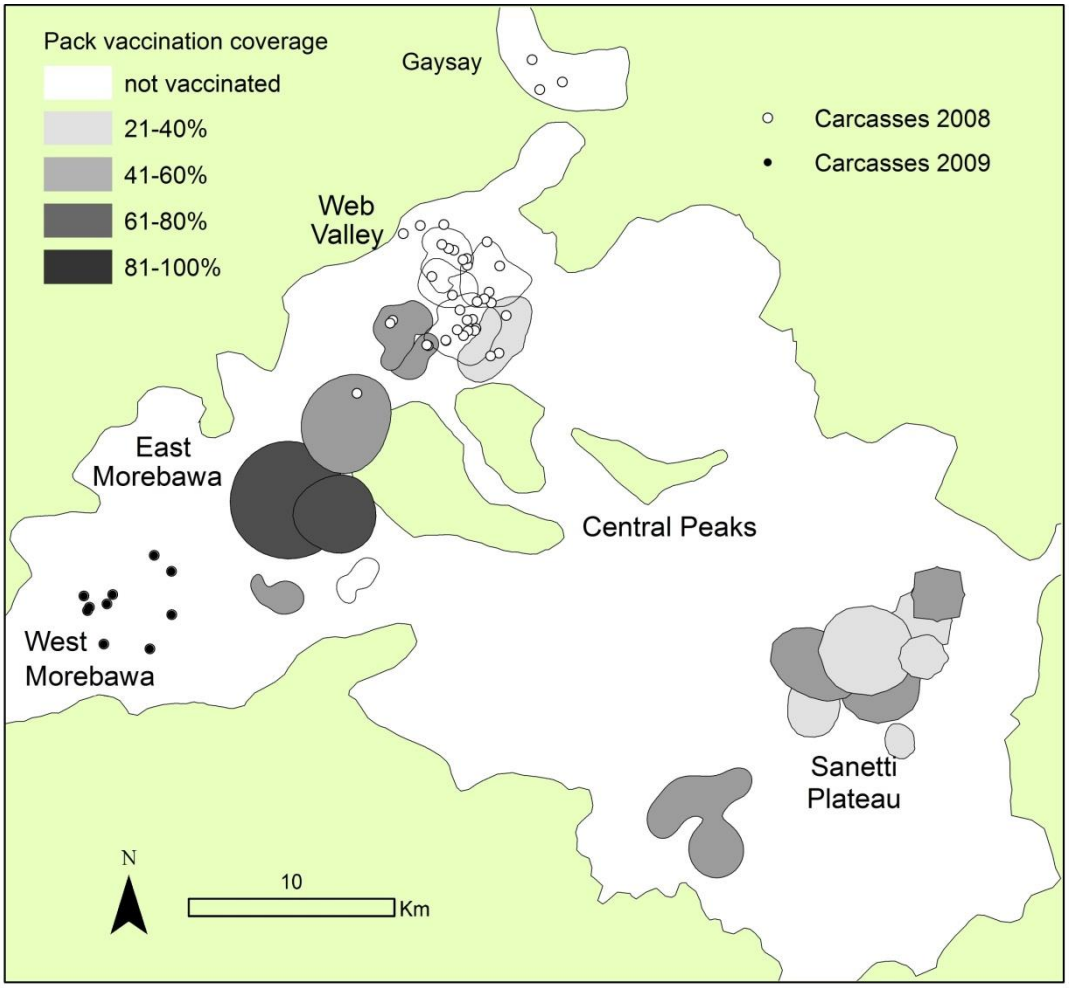

Figure 3.1: *Map showing wolf mortality from the 2008 rabies outbreak in Gaysay and Web Valley, and the 2009 rabies outbreak in West Morebawa. Polygons indicate the home ranges of focal and vaccinated packs. The 2008 intervention focused on packs between Web Valley and East Morebawa. The 2009 intervention focused on the Sanetti Plateau.*

If vaccinated packs are excluded from the analysis, an estimated 71% of wolves died or disappeared from six unvaccinated packs in Web Valley and Gaysay. In the three partially vaccinated Web Valley packs, 35% of wolves disappeared, but of those, only one individual had been vaccinated.

Table 3.1: *Summary of pack sizes pre- and post-outbreak, carcasses recovered in that pack's territory and mortality rate during the 2008 rabies outbreak.*

Pack	Population	Pack Size Pre-Outbreak	Carcasses Recovered	Pack Size Post-Outbreak	Mortality Rate (%)
Gaysay	Gaysay	5	3	2	60
Tarura	Web Valley	13	6	4	69
Darkeena	Web Valley	7	5	2	71
Mulamu	Web Valley	8	4	3	63
Meggity	Web Valley	16	13	3	81
Sodota	Web Valley	7	3	4	43
New Sodota	Web Valley	2	1	1	50
Kotera	Web Valley	3	1	1	67
Alandu	Web Valley	10	2	8	20
Genale	Morebawa	8	1	7	13
	TOTAL	**79**	**39**	**35**	**56**

Rabies Outbreak 2009

Eleven wolf carcasses were recovered from West Morebawa between 3rd and 17th May 2009 (Fig. 3.1), with an estimated 22 more wolves disappearing from 11 packs. A domestic dog carcass was recovered within Leliso pack's territory in West Morebawa on the 14th May 2009. Mortality rates were estimated at 49%, with one pack, Ariye, eradicated (Table 3.2). Of eleven recovered carcasses, one was sampled as the rest were too badly decomposed, being about at least three weeks old. The sample returned a positive rabies result.

Table 3.2: *Summary of pack sizes pre- and post-outbreak, carcasses recovered in that pack's territory, and mortality rate during the 2009 rabies outbreak in West Morebawa.*

Pack	Population	Pack Size Pre-Outbreak	Carcasses Recovered	Pack Size Post-Outbreak	Mortality Rate (%)
Ariye	West Morebawa	6	1	0	100
Gurati	West Morebawa	10	2	3	70
Duna	West Morebawa	6		4	33
Chalalaka	West Morebawa	6	2	2	67
Chokisa	West Morebawa	4		3	25
Horabache	West Morebawa	6		3	50
Waotta	West Morebawa	7	2	4	43
Leliso	West Morebawa	7	4	4	43
Kumbuta	West Morebawa	2		6	0
Rogicha	West Morebawa	9		3	67
Bura	West Morebawa	4		2	50
	TOTAL	**67**	**11**	**34**	**49**

Vaccination Intervention 2008

A total of 50 wolves from 11 packs in Web Valley and East Morebawa, including one pack in the habitat isthmus between these populations (Fig. 3.1), were captured for vaccinations between 20th October and 12th November 2008. The operation required 1,224 hours of trapping effort, averaging 24 hours 30 minutes per new wolf captured. On average, 22 hours of trapping effort per pack was needed to trap the required 40% of individuals in each pack, although this effort dropped to 17.5 hours per pack if Wassema pack was removed from analysis. Vaccination coverage ranged from 40-88% per pack, and averaged 70% for 11 packs (Table 3.3). The sex ratio of captured wolves was skewed towards males by 60:40, while 62% were adults and 38% sub-adults, reflecting the age/sex structure of Ethiopian wolf populations. There is no evidence that age (Wald χ^2_1(N=71) = 2.41, p=0.12) or sex (Wald χ^2_1(N=71) = 0.15, p=0.7) were significant demographical factors during these interventions. Female wolves were caught in eight of 11 packs where trapping occurred.

There were 21 recaptures, comprising 13 wolves, with one sub-adult male from Weshema pack caught five times. Recaptures were slightly skewed towards sub-adults (AD:SA 6:7) and were male biased (8:5). There is no evidence that age (Wald χ^2_1(N=71) = 3.26, p=0.07) or sex (Wald χ^2_1(N=71) = 0.15, p=0.7) were significant demographical factors affecting the composition of wolves caught more than once during the 2008 interventions, when compared to the total Web Valley wolf population.

Table 3.3: *Summary of vaccination effort during the 2008 emergency intervention for rabies.*

Pack	Population	Pack Size	No. Vaccinated	Vaccination Coverage (%)
Sodota	Web Valley	7	4	57
Kotera	Web Valley	3	2	67
Alandu	Web Valley	10	8	80
Genale	Web Isthmus	8	5	63
Weshema	Web Isthmus	8	7	88
Fotora	Morebawa	5	2	40
Fulbana	Morebawa	7	6	86
Huke	Morebawa	8	5	63
Osole	Morebawa	6	5	83
Horgoba	Horgoba Valley	5	4	80
Wassama	Central Peaks	4	2	50
	TOTAL	**71**	**50**	**70**

Vaccination Intervention 2009

A total of 48 wolves were vaccinated in nine Sanetti packs (Fig. 3.1) between 26th May and 9th June 2009, requiring 690 hours of trapping effort, and averaging 14 hours 20 minutes per new wolf. On average, 13.3 hours of trapping effort per pack was needed to vaccinate the required 40% of individual wolves in each pack. Overall, vaccination coverage ranged from 44-80% per pack, and averaged 58% for the nine packs (Table 3.4). Female wolves were trapped in seven of nine packs. The sex ratio of captured individuals was skewed towards males (77:33) and the age structure of those caught was 56% adults, 29% sub-adults and 15% juveniles. Both age (Wald $\chi^2_2(N=83) = 11.76$, $p<0.005$) and sex (Wald $\chi^2_1(N=83) = 11.32$, $p<0.005$) played a significant role in determining the composition of vaccinated wolves during the 2009 interventions, when compared to the demography of the total Sanetti wolf population.

There were seven recaptures of wolves caught more than once. Recaptured individuals were all males and skewed towards adults (AD:SA:JU 5:1:1). There is no evidence that age (Wald $\chi^2_2(N=83) = 2.07$, $p=0.35$) or sex (Wald $\chi^2_1(N=83) < 0.00$, $p=0.99$) were significant demographical factors affecting the composition of wolves caught more than once during the 2009 interventions, reflecting the age/sex structure of the Sanetti wolf population.

Table 3.4: *Summary of vaccination effort during the 2009 emergency intervention for rabies in Sanetti.*

Pack	Population	Pack Size	No. Vaccinated	Vaccination Coverage (%)
Badagassa	Sanetti	9	4	44
Batu	Sanetti	8	5	63
BBC	Sanetti	13	8	62
Bilisa	Sanetti	9	5	56
Dumal	Sanetti	9	4	44
Garba Guracha	Sanetti	9	5	56
Nyala	Sanetti	9	6	67
Quarry	Sanetti	12	7	58
Yadot	Sanetti	5	4	80
	TOTAL	**83**	**48**	**58**

Disease Epizootics affect Population and Pack Dynamics

Between 2002 and 2013, Web Valley focal packs were affected by two rabies epizootics (2003 and 2008/09), with a canine distemper virus (CDV) epizootic observed in 2010 (Chapter Four), just 20 months after the rabies outbreak (Fig. 3.2). Mortality rates were 76% and 61% for the two rabies epidemics respectively, and 68% for the CDV outbreak. One pack disappeared from West Morebawa during the 2009 outbreak, while a Web Valley pack lost all of its females during the 2008 epizootic. The brevity of the second inter-epizootic interval meant that wolf numbers had only just started to recover following the 2008/09 rabies epidemic before the CDV outbreak began. Consequently, this CDV epizootic eradicated four out of seven Web packs.

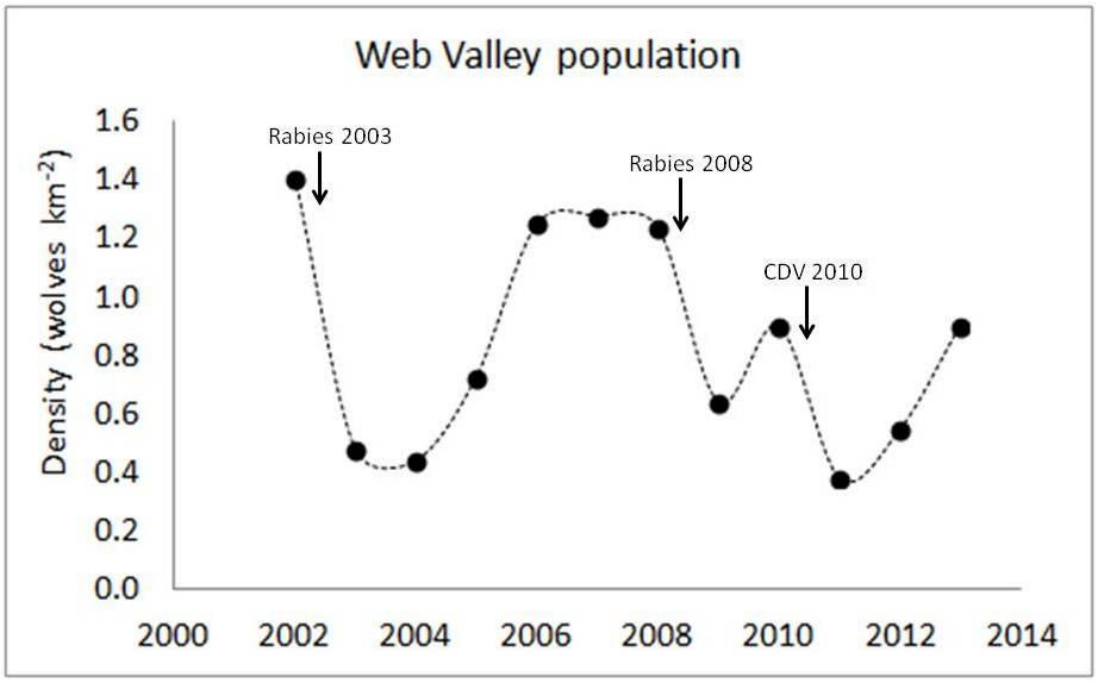

Figure 3.2: *Time series of wolf density (adults and sub-adults) in Web Valley between 2002-2013. The graph indicates timings of rabies and CDV epizootics in this area. Four of seven packs went extinct following the 2010 CDV outbreak, while two new packs were formed in 2011.*

Recovery after the 2003 rabies outbreak was so successful that five years later, immediately prior to the 2008 outbreak, wolf densities had almost returned to pre-outbreak levels. Two packs in Web Valley bred two months later than expected during the 2008/09 breeding season, immediately after the epizootic had ceased. This breeding delay, and the subsequent birth of pups just three months after the epizootic, enhanced recovery in the Web Valley population. Recruitment to sub-adult status of these two litters was high (78%).

Discussion

Rabies caused two large-scale epizootics in the Bale Mountains over a seven-year period between 2002 and 2009. In fact, the frequency of rabies epizootics in Bale has been seen more regularly than the eight-year cycles that were previously suspected (K. Laurenson, pers. comm.). Models suggest that a similarly-sized wolf population can recover from five year intervals between epizootics, but that the probability of extinction increases as this interval reduces (Haydon *et al*., 2002; 2006). As an example from the Web Valley, rabies in 2008 was followed CDV in 2010 (Chapter Four), resulting in four of seven packs going extinct.

Such intense and repeated rabies epizootics have occurred despite vaccinating over 68,000 dogs in Bale against rabies from 1996 to present (EWCP, 2012). The failure of dog vaccination campaigns to prevent these outbreaks in the Bale dog population was probably due to low vaccination coverage because of rapidly expanding dog populations and an insufficient coverage of seasonal herders' dogs (Randall *et al*., 2006), and possibly because of the distance over which rabid dogs can move occasionally (Hampson *et al*., 2007). Rural dog population growth in Bale was estimated at 7.5% per annum before vaccination campaigns started in 1996, with dog population turnover estimated at around 25% per annum (Laurenson *et al*., 1997). With such high turnover and growth, vaccination coverage would decline over time without increased vaccination effort, making it harder to maintain the 70% required coverage (Cleaveland *et al*., 2003; Coleman & Dye, 1996). This rapid growth in the dog population will likely increase the incidence of rabies, increasing transmission risk to wolves, and potentially further reducing time intervals between epizootics. The distance moved by infectious dogs may be exacerbated by the movement of seasonal herders, who bring their livestock and dogs into BMNP seasonally, usually between May and September, to maximise grazing opportunities when grazing is poor around their communities. This timing coincides with the index case seen in the first rabies outbreak in Web Valley in August 2008.

The virus nucleoprotein-coding sequences from the 2008 and 2009 samples were identical to each other in the 405bp N gene region (Johnson *et al*., 2010), indicating a single rabies

epizootic. It is unclear whether the 2009 rabies case occurred due to wolf-to-wolf transmission from the earlier outbreak in Web Valley, despite the barrier created by the emergency vaccinations, or was an additional spillover from the dog population (Johnson *et al*., 2010). The carcass of a domestic dog found in West Morebawa suggests that it was more likely an additional spillover from the dog population. A sequence from an Ethiopian wolf isolated in 2003 (ETH2003, AY500827) (Randall *et al*., 2004) was 99% identical to the 2008 and 2009 sequences. However, it is impossible that the virus has been maintained in the wolf population since 2003. Instead, domestic dogs were the likely source of the epizootic, at the time seasonal pastoralists bring their livestock and dogs into BMNP. The Adaba area, close to both the Web Valley and West Morebawa, appears to be the same approximate point of origin as the 2003 rabies introduction into the Web Valley population (Randall *et al*., 2004).

Mortality rates during 2008 in Web Valley were approximately 61%, when vaccinated and unvaccinated wolves are considered, whereas an estimated 71% of wolves in unvaccinated packs died or disappeared in Web Valley, comparable to mortality rates seen in 1991/92 and 2003 (Randall *et al*., 2004; Sillero-Zubiri *et al*., 1996b). Where non-focal packs in West Morebawa were affected in 2009, approximated mortality rates were lower than in Web Valley, most likely because of lower wolf densities in West Morebawa. Much literature on the relationship between density and disease transmission comes from models (see Sterner & Smith, 2006), while Hochachka and Dhont (2000) showed a density-dependent decline in abundance of the host species during a *Mycoplasma* epizootic.

Emergency vaccination interventions were conducted to stop the spread of rabies during both outbreaks. Trapping success in 2009 in Sanetti was higher than in 2008, most likely because Sanetti trapping occurred during the rainy season, when rodent prey is scarce and wolves are likely to be more receptive to the meat bait used for trapping. For both 2008/09 interventions, trapping effort was considerably lower than in the 2003 intervention (Knobel *et al*., 2008). Vaccination coverage attained and exceeded the target of 40% vaccination coverage (Haydon *et al*., 2002) in all but one pack, prescribed to ensure pack persistence. Female wolves were vaccinated in three-quarters of packs, ensuring the maintenance of a

breeding pair in those packs. Because reproduction is monopolised by the dominant female (Sillero-Zubiri *et al*., 1996a), a reduced number of breeding units can slow population growth and recovery (Marino *et al*., 2006; 2013), such as seen with one pack disappearing from West Morebawa, and another pack losing all its females in Web Valley. The geographical bottleneck between Web Valley and Morebawa acts as a natural restriction to disease spread from one sub-population to another. To maximise the strategic importance of this corridor, the 2008 intervention focused on protecting wolves in and around the area, a strategy that proved successful in 2003 (Knobel *et al*., 2008), and because previous models had shown that vaccination of individuals in corridors between sub-populations would reduce the probability of catastrophic metapopulation reduction (Haydon *et al*., 2006). The geographic structure of the Bale Mountains, and the vaccination interventions, helped to minimise the extent of the rabies epizootic in 2008/09.

Following the epizootic, a likely increase in rodent prey biomass per wolf capita, available in areas such as Web Valley, may have influenced fecundity and pup survival. After the 2008 outbreak in Web Valley, recruitment to sub-adult status of two litters of pups from the 2008/09 breeding season were higher than expected in both high density wolf areas (63%) and even in recovering wolf populations (71%) (Marino *et al*., 2013).

Rabies remains a major and immediate threat to this species. Despite the evident ability of the wolf population to recover after epizootics, it is likely that outbreaks will be more frequent, more widespread and with higher mortality rates without the emergency rabies vaccination interventions. Regardless of these interventions, repeated population crashes could reduce genetic diversity of a population that numbers only 250 wolves, although enhanced genetic drift due to population bottlenecks may be countered by increased migration into disease-affected subpopulations (Randall *et al*., 2010). These latest outbreaks highlight the need to assess and improve rabies control policies to reduce rabies prevalence in both wolves and dogs, and ultimately, to protect Ethiopian wolves.

A long term disease management plan has been developed for addressing rabies as a threat to Ethiopian wolves (EWCA, 2011; Randall *et al*., 2006). Vaccination of both host and target

populations will continue to be important strategies for disease management (Randall *et al*., 2006; Sillero-Zubiri & Macdonald, 1997). The high prevalence of rabies in the surrounding dog population and the frequent incursion of rabies into the BMNP wolves, even considering the dog parenteral vaccination efforts, makes the development of an effective oral vaccination protocol all the more urgent, as suggested previously by EWCA (2011), Haydon *et al*. (2006), Knobel *et al*. (2008) and Randall *et al*. (2006). This disease threat is intensified in the smaller wolf populations (50 or fewer individuals) where additional mortality could be catastrophic (Haydon *et al*., 2002).

Trials have proven the safety and efficacy of oral rabies vaccines in a number of wild canids (e.g. Artois *et al*., 1993; Aubert & Mutinelli, 2002; Brochier *et al*., 1996; Fearneyhough *et al*., 1998) as well as in domestic dogs (Cliquet *et al*., 2007; Fekadu *et al*., 1996). Most importantly, oral vaccination increases the effectiveness of delivery to both wolves and dogs, reduces the stresses to wolves of handling when reactively vaccinating parenterally, and offers a cost effective solution, in terms of both financial and human resources. These oral vaccines present the opportunity to deliver vaccines non-invasively, directly and pro-actively to wolves, while potentially increasing vaccination coverage in semi-feral dogs in and around wolf habitats. Furthermore, oral vaccination with sufficient coverage would prevent disease transmission through habitat corridors in the Bale Mountains. The theoretical potential of these oral vaccines demand immediate trials in both wolves and dogs. The use of oral vaccines could help ensure that disease management moves from a crisis discipline to one in which proactive planning leads to effective disease control (Randall *et al*., 2006). This is critical in endangered populations such as the Ethiopian wolf, for which the ultimate consequence of severe infectious disease outbreaks is extinction.

