Wildlife species: Natural treasures, disease reservoirs or victims of crime?

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Abstract

This thesis comprises three separate projects, each with a wildlife component. The first two involve wildlife species as reservoirs of disease, for domestic animals and for humans. The third project looks at wildlife species targeted during suspected wildlife crimes.

Chapter 1

A population of horses kept for wildlife tourism near the Serengeti in Tanzania was monitored for trypanosome infections for five years, from 2005-2010. Clinical observations and microscopic and PCR data were analysed to assess if there was a pattern of clinical signs associated with different trypanosome species infections, to calculate the incidence rate and describe the mortality in this population (n=41). The incidence rate in the population was 0.11 trypanosome cases/month at risk. Eleven horses died during the study period and the trypanosome case specific mortality was 78%. A number of single species and mixed species infections with *T. brucei* and *T. congolense* were diagnosed, but in contrast to studies in equine populations from West Africa, no *T. vivax* infections were recorded. Multilocus genotyping of *T. brucei* isolates confirmed that these cases were as a result of spill-over from the resident wildlife population. No pattern of clinical signs was significantly associated with a particular trypanosome species infection. However, *T. brucei* was the only trypanosome detected in the brain and cerebrospinal fluid of fatal cases. Lymphoplasmacytic meningoencephalitis was seen histologically in the two cases which were post mortemed. Interestingly a high proportion of horses infected with trypanosomes had no clinical signs recorded in the two weeks prior and following PCR diagnosis. While a spectrum of clinical signs has been reported previously in human cases of trypanosomiasis caused by the closely related parasite *T. brucei rhodesiense*, this is the first report of this in horses. These natural infections in equidae offer the opportunity to study the host parasite relationship and pathogenesis of *T. brucei* which will be of value to both human and animal trypanosomiasis research.

Chapter 2

A literature review was conducted to collate known information on vertebrate reservoir hosts for *Borrelia burgdorferi* s.l. in Scotland. Incompetent reservoir hosts and hosts of unknown reservoir status were also identified.
Host blood meal analysis to identify the important reservoir hosts for *B. burgdorferi* s.l. was adapted for use in ticks in Scotland by amplifying a ~ 145bp fragment from vertebrate mitochondrial 12S rDNA and sequencing the product. This technique successfully identified host DNA from engorged *Ixodes ricinus* larvae and nymphs of known host origin. Previous methods of establishing reservoir hosts by xenodiagnosis have been limited to rodents and birds due to logistical reasons. By sampling the questing tick population, these potential biases are eliminated. New information on *I. ricinus* biology which challenges previous dogma such as taking of multiple blood meals and possible co-feeding transmission of *B. burgdorferi s.l.* have been found in previously published reports. Due to the sensitivity and non specific nature of the primers, contamination is a major challenge with this technique. Detection rates in questing ticks can be low and the reasons for this need to be investigated further.

Chapter 3

Wildlife crimes have negative effects on conservation, animal welfare and may be a risk to human and domestic animal health from the misuse and abuse of pesticides and firearms in the countryside. This study aims to describe the occurrence of wildlife crimes which included forensic examination as part of the investigation. Information on suspected cases of wildlife crime submitted to the Scottish Agricultural Centre (SAC) Consulting: Veterinary Services Veterinary Investigation Centres, Science and Advice for Scottish Agriculture (SASA), and the University of Glasgow was retrieved between January 1 – December 31 2010.The location of suspected cases, the species targeted, cause of death, types of crime and where crimes are committed were summarized across Scotland. A total of 188 suspected wildlife crime cases were included in the study. Birds were most likely to be submitted and included 114 raptors, 14 waterfowl and 22 cases from other 'bird species'. Mammals were the second most likely group to be submitted, and included 12 badgers, 8 foxes, 7 deer, 4 hares, and 7 'other mammals'. The cause of death was determined in 124 cases: malicious or accidental trauma was the most likely cause of death in 72 cases and 33 were poisoned. Of the 91 cases which stated a suspected cause of death, poisoning was suspected in 58 of these and confirmed in 31%. Outcomes of cases in which a crime was suspected were not generally reported back to the laboratory conducting the forensic examination. Further work to follow outcomes of suspected crimes with agencies including the SSPCA, RSPB and Police Forces is underway. Cases submitted to the Fiscals Office will be followed to see if prosecution was successful.