CHAPTER IV

The emergence of canine distemper virus as a threat to Ethiopian wolves

Abstract

Disease is a major threat to the endangered Ethiopian wolf, with well-documented rabies epizootics affecting critical populations in the Bale Mountains in 1991/92, 2003 and 2008/09. Other canine pathogens may affect Ethiopian wolf populations, but no associated mortality has been recorded until now. Canine distemper virus (CDV) affects all carnivore families, with reports of mortality in five other canid species. This chapter describes two recent CDV epizootics affecting Ethiopian wolves in Bale in 2005/06 and 2010, resulting in extensive mortality. Mortality rates ranged from 43-68% in affected sub-populations, and were higher in sub-adults (83-87%) than in adults (34-39%). Breeding success was reduced in the breeding seasons during or immediately following CDV outbreaks, but subsequent growth rates were high. In the Web Valley in 2010, the CDV outbreak started 20 months after a rabies outbreak, before the population had fully recovered, with four of seven packs completely disappearing. The combined impact of both diseases increased the chance of pack extinction, exacerbating the slow recovery of wolf populations. Indeed, previous modelling predicted that the impact of epizootics would increase with reduced inter-epizootic intervals. These same models suggested that extinction probabilities for rabies alone were higher than for rabies and CDV, although estimated CDV mortality rates in those models were much lower than observed CDV mortality. The conservation implications of this emerging threat are discussed in the context of the dynamics of packs and populations, whilst also considering the fragility of smaller wolf populations. The potential relevance of a CDV control programme, alongside current rabies management efforts, is considered.

Introduction

Rapidly expanding human populations increase domestic dog contact with wild canids (Butler *et al*., 2004; Sillero-Zubiri & Switzer, 2004). While these interactions may affect wild canid populations through competition for food or even hybridisation (Gottelli *et al*., 1994), the major threat to wild canids is disease, as infectious viruses are readily transmitted between species because of their shared receptivity to numerous pathogens (Cleaveland *et al*., 2000; Woodroffe *et al*., 2004). Infectious diseases are a significant cause of population declines in wildlife (Dobson & Foufopoulos, 2001). Rabies is the most common cause of outbreaks in wild canids (Funk *et al*., 2003; Woodroffe *et al*., 2004), while other viral diseases, such as canine distemper virus (CDV) and canine parvovirus (CPV), have also resulted in losses (Banyard *et al*., 2012).

CDV is a single stranded RNA morbillivirus that affects all carnivore families, resulting in high mortality in wildlife populations, second only to rabies (Deem *et al*., 2000). Highly contagious virus particles are aerosolised and transmitted (Greene & Appel, 1990), with infected animals showing respiratory symptoms such as pneumonia (Deem *et al*., 2000), ocular symptoms such as optic neuritis (Gellatt *et al*., 1985) and signs of jaundice, anorexia, diarrhoea and neurological abnormalities (Banyard *et al*., 2012; Deem *et al*., 2000). CDV may be lymphotropic, and as such, infection might induce a profound immunosuppression that often leads to secondary opportunistic infections and usually death (von Messling *et al*., 2004). During a CDV outbreak in 1993 that affected lions (*Panthera leo*), other wildlife and the surrounding dog population in the Serengeti, Tanzania, virus isolates were serologically indistinguishable between the infected wildlife and dogs (Roelke-Parker *et al*., 1996).

CDV has been identified in 12 of 35 wild canid species (Deem *et al*., 2000), with mortality reported in five of these species. Significantly, CDV killed 90% of the Endangered island fox (*Urocyon littoralis*) on Santa Catalina Island, California (Timm *et al*., 2009), a large number of black-backed jackals (*Canis mesomelas*) in Kenya's Maasai Mara in 1978 (Alexander *et al*., 1994), and 61-94% of African wild dog (*Lycaon pictus*) populations in three separate outbreaks in Botswana and Tanzania (Alexander *et al*., 1996; Goller *et al*., 2010; van de Bildt *et al*., 2002). There is a report of one grey wolf (*C. lupus*) dying from CDV in Canada (Stronen

et al., 2011), while another study correlated peaks in CDV seroprevalence in Yellowstone wolves with high wolf pup mortality, suggesting that CDV contributed to the observed mortality (Almberg *et al*., 2009). Additionally, CDV-related mortality has been seen in bat-eared foxes (*Otocyon megalotis*) in Tanzania (Moehlman, 1983; Roelke-Parker *et al*., 1996).

This chapter reports the first CDV mortalities in Ethiopian wolves (*Canis simensis*). There are approximately 450 adult and sub-adult wolves remaining in half a dozen suitable Afroalpine habitats, with the largest population found in the Bale Mountains National Park (BMNP), where wolves reach densities of up to 1.4 adults and sub-adults/km² (Marino *et al*., 2011; 2013). Ethiopian wolves have a strong social structure, with cooperative breeding and territorial defence contrasted by solitary foraging (Sillero-Zubiri *et al*., 2004). Family groups contain on average six adult and sub-adults (2-20) protecting a home range of approximately 6 km² (Sillero-Zubiri *et al*., 2004). Such high wolf densities and large packs increase disease transmission risks, as predicted by mathematical models (Sterner & Smith, 2006). Wolves in Bale have seen dramatic population declines due to rabies in 1991/92 (Sillero-Zubiri *et al*., 1996b), 2003 (Randall *et al*., 2004), and 2008/09 (Johnson *et al*., 2010; Chapter Three). During these epizootics, 45-75% of affected sub-populations were lost. In Africa, dogs act as the principal reservoir for rabies (Talbi *et al*., 2009), and this is true around wolf populations (Laurenson *et al*., 1998; Mebatsion *et al*., 1992; Randall *et al*., 2004; Chapter Three).

While no CDV outbreaks have been previously recorded in wolves, there is serological evidence that the disease is present, with 30% of 30 samples taken in 1989-92 showing seropositivity (Laurenson *et al*., 1998). CDV was also prevalent in dogs at that time, with all dogs over two years seropositive, while dogs under two years were seronegative (Laurenson *et al*., 1998). These results, and anecdotal evidence of a non-rabies disease outbreak in dogs, strongly suggest a CDV epizootic in dogs in 1993/94, although it was not possible to assess whether there was mortality in the wolf population and whether it accounted for its slow recovery following the 1991/92 rabies epizootic (Laurenson *et al*., 1998).

Previous Population Viability Analyses (PVA) have been used to predict the effect of epizootics on wolf population dynamics (Haydon *et al*., 2002; Mace & Sillero-Zubiri, 1997). These suggested that periodic CDV epizootics play a relatively minor role in wolf population persistence, even when modelled together with rabies. One model suggested that when CDV was absent and only rabies present, extinction probabilities may be greater than when both diseases were present, possibly because CDV acted as a population thinning agent, lowering wolf densities and disease transmission rates (Haydon *et al*., 2002). However, estimated CDV mortality rates in these models were low (15-20%), with the authors noting that population impacts should be re-assessed if CDV mortality rates were 40% or higher (Haydon *et al*., 2002). Crucially, models predicted that Ethiopian wolf populations could recover from outbreaks of rabies or CDV, but that if intervals between disease outbreaks fell below 30 months, the likelihood of local extinction would be high in the absence of low-coverage parenteral vaccination campaigns (Haydon *et al*., 2006).

This chapter describes two separate CDV epizootics in the Bale Mountains' wolf population in 2005/06 and 2010, confirming the threat posed by CDV to this species. The source of CDV epizootics are investigated in dogs in villages close to the geographic onset of outbreaks, comparing CDV mortality between domestic and wild canids. The effect of CDV on pack and population dynamics is examined, and the combined impact of rabies and CDV discussed.

Methodology

The Bale Mountains National Park, situated in south-central Ethiopia (6° 54' N, 39° 42' E), contains the largest remaining continuous Afroalpine habitat (Yalden, 1983), upon which Ethiopian wolves are dependent (Sillero-Zubiri, 1994). Wolves in the BMNP are found in three major sub-populations, all linked by narrow corridors, namely Morebawa, the Web Valley and Sanetti (Fig. 4.1). Wolves occur at high density in these sub-populations, but are found throughout the Afroalpine range.

Since 2001, the Ethiopian Wolf Conservation Programme (EWCP) has closely monitored focal wolf packs in these three sub-populations, providing robust demographic data over

time. At the time of the CDV epizootics in 2005/06 and 2010, 18 packs were intensively monitored in the three focal areas, with 41 further packs monitored at lower intensity elsewhere in Bale (Gordon *et al*., 2012), in areas such as the Worgona Valley and Chafadalacha (Fig. 4.1). This intensive monitoring provides detailed pack compositions in focal wolf packs. Time series of wolf pack densities of the focal packs in Sanetti and Web Valley were calculated at six-monthly intervals, incorporating intensive monitoring data and disease information. Wolves are classified as juveniles up to one year, sub-adults up to two years, while full adult appearance and sexual maturity is attained at two years. Long-term population trends were calculated annually and presented as densities of adult and sub-adult animals around the time of breeding. To calculate densities, the area occupied by each population was calculated as the 95% kernel of all wolf sightings during the breeding season between October to March.

Intensive monitoring facilitated the timely recovery of wolf carcasses and the detection of disease epizootics, while detailed pack composition data allowed for the recognition of missing wolves, even when their carcasses were not found. The lower monitoring effort in non-focal areas reduced the accuracy of information on wolf losses and mortality rates in these areas. The disappearance of wolves coinciding with the discovery of carcasses provided evidence that missing wolves died of the same causes as the carcasses. Wherever possible, wolf carcasses were subjected to detailed post-mortem examination and sampling, following established protocols (Newey & Sillero-Zubiri, 2002). Samples including lymph node, lungs, spleen and brain tissue were preserved in 96% ethanol where possible, while brain samples were also stored in glycerol saline.

Interviews were conducted in 62 households in Ayida village following reports of a disease outbreak in this area. Dogs that were suspected to have recovered from the virus were captured where possible. Blood sampling involved capturing the dogs using dog sticks, muzzling the individual, and drawing blood.

Wolf and dog tissue and serum samples were coded and shipped to the United Kingdom for analysis at the Animal Health and Veterinary Laboratories Agency (AHVLA). Tissue samples

were analysed for CDV antibody presence using a semi-quantitative solid-phase ELISA (ImmunoComb®, Biogal, Galed Labs, Israel). Where possible, tissues were macerated and total cellular RNA was extracted using the Trizol® technique (InVitrogen). Reverse transcription polymerase chain reactions (RT-PCR) were performed using SuperScript III Reverse transcriptase (Invitrogen) and random hexanucleotide primers (RHPs). PCR was performed using KOD Hot Start polymerase (Novagen) as detailed below. Amplification of a segment of the phosphoprotein (P) gene was initially carried out using universal morbillivirus primers P1 (5' ATGT TTAT GATC ACAG CGGT 3') and P2 (5' ATTG GGTT GCAC CACT TGTC 3') (Forsyth & Barrett, 1995). Positive reaction products of the correct size (429bp) and the resulting products were sequenced in their entirety with primer sequences being removed from the consensus. Amplification of a section of the H gene was carried out using the same RHP primed RT protocol and CDV specific primers (CDVF1 5' TTAGGGCTCAGGTAGTCCAACA 3') to CDV R1 5' GACAAGGCCGACTCCAGACAA 3') which prime genome positions 7057-7078 and 8206-8225, respectively. Amplification with these primers yielded a product of 1122bp that was then sequenced in its entirety. Both P gene (334 nucleotides) and H gene (1034 nucleotides) data were aligned with available data using MEGA4 (Tamura *et al*., 2007). Samples were also analysed for rabies by nested RT-PCR, using methods described in Johnson *et al*. (2004, 2010).

Analysis of blood sera samples followed methods described in detail in Chapter Five and Six. Samples were analysed for the presence of CDV antibodies using standard ImmunoComb ELISA tests, and for rabies antibody titres using the FAVN test.

Chi-squared tests were used to test the difference between observed age category mortality against expected natural mortalities, while a contingency table was used to test the difference between observed adult and sub-adult mortalities.

Results

CDV Outbreak 2005/06

In July 2005, 65 domestic dog deaths were reported in Ayida village (Fig. 4.1), just 2km from the nearest Ethiopian wolf pack in Worgona Valley. In 62 households surveyed, 49% (n=132) of dogs had died. Owners commonly reported that infected dogs showed symptoms consistent with a CDV infection such as discharge at the eyes, convulsive head nodding and later, a complete loss of appetite leading to death. An additional 37 dogs were sick but recovered, implying possible CDV prevalence in as many as 77% of the village dogs (n=132). Serum samples were collected from 16 of the dogs that had recovered, of which 9 (56%) were positive for CDV antibodies.

On 15th September 2005 a sick wolf was observed in Worgona Valley. Symptoms observed included limping with some hind leg ataxia, hunching of the back, hair loss, and lethargy with little attempt to flee. A wolf carcass was subsequently discovered in September 2005, with one more juvenile carcass found in December. Seven known wolves disappeared from four packs in the Worgona Valley over a four month period from September to December. During this outbreak, there was a presumed 47% mortality rate of adult and sub-adult wolves in these four packs (nine of 19 wolves died or disappeared). One known female wolf emigrated in November 2005 from Shiya pack in Worgona to Garba Guracha pack in Sanetti (approximately 5km apart).

In January 2006, a wolf carcass was discovered in Garba Guracha in Sanetti, the same pack that the emigrated female had joined. Between January and April, 13 further carcasses were recovered in Sanetti (Fig. 4.1) and ten wolves were observed with clinical signs consistent with CDV, four of which recovered and survived the outbreak. Samples were collected from three of 14 carcasses. Another 17 known wolves disappeared from Sanetti during the same period, bringing suspected wolf mortality to 54% (31/58) from nine packs. Sub-adults showed higher mortality rates than adults (83% cf. 34%).

Table 4.1: *Wolf numbers monitored from seven packs in Sanetti before, during and after the CDV outbreak in 2005-06, indicating age-sex composition: adult males (AM), adult females (AF) and sub-adults (SA).*

	November 2005				April 2006				November 2006			
	AM	AF	SA	**Total**	AM	AF	SA	**Total**	AM	AF	SA	**Total**
Badagassa	4	3	3	**10**	2	2	0	**4**	2	2	0	**4**
Batu	3	2	4	**9**	2	2	0	**4**	2	2	0	**4**
BBC	4	1	7	**12**	4	1	3	**8**	2	1	0	**3**
Garba Guracha	2	2	4	**8**	2	1	1	**4**	2	1	1	**4**
Nyala	3	3	2	**8**	1	1	1	**3**	1	1	1	**3**
Quarry	3	2	3	**8**	3	1	2	**6**	3	1	2	**6**
Bilisa	2	1	0	**3**	2	1	0	**3**	2	1	0	**3**
Total	21	14	23	**58**	16	9	7	**32**	14	9	4	**27**

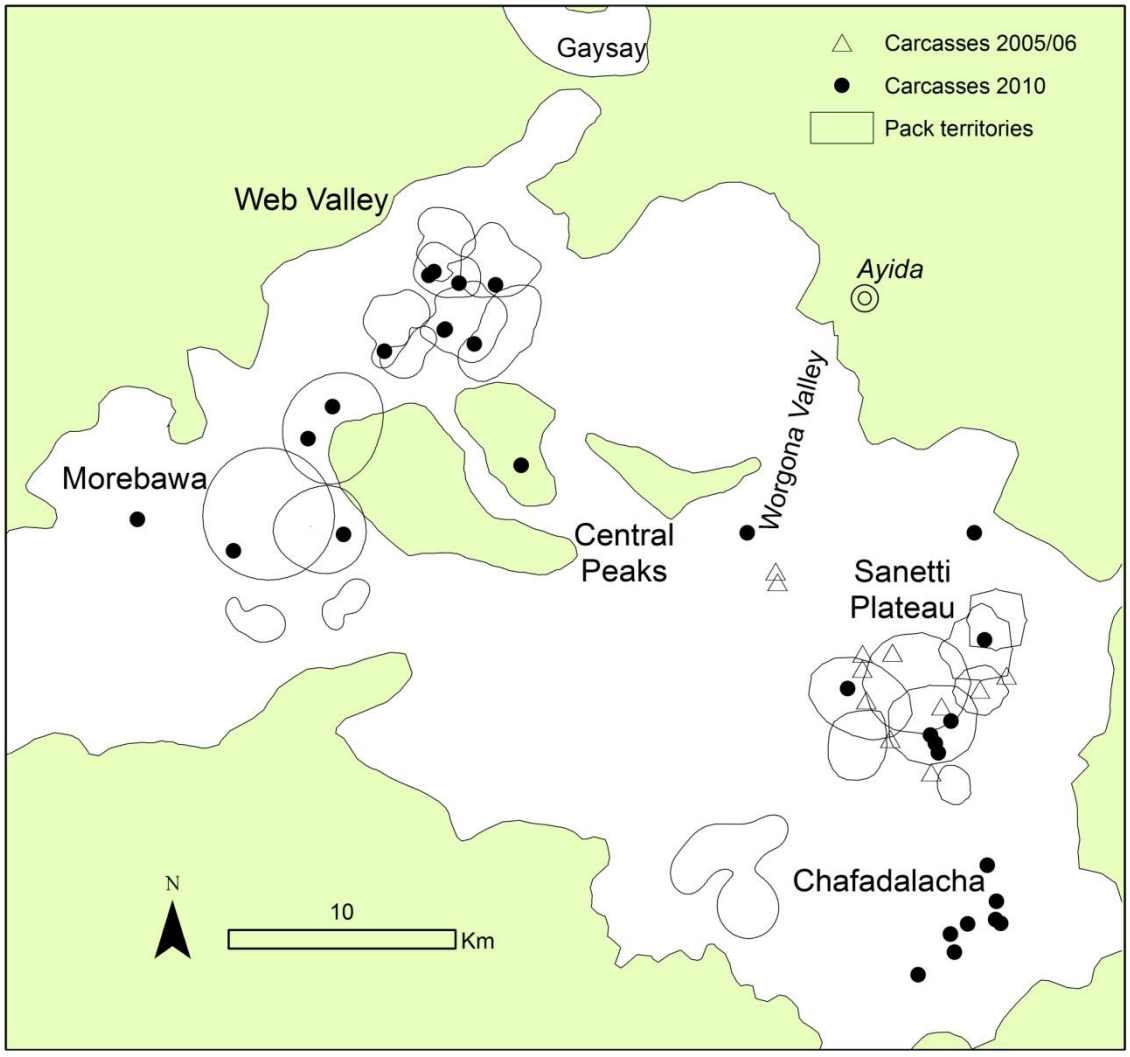

Figure 4.1: *Map showing wolf mortality from the 2005/06 CDV outbreak in Worgona Valley and Sanetti, and the point of origin, Ayida village. The 2010 CDV outbreak spread through the Bale Mountains.*

CDV Outbreak 2010

A second disease epizootic affected the Bale Mountains' wolves during 2010. The first three carcasses were discovered in Web Valley and Morebawa in April 2010. There was a period of no detected mortality before nine more carcasses were discovered in Web Valley and Morebawa in July and August. Five carcasses were also found in Chafadalacha in September, with another five detected in Sanetti in November. A total of 31 carcasses were recovered, as well as three dog carcasses. Seven samples were collected for analysis.

Eight of the 31 carcasses were found in Web Valley while another 13 wolves disappeared from the area, presumed dead, giving a mortality rate of 68% (21/31 wolves in Web died or disappeared) from seven packs (Table 4.2). In Sanetti, eight carcasses were recovered while a further 19 wolves went missing between October and December, giving a mortality rate of 43% (27/63) from seven packs (Table 4.2). In Morebawa, five wolf carcasses were found, and a further 11 wolves went missing between May and August, indicating a mortality rate of 47% (16/34) from six packs. Sub-adults showed a higher mortality rate in focal areas: 87% (20/23), compared to 39% in adults (28/71), while juvenile mortality was 93% (27/29). Ten carcasses were recovered in non-focal wolf areas such as Chafadalacha and Central Peaks.

Table 4.2: *Wolf numbers monitored from seven packs in Sanetti and seven packs in the Web Valley before, during and after the CDV outbreak in 2010, indicating age-sex composition: adult males (AM), adult females (AF) and sub-adults (SA).*

	April 2010				**November 2010**				**April 2011**			
	AM	AF	SA	**Total**	AM	AF	SA	**Total**	AM	AF	SA	**Total**
Badagassa	3	1	3	**7**	5	2	1	**8**	3	1	0	**4**
Batu	4	2	0	**6**	3	1	0	**4**	4	1	0	**5**
BBC	11	3	3	**17**	7	2	1	**10**	4	2	1	**7**
Garba Guracha	4	1	3	**8**	4	1	2	**7**	4	1	1	**6**
Nyala	2	3	2	**7**	2	1	0	**3**	1	1	0	**2**
Quarry	5	3	3	**11**	4	3	0	**7**	4	3	0	**7**
Bilisa	3	2	2	**7**	3	2	2	**7**	4	1	0	**5**
Sanetti Total	**32**	**15**	**16**	**63**	**28**	**12**	**6**	**46**	**24**	**10**	**2**	**36**
Darkeena	1	1	0	**2**	0	0	0	**0**	0	0	0	**0**
Mulamu	3	2	1	**6**	0	0	0	**0**	0	0	0	**0**
Meggity	3	1	0	**4**	2	1	0	**3**	1	1	0	**2**
Kotera	1	0	0	**1**	0	0	0	**0**	0	0	0	**0**
Sodota	2	2	6	**10**	1	1	0	**2**	0	0	0	**0**
Alandu	3	2	0	**5**	3	1	0	**4**	3	1	1	**5**
Tarura	2	1	0	**3**	2	1	0	**3**	2	1	0	**3**
Web Valley Total	**15**	**9**	**7**	**31**	**8**	**4**	**0**	**12**	**6**	**3**	**1**	**10**

Adult mortality during both outbreaks combined (37.7%) was significantly more than the expected natural mortality of 15% per year (Randall *et al*., 2004) (χ^2_1(N=106) = 42.98, p<0.001). Additionally, sub-adult mortality during both outbreaks combined (84.8%) was significantly more than the expected natural mortality of 15% (Randall *et al*., 2004) (χ^2_1(N=46) = 175.69, p<0.001), while significantly more sub-adults than adults died or disappeared during the combined outbreaks (χ^2_1(N=152) = 28.45, p<0.001). Juvenile mortality during the 2010 CDV outbreak (93.1%) was significantly more than the expected natural mortality of 37% per year (Marino *et al*., 2013) (χ^2_1(N=29) = 39.16, p<0.001).

CDV Diagnosis

Serological analysis of tissues from three wolves sampled during the 2005/06 epizootic returned two positive samples for CDV, while both were negative for rabies. The third sample returned inconclusive results, possibly because the sample had been taken one week after death. While we were able to generate the P gene PCR product from the cDNA in one sample, there was not enough cDNA to generate an H gene PCR. The P gene was most closely associated with previous CDV records in the USA and Germany (Fig. 4.2).