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Project 1

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Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:

Name: Caroline L. Millins

Abbreviations

ACPOS	Association of Chief Police Officers in Scotland
CSF	Cerebrospinal fluid
COPFS	Crown Office and Procurator Fiscal Service
DDE	Dichlorodiphenyldichloroethylene
IUCN	International Union for the Conservation of Nature
LAMP	Loop-mediated Isothermal Amplification
MLG	Multilocus genotyping
NWCU	National Wildlife Crime Unit
PAWS	Partnership for Wildlife Crime Scotland
RLB	Reverse line blot
RSPB	Royal Society for the Protection of Birds
SAC	Scottish Agricultural College
SARS	Severe acute respiratory syndrome
SASA	Science and Advice for Scottish Agriculture
SSPCA	Scottish Society for the prevention of cruelty to animals
WLEWG	Wildlife Law Enforcement Working Group

Introduction

Introduction

There are many qualitative and quantitative ways to ascribe value to ecosystems and the wildlife species they contain. Many wildlife species are integral to ecosystem functioning, providing direct benefits for people. Some communities rely on wildlife species as food, and commercial harvesting of wild species is common across many parts of the world. Nature tourism is estimated to contribute £1.4 billion annually to the Scottish economy alone[1], and much of this is to experience wildlife in natural habitats.

However, whilst there are very valid reasons to value wildlife species, there are also risks to humans and domestic animals from diseases transferred from wildlife populations. An estimated 60% of emerging diseases affecting humans originate from animals and 73% of these emerge from wildlife species[2]. These have been responsible for high profile disease outbreaks and threats of pandemics in people including severe acute respiratory syndrome (SARS) and highly pathogenic avian influenza (H5N1). Underlying drivers for disease emergence include continued human population expansion and land use change, global movement of animals and people and climate change which contribute to changes in the ecology of a host, pathogen or both[3]. Significant amounts of funding are being focused on ways to forecast which pathogens could result in future outbreaks of disease such as the PREDICT program run by the EcoHealth Alliance and funded by USAid, United States government development funding.

In the first chapter I describe a parasitic disease in a domestic species, occurring due to movement of a host (domestic horses) into a different environment, with resulting spill-over of disease from the resident wildlife population. The second project describes research into the ecology of lyme disease, a zoonoses which is considered to be emerging in Scotland,[4] and has a number of wildlife species as reservoir hosts.

Wildlife populations can also be at risk from humans and domestic animals. The transfer of disease from domestic animals to wildlife is well documented[3], examples include the introduction of brucellosis into bison in Yellowstone National Park, and sarcoptic mange into foxes in Europe. In some instances this

can threaten wildlife populations, for example the intermittent introduction of rabies and canine distemper into African wild dogs (*Lycaon pictus*) in the Serengeti. Wildlife can also be affected by human activity as the targets of crime, often as a result of perceived conflict over resources. For example the persecution of raptors has been linked to grouse moors in Scotland[5]. Wildlife are also affected by illegal poaching and as targets for abuse, for example badger baiting. This topic is the subject of the third project, where I report the results of a survey of wildlife crime in Scotland. By summarizing and analyzing forensic pathology reports, I describe the types of crimes, species affected, locations of crimes and outcomes of wildlife crime investigations in Scotland in 2010.

Chapter 1

An Epidemiological and Genetic Study of Trypanosome Infections in Horses from the Serengeti, Tanzania

1.1. Introduction

Trypanosome infections are responsible for a heavy burden of disease in humans and domestic animal species in Africa[6]. Low numbers of equidae have traditionally been kept in areas with high tsetse densities and thus trypanosome challenge. Our knowledge of the clinical syndrome and outcome of trypanosome infections in equidae is limited to a few observations of natural infections from Tanzania, Kenya, Zambia and Brazil[7-11], experimental infections[12-15], and cross sectional studies on horse and donkey populations from the Gambia[16-18]. These studies are widely separated geographically and over more than 50 years, in which time molecular methods for trypanosome identification have been developed.