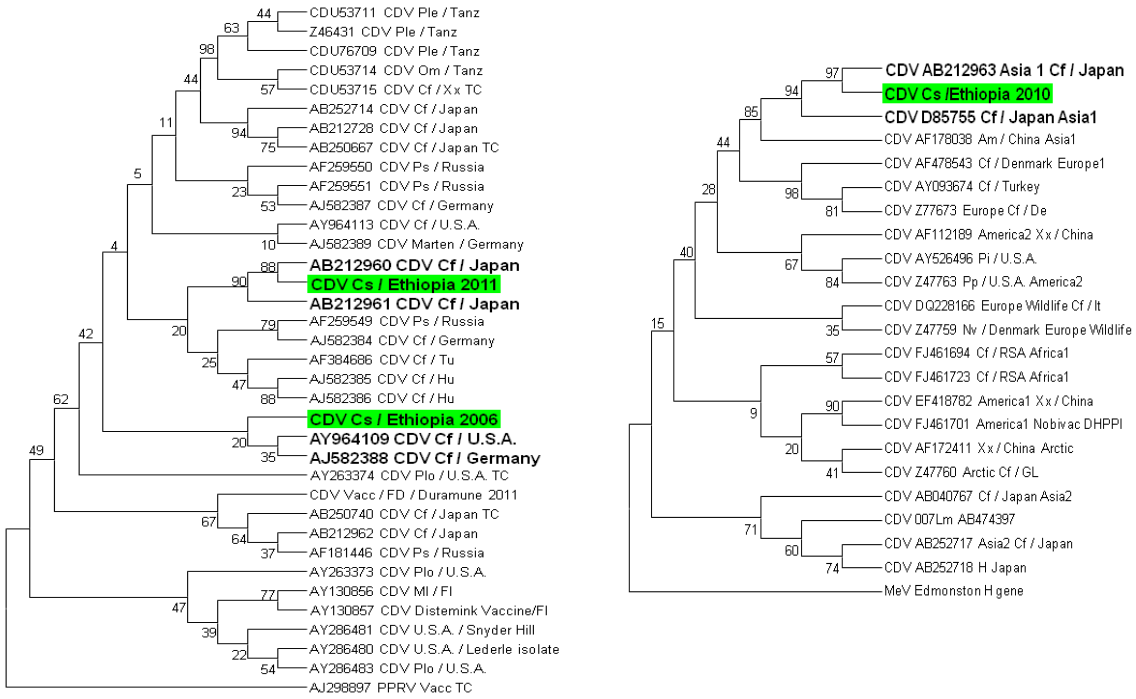

Figure 4.2: *Trees showing CDV isolates of the P gene at 331 nts for both 2006 and 2011 CDV samples (left) and the H gene at 1334 nts for the 2010 CDV sample (right). Both trees are NJ with bootstraps detailed. Ethiopian wolf samples are highlighted.*

Five of seven wolves sampled and analysed during the 2010 epizootic tested positive for CDV, while all were negative for rabies. One sample generated H gene data. Additionally, P gene data was generated from the blood sample of an adult female who survived the CDV outbreak, and was captured in Tarura pack in the Web Valley in November 2011. Both of these P and H gene samples were most closely associated with previous CDV epizootics that originated in Asia (Fig. 4.2). Four additional wolves sampled in 2011 were born after the 2010 CDV outbreak, and tested CDV negative.

CDV Effects on Population and Pack Dynamics

Between 2002 and 2013, focal packs in the Sanetti sub-population were affected by two CDV epizootics (2006 and 2010), while no rabies epizootics were observed. Wolf numbers fluctuated in Sanetti in response to CDV, with an inter-epizootic interval of four years (Fig. 4.3). Following both epizootics, there was an immediate lull in population growth. In 2006, two packs in Sanetti, BBC and Lencha, coalesced to form one pack, effectively meaning one pack went extinct. Breeding success during or immediately after epizootics was also affected. Only four packs (44%) bred in Sanetti in 2005/06, three (38%) in 2006/07, and four (57%) in 2010/11. During the 2005/06 breeding season in Sanetti, four pups survived from three packs, while all four pups from a fourth pack, Badagassa, died in February during the epizootic. The other five packs in Sanetti did not breed in that 2005/06 season. Breeding remained suppressed in the 2006/07 breeding season as only three of eight packs produced pups, of which ten survived to independence at six months. During the 2010/11 breeding season, three of seven focal packs did not breed, while a further two packs bred but lost their pups before emergence. Four of nine pups from the other two packs died before they reached independence.

During the interval between the two CDV epizootics, wolf numbers recovered strongly after this initial two–year lull, to the extent that by the second outbreak, population numbers and densities had surpassed pre-CDV outbreak levels. Wolf densities in Sanetti focal packs were seen to more than double during the period between 2007 and 2010 (Fig. 4.3).

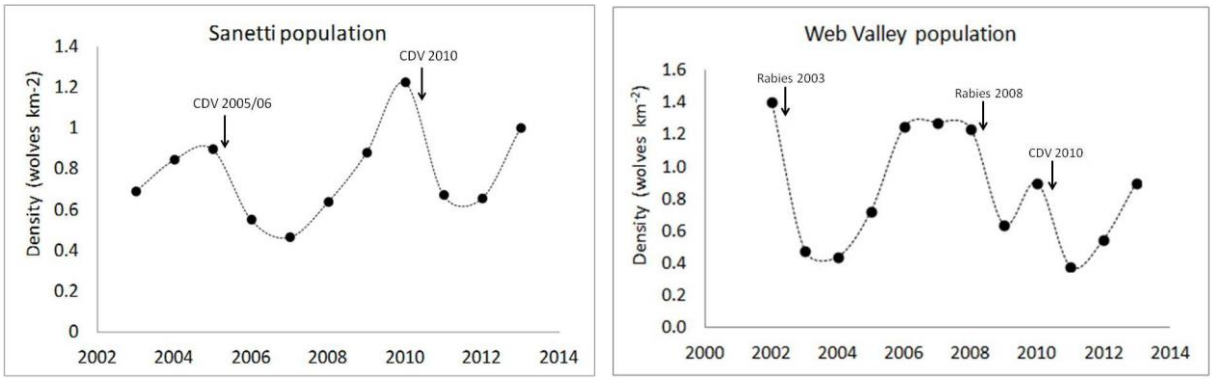

Figure 4.3: *Time series of wolf densities (adults and sub-adults) in Sanetti (left) and Web Valley (right) between 2002-2013. The graphs indicate timings of CDV and rabies epizootics in these areas. Four of seven packs in Web Valley went extinct following the 2010 CDV outbreak, while two new packs formed in this area in 2011.*

Between 2002 and 2013, all of the Web Valley wolf packs were affected by rabies epizootics in 2003 and 2008/09, followed by a CDV epizootic in 2010 (Fig. 4.3). Mortality rates were 62% and 54% for the two rabies epizootics respectively (Chapter Three), and 68% for the CDV epizootic. The brevity of the second inter-epizootic interval of just 20 months meant that wolf numbers had only just started to recover following the 2008/09 rabies epizootic before the CDV epizootic began. This CDV epizootic eradicated four out of seven Web Valley packs, including 86% of the sub-adult wolves that were born during the 2008/09 breeding season (Chapter Three). Two new packs formed in the Web Valley in 2012, explaining the recovery of wolf densities since that time, as seen in the time series in Figure 4.3.

Discussion

CDV Effects on Population and Pack Dynamics

The epizootics reported here confirm CDV as a serious threat to the Ethiopian wolf. The detailed mortality information gathered from both epizootics contradicts the predictions of previous PVA models, which were based on lower estimated CDV mortality rates (15-20%). These models indicated that CDV would have little impact on wolf population persistence or pack size (Haydon *et al*., 2002; Mace & Sillero-Zubiri, 1997). The observed mortality rates in the two epizootics were two to four times higher than those estimated mortality rates. Haydon *et al*. (2002) did however suggest that CDV might be important if mortality rates

exceeded 40%. The observed high mortality rates rapidly alter population and pack dynamics, when compared to natural mortality rates of approximately 15% of adult and sub-adult wolves per year (Randall *et al*., 2004). The missing adult and sub-adult wolves in Sanetti in 2005/06 and 2010, and Web in 2010 all constituted more than the 15% natural mortality rate (29%, 35%, and 42% respectively), providing further confirmation that these missing individuals had died from the CDV epizootics.

Wolf mortality rates from CDV were comparable to mortality rates in dogs in Ayida village. The genetic identity of the virus and the close proximity between the village and wolf habitat makes it almost certain that the village dogs were the source of the epizootic in wolves in 2005/06, supporting the assertion that CDV is transmitted to wild canids by domestic dogs (Cleaveland *et al*., 2000). Some dogs in Ayida recovered from CDV, which when combined with numbers of dead dogs, suggests CDV prevalence during this epizootic was much higher than reported mortality. Wolves can also survive exposure to CDV, as evidenced by blood titres from one wolf sampled in 2011 who survived the 2010 CDV epizootic, and the seropositive wolves in 1989-92 (Laurenson *et al*., 1998).

Surveys of people living in and around the BMNP indicate that there are approximately 20,000 domestic dogs in this area (K. Laurenson, pers. comm.) with a 25% turnover per year (Laurenson *et al*., 1997). Once disease is transmitted from a domestic dog to an individual wolf, the intense social behaviour of packs allows pathogens to spread almost instantly within that pack. Adjacent packs interact at territorial boundaries permitting further transmission through the population. The two epizootics showed different transmission patterns across sub-populations (Fig. 1). The 2005/06 epizootic spread a relatively short distance from the lower density wolf habitats of Worgona Valley to the adjacent high density habitats of Sanetti, where the disease died out. In contrast, the 2010 epizootic moved temporally and geographically from Morebawa to both Web Valley and Sanetti, crossing through geographical bottlenecks and lower wolf density areas between these sub-populations. Inter-pack contact rates are reduced by low connectivity between packs within geographical bottlenecks, reducing the probability of disease transmission through these areas. This fact only serves to reinforce the severity of the 2010 epizootic, with 64 wolves

dead or missing from the three focal sub-populations. Monitoring efforts were lower in the non-focal, lower density wolf areas that were also affected. It was consequently difficult to gauge mortality in these areas. The reported losses from focal packs, combined with unknown mortality from lower density areas, suggest that this was the single most catastrophic disease event for Ethiopian wolves reported to date, the losses outnumbering reports from all previous rabies epizootics, because the disease spread to all areas of the BMNP (Johnson *et al*., 2010; Randall *et al*., 2004; Sillero-Zubiri *et al*., 1996b; Chapter Three).

CDV had a considerable impact on younger wolves, with sub-adult mortality more than twice as high as adult mortality. Lower mortality rates in adult wolves will aid recovery of packs by keeping breeding units (packs) intact, assuming survival of at least one adult female. While juvenile wolves usually have natural mortality rates of 37% during high density periods, and 29% during periods of population recovery (Marino *et al*., 2013), juvenile mortality rates were three time these levels after the 2010 epizootic. Lower mortality in adult wolves may reflect previous low-level exposure, and thus immunity.

While the mechanism by which CDV affects reproduction is uncertain, both CDV outbreaks clearly affected breeding success and pup survival in Sanetti. Typically, 75% of packs breed successfully in periods of high wolf densities, while 83% of packs would breed during periods of population recovery (Marino *et al*., 2013), but less than half of Sanetti packs bred successfully immediately after both epizootics. This unexplained breeding suppression can be seen in the population lull in the time series graphs. Following this lull, breeding was not impaired, and once the juveniles were recruited into the population, growth rates were rapid in Sanetti, with wolf densities doubling over a three year period. With the exception of the two packs coalescing, all breeding packs in Sanetti were maintained, with at least one adult female surviving in each pack. Four years after the 2005/06 outbreak, wolf densities had recovered above pre-outbreak levels, and initial signs suggest that the same pattern will be observed following the 2010 outbreak.

Wolf populations can recover from CDV epizootics, but the capacity to recover will be impaired with low inter-epizootic intervals, as was seen in the Web Valley when just 20

months separated the 2008 rabies outbreak from the 2010 CDV epizootic. After the 2010 CDV outbreak, mortality was high, with four of seven packs eradicated. These pack extinctions confirmed modelling predictions that the probability of pack extinctions are greatly increased as inter-epizootic periods decline (Haydon *et al*., 2002).

The loss of breeding units can slow population growth as pack fission is rare in Ethiopian wolves (Sillero-Zubiri *et al*., 1996a), even though large litter sizes and high juvenile survival may occur following a decrease in population density (Vucetich *et al*., 1997), as described for Ethiopian wolf populations (Marino *et al*., 2013). Dispersal movements for Ethiopian wolves are constrained by the scarcity of suitable, unoccupied habitat, although some subordinate females disperse once recruited to adult status (Sillero-Zubiri *et al*., 1996a). Packs will expand their territory if opportunities arise, usually following the disappearance of a neighbouring pack (Marino *et al*., 2006). Following the 2010 CDV epizootic, available habitat in optimal Afroalpine areas was abundant in the Web Valley, and in 2012, two new packs formed following the loss of four packs in 2010, hinting at the resilience of this species. Several of the founder members of these new packs came through the corridor from Morebawa, joining surviving solitary wolves from extinct Web packs, thereby confirming that corridors facilitate migration and recovery after epizootics (Hess, 1996), with migration critical for minimizing genetic drift from bottlenecks (Randall *et al*., 2010). Such mechanisms indicate that individual wolves take advantage of breeding opportunities and low population density, filling available habitat to form new packs. The size and structure of Bale Mountains' Ethiopian wolf population lends robustness to wolf numbers and provides greater resilience against disease catastrophes (Haydon *et al.*, 2002).

Conservation Implications

CDV is a major threat to the Ethiopian wolf. A long term disease management plan has been developed for addressing this threat (EWCA, 2011). Vaccination of both host and target populations remain important strategies for disease management in general (Randall *et al*., 2006), with the World Health Organisation (WHO) recommending the use of parenteral vaccines on the dog reservoir population as a disease control method where possible (WHO, 2004). Additionally, vaccination of dogs brings associated human and livestock health

benefits (Laurenson *et al*., 1997). Parenteral vaccination of dogs against CDV has consequently restarted following the 2010 CDV epizootic, focusing on dog populations living inside wolf range. Even with incomplete CDV control in dogs, any reduction in disease incidence should have a beneficial effect on wolf persistence.

Population viability models indicate that disease-induced population fluctuations and extinction risks can be markedly reduced with the vaccination of a small proportion of wolves (Haydon *et al*., 2002; 2006). However, CDV vaccines for wild species are not currently at the same stage of development as rabies vaccines. Monovalent canarypox-vectored CDV recombinant vaccines hold the greatest promise for protection of wild canids against CDV (Deem *et al*, 2000), while trials of the Nobivac D and P antigens have also been conducted on wildlife (Kock *et al*., 1998; D. Sutton, pers. comm.), with no adverse reactions. A small-scale trial on a handful of Ethiopian wolves would allow controlled testing of a parenteral CDV vaccine, should future emergency interventions against CDV be required. The high prevalence of CDV in the surrounding domestic dog population and the apparent frequent incursion of CDV into the BMNP wolves makes new disease control strategies all the more urgent, as suggested by EWCA (2011). This is particularly critical in the smaller wolf populations, where extinction probabilities are even higher with the reduction in inter-epizootic periods (Haydon *et al*., 2002). A failure to control these diseases could potentially result in the extinction of the Endangered Ethiopian wolf.

CHAPTER V

Trialling a live attenuated oral rabies vaccine on domestic dogs in Ethiopia

Abstract

Rabies is widespread in domestic dogs in Ethiopia, and has proven a serious threat to endangered Ethiopian wolves, with previous recurrent outbreaks having caused extensive mortalities. Parenteral vaccination of dogs in and around wolf populations is challenging since dogs in rural Ethiopia are notoriously difficult to handle, making injectable hand vaccinations impractical. Oral rabies vaccination offers an efficient and economical method to inoculate dogs against rabies, and was identified as a key tool for Ethiopian wolf conservation in the National Action Plan for this species. This chapter describes a trial of the oral vaccine *Rabigen® SAG2Dog* on rural dogs in Ethiopia under field conditions. In total, 142 oral vaccines were delivered to 116 dogs, using a variety of baits. With the exception of the commercial bait, the other four bait types had high uptake levels. Intestines had the highest uptake rates, although 38% were swallowed after less than five chews, while the meat bait was chewed longest, enhancing the potential for vaccine absorption in the oral mucosa.

Of 116 vaccinated dogs, 104 were captured and blood samples drawn. Three weeks later, 88% were recaptured for subsequent sampling. Nine percent of sampled dogs had positive baseline rabies titres at a 0.5 IU/ml protection threshold, and were excluded. Of the rest, only 11% had successfully sero-converted following vaccination. Three times as many dogs showed values above 0.2 IU/ml, achieving some level of rabies protection, and unpublished data suggest this titre level may provide adequate rabies protection for SAG2. Furthermore, there were a limited number of anamnestic responses to the vaccine in baseline positive dogs (an enhanced reaction in previously exposed dogs). This trial offered little evidence that this oral vaccine would provide effective rabies protection for dogs, even at the lower threshold. Combined with the logistical difficulties of maintaining the cold chain for this vaccine, this field trial would indicate that *Rabigen® SAG2Dog* is not a suitable vaccine for reducing rabies prevalence in rural domestic dogs in Ethiopia.

Introduction

Infectious diseases are a major reason for declines in wildlife populations (Dobson & Foufopoulos, 2001; Chapter 2), with rabies accepted as the most common cause for disease-related epizootics in wild canids (Funk *et al*., 2003; Woodroffe *et al*., 2004). Growing human populations have resulted in increased contact between wild canids and domestic dogs. Due to their shared receptivity of numerous pathogens, the most immediate threat to wild canids resulting from this contact is disease. Rabies has caused dramatic population declines and even the local extinction of African wild dogs (*Lycaon pictus*) (Gascoyne *et al*., 1993; Hofmeyr *et al*., 2004) and Blanford's foxes (*Vulpes cana*) (Geffen, 1994). Populations of the Ethiopian wolf (*Canis simensis*), the rarest canid in the world and Africa's most threatened carnivore (Marino *et al*., 2011; Sillero-Zubiri & Macdonald, 1997), are heavily impacted by rabies. Less than 450 adult and sub-adults remain, restricted to six enclaves above 3,000m in the Ethiopian highlands (Marino *et al*., 2011). Well documented rabies epizootics affected sub-populations in the Bale Mountains in 1991/92 (Sillero-Zubiri *et al*., 1996), 2003 (Randall *et al*., 2004) and 2008/09 (Johnson *et al*., 2010; Chapter Three). Wolf mortality during these outbreaks was high (65-75%) in affected areas. These recurrent rabies epizootics clearly demonstrate that the rabies virus remains a major and immediate threat to this species.

Disease outbreaks that threaten endangered species have raised awareness of the need to integrate veterinary epidemiology into carnivore conservation and management (Breed *et al*., 2009; Cleaveland *et al*., 2007; Delahay *et al*., 2009; Haydon *et al*., 2006). Throughout Africa, domestic dogs act as the principal reservoir for rabies (Talbi *et al*., 2009), and in Ethiopia, rabies remains endemic and widespread in dog populations (Fekadu, 1982; Mebatsion *et al*., 1992; Tefera *et al*., 2002) with evidence that these dogs are the main rabies reservoir in areas where Ethiopian wolves persist (Laurenson *et al*., 1998; Mebatsion *et al*., 1992; Randall *et al*., 2004). Mebatsion *et al*. (1992) found that rabies antibodies were present in 80% of dog (n=10) samples collected in Bale.

The World Health Organisation (WHO) recommends using parenteral vaccines on the domestic dog reservoir population as a disease control method where possible (WHO, 2004), which, in the case of rabies, brings added human and livestock health benefits

(Laurenson *et al*., 1997). In 1996, following the 1991/92 rabies epizootic in wolves, the Ethiopian Wolf Conservation Programme (EWCP) established a disease management strategy and began vaccinating the reservoir population of dogs in the Bale Mountains against rabies using injectable vaccines, in an attempt to create a *cordon sanitaire* of inoculated dogs in and around wolf territories (Laurenson *et al*., 1998). Models predict that 70% of dogs have to be vaccinated against rabies to prevent 95% of rabies epizootics (Coleman & Dye, 1996). Rural domestic dogs in Ethiopia tend to lead a semi-feral existence, particularly in the Bale Mountains, and are consequently difficult to capture and handle to enable vaccination. Furthermore, there are an estimated 20,000 dogs living in and around Bale, not to mention the dogs living close to the other five smaller and more vulnerable wolf populations elsewhere in the country. The logistical complexity of hand vaccinating the projected levels of 70% of dogs in these areas is evident.

Intense and repeated rabies epizootics in wolves have occurred despite vaccinating over 68,000 domestic dogs in the Bale Mountains since 1996 (EWCP, 2012), suggesting that the vaccination of dogs alone is not a suitable solution for managing the threat that rabies poses to wolves. Insufficient vaccination coverage in dogs can be attributed in part to the high turnovers and growth rates in the dog population (Laurenson *et al*., 1997). These factors likely increase the incidence of rabies in the area, escalating the risk of transmission to wolves, and potentially further reducing time intervals between epizootics.

Other approaches to managing disease in the Afroalpine ecosystem have been considered. For example, parenteral or injectable vaccination of wild canids has been shown to reduce losses brought about by an outbreak (Hofmeyr *et al*., 2004). The emergency parenteral interventions in 2003 and 2008/09 limited the spread of rabies in Ethiopian wolves when compared to the extent of the outbreak observed in 1991 (Randall *et al*., 2006; Knobel *et al*., 2008; Chapter Three). However, these emergency interventions are costly, and more seriously, cannot protect individuals who are already infected with rabies. Furthermore, due to their low numbers, capture and handling of wolves should be kept to a minimum, eliminating the routine utilization of parenteral vaccination as a viable option to directly protect the wolves. Based on current monitoring estimates in the Bale Mountains National

Park (Gordon *et al*., 2013), a minimum of 117 wolves from 58 packs would have to be handled for vaccination every 2-3 years to comply with modeling recommendations of 20% coverage in all packs (Haydon *et al*., 2002).

While the ideal intervention protocol has not been finalised, it is apparent that vaccination of both host and target populations will continue to be important strategies for disease management (Randall *et al*., 2006), with oral vaccination identified as a key conservation tool for Ethiopian wolf conservation in the 10-year National Action Plan (EWCA, 2011). Oral rabies vaccines have been used successfully in domestic dogs (Cliquet *et al*., 2007; Estrada *et al*., 2001; Fekadu *et al*., 1996; Hammami *et al*., 1999a; Orciari *et al*., 2001; Rupprecht *et al*., 1998; Schumacher *et al*., 1997), offering a more efficient and cost-effective vaccine delivery option to reach and maintain the 70% inoculation level required to reduce rabies prevalence for dogs in target areas. The WHO promotes co-ordinated field studies on oral vaccination of dogs to improve the vaccination coverage of inaccessible dogs (WHO, 2005). Delivering the vaccine orally could also expedite inoculation programmes, while allowing vaccination of even the most feral dogs (Aubert *et al*., 2000).

There are two types of oral rabies vaccines currently available for wildlife and dogs: modified live attenuated virus vaccines and genetically modified recombinant (GM) vaccines. In Ethiopia, current legislation dictates that GM vaccines cannot be imported and utilised in the country, and thus modified live attenuated virus vaccines are the only feasible oral rabies vaccine option. While several commercial vaccines appear similarly effective in protecting red foxes (*V. vulpes*) from rabies (Artois, 1993), SAG2 (Avirulent-Gif Street Alabama Dufferin), an attenuated double mutant vaccine strain, has been more widely used and has proved safe and effective (Aubert *et al*., 1994; Hammami *et al*., 1999a). SAG2 has been tested on more than 30 target and non-target species, including carnivores, primates, rodents and birds, and is one of only two vaccines recommended by WHO specifically for oral vaccination (WHO, 2005).

This chapter describes trials to test the feasibility of *Rabigen® SAG2Dog*, an oral rabies vaccine manufactured by *Virbac Laboratories* (Carros, France), on rural dogs in Ethiopia under field conditions. Such dogs may have different immunocompetence to specifically-

bred dogs in laboratory conditions, although the safety of SAG2 has been already proven for street dogs in India, Tunisia and South Africa (Schumacher *et al*., 1997; Hammami *et al*., 1999b; Cliquet *et al*., 2007). The objectives of this trial go hand-in-hand with the proposed disease management actions of the National Action Plan for Ethiopian wolves (EWCA, 2011).

Methodology

Trials were conducted in August 2012 in Giba Mikaela village near the Arsi Mountains of Southern Ethiopia, chosen for two reasons. First, the dogs in this village had not been previously vaccinated by EWCP or the Ethiopian authorities, and second, contrary to practice in the rural areas of Bale where EWCP focuses disease control efforts, dog owners in Arsi handle their animals more often, thereby increasing the chance of capture.

Oral rabies *Rabigen® SAG2Dog* vaccines were delivered to dogs in a variety of baits (Fig. 5.1), including a commercial liver-based bait and four local baits: bread and *ambash*a [a local dense bread], both regularly used to feed dogs in this area; chunks of goat meat; and boiled goat intestines, which were used successfully as a local bait for dogs in the Philippines (Estrada *et al*., 2001). *Rabigen® SAG2Dog* vaccines (batch DI159), with an indicated titre of $10^{8.6}$ $TCID_{50}$/ml (Virbac Laboratories, Carros, France), were shipped on dry ice to Ethiopia, where the vaccine was received frozen and stored at -30°C until the week of the trials. Thereafter, they were kept in cool boxes with ice bricks until each vaccine was prepared and delivered. Where necessary, the sachet was removed from the commercial bait matrix and wrapped in the local bait. For the meat bait, the sachet was sewn into the bait using absorbable sutures.

Figure 5.1*: Three of the baits (from left to right): commercially-available placebo bait (Rabigen® SAG2Dog); the placebo sachet inserted into boiled intestines; and inserted into a chunk of goat meat.*

Baits were chosen and delivered following a pre-determined random sequence. Successful bait delivery involved the target dog chewing the bait to pierce the sachet, and maximising how long the sachet was in the mouth so that the vaccine could be absorbed in the oral mucosa (Orciari *et al*., 2001). Some dogs discarded the sachet while chewing the bait, and in these cases, it was recorded as to whether the sachet remained unpierced and full of vaccine, or pierced and empty. The number of discarded sachets that were empty and the number of consumed baits were used to calculate vaccine delivery rates.

Figure 5.2*: capturing a domestic dog in Arsi to draw a blood sample to test for rabies-neutralising antibody titres.*

Dogs were captured immediately after eating the bait and sampled to provide baseline rabies-neutralising antibody titres, and then re-captured three weeks post delivery to provide a post-vaccination titre. Dogs were captured using dog sticks, muzzled, and blood drawn. Different hair patches were shaved on dogs to facilitate identification for the post vaccination captures. The bait delivery and baseline captures were conducted during 7th-12th August 2012. The post-vaccination captures were completed during 27th-30th August 2012.

Blood and serum samples were coded and shipped to the United Kingdom for analysis at the Animal Health and Veterinary Laboratories Agency (AHVLA). The rabies neutralizing titres of the sera samples were assessed using the gold standard fluorescent antibody virus neutralization (FAVN) test (Cliquet *et al*., 1998). A constant volume of rabies virus (CVS-11, 100 $TCID_{50}$/50µl) was added to serial dilutions of serum in quadruplicate. The 50% endpoint dilution, where neutralization of the virus ceased, was calculated with the Spearman-Kärber method. The virus dose was checked by back-titration on every test and results were rejected if virus dose was outside pre-determined limits. Serum titres were converted into international units (IU/ml) by comparison with a standard control serum. The conservative threshold for the sero-conversion of rabies-neutralising antibodies is internationally recognised to be 0.5 IU/ml (OIE, 2011). However, there is unpublished evidence to suggest that a titre of 0.2 IU/ml may be linked to adequate rabies protection with the Rabigen® SAG2Dog vaccine (P. Mahl, pers. comm.).

Additionally, samples from two dead dogs, two sick dogs and one other dog (the sibling of one of the dead dogs) were analysed for the presence of canine parvovirus (CPV) and canine distemper virus (CDV) antibodies using standard ImmunoComb ELISA tests.

The titres were logged to normalise the data. Univariate ANOVAs were conducted to test hypotheses concerning the difference between baseline and post-vaccination samples. The analyses were carried out with SPSS 14.0.

Results

Vaccine Delivery and Baseline Capture

In total, 142 *Rabigen® SAG2Dog* oral vaccines were delivered to 116 domestic dogs from 88 households, using a variety of baits (Table 5.1). Ninety dogs received single doses of vaccine, while 26 dogs received double doses. Samples of blood were drawn from 104 of 116 dogs during baseline captures. Of these, 78 received a single dose and 26 received double doses.

Table 5.1: *Oral vaccines and dosage delivered to dogs within different baits. Numbers within brackets represent dogs which were captured and baseline blood samples taken.*

	Single Dose	Double Dose	**Total**
Commercial	5 (4)	2 (2)	**7 (6)**
Bread	24 (17)	6 (6)	**30 (23)**
Ambasha	19 (17)	6 (6)	**25 (23)**
Meat	26 (24)	8 (8)	**34 (32)**
Boiled Intestine	16 (16)	4 (4)	**20 (20)**
Total	**90 (78)**	**26 (26)**	**116 (104)**

The 142 *Rabigen® SAG2Dog* vaccines were delivered using a total of 212 baits, as summarised in Table 5.2. More baits were delivered than vaccines as the vaccine sachet could be re-used in a new bait if discarded (spat out) unpierced. The commercial bait was the least popular with only 55% bait uptake (n=40). Bait uptake rates differed significantly as a function of bait type ($F_{4,207}$ = 10.88, p<0.001). Post-hoc Tukey's HSD tests showed that the commercial bait had significantly lower uptake than all other bait types (p<0.001). The four other local bait types had higher uptake rates (86.8-100%) than the commercial bait. All pairwise comparisons in these local bait types showed no significant difference in bait uptake.

Table 5.2: *Summary of the different baits delivered, showing bait uptake rates, vaccine delivery rates, and the average number of chews per bait.*

Bait	**Average Chews (±SD)**	**N**	**Bait eaten, vaccine consumed**	**Bait eaten, sachet discarded**	**Bait refused**	**Bait Uptake %**	**Vaccine Delivery %**
Commercial	11.1±5.8	40	9	13	18	55	22.5
Bread	7.6±6.4	53	37	9	7	86.8	69.8
Ambasha	10.5±5.2	48	32	13	3	93.75	66.7
Intestine	10.3±9.7	24	24			100	100
Meat	16.3±15.4	47	42	1	4	91.5	89.4

Effective delivery of the vaccine requires contact between the liquid oral vaccine and the oral mucosa. This contact could be prolonged with a bait that requires more chewing, which works to pierce the sachet, and maximises time of the vaccine in the mouth. The bread bait was swallowed after the fewest chews, with an average of 7.6±6.4 (±SD) chews. Three baits averaged approximately ten chews each, with the commercial bait eaten in 11.1±5.8 chews, ambasha in 10.5±5.2 chews, and boiled intestine in 10.3±9.7 chews, although 38% of these intestine baits were swallowed after less than five chews. The meat bait (n=43) was the most successful, and was swallowed after an average of 16.3±15.4 chews. The number of chews taken differed significantly as a function of bait type ($F_{4,137}$ = 3.69, p<0.05). Post-hoc Tukey's HSD tests showed that the meat bait was chewed significantly more than the bread bait (p<0.05). All other pairwise comparisons were not significant.

Five dogs were seen to spill some vaccine while consuming their baits (two bread, one *ambasha*, two meat), but otherwise, all dogs that chewed and swallowed the bait appeared to pierce the sachet while chewing. Of the commercial baits that were consumed, 65% (13/20) crumbled as they were chewed; the hard plastic vaccine sachet falling to the floor, usually unpierced and therefore entirely ineffective. As a result of this, and the number of baits refused, there was only 22.5% vaccine delivery for the commercial bait. Bread and ambasha had similar vaccine delivery rates of 70% and 67% respectively. The meat bait had an 89% vaccine delivery rate, while 100% of intestine baits were chewed and swallowed. Vaccine delivery rates differed significantly with bait type ($F_{4,207}$ = 20.07, p<0.001). Post-hoc Tukey's HSD tests showed that the commercial bait had significantly lower delivery rates than all other bait types (p<0.001). Additionally, post-hoc Tukey's HSD showed that there was significantly higher vaccine delivery by intestines than by ambasha (p=0.009). All other pairwise comparisons showed no significant difference in vaccine delivery rate.

Post-vaccination Capture

Post-vaccination blood samples were taken from 95 dogs, including 91 that had provided baseline samples (Table 5.3), giving a recapture rate for the post-vaccination captures of 88% (91/104). Two dogs, a 1 year old female and an 8 month old male, died after vaccination, four and five days after delivery respectively, and thus were not sampled for a

second time. Both dogs had eaten single doses of vaccine in the intestine bait. Dog owners described symptoms where the dog refused to eat, and became lethargic. A further two dogs were described by their owners as "sick" when captured for the second time; one was visibly lethargic and refused food when offered. Of the other 11 dogs that were not sampled, five could not be caught, three were not caught as the owner was not home to permit recaptures, two dogs were not at the homestead, and one was pregnant.

Table 5.3: *Domestic dogs sampled for post-vaccination rabies antibody titres, including information on vaccine dosage and bait type. The numbers within brackets represent dogs for which no blood samples had been taken.*

	Single Dose	Double Dose	Not Caught	**Total**
Commercial	3	2	1	**6**
Bread	19(4)	6	2	**27**
Ambasha	17	6	-	**23**
Meat	20	7	5	**32**
Boiled Intestine	11	4	5	**20**
Total	**70 (4)**	**25**	**13**	

Seroconversion response to oral vaccination

Nine out of 104 (9%) dogs had positive baseline rabies-neutralising antibody titres of at least 0.5 IU/ml. Post-vaccination, 14% (13/94) were above the threshold. Nine dogs had negative baseline antibody titres rising to positive post-vaccination titres, and four dogs had positive baseline antibody titres falling to negative post-vaccination levels. There was no evidence for any significant difference in titres comparing baseline and post-vaccination samples ($F_{1,89}$ = 0.003, p=0.954). Twenty-five percent (n=8) of baseline positive dogs had anamnestic responses to the vaccine, showing higher post-vaccination titres.

There is no significant difference between single and double doses of vaccine, because vaccine reactions work according to logarithmic scales. Dosage was consequently removed from further analysis. All bait types had similar percentages (13-16%) of individuals with positive titre levels post-vaccination (Table 5.4), with the exception of commercial baits which had no seropositive individuals post-vaccination.

When dogs with positive baseline levels are removed from the analysis, 11% of dogs successfully sero-converted rabies-neutralising antibody titres at the 0.5 IU/ml threshold, following vaccination (Table 5.4). Even with the removal of baseline positive dogs, there was no significant difference between titres for comparisons of baseline and post-vaccination samples in each individual dog ($F_{1,89}$ = 0.768, p = 0.384). Again, all bait types had similar percentages (10-14%) of individual dogs with positive titre levels post-vaccination, with the exception of commercial baits which had no seropositive individuals.

Table 5.4: *Positive rabies antibody levels for baseline samples and post-vaccination samples, set at the internationally recognised conservative protection threshold of 0.5 IU/ml. Each bait type is individually summarised.*

	All Samples		**Positives Removed**	
	Positive Baseline samples	**Positive Post-vaccination samples**	**Positive Baseline samples**	**Positive Post-vaccination samples**
Commercial	17% n=6	0% n=5	0% n=4	0% n=4
Bread	9% n=23	16% n=25	0% n=19	11% n=19
Ambasha	4% n=23	14% n=22	0% n=21	10% n=21
Intestine	10% n=20	13% n=15	0% n=14	14% n=14
Meat	9% n=32	15% n=27	0% n=24	13% n=24
TOTAL	**9% n=104**	**14% n=94**	**0% n=82**	**11% n=82**

If a lower titre threshold of 0.2 IU/ml is used to define 'positive', 40% (42/104) of dogs had positive baseline titres. Post-vaccination, 37% (35/94) of dogs had positive antibody titres. Seventeen dogs had negative baseline levels rising to positive post-vaccination levels, while 21 dogs had positive baseline levels falling to negative post-vaccination levels (Table 5.5). There was no significant difference between the titres for comparisons of baseline and post-vaccination samples ($F_{1,89}$ = 0.003, p = 0.954). Additionally, 24% (n=38) of baseline positive dogs showed anamnestic responses to vaccination, returning higher post-vaccination titres.

Three of five bait types produced negative results at this reduced titre threshold, whereby there were higher percentages of dogs with positive baseline levels than there were with positive post-vaccination levels (Table 5.5). However, bread showed signs of effectiveness at

this reduced threshold, as 13% (n=23) of dogs that ate the bread bait had baseline positive rabies titres, which rose to 36% (n=25) positive titres, post-vaccination.

Table 5.5: *Positive rabies antibody levels for baseline samples and post-vaccination samples, set at the reduced protection threshold titre of 0.2 IU/ml. Each bait type is individually summarised.*

	All Samples		Positives Removed	
	Positive Baseline samples	**Positive Post-vaccination samples**	**Positive Baseline samples**	**Positive Post-vaccination samples**
Commercial	50% n=6	20% n=5	0% n=3	33% n=3
Bread	13% n=23	36% n=25	0% n=19	37% n=19
Ambasha	43% n=23	45% n=22	0% n=12	33% n=12
Intestine	55% n=20	33% n=15	0% n=7	14% n=7
Meat	53% n=32	37% n=27	0% n=11	36% n=11
TOTAL	**40% n=104**	**37% n=94**	**0% n=52**	**33% n=52**

If we remove dogs with positive baseline titres from our analysis, then at the reduced 0.2 IU/ml threshold, we find that 33% of dogs (n=52) successfully sero-converted after vaccination. There was a significant difference during comparisons of baseline titres and post-vaccination titres for each individual dog ($F_{1,51} = 6.98$, $p<0.05$) when baseline positive dogs were removed from analysis at this reduced threshold. Seroconversion of the vaccine in the intestine bait was low (14%, n=7). The other four bait types showed similar seroconversion rates to each other (33-37%).

Of the two dogs that died post vaccination, one showed positive levels of CPV, with the positive threshold recognised as being any sample above 3 IgG. That dog returned negative results for both rabies and CDV. The other dead dog returned negative samples for all three diseases. The two “sick” dogs tested positive for CPV for both the baseline and post-vaccination levels. One of these dogs was positive for CDV in both the baseline and post-vaccination levels.

Discussion

These oral vaccine trials on domestic dogs showed a successful engagement with dog owners in the Arsi Mountains, as there were only a handful of owners who chose not to take part in the study. The commercial baits had poor uptake rates by domestic dogs in this study, and poor vaccine delivery rates, due to the crumbling texture of the bait matrix, with the vaccine sachet often spat out, usually unpierced. This would rule out the use of the commercial bait matrix in any future interventions. There was no significant difference in uptake rates for the other local bait types, with the intestine bait scoring highest with a 100% uptake rate. Attenuated rabies viruses, such as SAG2, replicate in local tissues of the oral cavity, and can be cleared relatively quickly, leading to protective immunity (Orciari *et al*., 2001). Therefore, in order to ensure maximum absorption of the vaccine, it is ideal that the pierced sachet remains in the oral cavity for as long as possible. All baits except meat were swallowed after an average of 8-11 chews, although over a third of the intestine baits were swallowed after less than five chews, reducing the chance for vaccine absorption in the oral cavity. Meat baits were chewed for longer than any other bait at an average of 16 chews, maximising the opportunity for absorption.

Capture and recapture rates for domestic dogs during this study were high. Furthermore, the serum samples taken during these captures were high quality field samples with minimal haemolysis or contamination (D. Horton, pers. comm.), proving the effectiveness of the sampling techniques and protocols used under field conditions.

The results for seroconversion of rabies antibodies at the conservative threshold of 0.5 IU/ml were poor. At this threshold, there is little evidence that these oral vaccines provide effective vaccination protection for domestic dogs. This study showed high rabies seroprevalence levels in the Arsi dog population, reducing the effectiveness of the trial. Nine percent of dogs returned baseline positive rabies antibody titres, suggesting that the dogs currently had, or had been previously exposed to, rabies, or that they had been vaccinated before, but this latter possibility was ruled out through questionnaires of dog owners. Therefore, the most plausible explanation is that the dog population had been affected by a rabies epizootic. Recent studies have challenged the classic description of rabies infection,

demonstrating rabies seropositivity in a proportion of healthy, unvaccinated domestic dogs in rural Africa (Cleaveland *et al*., 1999), suggesting that seropositive dogs were unlikely to have current rabies infections, but that seropositivity was more likely consistent with an aborted infection (Fekadu, 1991).

Furthermore, three out of five dogs tested for CPV were positive, and two out of five were positive for CDV, highlighting the prevalence of various canine pathogens in this landscape. As a comparison, tests on domestic dogs in the Bale Mountains reported 100% of dogs were sero-positive for CPV (although a recent outbreak of CPV at that time could not be precluded), while 32% of dogs were sero-positive for CDV (Laurenson *et al*., 1998).

We were unable to demonstrate what had killed the two dogs, as serum tests only provide levels of antibodies. Tissue tests would have definitively shown which disease had caused death, but unfortunately the carcasses had been dragged away by scavengers by the time we returned to the village. One of the dead dogs had baseline blood levels that were positive for CPV. SAG2 is a live attenuated vaccine, and may be pathogenic for immuno-compromised dogs, although no deaths occurred in other trials of this vaccine in dogs of a similar immuno-status (Cliquet *et al*., 2007; Hammami *et al*., 1999b). The clinical signs described by owners, that sick dogs were lethargic and had a loss of appetite, were consistent with CPV (Nandi & Kumar, 2010), CDV (Perpinan *et al*., 2008) or rabies (M. Aubert, pers. comm.) infections. It is unlikely that these two deaths were caused by the vaccine as the dogs died four and five days after delivery respectively, a more rapid death than rabies usually inflicts (WHO, 2005).

Why do some samples show positive baseline rabies-neutralising antibody levels but negative post-vaccination levels? One explanation is that those dogs had existing genuine rabies-neutralising antibodies above the titre threshold, induced by previous exposure to the virus, and the changes seen post-vaccination were caused by a natural variation in serum antibody titres over time. However, this explanation goes against traditional understanding of the clinical course of the rabies virus in dogs (D. Horton, pers. comm.). These individuals could also have had false positive baseline levels, although this is an

unlikely scenario given the quality of the serum samples. If the SAG2 vaccine was working sufficiently for some negative baseline individuals to sero-convert rabies-neutralising antibody titres, one would expect to see an anamnestic "memory" response in all dogs that were already rabies positive, where previously exposed individuals produce higher rabies titres post-vaccination than naive individuals, the vaccination stimulating an increase in antibodies that are already familiar. However, this expected anamnestic response and subsequent rise in titre levels was only seen in two of eight positive baseline individuals at the 0.5 IU/ml threshold, and nine of thirty-eight positive baseline individuals at the 0.2 IU/ml threshold.

If we remove dogs that had positive baseline titres at the conservative 0.5 IU/ml threshold from our analysis, we find that 11% of dogs sero-converted the vaccine, with effectiveness seen in a few bait/dose combinations. Even at the reduced threshold of 0.2 IU/ml, it is difficult to draw conclusions from these samples as there were similar percentages of dogs that had positive rabies-neutralising antibody titres in both baseline and post-vaccination samples. If we remove the dogs that had positive baseline levels from our analysis, then at the 0.2 IU/ml threshold, 33% of dogs successfully sero-converted the vaccine, while four of the baits had similar levels of seroconversion, more than double the levels produced by the intestine baits. Seroconversion by the intestine baits may have been reduced because over a third of the baits were swallowed after less than five chews.

After baseline positive (0.2IU/ml) individuals were removed, there was a significant difference between baseline logged titres and post-vaccination logged titres in individual dogs, indicating that the vaccine had a small effect on titre levels. This result indicates that *Rabigen® SAG2Dog* vaccine can provide rabies protection, although there are still two major stumbling blocks: first, a large percentage (67%) of baseline negative dogs did not sero-convert the vaccine even after the positive baseline individuals were removed from the analysis; and second, an anamnestic response to the vaccine was seen in less than a third of positive baseline individuals.

The *Rabigen® SAG2Dog* vaccine was delivered to the dogs two months prior to the expiry date, which might have meant that the vaccine titres had already started to reduce. Unfortunately, there was no remaining vaccine after the trials that we could use to test titre levels. With both the internationally recognised 0.5 IU/ml threshold and the reduced 0.2 IU/ml threshold, vaccination with *Rabigen® SAG2Dog* did not convert widespread rabies-neutralising antibody titres in the domestic dog population of the Arsi Mountains.

All previous trials of the SAG2 virus strain have been conducted on dogs under laboratory conditions using purpose-bred dogs (Fekadu *et al*., 1996; Orciari *et al*. 2001; Rupprecht *et al*., 1998) or street dogs (Schumacher *et al*., 1997; Hammami *et al*., 1999a; 1999b; Cliquet *et* al., 2007) (Table 5.6). The variability of the immune response following oral vaccination with SAG2 in these previous trials was amplified when working with street dogs of undefined origin and health status (Schumacher & Aubert, 1996). Furthermore, Tunisian street dogs generally produced lower antibody titres than pet dogs in Europe, and lower titres still than laboratory dogs (Blancou *et al*., 1996). As the Ethiopian rural dogs likely have a similar immuno-status, this provides one explanation for the low antibody titres seen post-vaccination in this study.