Horses are susceptible to infection with *Trypanosoma brucei, T. congolense* and *T. vivax* which are transmitted cyclically by a number of species of tsetse flies (*Glossina* spp.). *T. vivax* can also be transmitted by biting flies. Additionally, *T. evansi* and *T. equiperdum* also infect equidae. These species are essentially mutants of *T. brucei* that have evolved alternate transmission pathways enabling them to exist outside of the range of the tsetse fly vector[19]. *T. evansi* is mechanically transmitted by blood feeding insects and *T. equiperdum* is transmitted venereally. Confirming the presence of *T. evansi* or *T. equiperdum* within the tsetse belt is problematic as they are genetically very closely related to *T. brucei brucei* and currently there are no molecular tools that will adequately allow discrimination between the three variants[20]. This is particularly problematic in areas where they may co-exist, as the markers that do exist rely on confirming a negative (i.e. absence of genetic marker), which is less than ideal when working with field samples.

The clinical presentation and outcome of infection with a trypanosome species is known to vary depending on the host species, breed, nutritional status, age, co-infections including other trypanosome species, and the strain and dose of trypanosome[21,22]. Comparative studies in a number of host species have found that *T. congolense* is confined to the vascular system whereas *T. brucei* and *T. vivax* are able to leave the vascular system and invade into tissues[21]. In horses, *T. brucei* is considered to cause the most severe disease, and presentations vary

from acute to chronic but disease is thought to be invariably fatal if untreated[22]. *T. congolense* and *T. vivax* infections are reported to result in mild to moderate disease[21-23]. Clinical signs described in infections with all three species include pyrexia, mild jaundice, lethargy, weakness, anaemia and subcutaneous oedema of the legs and ventral abdomen. Additionally, in chronic infections with *T. brucei*, keratitis, genital swelling, ataxia and paresis may be seen[21,23]. A number of reports of natural *T. brucei* infections have documented relapse of infection following treatment leading to death in some cases[9,11]. Relapse of infection following treatment has also been reported in some *T. vivax* cases[7,11], whereas horses infected with *T. congolense* alone recovered following treatment with trypanocidal drugs[9]. The reasons for this are unclear but may relate to the varying abilities of different trypanosome species to invade from the vasculature into tissues and also trypanocidal drug efficacy. The timing of treatment is also important because early diagnosis and treatment can lead to a clinical cure for infections with *T. congolense* and *T. brucei*. However, horses showing signs of chronic infection such as anaemia and ataxia had a poor prognosis and did not respond as well to treatment[8].

Recently, a demand for horse safaris in East Africa has introduced horses to areas inside the tsetse belt with a high risk of trypanosome infections. Following a preliminary study[8], a population of horses kept for wildlife tourism near the Serengeti, Tanzania have been continuously monitored for five years. Using this data set we aimed to characterize the pattern of clinical signs seen with different trypanosome species in a field setting, estimate the incidence of trypanosome infections and describe trypanosome associated mortality in this population.

1.2. Material and methods

1.2.1 Study Area

The study population of horses are owned by Singita-Grumeti Reserves Ltd and kept on a 2500ha safari lodge adjacent to the Grumeti Game Reserve, a wildlife protected area in northern Tanzania (Figure 1). The local area is dominated by acacia wooded grassland with high densities of *Glossina swynnertoni and*

Glossina pallipides[8]. Horses are used for horseback safaris by tourists staying on the property.

1.2.2 Health Monitoring and Treatment regime

Health monitoring was carried out from January 2005 to December 2010. As described previously[8], horses were treated with a prophylactic drug (isometamidium chloride (Trypamidium-Samorin®, Merial, France) or quinapyramine (Triquin, Wockhardt Europe Ltd, Ireland), at approximately 3 month intervals. Quinapyramine was also used as the preferred drug to treat confirmed cases of trypanosomiasis. Horses were examined daily and any animals considered to be unwell were blood sampled and a fresh blood smear and Giemsa stained buffy coat smear examined for trypanosomes. Preventative measures to reduce exposure to tsetse flies were continued as described previously[8].