Table 5.6: *A summary of seven laboratory-based trials of SAG2 and lypholised SAG2 on purpose-bred and street dogs. The number of dogs that successfully sero-converted rabies-neutralising antibodies above the 0.5 IU/ml threshold is summarised, in addition to the response of vaccinated and control individuals that were rabies positive following a rabies challenge.*

Dogs	Vaccine	0.5 IU/ml Seroconversion	Response of Vaccinated Dogs after Rabies Challenge	Response of Control Dogs after Rabies Challenge	
street	lypholised SAG2	4 of 13 at d28	0 of 9 positive	5 of 5 positive	Cliquet *et al*., 2007
bred	SAG2	33 of 40 at d28	0 of 40 died	8 of 10 died	Fekadu *et al*., 1996
street	SAG2	2 of 5 at d150			Hammami *et al*., 1999a
street	lypholised SAG2	4 of 7 at d40	2 of 7 died	5 of 7 died	Hammami *et al*., 1999b
bred	lypholised SAG2	4 of 12 at d28	2 of 12 died	6 of 6 died	Orciari *et al*., 2001
bred	SAG2 & lypholised SAG2	14 of 26 at d14	7 of 26 died	5 of 5 died	Rupprecht *et al*., 1998
street	SAG2	10 of 10 at d30	0 of 10 positive	0 of 1 positive	Schumacher *et al*., 1997

The conversion of rabies-neutralising antibody titres in this Ethiopian trial was generally poor, with 11% of baseline-negative individuals sero-converting over the 0.5 IU/ml threshold at day 18-20 post-vaccination. These rates were lower than in seven other SAG2 vaccine studies on domestic dogs elsewhere, where samples taken between day 14-40 showed that seroconversion above the 0.5 IU/ml threshold ranged from 31-100% of dogs (Table 5.6). Previous trials of SAG2 showed slowly increasing serological responses over six months post-vaccination in laboratory beagles (Fekadu *et al.*, 1996). Further studies compared two methods of immunisation (oral and parenteral) with the same dose of SAG2 vaccine in 20 street dogs in South Africa. Antibody titres peaked at day 30 for injected dogs, whereas they peaked at day 60 for orally vaccinated dogs (Schumacher *et al*., 1997). Additional serum samples taken at later stages post-vaccination might have revealed higher seroconversion rates in this Ethiopian study.

In Tunisian stray dogs, only 57% (n=7) of dogs sero-converted at day 40 following vaccination, and yet 71% of dogs survived a rabies challenge (Hammami *et al*., 1999b). A second study with purpose-bred beagles showed that only 33% (n=12) of dogs produced rabies-neutralising antibody titres at day 28, but 83% of vaccinated dogs survived a rabies challenge (Orciari *et al*., 2001). In a third study in Indian stray dogs, just 31% (n=13) of dogs sero-converted titres at day 28, and yet in post-mortem samples on euthanized dogs taken after a rabies challenge, 0/9 of these vaccinated dogs were rabies positive while 5/5 control dogs were rabies positive (Cliquet *et al*., 2007). Under field conditions in Ethiopia, we were, for obvious reasons, unable to deliver a rabies challenge to the study dogs. As such, it is not clear whether the SAG2 vaccine's effectiveness was more far-reaching than the low percentage of dogs with titres above the threshold might suggest.

The majority of previous SAG2 vaccine trials on dogs delivered a concentrated liquid form of SAG2 directly onto the tongue, or utilised the now defunct freeze-dried lypholised SAG2 strain in baits. The logistical difficulties of maintaining the cold chain for this *Rabigen® SAG2Dog* vaccine at the required -30°C level render this vaccine a poor option for a widespread campaign to reduce rabies prevalence for rural dogs in the Ethiopian context. Additionally and ultimately, the seroconversion results in this Ethiopian trial were much

lower than in all previous laboratory-based trials. Despite these results, there are some positives to be taken from this trial including the participation of the community in the trials, the excellent uptake rates of four of the bait types, the high capture/recapture rates, and the quality of the serum collected under field conditions. While these results do not entirely rule out SAG2 as a practical solution, a trial of a thermo-stable GM oral rabies vaccine, such as VRG, on dogs in Ethiopia, would offer a more realistic method for achieving widespread vaccination coverage of dogs in and around threatened populations of Ethiopian wolves, a goal that has been recently highlighted in the National Action Plan for this species (EWCA, 2011). The resources currently dedicated to domestic dog vaccinations in Ethiopia have not been able to prevent recurrent rabies epizootics in wolves in the Bale Mountains. Only by improving vaccination coverage, and the efficiency of vaccination effort, will disease prevalence decrease in Bale and around the smaller, more vulnerable wolf populations.

CHAPTER VI

Testing the suitability of the live attenuated oral rabies vaccine *Rabigen® SAG2Dog* to protect endangered Ethiopian wolves

Abstract

Wild canids are resilient, but despite their adaptability, diseases are still a major cause of population declines in several canid species. The Bale Mountains population of the Endangered Ethiopian wolf has suffered repeated population crashes from rabies and canine distemper. To date, vaccination efforts to protect wolves from rabies have targeted the domestic dog reservoir. Disease interventions have twice involved capture and vaccination of the wolves in response to rabies outbreaks, but only once significant losses had occurred. Oral rabies vaccination potentially offers a more cost efficient and proactive approach to protecting wolves against rabies. This chapter reports on the first in-situ trial of an oral rabies vaccine in wolves to test its effectiveness. Different bait types and delivery methods were tested to maximise bait uptake. Commercial baits were never taken by wolves and rodent baits also had poor uptake rates (17-22%), while actual vaccine ingestion for the intestine baits (18-25%) was lower than bait uptake rates as the wolves easily removed the vaccine sachet from the bait. Goat meat baits had the highest uptake rates (50-53%). Targeted delivery from horseback had better uptake rates than when baits were randomly distributed throughout the pack territory, whilst also allowing for easy monitoring of which individuals ate the bait, and the reducing of delivery to non-target species.

The oral vaccine *Rabigen® SAG2Dog* was delivered to one wolf pack, using the favoured meat bait and the targeted delivery method. In total, 21 oral vaccines were consumed by five out of eight wolves, and rabies-neutralizing antibody titres in sera samples were assessed using the FAVN test. Two of four wolves that had consumed the vaccine returned positive rabies titres (>0.5 IU/ml), with a third wolf returning raised levels. All vaccinated wolves were alive 18 months after vaccine delivery. This small, controlled trial confirms the potential for *Rabigen® SAG2Dog* to effectively protect Ethiopian wolves against rabies. Due to the small size of the experiment, further extended trials will be required before oral rabies vaccination can be implemented as a proactive disease management approach.

Introduction

Rapidly expanding human populations have increased contact between wild canids and domestic dogs (Sillero-Zubiri & Switzer, 2004), with disease being the most immediate threat to wild canids, due to their shared receptivity of numerous pathogens (Woodroffe *et al*., 2004). Rabies is recognized as the most common cause of disease outbreaks in wild canids (Funk *et al*., 2003; Woodroffe *et al*., 2004), having caused dramatic population declines of African wild dogs (*Lycaon pictus*) (Gascoyne *et al*., 1993; Hofmeyr *et al*., 2004) and Blanford's foxes (*Vulpes cana*) (Geffen, 1994). Rabies has also impacted upon Ethiopian wolves (*Canis simensis*) in the Bale Mountains with devastating effects. Large epizootics were reported in 1991/92 (Sillero-Zubiri *et al*., 1996b), 2003 (Randall *et al*., 2004) and 2008/09 (Johnson *et al*., 2010; Chapter Three), killing 65-75% of affected sub-populations. These recurring epizootics demonstrate that rabies remains a major and immediate threat to the endangered wolves. With less than 450 adults and sub-adults remaining in just six enclaves in the Ethiopian highlands, Ethiopian wolf populations are susceptible to dramatic declines and even local extinction (Haydon *et al*., 2002; Marino *et al*., 2011).

Disease outbreaks that threaten endangered carnivores have highlighted the need to integrate disease epidemiology with carnivore conservation (Breed *et al*., 2009; Delahay *et al*., 2009; Cleaveland *et al*., 2007; Haydon *et al*., 2006). Throughout Africa, domestic dogs act as the principal reservoir for rabies (Talbi *et al*., 2009), with the World Health Organisation (WHO) recommending parenteral or injectable vaccination of dog reservoirs where possible (WHO, 2004). In 1996, following the 1991/92 rabies epizootic in wolves, the Ethiopian Wolf Conservation Programme (EWCP) began a vaccination campaign against rabies, targeting the dog reservoir in Bale using parenteral vaccines in an attempt to create a *cordon sanitaire* of inoculated dogs in and around wolf habitats (Laurenson *et al*., 1998). However, intense and repeated rabies epizootics in wolves have occurred despite vaccinating over 68,000 dogs since 1996 (EWCP, 2012), suggesting that the vaccination of dogs alone is not a suitable solution for managing the threat that rabies poses to this species.

Other approaches to managing disease have been considered. For example, parenteral vaccination of wild canids (Hofmeyr *et al*., 2004) has been shown to reduce losses during an

outbreak. The emergency parenteral interventions of Ethiopian wolves in 2003 and 2008/09 limited the spread of rabies in this species when compared to observed disease spread and losses in 1991 (Randall *et al*., 2006; Knobel *et al*., 2008; Chapter Three). However, these interventions are costly and, more critically, cannot protect individuals once they have been infected. Furthermore, due to their low numbers, capture and handling of wolves should be kept to a minimum, eliminating routine parenteral vaccination of wolves as a serious option to directly protect them.

The extinction risks faced by small Ethiopian wolf populations due to rabies are extremely high (Haydon *et al*., 2002), and any policy to secure their long term future would necessitate proactive disease control management. Similarly, intensive disease management has been required to protect the Critically Endangered island fox (*Urocyon littoralis*) against canine distemper virus through proactive vaccination (Timm *et al*., 2000). Oral rabies vaccination was identified as a key conservation tool for Ethiopian wolves in the 10-year National Action Plan (EWCA, 2011, Randall *et al*., 2006; Sillero-Zubiri & Macdonald, 1997), as it could allow wolves to be vaccinated proactively and cost-effectively, whilst reducing unnecessary handling of a significant proportion of a very rare and endangered species. Based on current monitoring estimates in the Bale Mountains National Park (BMNP) (Gordon *et al*., 2013), a minimum of 117 wolves from 58 packs would have to be handled for parenteral vaccination every 2-3 years to comply with modeling recommendations of 20% coverage (Haydon *et al*., 2002).

Oral rabies vaccines have been extensively and successfully used to control rabies in red foxes (*V. vulpes*) in Europe (Winkler & Bogel, 1992), as well as for other wildlife species (Bingham *et al*., 1999; Follmann *et al*., 1992; Hammami *et al*., 1999a). Two types of oral rabies vaccine are available; modified live attenuated virus vaccines and genetically modified recombinant (GM) vaccines. In the Ethiopian context, current legislation dictates that GM vaccines cannot be imported and utilised in the country, and thus modified live attenuated virus vaccines are the only feasible oral vaccine solution. While all types of commercially produced vaccines appear similarly effective in protecting red foxes from rabies (Artois, 1993), one attenuated double mutant vaccine strain, SAG2 (Avirulent-Gif Street Alabama Dufferin), has been widely used, and has proved safe and effective (Aubert *et al*., 1994; Hammami *et al*., 1999a). SAG2 has been

tested on more than 30 target and non-target species, including carnivores, primates, rodents and birds, and is one of only two vaccines, along with the recombinant VRG vaccine, to be recommended by the WHO specifically for oral vaccination (WHO, 2004).

This chapter describes a field trial to test the feasibility for Ethiopian wolves of the oral *Rabigen® SAG2Dog* vaccine, manufactured by *Virbac* (Carros, France). In order to determine whether oral vaccination is feasible in Ethiopian wolves, trials were conducted to determine preferences and vaccine delivery potential for a range of commercial and locally available baits. The preferred bait option and delivery method were then used to test the uptake rate and effectiveness of the oral vaccine *Rabigen®* SAG2Dog in one pack of wolves as a pilot experiment. Success would provide a basis for a more extended trial to assess the feasibility of using a full-scale oral vaccination programme to protect Ethiopian wolves from rabies, in accordance with Ethiopian Government permissions.

Methodology

The Bale Mountains of South-Eastern Ethiopia harbour the largest population of Ethiopian wolves, distributed across three major sub-populations (Fig. 6.1). Bait preference trials were conducted in the sub-populations of Web Valley, Sanetti and Morebawa, where wolves occur at high densities of up to 1.4 adults and sub-adults/km² (Marino *et al*., 2013). Wolves are classed as juvenile up to one year and sub-adult up to two years, while full adult appearance is attained at two years (Sillero-Zubiri *et al*., 2004). The Tarura pack selected for this vaccine trial is found in the Web Valley (Fig. 6.1).

Testing Bait Preference

Rabigen® SAG2Dog is produced as a vaccine sachet within a commercial liver-based bait matrix, specifically designed to maximise uptake in domestic dogs. The liver-based composite of these baits is not a usual component of the wolf diet, which feed almost exclusively upon diurnal small rodents of the Afroalpine grassland community (Sillero-Zubiri & Gottelli, 1995a). Wolves will scavenge on occasion, but dogs and jackals tend to monopolize carcasses (Sillero-Zubiri & Gottelli, 1995a).

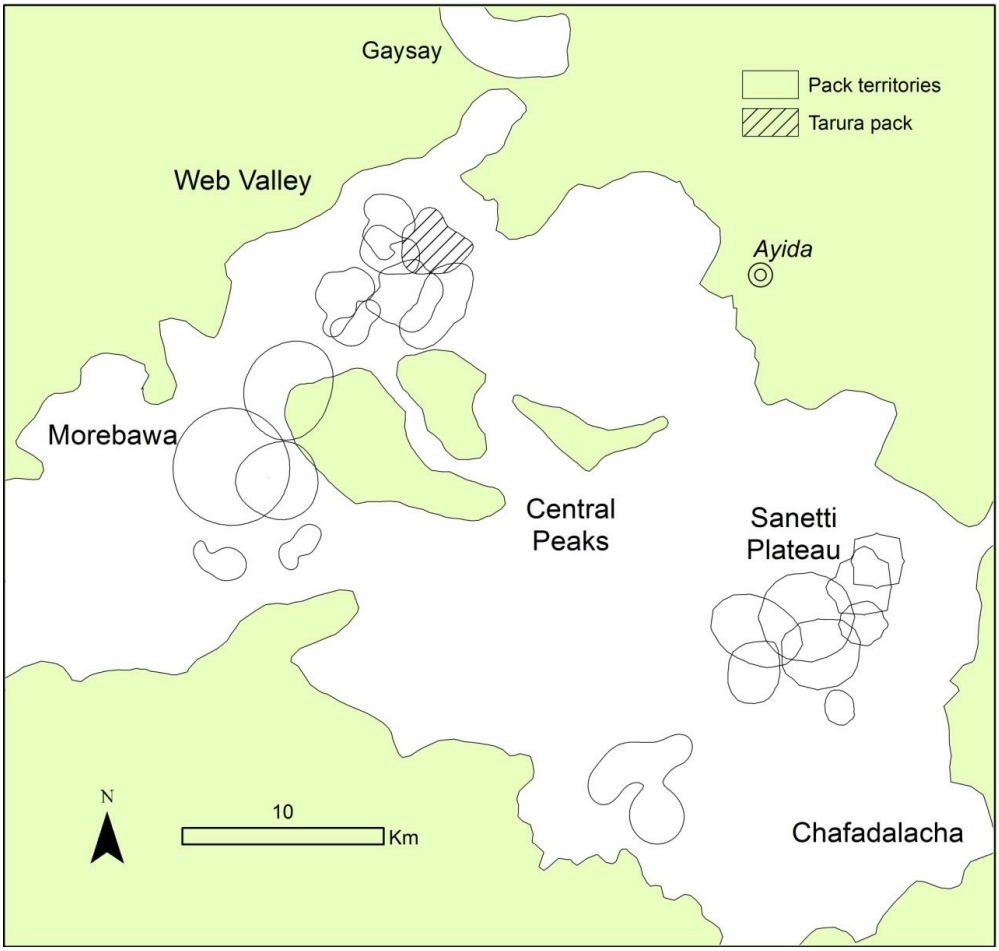

Figure 6.1: *The Bale Mountains National Park, showing polygons of focal pack territories in high density wolf sub-populations, including Tarura, the trial pack in Web Valley.*

We tested the commercial bait against three local baits (Fig. 6.2): goat meat (successfully used for trapping wolves - Sillero-Zubiri, 1996); boiled intestines (Estrada *et al*., 2001); and grass rats, *Arvicanthis blicki*, killed humanely using the *Procter Pest-Stop™* metal rat trap (Baker *et al*., 2011). This is a locally abundant species which is well represented in the wolves' diet (Sillero-Zubiri & Gottelli, 1995a). All baits contained placebo vaccine sachets, which were inserted into the rats' thorax, and into the goat meat, with the openings closed using absorbable sutures. Bait uptake rates were compared between two delivery methods: baits delivered at random in the landscape, and targeted delivery from horseback.

Delivering baits

First, baits were distributed at random in the landscape in eleven pack territories, between June-September 2011, including seven packs from Sanetti, three from Web Valley and one

from Morebawa. Bait stations were chosen at random in an open area within the range of a chosen pack, at least 200m apart to reduce the likelihood that the scent of one bait would attract the target animal to another bait, while still ensuring that all baits could be observed from a vantage point. Following a randomly generated order, each of the four bait types was placed in the open within 5m of a wooden bait station marker, which acted as a reference point. Once all baits had been placed, they were watched over a four hour period by an observer, or until all baits had been consumed.

***Figure 6.2**: (clockwise from top left) commercially-available placebo bait (Rabigen® SAG2Dog); placebo inserted into boiled intestines; inserted into a chunk of goat meat; inserted into Arvicanthis blicki.*

Any terrestrial carnivore (including dogs) or raptor that passed within 20m of the bait was recorded with time noted, their response to the bait (ignored/seen/sniffed/licked/eaten), and whether there was any delay in consumption. Successful vaccine delivery involves the sachet being pierced, and kept in the mouth for the longest possible period, so that the vaccine can be absorbed in the oral mucosa (Orciari *et al*., 2001). To capture this information, the time taken to chew a bait was noted.

A second delivery method, termed targeted delivery, was tested, with baits delivered from horseback directly to individual wolves. The three local bait types were used, but the commercial bait was dropped from this experiment owing to its poor uptake in the random delivery trial. Transects of 1-3km were ridden in parallel by 2-3 observers through a wolf pack's territory. When a wolf was found, an observer approached to within 20m of the wolf with a pre-determined bait, chosen by a randomly generated order, and presented the bait to the wolf, before the deliverer retreated. A second observer recorded the interaction from a distance of 50m, noting the wolf's reaction to the bait including whether it approached to within 5m, whether it sniffed or ate the bait, and recording the wolf's age, sex and individual identification if known.

Delivering Rabigen® SAG2Dog to the wolves

The trial delivery of *Rabigen® SAG2Dog* to Ethiopian wolves was conducted on Tarura pack in November 2011. At the time of the trials, Tarura was composed of eight wolves: two adult males, an adult female, four sub-adult males and a sub-adult female. This pack was chosen for several reasons. Tarura is an out-lying pack of the Web Valley, sharing a small border with only one other pack, thus further minimising the negligible risk of transmission to other wolves if, under very unlikely circumstances, the vaccine reverted to virulent form. Furthermore, only one individual in the pack had previously been vaccinated against rabies during the emergency interventions in 2008 (Chapter Three), while the five sub-adults were not alive during the 2008 rabies outbreak and would thus likely be naive to the rabies virus.

The *Rabigen® SAG2Dog* oral vaccines were stored at -30°C until the week of the trials, thereafter they were kept in cool boxes with ice bricks until each vaccine was prepared. Fresh bait/vaccine combinations were prepared each day for delivery using the preferred meat bait (Fig. 6.3). The vaccine sachet was removed from the commercial bait matrix, inserted into the meat bait, with the opening sealed using absorbable sutures. A serum biomarker, iophenoxic acid, was injected into the bait surrounding the vaccine sachet before delivery to confirm ingestion of the bait, as has been used in other field trials of oral vaccines (Fletcher *et al*., 1990; Fry & Dunbar, 2007). The biomarker was dissolved in 20% ethanol, with 3ml of the

solution injected into each bait. Baits were delivered to wolves by two methods: delivery at a rendezvous area at a wolf den where the pups had very recently emerged (~3 weeks old), and a targeted delivery from horseback or vehicle. Vaccines were monitored at all times once delivered, and were removed and disposed of safely if not consumed.

***Figure 6.3**: Preparation of oral rabies vaccines (Rabigen® SAG2Dog) within the preferred meat bait: (clockwise from top left) removing the vaccine sachet from the commercial matrix; inserting the vaccine sachet into the meat bait; sealing the opening with absorbable sutures; and injecting the bait with a serum biomarker.*

Post-Vaccination rabies titres

Efficacy of the vaccine, uptake rates and bait consumption were calculated by analysing blood sera for rabies-neutralising antibody titre levels and serum biomarker levels. Two weeks after vaccine delivery, wolves were captured following detailed standard trapping protocols (Sillero-Zubiri, 1996; Knobel *et al*. 2008) with blood samples taken. Rubber-lined *Soft Catch*™ leg-hold traps (Woodstream Corporation, PA, USA) were deployed in sets of three, and checked every 2–3 hours day and night. Trapped wolves were immediately covered with a blanket to induce passivity and subsequently immobilized using a Ketamine/Medetomidine

combination with Atipamezole reversal. Once sedated, 5-10ml of blood was taken from each individual. All captured wolves were ear-tagged to prevent multiple anaesthetisations, and for monitoring identification. Any naive individuals that had not been delivered the oral rabies vaccine, were inoculated with a 2ml dose of *Nobivac* rabies vaccine to provide rabies protection (Knobel *et al*., 2008). Trapped animals were closely observed to monitor health post-capture. After collection, blood samples were spun in a centrifuge and separated into serum and blood clot.

Blood and serum samples were coded and shipped to the United Kingdom for analysis at the Animal Health and Veterinary Laboratories Agency (AHVLA). Rabies-neutralizing antibody titres of sera samples were assessed using the gold standard fluorescent antibody virus neutralization (FAVN) test (Cliquet *et al*., 1998). A constant volume of rabies virus (CVS-11, 100 $TCID_{50}$/50µl) was added to serial dilutions of serum in quadruplicate. The 50% endpoint dilution, where neutralization of the virus ceased, was calculated with the Spearman-Kärber method. The virus dose was checked by back-titration on every test and results were rejected if virus dose was outside pre determined limits. Serum titres were converted into international units (IU/ml) by comparison with a standard control serum. The threshold for adequate response to rabies vaccination is internationally recognised to be 0.5 IU/ml (OIE, 2011). Additionally, samples were analysed for the presence of canine parvovirus (CPV) and canine distemper virus (CDV) antibodies using standard ImmunoComb ELISA tests.