1.2.3 Molecular Analysis

Species specific PCR reactions

From January 2006 to December 2007, 182 samples of heparinised blood from 24 horses were collected, applied to Whatman Classic FTA Cards, air dried, placed in foil lined envelopes containing dessicant and transported to the UK for PCR testing. Horses were sampled between 1- 18 times (median 9) on different dates. The sample set included both horses showing clinical signs of illness, horses sampled post treatment for trypanosomes (recovery period) as well as routine samples from healthy horses. Additionally, a small number of horses which died had cerebrospinal fluid or brain tissue applied to FTA cards. These were washed and tested as for blood. For *T. brucei* and *T. congolense* PCRs, DNA was eluted from several punches of the FTA card and used in the PCR reaction in an attempt to avoid problems caused by heterogenous distribution of parasite DNA[24]. Five 3mm punches from each card were made using a clean 3mm Harris unicore punch, and then washed to reduce PCR inhibitors found in blood[25]. The discs were washed twice with 1ml of FTA purification reagent (Whatman) and then twice with 1ml of 1mM TE (10mMTris, 1mM EDTA) buffer pH 8.0, washing for 15 minutes at each step. Discs were then dried before 100µl of 5% Chelex

100 solution (Sigma-Aldrich Ltd) was added, and the mixture heated to 90°C in a Peltier thermal cycler for 30 minutes to create the eluate. For *T. brucei*, a 25µl reaction mix was prepared containing 16mM $(NH_4)_2SO_4$, 67 mM Tris H-Cl (pH 8.8 at 25ºC), 0.01% tween-20, (collectively 10x NH_4 Reaction Buffer, Bioline, London), 1.5mM of each of the four dNTPs (Bioline, London), 5µl of eluted sample DNA, 0.7 units of Redtaq (Bioline, London), 0.2mM of each of the four dNTPs (Bioline, London), and 0.8µM of each of the primers TBR1 and 2 (MWG Biotech, London)[26] and water. The PCR conditions were 94ºC for 3 minutes followed by 35 cycles of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 30 seconds and a final extension step of 72ºC for 5 minutes in a Peltier thermocycler. For *T. congolense* Savannah, a 25µl reaction mix was prepared containing 5µl of eluted sample DNA in 10x NH_4 reaction buffer (Bioline, London), 1.5mM $MgCl_2$ (Bioline, London) 0.2mM of each dNTP, TCS1 and 2 primers at a concentration of 1µM and 1 unit of REDtaq (Bioline, London)[26]. PCR conditions were an initial denaturation step of 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 1 minute, 60ºC for 2 minutes and 74ºC for 30 seconds. Products were run on a 1.5% agarose gel, and visualized using a Geldoc imaging machine and BioRad GelDoc software package. For *T. vivax* there was insufficient eluate left for all samples, and also insufficient disc material left for all samples to prepare further eluates as described above, so a single 2mm punch from each case was placed directly in the PCR reaction following washing of the disc to remove inhibitors. Disc washing was carried out three times with 200µl of FTA buffer and twice with 200µl of TE buffer, each with a 5 minute incubation period. A 20µl reaction mix was prepared composed of 2µl of primer A, 2µl of primer B[27], 2µl of PCR buffer (45mM TrisHCl, pH8.8, 11mM $(NH_4)_2SO_4$ 4.5mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4µM EDTA, 113 µg per ml BSA, 1mM of each deoxyribonucleotide triphosphates), 0.2µl of *Taq* polymerase (ThermoFisher Scientific) and 13.8µl of water and seeded with either a washed disc or 1µl of eluate. The PCR conditions were 95ºC for 50 seconds, 60ºC for 50 seconds, and 65ºC for one minute, for 30 cycles on a Robocycler Gradient 96 (Stratagene). The reaction volume was doubled to 40µl for a subset of the samples to reduce the effect of inhibitors. Additionally, a subset of samples were tested with two additional sets of primers for *T. vivax*[28,29]. The PCR products were separated by electrophoresis on 3% Nusieve (Cambrex) agarose gels and visualized under a UV camera (FluoroChem 5500™, Alpha Innotech).

A proportion of the samples which tested positive for *T. brucei* were tested for the serum resistance gene (*SRA*), a single copy gene which differentiates *T. brucei rhodesiense*, the human infective subspecies, from *T. brucei brucei*, which while otherwise promiscuous in its mammalian host choice, is unable to infect humans[30].