Tests were run to detect iophenoxic acid (IPA) to confirm ingestion of the bait. A detection method for IPA was developed using an Agilent 6410 Triple Quadrupole mass spectrometry system equipped with Agilent 1200 nanoHPLC system and HPLC chip. Calibration standards were developed based on human serum spiked with known quantities of IPA, ranging from 6 ng-6µg/ml serum (α-Ethyl-3-hydroxy-2,4,6-triiodohydrocinnamic acid, 361046 - 97%, SigmaAldrich). Each serum sample was processed in triplicate (100 µl). Samples were prepared as previously described (Jones, 1994; Wiles & Campbell, 2006; Ballesteros *et al*., 2010). Briefly, to each 100µl serum aliquot, 0.6ml of acetonitrile and 0.2ml of 0.33M sulphuric acid were added, followed by 0.2ml 10% sodium tungstate. Samples and standards were left to stand for 15 minutes at room temperature then frozen overnight at -20˚C.

Samples were then centrifuged and 1ml of supernatant from each sample was transferred to a clean tube. Supernatants were re-centrifuged to remove any remaining particulate matter. From each supernatant, 10µl was taken and mixed with 30µl 5mM ammonium formate in an autosampler vial. From the resulting mixtures, 1µl was injected onto the HPLC chip for IPA detection by mass spectrometry. Data were analysed using MassHunter Quantitative Analysis Version B.03.01, and IPA concentrations in samples were calculated by extrapolation from the calibration standards using Prism 5 for Windows v5.01.

Statistical Analysis

Statistical analyses were run in SPSS 14.0. Contingency tables were used to test the significance of observed differences between observed consumption of baits and species. Wald chi-squared values were calculated for testing the effect of bait and species on bait consumption (a binary response) using logistic regression.

Results

Random bait delivery

A total of 116 experiments to deliver baits at random in the landscape were conducted within the territory of eleven wolf packs, using four different placebo bait types. Once deployed, each bait type was observed for between 293-330 hours (Table 6.1).

***Table 6.1**: Summary of the experiments offering four different placebo bait types during random delivery trials and the responses of various species. Time represents the total time that particular bait type was observed while deployed.*

Bait	Time	Ethiopian wolf			Domestic dog			Raptor	
	(Hours)	Walk Only	Walk and Sniff	Walk and Eat	Walk Only	Walk and Sniff	Walk and Eat	Walk Only	Walk and Eat
Commercial	330.4	5	1 (17%)	0 (0%)	0	1 (50%)	1 (50%)	1	0 (0%)
Intestine	324.6	0	5 (71%)	2 (29%)	2	0 (0%)	3 (60%)	0	2 (100%)
Meat	313.2	4	0 (0%)	4 (50%)	0	0 (0%)	2 (100%)	0	3 (100%)
Rodent	293.5	6	1 (11%)	2 (22%)	1	0 (0%)	1 (50%)	1	1 (50%)

Six wolves passed within 20m of the commercial bait, one of them stopping to sniff the bait (16.6%), but none of these baits were eaten. Significantly more dogs than wolves sniffed or

consumed the commercial bait during the trials (Table 6.1, $\chi^2_1(N=8) = 4.44$, $p<0.05$). Intestine baits were sniffed by all seven wolves that passed within 20m. Two wolves ate these baits, but both dropped the placebo sachet unpierced during consumption. Intestine baits were consumed by 60% of dogs (n=5), although two of the dogs dropped the sachet from the intestines, untouched. Of the eight wolves that walked within 20m of a meat bait, four wolves sniffed and consumed the baits (50%), while only 33% of wolves that passed within 20m (n=9) sniffed a rodent bait, and 22% ate one. When analysing only wolf encounters, the meat bait was consumed most, and the commercial bait had lowest uptake (Wald $\chi^2_3(N=62) = 7.31$, $p=0.06$). When analysing all encounters, both the bait type (Wald $\chi^2_3(N=95) = 13.77$, $p<0.005$) and which species encountered the bait (Wald $\chi^2_2(N=95) = 15.47$, $p<0.005$) had significant effects on bait consumption. Raptors consumed the most baits per encounter, while wolves consumed the least (Wald $\chi^2_1(N=73) = 11.41$, $p<0.005$). The meat bait had the highest encounter to consumption rate, while the commercial bait had the lowest. There were also significant differences between the consumption of meat and rodent baits (Wald $\chi^2_1(N=34) = 5.72$, $p<0.05$).

No commercial baits were eaten by wolves during 330h of observation (Table 6.1). Intestine baits took an average of 162h field observation to be consumed by a wolf, compared with 147 hours for a rodent bait, and 78 hours for a meat bait.

Targeted bait delivery

Eighty-three transects were surveyed in the territories of nine Web Valley and Morebawa packs over 40h, covering 172km at an average of 2.1km per transect. A total of 68 wolves were spotted, with pre-selected placebo baits delivered to 44 of these.

***Table 6.2**: Uptake of three bait types delivered from horseback during the trial.*

	Total wolves	**Approach <5m**	**Sniff**	**Eat**
Intestine	15	11 (73.3%)	4 (26.7%)	6 (40%)
Meat	17	13 (76.5%)	4 (23.5%)	9 (52.9%)
Rodent	12	7 (58.3%)	3 (25%)	2 (16.7%)
Total	44	31 (70.4%)	11 (25%)	17 (38.6%)

Overall, the evidence was weak for differences in uptake rates across bait types (χ^2_2(N=44) = 3.9, p=0.14). Meat baits had the highest successful uptake rate (53%), as well as the highest rate of approach (77%). Seventy-three percent of wolves (n=15) approached intestine baits, with six wolves (40%) eating one, but of these, one wolf spat out the placebo sachet. Fifty-eight percent of wolves (n=12) approached the rodent bait within 5m but only 17% ate the rodent bait. There was significantly higher consumption of meat baits during targeted delivery than rodent baits (χ^2_1(N=29) = 3.93, p<0.05). A meat bait was consumed every 1.7h, while an intestine bait was eaten every 2.3h, and a rodent bait every 5.5h.

Oral vaccine delivery trial

The *Rabigen® SAG2Dog* oral rabies vaccine was delivered within the preferred meat bait to the Tarura pack. Vaccine baits were delivered successfully to five of eight adult and sub-adult wolves between 3rd-8th November 2011, an adult female and four sub-adult males (Table 6.3). Baits were rejected by both adult males, while there was no opportunity to deliver a bait to the sub-adult female. One sub-adult male (TAR05) cached two baits, and was seen retrieving and consuming them three days later. Delivery at the den had to be abandoned due to one sub-adult male (TAR07) monopolising the consumption of all baits, and was replaced with targeted delivery from vehicle and horseback.

***Table 6.3**: Summary of oral vaccine bait delivery to Tarura pack wolves during a pilot trial in Web Valley. Summary of rabies-neutralising antibody titres and levels of serum biomarker iophenoxic acid (IPA) after trapping. Internationally recognized threshold for adequate response to rabies vaccination is 0.5 IU/ml (OIE, 2011).*

Wolf Individual	Rabies FAVN (IU/ml)	IPA (ug/ml)	Baits Eaten	Comments
AM (TAR01)			0	Refused baits
AF (TAR02)	0.29	61.2	2	Ate at den
AM (KOT31)			0	Refused baits
SAM (TAR03)	1.14	43.0	5	Ate five baits
SAM (TAR05)	0.06	17.7	4	Ate two, cached two and ate 3 days later
SAM (TAR07)	0.87	75.4	9	Ate eight, one sachet not pierced when eaten
SAM (TAR09)			1	Ate one bait
SAF (TAR04)	0.06	0.2	0	Only seen for 5 minutes, bait not delivered.

Post-vaccination titres

Tarura wolves were trapped between the 21st-24th November 2011 using five trap sets totalling 311 hours of trapping effort. Five individual wolves were caught (one adult female, three sub-adult males and one sub-adult female) and blood samples collected, with three of these individuals re-captured. On average, it took 62.2 trap hours to catch an individual wolf, and 38.9 trap hours per wolf capture. The sub-adult female (TAR04) had not ingested any baits, and served as a "naive" control.

Positive levels of IPA in all four individuals that consumed baits during the oral vaccine delivery confirmed bait uptake; control TAR04 tested negative as expected (Table 6.3). IPA levels in one of the sub-adult males (TAR05) were lower than in the other three wolves. Two wolves returned positive results for rabies antibodies, two returned negative results (including TAR04) and one showed raised antibody levels. There was a correlation between bait uptake, as demonstrated by serum biomarker levels, and vaccine seroconversion, as shown by rabies-neutralising antibody titres (r=0.62, p=0.2) (Fig. 6.4).

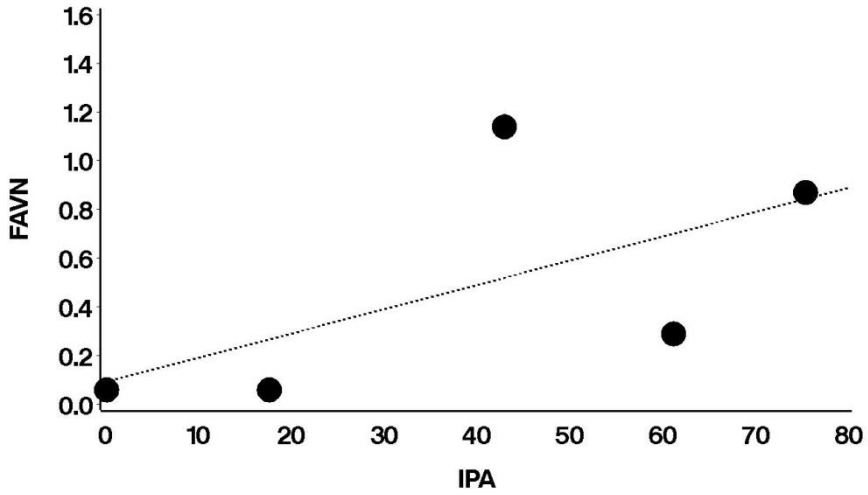

Figure 6.4: *Levels of the serum biomarker (IPA) and rabies-neutralising antibody titres (FAVN) in blood sera from five sampled wolves from Tarura pack.*

All five wolves returned positive results for CPV (S>3 IgG), while one wolf (TAR02) returned positive results for CDV (S>3 IgG). All wolves that consumed oral vaccines, and were subsequently captured, are still alive and healthy 18 months after consuming the vaccine.

Discussion

The rapid and successful delivery of oral rabies vaccines to free-ranging large carnivores requires a suitable bait as the delivery medium. Thus, in preparation for this field trial, different bait types were tested to identify the most efficient one. The liver-based matrix of commercial (*Rabigen® SAG2Dog*) baits is not a usual component of the wolf's diet (Sillero-Zubiri & Gottelli, 1995a), which would partly explain why there was no uptake of this bait by wolves in 330h of field offer. The texture of the bait was also an important consideration as the commercial liver-based matrix easily crumbled when bitten, with the vaccine sachet often discarded (Chapter Five). As a result, the commercial bait was removed from the targeted trials. During both random and targeted trials, wolves removed the vaccine sachet from the intestine bait without biting and piercing the sachet. From both trials combined, the vaccine sachet was not pierced for 37.5% of intestine baits consumed by wolves. This reduced that bait's ability to successfully deliver the vaccine, with the estimated uptake rate of the vaccine reduced from 29-40% to 18-25%.

Some wolves struggled to locate rodent baits during the random delivery trials, as the baits did not seem to give off a strong odour. Even during the targeted trials, 58% of wolves approached the rodent bait with just 25% sniffing the bait, and only 17% consuming it. Meat baits scored highest of all bait types, and were located fastest during both random and targeted trials. Furthermore, meat baits were eaten by 50-53% of wolves that approached them. Both delivery methods showed significantly higher uptake of meat baits than rodent baits. The meat baits were consequently chosen as the preferred medium for delivering the oral rabies vaccine to the wolves during the oral vaccine trial.

Rabigen® SAG2Dog vaccines require a cold chain of at least -20˚C (P. Mahl, pers. comm.). Once the vaccines are removed from the cold chain, the vaccine's rabies titre levels rapidly reduce within one week (Aubert & Mutinelli, 2002). Targeted horseback delivery saw significantly better uptake times for all three bait types, with baits consumed in a matter of hours rather than days. Delivery of oral vaccines at the den was abandoned due to one sub-adult male in particular monopolising this "freely-available" food resource, consuming a total of nine vaccine baits. While the vaccine manufacturers have conducted overdose trials

on this vaccine in other species (P. Mahl, pers. comm.), it was considered important to avoid any potential issues in such an endangered carnivore. Delivery therefore reverted to targeted delivery via horseback or vehicle, which allowed improved observation of which individuals consumed the bait, minimizing the number of baits eaten by the same wolf while allowing monitoring of the percentage of vaccination coverage in each pack. Modelling shows that pack persistence can be ensured with vaccination of over 20-40% of wolves in each pack, depending on the population size (Haydon *et al*., 2002). Targeted delivery also reduced the number of baits consumed by non-target species, such as raptors and domestic dogs, to zero.

Attenuated rabies viruses, such as SAG2, replicate in local tissues of the oral cavity, and can be cleared relatively quickly, leading to protective immunity (Orciari *et al*., 2001). To ensure maximum absorption of the vaccine, the pierced sachet must remain in the oral cavity for as long as possible so as to maximise the chance that the liquid vaccine comes into contact with the oral mucosa. From the bait preference trials, the meat bait presented the longest opportunity for vaccine absorption in the oral mucosa. The wolf occasionally noticed the presence of an alien sachet in the bait and would manoeuvre the bait to spit out the sachet. The delivery of the oral vaccine could be improved by further securing the sachets directly to the meat bait with absorbable sutures piercing the sachet rim, making it harder to spit out the sachet, and thus lengthening the potential for vaccine contact on the oral mucosa.

Three of the five sampled wolves had titres below the 0.5 IU/ml threshold: control TAR04 as expected, and TAR05 both had titres of effectively zero. The latter returned low levels of iophenoxic acid (a measure of bait uptake) and might not have pierced the vaccine sachet during consumption. Serum biomarker (iophenoxic acid) levels and vaccine titres were correlated (Figure 6.4). TAR05 had been seen to cache two vaccine baits, and consumed two baits. TAR02 had a raised rabies-neutralising antibody titre of 0.29 IU/ml, while TAR03 and TAR07 both returned positive rabies-neutralising antibody titres above the threshold. Although TAR02 had a lower titre than the threshold, this may still represent a response to the vaccine, or consumption of lower levels of vaccine. Regarding the threshold for protection, there is unpublished evidence that a titre of 0.2 IU/ml, following vaccination

with *Rabigen® SAG2Dog*, may provide adequate rabies protection for dogs (P. Mahl, pers. comm.). At this reduced threshold, 75% rather than 50% of wolves that consumed the vaccine were likely to have received adequate protection against rabies infection.

It is worth noting that in similar trials of SAG2 in dogs, some animals that had consumed the vaccine but had titres below the 0.5 IU/ml threshold were still protected against a rabies challenge (Cliquet *et al*., 2007). In a study in Indian stray dogs, just 31% (n=13) of dogs' sero-converted titres at day 28, and yet in nine post-mortem samples from euthanized dogs taken after a rabies challenge, none were rabies positive while 100% of five control dogs were rabies positive (Cliquet *et al*., 2007). Trialling the vaccine under field conditions on a very rare endangered species, we were obviously unable to deliver a rabies challenge to Ethiopian wolves. As such, it is not clear whether the SAG2 vaccine's effectiveness was more far-reaching than the 50% positive samples suggest. Given the Ethiopian wolf's genetic similarity with dogs, as evidenced by fertile hybrids between the two (Gottelli *et al*., 1994), it is likely that wolves may respond similarly to dogs from an immunogenic perspective.

All five wolves returned positive results for CPV, suggesting a potentially higher rate of CPV prevalence in Ethiopian wolves than initially described by Laurenson *et al*. (1998). One wolf (TAR02) returned positive results for CDV. This female was the only individual of the five that was alive during the CDV epizootic in 2010, and this result confirms that she was exposed to, but survived, the virus during this time, as discussed in Chapter Four.

It is essential that any vaccine proposed for immunising an endangered species must meet a rigorous safety protocol. The SAG2 rabies vaccine used in the pilot study proved both safe and effective. All wolves that consumed the oral vaccines were still alive and healthy more than 18 months later. At least two wolves produced antibodies against rabies after ingestions of the oral rabies vaccine, with a third wolf showing a detectable but lower antibody response. These results confirm the potential of *Rabigen® SAG2Dog* as a successful vaccination method against rabies for the Ethiopian wolf. These promising outcomes and the lack of any apparent negative impact of vaccine bait consumption open the way for an extended trial in the Bale Mountains. We propose an extended, thorough trial on 35 wolves across ten packs,

with baseline and post-vaccination collection of blood samples to test for before and after changes in rabies-neutralising antibody titres. Ideally, future trials would test the safety and efficacy of VRG, in addition to SAG2. The recombinant VRG vaccine has passed WHO safety requirements (WHO, 2004), and if effective in wolves, provides logistical and cost benefits to the live attenuated vaccine. This second trial will establish relevant demographic and epidemiological parameters, as well as financial considerations around vaccine delivery, to inform future population-level oral rabies vaccination. Once a full trial confirms its usefulness, oral vaccination will enable a shift in the rabies management paradigm for Ethiopian wolves. Reactive responses that limit disease-related mortalities may be replaced by proactive actions for effective disease control and higher wolf survival**.**

CHAPTER VII

General Discussion

Discussion

Rabies poses an immediate and present threat to Ethiopian wolves in the Bale Mountains and possibly elsewhere in a handful of small, more vulnerable populations. The rabies epizootics in 2008 and 2009 caused mortality levels comparable to those in previous major epizootics (Randall *et al*., 2004; Sillero-Zubiri *et al*., 1996b; Chapter Three), and raised concerns that these recurrent epizootics can be more frequent than previously thought, with important consequences for wolf population dynamics. Such intense and repeated rabies epizootics have occurred despite the vaccination of over 68,000 domestic dogs against rabies in Bale from 1996 to present, aimed at protecting nearby Ethiopian wolves (EWCP, 2012). The recent rabies outbreaks reported here most likely resulted from two separate introductions of the same rabies virus strain from the domestic dog reservoir to the sympatric wolves, as indicated by virus nucleoprotein-coding sequencing (Johnson *et al*., 2010). During both outbreaks, intensive monitoring allowed for the early detection of wolf carcasses, sampling and subsequent diagnosis.

Emergency interventions, involving the capture and parenteral vaccination of 98 wolves in 20 packs adjacent to infected packs, achieved the intended objective of slowing, or even stopping, the spread of rabies in the wolf population. Vaccination coverage attained or exceeded the 40% vaccination coverage target recommended by simulation models to ensure pack persistence (Haydon *et al*., 2002), in all but one pack. Female wolves were vaccinated in 15 of 20 packs, meeting the minimum objective of protecting at least a breeding pair in each pack (Chapter Three).

The narrow valley connecting the Web Valley and Morebawa to the South is of strategic importance when considering disease transmission, and the 2008 intervention focused on vaccinating wolves around this corridor to prevent the expansion of the epizootic that originated in the Web. This was a strategy that proved successful in 2003 (Knobel *et al*., 2008), as confirmed by post-hoc models that showed that vaccination of individuals in corridors had reduced the probability of a catastrophic meta-population impact (Haydon *et al*., 2006). Detailed demographic and epidemiological data, coupled with quantitative analyses, provided the practical guidance needed for successful control interventions, and

showed that even low levels of vaccination were effective in controlling the spread of rabies (Knobel *et al*., 2008; Haydon *et al*., 2006).

CDV has also been suspected of affecting domestic dogs and wolves in Bale (Laurenson *et al*., 1998). CDV epizootics were reported in 2005/06 and 2010 (Chapter Four), with mortality rates of 43-68% confirming its relevance as an additional threat that may compound the impact of rabies on population dynamics. The genetic identity of the virus and the close proximity between wolf range and the nearby Ayida village, where CDV affected dogs, makes it likely that the epizootic in wolves in 2005/06 originated in this village, supporting the assertion that domestic dogs are the reservoir host for the canine distemper virus (Cleaveland *et al*., 2000).

The 2010 CDV epizootic spread through all areas of the Bale Mountains, moving temporally and geographically from Morebawa North to the Web Valley and East to Sanetti, across narrow valleys that act as geographical bottlenecks, and through lower wolf density areas between these sub-populations. Inter-pack contact rates are reduced by low connectivity between packs within geographical bottlenecks, reducing the probability of disease transmission through these areas (Sterner & Smith, 2006). That the CDV virus managed to spread from Morebawa to Sanetti serves to confirm the severity of the 2010 epizootic. The confirmed CDV mortalities, combined with unknown mortality from lower density areas, points at this epizootic as the single most catastrophic disease event recorded for Ethiopian wolves to date.

Detailed demographic data and extensive monitoring during disease outbreaks allows an understanding of pack and population dynamics in response to increased epizootic frequencies as well as the occurrence of rabies and CDV epizootics in rapid succession (Marino *et al*., 2006; 2013). Mortality rates during the CDV epizootics were three times higher in sub-adults than adults, and higher still in juveniles, although mortality in this age class is counteracted by typically high natural mortality. The high CDV mortality observed exceeded by two to four times (Chapter Four) the predictions of earlier PVA and simulation models, which were based on lower mortality rates (15-20%), and which suggested that CDV

would have little impact on wolf population or pack persistence (Haydon *et al*., 2002; Mace & Sillero-Zubiri, 1997). Haydon *et al*. (2002) did however suggest that the impact of CDV might become more important if mortality rates exceeded 40%. While the mechanism by which CDV affects wolf reproductive behaviour is unclear, the CDV outbreaks described here reduced breeding success in Sanetti, with fewer packs breeding successfully after both the 2005/06 and 2010 epizootics, slowing the initial recovery of the population. The 2008 rabies outbreak in Web Valley was followed by the 2010 CDV outbreak, before the population had fully recovered, and four of seven wolf packs there went extinct. Thus, it would seem that the combined impact of both diseases severely affected pack persistence, exacerbating the typically slow recovery pattern of wolf populations (Marino *et al*., 2013; Chapter Four). The observed pack losses confirmed the predictions of Haydon *et al*. (2002) that the probability of pack extinctions would be greatly increased as inter-epizootic periods decline.

Because reproduction in Ethiopian wolf packs is monopolised by the dominant female and because pack fission is rare (Sillero-Zubiri & Gottelli, 1995b; Sillero-Zubiri *et al*., 1996a), a reduced number of breeding units as a consequence of an epizootic can slow population growth and recovery (Marino *et al*., 2006; 2013), even though large litter sizes and high juvenile survival may occur following a decrease in population density (Marino *et al*., 2013; Vucetich *et al*., 1997). This slow population growth is exacerbated by the delay resulting from a time lag in pack formation. The size and structure of the Bale Mountains' wolf population, which accounts for over half of the species' global population, lends robustness to wolf numbers and provides greater resilience against disease catastrophes (Haydon *et al.*, 2002). Two new packs formed in the Web Valley following the 2010 CDV outbreak, with several of the founder members coming through the corridor from East Morebawa, confirming that corridors facilitate migration and recovery after epizootics (Hess, 1996) and minimize genetic drift (Randall *et al*., 2010). Repeated population crashes may eventually reduce genetic diversity of the 250-strong Bale wolf population, but more worryingly, have the potential to decimate and even extirpate smaller wolf populations elsewhere.

The severity of recurrent disease epizootics in the Bale wolf population, and the fragility of Ethiopian wolf populations elsewhere, highlight the need to improve current disease control

policies in the context of Ethiopian wolf conservation. Despite the apparent ability of the wolf population in Bale to recover from recurrent epizootics, there is a distinct possibility that outbreaks would become more frequent, more widespread and would show higher mortality rates in the absence of any disease management and control actions. A long-term disease management plan has been developed for tackling both rabies and CDV as threats to the Ethiopian wolf (EWCA, 2011; Randall *et al*., 2006), with vaccination of both host and target populations central to the effectiveness of this strategy (EWCA, 2011; Randall *et al*., 2006; Sillero-Zubiri & Macdonald, 1997).

The relationship between disease reduction in dogs and wolf population persistence appears approximately linear in model predictions over 50 years (Haydon *et al*., 2002). To date, efforts to protect wolves from disease have concentrated on the parenteral vaccination of the domestic dog reservoir, combined with emergency vaccination of wolves in response to rabies outbreaks. The World Health Organisation (WHO) recommends using parenteral rabies vaccines on the domestic dog reservoir as a disease control method where possible (WHO, 2004). Parenteral vaccination of dogs against CDV restarted in Bale following the 2010 CDV epizootic. CDV vaccines for wild species are not currently at the same stage of development as rabies vaccines, although trials of the Nobivac D and P antigens have been conducted on wild carnivores, including lions (*Panthera leo*) (Kock *et al*., 1998), red foxes (*Vulpes vulpes*) and northern raccoons (*Procyon lotor*) (D. Sutton, pers. comm.), with no adverse effects recorded. Parenteral vaccination of endangered wild canids can control spillover infections and lessen the severity of outbreaks (Hofmeyr *et al*., 2004; Knobel *et al*., 2008). A small-scale trial on a handful of wolves would allow controlled testing of a CDV vaccine, and determine its feasibility should future emergency interventions against CDV be required.

Rabies prevalence in rural dogs in Ethiopia is high (Fedadu, 1982; Tefera *et al*., 2002). As dogs in these areas are primarily kept to protect livestock (Sillero-Zubiri & Switzer, 2004), and often are not fed properly, they have the propensity to roam in wolf range, competing with wolves for rodent prey (Gottelli *et al*., 1994), and coming into contact with their wild relatives. The high prevalence of rabies in dogs, and the frequent incursion of rabies into the

wolf population, even with ongoing large scale dog vaccination efforts, makes the development of an effective oral vaccination protocol all the more urgent. PVA models indicate that disease-induced population fluctuations and extinction risks could be markedly reduced with the vaccination of a relatively small proportion (20-60%) of wolves (Haydon *et al*., 2002), the required percentage dependent on the size of the wolf population. Oral rabies vaccination offers a potentially more efficient and economical method to inoculate both dogs and wolves against rabies, as suggested by EWCA (2011), Haydon *et al*. (2006), and Randall *et al*. (2006). Oral vaccines present the opportunity to deliver protection non-invasively, directly and pro-actively to Ethiopian wolves, as well as increasing the vaccination coverage in the "semi-feral" domestic dogs while reducing costs.

Trials of the oral vaccine *Rabigen® SAG2Dog* aimed to determine whether this vaccine would provide a realistic solution for protecting dogs in rural Ethiopia against rabies (Chapter Five). The first step in these trials involved the selection of a suitable bait medium for delivering the vaccine. In our tests, the commercial bait offered by the vaccine manufacturer was not suitable, as its liver-based matrix crumbled and easily disintegrated when chewed, allowing dogs to spit out the sachet containing the vaccine. In contrast, four local bait types had high uptake rates by dogs, with goat/sheep meat baits chewed longer than all other baits, maximising the opportunity for puncturing of the sachet and absorption of the vaccine in the oral mucosa (Orciari *et al*., 2001). While bait uptake was satisfactory, seroconversion of rabies antibodies at the conservative threshold of 0.5 IU/ml was poor, with little evidence that oral vaccines provide effective protection for domestic dogs. Effectiveness of the vaccine was investigated at a reduced protection threshold of 0.2IU/ml, based on unpublished evidence lower titres provided rabies protection after vaccination with SAG2 (P. Mahl, pers. comm.). While the majority of dogs did not sero-convert titres above the reduced threshold, there was a significant difference in baseline logged titre levels and post-vaccination logged titres in these individuals, indicating that the vaccine did have a small effect as reflected by titre levels.

The study showed high rabies seroprevalence levels in dogs prior to vaccine delivery, suggesting that they had previously been exposed to rabies. The expected anamnestic

response - an enhanced reaction when an individual has previously encountered particular antibodies - was only seen in a quarter of baseline positive individuals at either protection threshold. All considered, the low response, combined with the logistical difficulties of maintaining a -20°C cold chain for this vaccine, offered little support for *Rabigen® SAG2Dog* as an effective rabies vaccine for dogs. Previous trials of SAG2 in laboratory beagles showed slowly increasing serological responses over six months post-vaccination (Fekadu *et al.*, 1996), indicating that it might be possible that some of our trial dogs might show increased titres over time. There is evidence from other trials that SAG2 can still provide rabies protection despite low titre levels (Cliquet *et al*., 2007). Typically this is measured by introducing a rabies challenge to inoculated animals, an experiment precluded here under field conditions by the potential risks of infection and spread to non-target animals. Taking these considerations into account, it is not clear whether the vaccine's effectiveness was more far-reaching than that suggested by the low percentage of dogs with titres above the accepted threshold.

While these results do not entirely rule out SAG2 as a suitable vaccine to control rabies in rural dogs in and around wolf ranges, there are other alternatives that should be considered and tested beforehand. For instance, a trial of a thermo-tolerant recombinant oral rabies vaccine, such as VRG (virus-rabies glycoprotein), on dogs in Ethiopia has been recommended and would potentially offer a more realistic method for achieving widespread vaccination coverage of dogs in and around threatened populations of Ethiopian wolves. This was highlighted in the National Action Plan for this species (EWCA, 2011). VRG is one of two oral rabies vaccines approved by the WHO (WHO, 2004), and has been safely used to protect red foxes, coyotes (*Canis latrans*), and northern raccoons (Aubert *et al*., 1994; Brochier *et al*., 1996; Fearneyhough *et al*., 1998; Robbins *et al*., 1998).

There is evidence of variation in rabies-neutralising antibody titres for the SAG2 vaccine (Aubert & Mutinelli, 2002), and thus, despite the poor results in dogs (Chapter Five), this vaccine was also trialled in Ethiopian wolves (Chapter Six). The rapid and successful delivery of oral rabies vaccines to free-ranging large carnivores requires a suitable bait, and both a commercial bait and three local bait types were tested during this study. The commercial

liver-based bait had no uptake by wolves during 330 hours of field offer, and the earlier bait trials for dogs had also identified texture weaknesses in this bait. Of the local bait types, wolves removed the vaccine sachet from the intestine baits without piercing the sachet. Of all the local baits tested, goat meat had the highest uptake rates (50-53%) by wolves, and presented the longest opportunity for vaccine absorption in the oral mucosa due to the time recipients spent chewing the meat. Targeted delivery from horseback had better uptake rates than random delivery, while also reducing unintentional delivery to non-target species such as raptors and rodents.

Using the goat meat bait and the targeted delivery method, *Rabigen® SAG2Dog* was delivered to one pack of eight adult and sub-adult wolves in the Web Valley. Two of four wolves that consumed baits were later caught and sampled, and returned positive rabies antibody titres (>0.5 IU/ml), while a third wolf showed raised levels. There was evidence of correlations between bait consumption and vaccine seroconversion. Trialling the vaccine under field conditions on a very rare and threatened species, it was not possible to consider a rabies challenge to validate protection status and vaccine effectiveness. It is essential that any vaccine proposed for immunising a threatened, free-ranging species such as Ethiopian wolves, must meet a rigorous safety protocol. The SAG2 rabies vaccine was both safe and effective; all wolves that consumed oral vaccines were still alive and healthy 18 months later. This small, controlled trial confirms the potential of *Rabigen® SAG2Dog* as a successful vaccination method to protect wolves against rabies, opening the way for an extended trial in Bale. As a follow up to this first trial, an extensive trial on 35 wolves across ten packs was proposed, with baseline and post-vaccination collection of blood samples to test for before and after changes in rabies-neutralising antibody titres. This trial will establish relevant demographic and epidemiological parameters to inform future population-level oral rabies vaccination. Ideally, future trials would test the safety and efficacy of VRG, in addition to SAG2. If this recombinant VRG vaccine proves effective, this vaccine provides logistical and cost benefits to the live attenuated vaccine for a large-scale vaccination campaign in all wolf habitats.

Implementing a practical and effective oral vaccination campaign to reduce disease prevalence in Ethiopian wolves has significant conservation implications for other

endangered carnivores as well. While oral vaccines have been extensively used on relatively common wild carnivores, such as red foxes and coyotes (Artois *et al*., 1993; Aubert *et al*., 1994; Fearneyhough *et al*., 1998), their uses in rare, threatened carnivore species are minimal. These two preliminary trials on wolves and dogs have provided significant baseline knowledge, and represent a major step forward for protecting wolves from rabies. Once the safety and usefulness of either of the oral vaccines are further verified by an extensive field trial, oral vaccination will enable a shift in our approach to rabies management in Ethiopian wolves. Reactive responses that limit disease-related mortalities may be replaced by proactive actions for effective disease protection and greater wolf survival**.**

REFERENCES

Alexander KA, Kat PW, Wayne RK and Fuller TK. 1994. Serologic survey of selected canine pathogens among free-ranging jackals in Kenya. *Journal of Wildlife Diseases* 30: 486-491.

Alexander KA, Kat PW, Munson LA, Kalake A and Appel MJG. 1996. Canine distemper-related mortality among wild dogs (*Lycaon pictus*) in Chobe National Park, Botswana. *Journal of Zoo and Wildlife Medicine* 27: 426-427.

Almberg ES, Mech LD, Smith DW, Sheldon JW and Crabtree RL. 2009. A serological survey of infectious disease in Yellowstone National Park's canid community. *PLoS One* 4(9): e7042.

Appel MJG, Cooper BJ, Greisen H and Carmichael LE. 1978. Status report: canine viral enteritis. *Journal of the American Veterinary Association* 173: 1516-1518.

Artois M, Masson E, Barrat J and Aubert MFA. 1993. Efficacy of three oral rabies vaccine baits in the red fox- a comparison. *Veterinary Microbiology* 38: 167-172.

Atickem A, Bekele A and Williams SD. 2010. Competition between domestic dogs and Ethiopian wolf (*Canis simensis*) in the Bale Mountains National Park, Ethiopia. *African Journal of Ecology* 48: 401-407.

Aubert MFA, Cliquet F and Hammami S. 2000. De la vaccination orale du renard contre la rage à l'élimination de la rage canine. *Bull. Soc. Vét. Prat. de France* 84(1): 17-28.

Aubert MFA, Flamand A and Kieny MP. 1994. Will rabies soon disappear from western Europe? *Recherche* 25: 582-584.

Aubert MFA and Mutinelli F. 2002. *Wildlife vaccination against rabies in difficult and emergency situations and its potential impact on the environment.* FAIR CT 97 – 3515 - Final Report, 2nd edition. 24pp.

Baker SE, Ellwood SA and Macdonald DW. 2011. *A Review of Rodent and Mole Traps: Part 1 – a study on the mechanical performance of lethal spring traps.* A report to the RSPCA, March 2011. The Wildlife Conservation Research Unit, Department of Zoology, University of Oxford.

Ballesteros C, Camarero PR, Cristofol C, Vicente J, Gortazar C, de la Fuente J and Mateo R. 2010. Analysis by LC/ESI-MS of iophenoxic acid derivatives and evaluation as markers of oral baits to deliver pharmaceuticals to wildlife. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* 878(22): 1997-2002.

Bandai C, Ishiguro S, Masuya N, Hohdatsu T and Mochizuki M. 1999. Canine coronavirus infections in Japan: virological and epidemiological aspects. *Journal of Veterinary Medicine Sci* 61: 731–736.

Banyard AC, Gordon CH, Fooks AR, Sillero-Zubiri C and Johnson N. 2011. *Conservation of Canids: The Impact of Infectious Viral Disease.* In Maia AP and Crussi HF (Eds.). Wolves: Biology, Behavior and Conservation. Nova Science Publishers, New York, USA.

Barrett T. 1999. Morbilivirus infection, with special emphasis on morbiliviruses of carnivores. *Veterinary Microbiology* 69: 3-13.

Bartz CR and Montali RJ. 1987. *Canine Coronavirus.* Pp. 429-430 in Appel MJ (Ed.). Virus infections of carnivores. Elsevier Science Publishers, New York, USA.

Bingham J, Kappeler A, Hill FWG, King AA, Perry BD and Foggin CM. 1995. Efficacy of SAD (Berne) rabies vaccine given by the oral route to two species of jackal (*Canis mesomelas* and *Canis adustus*). *Journal of Wildlife Diseases* 31: 416-419.

Blancou J, Aubert MFA, Prave M and Haddad N. 1986. Influence du statut sanitaire des carnivores sur leur capacité à s'immuniser contre la rage. *Sci. Tech. Anim. Lab.* 11: 237-242.

Breed AC, Plowright RK, Hayman DTS, Knobel DL, Molenaar FM, Gardner-Roberts D, Cleaveland S, Haydon DT, Kock RA, Cunningham AA, Sainsbury AW and Delahay RJ. 2009. Disease Management in Endangered Mammals. In Delahay RJ *et al.* (Eds.). *Management of Disease in Wild Mammals.* Springer.

Brochier B, Blancou J, Thomas I, Languet B, Artois M, Kieny MP, Lecocq JP, Costy F, Desmettre P, Chappuis G and Pastoret PP. 1996. Field use of recombinant vaccinia-rabies glycoprotein virus for oral vaccination of wildlife against rabies: innocuity to several non-target bait consuming species. *Journal of Wildlife Diseases* 25: 540-547.

Butler JRA, du Toit JT and Bingham J. 2004. Free-ranging domestic dogs (*Canis familiaris*) as predators and prey in rural Zimbabwe: threats of competition and disease to large wild carnivores. *Biological Conservation* 115: 369–378.

Cabasso VJ. 1981. *Infectious canine hepatitis virus.* Pp. 191-195 in Davis JW, Karstad LH and Trainer DO (Eds.). Infectious diseases of wild mammals. Iowa State University Press, Ames, Iowa, USA.

Choquette LPE and Kuyt E. 1974. Serological indication of canine distemper and of infectious canine hepatitis in wolves *(Canis lupus* L.) in northern Canada. *Journal of Wildlife Diseases* 10: 321-324.

Cirone F, Elia G, Campolo M, Friedrich K, Martella V, Pratelli A and Buonavoglia C. 2004. Immunogenicity of an inactivated oil-emulsion canine distemper vaccine in African wild dogs. *Journal of Wildlife Diseases* 40: 343-346.

Cleaveland S. 1998. Epidemiology and control of rabies: The growing problem of rabies in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92: 131-134.

Cleaveland S, Appel MJG, Chalmers WS, Chillingworth C, Kaare M and Dye C. 2000. Serological and demographic evidence for domestic dogs as a source of canine distemper virus infection for Serengeti wildlife. *Veterinary Microbiology* 72: 217-227.

Cleaveland S, Barrat J, Barrat MJ, Selve M, Kaare M and Esterhuysen J. 1999. A rabies serosurveys of domestic dogs in rural Tanzania: Results of a rapid fluorescent focus inhibition test (RFFIT) and a liquid-phase blocking ELISA used in parallel. *Epidemiology and Infection* 123: 157-164.

Cleaveland S and Dye C. 1995. Maintenance of a microparasite infecting several host species: rabies in the Serengeti. *Parasitology* 111: S33-S47.

Cleaveland S, Kaare M, Tiringa P, Mlengeya T and Barrat J. 2003. A dog rabies vaccination campaign in rural Africa: impact on the incidence of dog rabies and human dog-bite injuries. *Vaccine* 21: 1965–1973.

Cleaveland S, Mlengeya T, Kaare M, Haydon DT, Lembo T, Laurenson MK and Packer C. 2007. The conservation relevance of epidemiological research into carnivore viral diseases in the Serengeti. *Conservation Biology* 21: 612-622.

Cliquet F, Aubert MFA and Sagne L. 1998. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *Journal of Immunological Methods* 212: 79-87.

Cliquet F, Gurbuxani JP, Pradhan HK, Pattnaik B, Patil SS, Regnault A, Begouen H, Guiot AL, Sood R, Mahl P, Singh R, Meslin FX, Picard E, Aubert MFA and Barrat J. 2007. The safety and efficacy of the oral rabies vaccine SAG2 in Indian Stray Dogs. *Vaccine* 25: 3409-3418

Coke RL, Backues KA, Hoover JP, Saliki JT, Ritchey JW and West GD. 2005. Serological responses after vaccination of fennec foxes (*Vulpes zerda*) and meerkats (*Suricata suricatta*) with a live, canarypox-vectored canine distemper virus vaccine. *Journal of Zoo and Wildlife Medicine* 36(2): 326-330.

Coleman PG and Dye C. 1996. Immunization coverage required to prevent outbreaks of dog rabies. *Vaccine* 14: 185-186.

Deem SL, Spelman LH, Yates RA and Montali RJ. 2000. Canine distemper in terrestrial carnivores: A review. *Journal of Zoo and Wildlife Medicine* 31(4): 441-451.

Delahay RJ, Smith GC and Hutchings MR. 2009. The Science of Wildlife Disease Management. In Delahay RJ *et al.* (Eds.). *Management of Disease in Wild Mammals.* Springer.

Dobson A and Foufopoulos J. 2001. Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society of London B.* 356: 1001-1012.

Dutta JK. 1998. Rabies transmission by oral and other non-bite routes. *Journal of the Indian Medical Association* 96: 359.

Estrada R, Vos A and De Leon RC. 2001. Acceptability of local made baits for oral vaccination of dogs against rabies in the Philippines. *BMC Infectious Diseases* 1: 19.

Evermann JF, Abbott JR and Han S. 2005. Canine coronavirus-associated puppy mortality without evidence of concurrent canine parvovirus infection. *Journal of Veterinary Diagnostic Investigation* 17: 610-614.

EWCA. 2011. *National Action Plan for the Conservation of the Ethiopian wolf.* In Strategic plan for Ethiopian wolf conservation. EWCA, Addis Ababa, Ethiopia.

EWCP. 2004. *Review of activities and achievements, 2000-2004.* Ethiopian Wolf Conservation Programme Report, Bale Robe, Ethiopia.

EWCP. 2012. *EWCP Progress and Achievements 2007-2012.* Ethiopian Wolf Conservation Programme Report, Bale Dinsho, Ethiopia.

Fearneyhough MG, Wilson PJ, Clark KA, Smith DR, Johnston DH, Hicks BN and Moore GM. 1998. Results of an oral rabies vaccination program for coyotes. *Journal of the American Veterinary and Medical Association* 212: 498-502.

Fekadu M. 1982. Rabies in Ethiopia. *American Journal of Epidemiology* 115: 266-273.

Fekadu M. 1991. *Latency and aborted rabies.* Pp. 191-198 in Baer GM (Ed.). The natural history of rabies, 2nd ed. Boca Raton, CRC Press.

Fekadu M, Nesby S, Shaddock J, Schumacher CL, Linhart S and Sanderlin DW. 1996. Immunogenicity, efficacy and safety of an oral rabies vaccines (SAG-2) in dogs. *Vaccine* 14: 465-468.

Fletcher KC, Eugster AK, Schmidt RE and Hubbard GB. 1979. Parvovirus infection in maned wolves. *Journal of the American Veterinary Medical Association* 175: 897-900.

Follman EH, Ritter DG and Baer GM. 1996. Evaluation of the safety of two attenuated oral rabies vaccines, SAG1 and SAG2 in six Arctic mammals. *Vaccine* 14: 270-273.

Fooks AR, Johnson N, Phillpotts R, Vos A, Muller T and Rupprecht CE. 2006. Non-bite transmission of rabies virus. *International Conference Rabies in the Americas, Ministry of Health Brazil, Series D, Meetings and Conferences* 1: 1–64.

Ford RB. 2003. *Infectious diseases: canine coronavirus.* Pp. 78-79 in Schaer M (Ed.). Clinical medicine of the dog and cat. Iowa State Press, Ames, Iowa, USA.

Foreyt JW and Evermann JF. 1985. Serologic survey of canine coronavirus in wild coyotes in the western United States, 1972-1982. *Journal of Wildlife Diseases* 21: 428-430.

Forsyth MA and Barrett T. 1995. Evaluation of polymerase chain reaction for the detection and characterisation of rinderpest and peste des petits ruminants viruses for epidemiological studies. *Virus Research* 39(2-3): 151-163.

Fry TL and Dunbar MR. 2007. A review of biomarkers used for wildlife damage and disease management. *Proceedings of the 12th Wildlife Damage Management Conference* 217-222.

Funk SM, Fiorello CV, Cleaveland S and Gopper ME. 2003. *The role of disease in carnivore ecology and conservation.* In Gittleman JL, Funk SM, Macdonald DW and Wayne RK (Eds.). Carnivore conservation. Cambridge University Press, Cambridge, United Kingdom.

Garcelon DK, Wayne RK and Gonzales BJ. 1992. A serological survey of the island fox (*Urocyon littoralis*) on the Channel Islands, California. *Journal of Wildlife Diseases* 28: 223-229.

Gascoyne SC, Laurenson MK, Lelo S and Borner M. 1993. Rabies in African wild dogs (*Lycaon pictus*) in the Serengeti region, Tanzania. *Journal of Wildlife Diseases* 29: 396–402.

Geffen E. 1994. *Vulpes cana. Mammalian Species* 462: 1-4.

Gellatt KN, Whitley RD, Samuelson DA and Garcia-Sanchez G. 1985. Ocular manifestations of viral diseases in small animals. *Compendium on Continuing Education for the Practising Veterinarian* 7: 968-976.

Ginsberg J and Macdonald DW. 1990. *Foxes, wolves, jackals and dogs. An action plan for the conservation of Canids.* IUCN/SSC Canid Specialist Group and Wolf Specialist Group. Gland, Switzerland.

Goller KV, Fyumagwa RD, Nikolin V, East ML, Kilewo M, Speck S, Muller T, Matzke M and Wibbelt G. 2010. Fatal canine distemper infection in a pack of African wild dogs in the Serengeti ecosystem, Tanzania. *Veterinary Microbiology* 146: 245-252.

Gordon CH, Hussein A, Marino J, Stewart AE and Sillero-Zubiri C. 2012. *Ethiopian Wolf Conservation Programme Annual Wolf Monitoring Report 2011-12.* Ethiopian Wolf Conservation Programme Report, Dinsho Bale, Ethiopia.

Gordon CH, Hussein A, Marino J, Stewart AE and Sillero-Zubiri C. 2013. *Ethiopian Wolf Conservation Programme Annual wolf monitoring report 2012-2013.* EWCP Report, Bale Dinsho, Ethiopia.

Gottelli D, Marino J, Sillero-Zubiri C and Funk SM. 2004. The effect of the last glacial age on speciation and population genetic structure of the endangered Ethiopian wolf (*Canis simensis*). *Molecular Ecology* 13: 2275-2286.

Gottelli D, Sillero-Zubiri C, Applebaum GD, Roy MS, Girman DJ, Garcia-Moreno J, Ostranders EA

and Wayne RK. 1994. Molecular genetics of the most endangered canid: The Ethiopian wolf *Canis simensis*. *Molecular Ecology* 3: 301-312.

Gottelli D, Sillero-Zubiri C, Marino J, Funk SM and Wang J. 2012. Genetic structure and patterns of gene flow among populations of the endangered Ethiopian wolf. *Animal Conservation.* doi: 10.1111/j.1469-1795.2012.00591.x

Gowtage-Sequeira S, Banyard AC, Barrett T, Buczkowski H, Funk SM and Cleaveland S. 2009. Epidemiology, pathology and genetic analysis of a canine distemper epidemic in Namibia. *Journal of Wildlife Diseases* 45: 1008-1020.

Green JS, Bruss ML, Evermann JF and Bergstrom PK. 1984a. Serologic response of captive coyotes *(Canis latrans)* to canine parvovirus and accompanying profiles of canine coronavirus titers. Journal of Wildlife Diseases 20: 6-11.

Green JS, Woodruff RA and Tueller TT. 1984b. Livestock-guarding dogs for predator control: Costs, benefits, and practicality. *Wildlife Society Bulletin* 12: 44-50.

Greene GE and Appel MJ. 1990. *Canine distemper*. Pp. 226-241 in Greene GE (ed.). Infectious Diseases of the Dog and Cat. W. B. Saunders, Philadelphia, Pennsylvania, USA.

Hammami S, Schumacher CL, Cliquet F, Barrat J, Tlatli A, Ben Osman R, Aouina T, Aubert A and Aubert MFA. 1999a. Safety evaluation of the SAG rabies virus mutant in Tunisian dogs and several non-target species. *Veterinary Research* 30: 353-362.

Hammami S, Schumacher CL, Cliquet F, Tlatli A, Aubert A and Aubert MFA. 1999b. Vaccination of Tunisian dogs with the lypholised SAG2 oral rabies vaccine incorporated into the DBL2 dog bait. *Veterinary Research* 30: 607-613.

Hampson K, Dushoff J, Bingham J, Bruckner G, Ali YH and Dobson A. 2007. Synchronous cycles of domestic dog rabies in sub-Saharan Africa and the impact of control efforts. *Proceedings of the National Academy of Sciences of the United States of America* 104(18): 7717-7722.

Harder T and Osterhaus AD. 1997. Canine distemper virus – a morbilivirus in search of new hosts? *Trends in Microbiology* 5: 120-124.

Haydon DT, Laurenson MK and Sillero-Zubiri C. 2002. Integrating epidemiology into population viability analysis: Managing the risk posed by rabies and canine distemper in the Ethiopian wolf. *Conservation Biology* 16: 1372-1385.

Haydon DT, Randall DA, Matthews L, Knobel DL, Tallents LA, Gravenor M, Williams SD, Pollinger JP, Cleaveland S, Woolhouse MEJ, Sillero-Zubiri C, Marino J, MacDonald DW and Laurenson MK. 2006. Low-coverage vaccination strategies for the conservation of endangered species. *Nature* 443: 692-695.

Hess G. 1996. Disease in metapopulation models: Implications for conservation. *Ecology* 77: 1617-1632.

Hochachka WM and Dhont AA. 2000. Density-dependent decline of host abundance resulting from a new infectious disease. *Proceedings of the National Academy of Sciences of the United States of America* 97(10): 5303-5306.

Hoelzer K and Parrish CR. 2010. The emergence of parvoviruses of carnivores. *Veterinary Research* 41: 39.

Hofmeyr M, Hofmeyr D, Nel L and Bingham J. 2004. A second outbreak of rabies in African wild dogs (*Lycaon pictus*) in Madikwe Game Reserve, South Africa, demonstrating the efficacy of vaccination against natural rabies infection. *Animal Conservation* 7: 193–198.

IUCN. 2012. IUCN Red List of Threatened Species. Version 2012.2. www.iucnredlist.org. Downloaded on 28 January 2013.

Johnson N, Mansfield KL, Marston DA, Wilson C, Goddard T, Selden D, Hemson G, Edea L, van Kesteren F, Shiferaw F, Stewart AE, Sillero-Zubiri C and Fooks AR. 2010. A new outbreak of rabies in rare Ethiopian wolves (*Canis simensis*). *Archives of Virology* 155: 1175-1177.

Johnson N, McElhinney LM, Ali YH, Saeed IK and Fooks AR. 2004. Molecular epidemiology of canid rabies in Sudan: evidence for a common origin of rabies with Ethiopia. *Virus Research* 104: 201-205.

Jones A. 1994. High performance liquid chromatographic determination of iophenoxic acid in serum. *Journal of Chromatography* 654: 293-296.

Keenan KP, Jervis HR, Marchwicki RH and Binn LN. 1976. Intestinal infection of neonatal dogs with canine coronavirus 1–71: studies by virologic, histologic, histochemical, and immunofluorescent techniques. *American Journal of Veterinary Research* 37: 247–256.

Kelly WR. 1993. *The liver and biliary system.* Pp. 364-365 in Kennedy CK, Jubb KVF & Palmer N (Eds.). Pathology of Domestic Animals, 4th edn. Academic Press, San Diego, California, USA.

Kennedy LJ, Randall DA, Knobel DL, Brown JJ, Fooks AR, Argaw K, Fekadu S, Ollier W, Sillero-Zubiri C, Macdonald DW and Laurenson MK. 2011. Major Histocompatability Complex diversity in the endangered Ethiopian Wolf (*Canis simensis*). *Tissue Antigens* 77: 118-125.

Knobel DL, Fooks AR, Brookes M, Randall DA, Williams SD, Argaw K, Shiferaw F, Tallents LA and Laurenson MK. 2008. Trapping and vaccination of endangered Ethiopian wolves to control an outbreak of rabies. *Journal of Applied Ecology* 45: 109-116.

Kock R, Chalmers WSK, Mwanzia J, Chillingworth C, Wambua J, Coleman PG and Baxendale W. 1998. Canine distemper antibodies in lions of the Masai Mara. *Veterinary Record* 142: 662-665.

Laurenson K, Shiferaw F and Sillero-Zubiri C. 1997. *Disease, domestic dogs and the Ethiopian wolf: the current situation.* In Sillero-Zubiri C and Macdonald DW (Eds.). The Ethiopian Wolf: Status Survey and Conservation Action Plan. IUCN, Gland, Switzerland and Cambridge, United Kingdom.

Laurenson MK, Sillero-Zubiri C, Thompson H, Shiferaw F and Malcolm JR. 1998. Disease threats to endangered species: patterns of infection by canine pathogens in Ethiopian wolves (*Canis simensis*) and sympatric domestic dogs. *Animal Conservation* 1: 273-280.

Macdonald DW. 1980. *Rabies and wildlife: A biologist's perspective.* Oxford University Press, Oxford, United Kingdom.

Macdonald DW. 1995. *European Mammals. Evolution and Behaviour.* Harper Collins Publishers, London, United Kingdom.

Macdonald DW and Sillero-Zubiri C. 2004. Wild canids – An introduction and *dramatis personae.* Pp. 3-36 in Macdonald DW & Sillero-Zubiri C (eds.) *The Biology and Conservation of Wild Canids.* Oxford University Press, Oxford, United Kingdom.

Mace G and Sillero-Zubiri C. 1997. *A Preliminary Population Viability Analysis for the Ethiopian Wolf.* In Sillero-Zubiri C and Macdonald DW (Eds.). The Ethiopian Wolf Status Survey and Conservation Action Plan. Gland, Switzerland and Cambridge, UK.

Malcolm JR. 1988. *A recensus of Simien jackals (Canis simensis) in Bale, Ethiopia.* External report to Ethiopian Wildlife Conservation Organisation. University of Redlands, California, USA.

Malcolm JR and Sillero-Zubiri C. 1997. *The Ethiopian Wolf: Distribution and Population Status.* Pp. 12-25 in Sillero-Zubiri C, Macdonald DW and the IUCN/SSC Canid Specialist Group (Eds.). *The Ethiopian wolf – Status Survey and Conservation Action Plan.* IUCN, Gland, Switzerland.

Marino J. 2003. *Spatial ecology of the Ethiopian wolf, Canis simensis.* D.Phil. Thesis, University of Oxford, Oxford, United Kingdom.

Marino J, Sillero-Zubiri C, Gottelli D, Johnson P and Macdonald DW. 2013. The fall and rise of Ethiopian wolves: lessons for conservation of long-lived, social predators. *Animal Conservation.* [Preprint]

Marino J, Sillero-Zubiri C and Macdonald DW. 2006. Trends, dynamics and resilience of an Ethiopian wolf population. *Animal Conservation* 9: 49-58.

Marino J, Stewart AE, Gordon CH, Gottelli D, Tefera Z, Laurenson MK and Sillero-Zubiri C. 2011. *Ethiopian wolf status review.* Pp. 1-50 in IUCN/SSC Canid Specialist Group (Eds.). Strategic planning for Ethiopian wolf conservation. Oxford, United Kingdom.

McCue PM and O'Farrell TP. 1988. Serological survey for selected disease in the endangered San Joaquin kit fox *(Vulpes macrotis mutica). Journal of Wildlife Diseases* 24: 274-281.

Mebatsion T, Sillero-Zubiri C, Gottelli D and Cox JH. 1992. Detection of rabies antibodies by ELISA and RFFIT in unvaccinated dogs and in the endangered Simien jackal (*Canis simensis*) of Ethiopia. *Journal of Veterinary Medicine, Series B* 39: 233–235.

Mech LD, Goyal SM, Bota CN and Seal US. 1986. Canine parvovirus infection in wolves (*Canis lupus*) from Minnesota. *Journal of Wildlife Diseases* 22: 104-106.

Mech LD and Goyal SM. 1995. Effects of canine parvovirus on gray wolves in Minnesota. *Journal of Wildlife Management* 59: 565-570.

Mech LD, Kurtz HJ and Goyal SM. 1997. Death of a wild wolf from canine parvoviral enteritis. *Journal of Wildlife Diseases* 33: 321-322.

Moehlman PD. 1983. *Socioecology of silver-backed and golden jackals (Canis mesomelas, C. aureus).* In J.F. Eisenberg and D.G. Kleiman (Eds.). Recent Advances in the Study of Mammalian Behaviour. Special Publication No. 7, American Society of Mammalogists, USA.

Montali RJ, Bartz CR, Teare JA, Allen JT, Appel MJG and Bush M. 1983. Clinical trials with canine distemper vaccines in exotic carnivores. *Journal of the American Veterinary Medical Association* 183(11): 1163-1167.

Nandi S and Kumar M. 2010. Canine Parvovirus: Current perspective. *Indian Journal of Virology* 21(1): 31-44.

Naylor MJ, Monckton RP, Lehrbach PR and Deane EM. 2001. Canine coronavirus in Australian dogs. *Australian Veterinary Journal* 79: 116–119.

Newey S and Sillero-Zubiri C. 2002. *Monitoring Ethiopian wolf populations: A field manual.* Wildlife Conservation Research Unit, Oxford University, UK.

OIE. 2011. *Chapter 2.1.13. Rabies.* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.13_RABIES.pdf

Orciari LA, Niezgoda M, Hanlon CA, Shaddock JH, Sanderlin DW, Yager PA and Rupprecht CE. 2001. Rapid clearance of SAG-2 rabies virus from dogs after oral vaccination. *Vaccine* 19: 4511-4518.

Pardo IDR, Johnson GC and Kleibocker SB. 2005. Phylogenetic characterization of canine distemper virus in naturally infected dogs in North America. *Journal of Clinical Microbiology* 43: 5009-5013.

Perpinan D, Ramis A, Tomas A, Carpintero E and Bargallo F. 2008. Outbreak of canine distemper in domestic ferrets (*Mustela putorius furo*). *Veterinary Record* 163: 246-250.

Randall DA, Marino J, Haydon DT, Sillero-Zubiri C, Knobel DL, Tallents LA, Macdonald DW and Laurenson MK. 2006. An integrated disease management strategy for the control of rabies in Ethiopian wolves. *Biological Conservation* 131: 151-162.

Randall DA, Pollinger JP, Kifle A, Macdonald DW and Wayne RK. 2010. Fine scale genetic structure in Ethiopian wolves imposed by sociality, migration and population bottlenecks. *Conservation Genetics* 11: 89–101.

Randall DA, Williams SD, Kuzmin IV, Rupprecht CE, Tallents LA, Tefera Z, Argaw K, Shiferaw F, Knobel DL, Sillero-Zubiri C and Laurenson MK. 2004. Rabies in endangered Ethiopian wolves. *Emerging Infectious Diseases* 10: 2214-2217.

Robbins AH, Borden MD, Windmiller BS, Niezgoda M, Marcus LC, O'Brien SJ, Kreindel SM, McGuill MW, DeMaria A, Rupprecht CE and Rowell S. 1998. Prevention of the spread of rabies to wildlife by oral vaccination of raccoons in Massachusetts. *Journal of the American Veterinary Medical Association* 213: 1407-1412.

Roelke-Parker ME, Munson L, Packer C, Kock R, Cleaveland S, Carpenter M, O'Brien SJ, Pospischil A, Hofmann-Lehmann R, Lutz H, Mwamengale GL, Mgasa MN, Machange GA, Summers BA and Appel MJ. 1996. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). *Nature* 379: 441-445.

Rupprecht CE, Shaddock JS, Sanderlin DW, Hanlon CA, Niezgoda M and Schumacher CL. 1998. Oral rabies vaccination of dogs. *Israel Journal of Veterinary Medicine* 53(4): 127-131.

Schumacher CL and Aubert A. 1996. *Progress in development of SAG-2 as an oral dog rabies vaccine.* In: VII annual international meeting. Advances towards rabies control in the Americas.

Schumacher CL, Hammami S, Chaparro F, Bishop G, von Teichman BF, Aubert MFA, Bingham J, Cliquet F and Aubert A. 1997. *Safety of the oral rabies vaccine SAG2 and efficacy of its delivery system DBL2 in indigenous dogs.* Proceedings of the Southern and Eastern African Rabies Group (SEARG) meeting, Session 5, Nairobi, Kenya.

Sillero-Zubiri C. 1994. *Behavioural ecology of the Ethiopian wolf, Canis simensis.* D.Phil. Thesis. University of Oxford, Oxford, United Kingdom.

Sillero-Zubiri C. 1996. Field immobilization of Ethiopian wolves (*Canis simensis*). *Journal of Wildlife Diseases* 32: 147-151.

Sillero-Zubiri C and Gottelli D. 1994. *Canis simensis. Mammalian Species* 485: 1-6.

Sillero-Zubiri C and Gottelli D. 1995a. Diet and feeding behavior of Ethiopian wolves (*Canis simensis*). *Journal of Mammalogy* 76: 531-541.

Sillero-Zubiri C and Gottelli D. 1995b. Spatial organisation in the Ethiopian wolf (*Canis simensis*): Large

packs and small stable home ranges. *Journal of Zoology, London* 237: 65-81.

Sillero-Zubiri C, Gottelli D and Macdonald DW. 1996a. Male philopatry, extra-pack copulations, and inbreeding avoidance in Ethiopian wolves (*Canis simensis*). *Behavioral Ecology and Sociobiology* 38: 331-340.

Sillero-Zubiri C, King AA and Macdonald DW. 1996b. Rabies and mortality in Ethiopian wolves (*Canis simensis*). *Journal of Wildlife Disease* 32: 80-86.

Sillero-Zubiri C and Macdonald DW. 1997. *The Ethiopian Wolf: Status Survey and Conservation Action Plan*. IUCN, Gland, Switzerland & Cambridge, United Kingdom.

Sillero-Zubiri C and Marino J. 2011. *Canis simensis*. In IUCN 2011. *IUCN Red List of Threatened Species*. Version 2011.2. www.iucnredlist.org.

Sillero-Zubiri C, Marino J, Gottelli D and Macdonald DW. 2004. *Afroalpine ecology, solitary foraging and intense sociality amongst Ethiopian wolves*. In Macdonald DW and Sillero-Zubiri C (Eds.). Biology and conservation of wild canids. Oxford University Press, Oxford, United Kingdom.

Sillero-Zubiri C and Switzer D. 2004. *Management of canids near people*. Pp. 256-266 in Sillero-Zubiri C, Hoffmann M and Macdonald DW (Eds.). Canids: foxes, wolves, jackals and dogs: status survey and conservation action plan, second edition. IUCN Canid Specialist Group, Gland, Switzerland and Cambridge, United Kingdom.

Sillero-Zubiri C, Tattersall FH and Macdonald DW. 1995. Bale mountains rodent communities and their relevance to the Ethiopian wolf (*Canis simensis*). *African Journal of Ecology* 33: 301-320.

Stephenson RO, Ritter DG and Nielson CA. 1982. Serologic survey for canine distemper and infectious canine hepatitis in wolves in Alaska. *Journal of Wildlife Diseases* 18: 419-424.

Sterner RT and Smith GC. 2006. Modelling wildlife rabies: Transmission, economics and conservation. *Biological Conservation* 131: 163-179.

Stronen AV, Sallows T, Forbes GJ, Wagner B and Paquet PC. 2011. Diseases and parasites in wolves of the Riding Mountain National Park region, Manitoba, Canada. *Journal of Wildlife Diseases* 47: 222-227.

Talbi C, Holmes EC, de Benedictis P, Faye O, Nakoune E, Gamatie D, Diarra A, Elmamy BE, Sow A, Adjogoua EV, Sangare O, Dundon WG, Capua I, Sall AA and Bourhy H. 2009. Evolutionary history and dynamics of dog rabies virus in western and central Africa. *Journal of Genetic Virology* 90: 783–791.

Tamura K, Dudley J, Nei M and Kumar S. 2007. **MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.** *Molecular Biology and Evolution* 24: 1596-1599.

Tefera G, Yimer E and Geyid A. 2002. Endemic existence of rabies in Ethiopia. *Ethiopian Medical Journal* 40: 163–170.

Thomson GR and Meredith CD. 1993. Rabies in bat-eared foxes in South Africa. *Onderstepoort Journal of Veterinary Research* 60: 399-403.

Timm SF, Stokely JM, Gehr TB, Peebles RL and Garcelon DK. 2000. *Investigation into the decline of island foxes on Santa Catarina Island.* Institute for Wildlife Studies, Arcata, California, USA.

Timm SF, Munson L, Summers BA, Terio KA, Dubovi EJ, Rupprecht CE, Kapil S and Garcelon DK. 2009. A suspected canine distemper epidemic as the cause of a catastrophic decline in Santa Catalina island foxes (*Urocyon littoralis catalinae*). *Journal of Wildlife Diseases* 45(2): 333-343.

van de Bildt MWG, Kuiken T, Visee AM, Lema S, Fitzjohn TR and Osterhaus ADME. 2002. Distemper outbreak and its effect on African wild dog conservation. *Emerging Infectious Diseases* 8(2): 211-213.

van Heerden J, Bingham J, van Vuuren M, Burroughs REJ and Stylianides E. 2002. Clinical and serological response of wild dogs (*Lycaon pictus*) to vaccination against canine distemper, canine parvovirus infection and rabies. *Journal of the South African Veterinary Association* 73(1): 8–12.

Vickers T, Garcelon D, Fritcher D and Christianson M. 2004. *Evaluation of an oral and food-based canarypox-vectored canine distemper vaccine in endangered Channel Island Foxes* (*Urocyon littoralis*). Pp. 89-90 in American Association of Zoo Veterinarians, Annual Proceedings.

von Messling V, Milosevic D and Cattaneo R. 2004. Tropism illuminated: lymphocyte-based pathways blazed by lethal morbillivirus through the host immune system. *Proceedings of the National Academy of Sciences, USA* 101: 14216-14212.

Vucetich JA, Peterson RO and Waite TA. 1997. Effects of social structure and prey dynamics on extinction risk in gray wolves. *Conservation Biology* 11: 957-965.

Whetstone CA, Draayer H and Collins JE. 1988. Characterization of canine adenovirus type 1 isolated from American black bears. *American Journal of Veterinary Research* 49: 778–780.

WHO. 2004. *WHO Expert Consultation on Rabies: First report.* WHO Technical Report Series; 931. Geneva, Switzerland.

Wiles MC and Campbell TA. 2006. Liquid chromatography-electospray ionization mass spectrometry for direct identification and quantification of iophenoxic acid in serum. *Journal of Chromatography B* 832: 144-157.

Wimsatt J, Biggins DE, Williams ES and Becerra VM. 2006. *The quest for a safe and effective canine distemper virus vaccine for black-footed ferrets.*Pp. 248-268 in Roelle JE, Miller BJ, Godbey JL and Biggins DE (Eds.). Recovery of the black-footed ferret – progress and continuing challenges. U.S. Geological Survey Scientific Investigations Report 2005-5293. Virginia, USA.

Winkler WG and Bogel K. 1992. Control of rabies in wildlife. *Scientific American* 266(6):86-92.

Woodroffe R, Cleaveland S, Courtenay O, Laurenson MK and Artois M. 2004. *Infectious diseases in the management and conservation of wild canids.* Pp. 123-142 in Macdonald DW and Sillero-Zubiri C. (Eds.). The Biology and Conservation of Wild Canids. Oxford University Press, Oxford, United Kingdom.

Yalden DW. 1983. The extent of high ground in Ethiopia compared to the rest of Africa. *Ethiopian Journal of Science* 6: 35-38.