Microsatellite PCRs

Samples which were *T. brucei* positive were selected for amplification of five single copy microsatellite loci. Washed 2mm^2 discs were used to seed nested PCR reactions. The nested PCR reaction involves two consecutive PCR reactions, the product of the first PCR seeding the second. Twenty microlitre reactions were prepared as described above. Primers for each microsatellite loci have been published previously[31-33]. The PCR products were separated by electrophoresis on 3% Nusieve (Cambrex) agarose gels and visualized using a UV camera (FluoroChem 5500^{TM}, Alpha Innotech). The PCR conditions were 95ºC for 50 seconds, 55ºC for 50 seconds, and 65ºC for one minute, for 28 cycles on a Robocycler Gradient 96 (Stratagene) for each round of PCR.
Allele size was determined incorporating a fluorescently tagged primer (FAM or HEX) as one of the second round primer pair, and running the product through a capillary based sequencer which allowed accurate sizing to within 1bp. (Dundee Sequencing Service http://www.dnaseq.co.uk/). The size of the DNA fragment was measured relative to ROX-labelled size standards (GS400HD markers; Applied Biosystems) included in the sequencer run, and the output was analyzed using Peak Scanner V1.0 software (Applied Biosystems). Each allele was given a number for each locus, and a multilocus genotype (MLG) for each isolate defined by the combination of alleles at each locus. Similarity analysis was carried out by analyzing MLGs with the Clustering Calculator program (http://www2.biology.ualberta.ca/jbrzusto/cluster.php). The unweighted arithmetic average was used as the clustering method and Jaccard's similarity coefficient to form a pairwise distance matrix. The output was used to generate a Phylip Drawtree string which was then converted into a dendrogram using the Treeview program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The clustering calculator program was used to calculate the bootstrap values for the branches of the dendrograms using 100 iterations.

1.2.4 Epidemiological analysis

During the study period January 1 2005 – December 31 2010, the lodge was managed to have around 16 horses on the property. However, due to horses leaving or arriving, a total of 41 horses were enrolled in the study. Horses arriving at the lodge came from outside the tsetse belt and were assumed to be naïve to trypanosome infection. Horses leaving the study either died, or were sold as unsuitable for pleasure riding and were lost to further follow up. A case of trypanosomiasis was defined as a positive case on microscopy and one other compatible clinical sign, including pyrexia, jaundice, anaemia, tachycardia, tachypnoea, leg or ventral oedema, head shaking or ataxia. The incidence rate was calculated using the first case for each horse, and the number of months at risk as the denominator[34]. The incidence rate was also calculated from 2005-2007 as described above and also using an alternate case definition of either microscopy or PCR positive, plus one other compatible clinical sign as listed above. Fishers's exact 95% confidence intervals were calculated in WinPepi (www.brixtonhealth.com/pepi4windows.html).

Clinical signs data were compiled by recording presence or absence of a pre-determined set of clinical signs, as listed above two weeks prior and post PCR diagnosis. A subset of samples which had been tested in triplicate for *T. brucei brucei* and *T. congolense* was used for this analysis to increase the sensitivity of detecting clinical cases. Control animals were classified as animals which never tested positive for trypanosomes on any test.

1.3. Results

1.3.1 Microscopy and species specific PCR

Microscopy and PCR results of samples collected routinely (n=132), from sick horses (n=35) and horses in the post treatment recovery phase (n=14) found that 28 samples tested PCR positive for *T. brucei*, 15 tested positive for *T. congolense*, and 5 were positive for both *T. brucei* and *T. congolense*. Eleven

samples were positive on microscopy, these were all from horses which were classified as sick (Table 1.1). No infections with *T. vivax* were recorded. None of the *T. brucei* PCR positive samples were positive for the *SRA* gene, indicating that these infections were due to *T. brucei brucei*. No infections with *T. vivax* were recorded (Table 1.1). A case of trypanosomiasis was 16 times more likely to be detected by PCR versus microscopy, if the date of first detection of a trypanosome infection by either technique was selected for each horse (Table 1.2).

1.3.2 Clinical signs with trypanosome infections

Pyrexia was the most consistent clinical sign recorded in trypanosome infections (Fig. 1.2). Some horses in the control group exhibited some of the clinical signs of interest including pyrexia, tachycardia and jaundice. Also, 71% of *T. brucei* PCR positive horses, 50% of *T. congolense* PCR positive and 25% of mixed *T. brucei* and *T. congolense* PCR positive horses had no clinical signs recorded. Robust statistical comparison of clinical signs between trypanosome species was hindered by small sample size.

1.3.3 Mortality due to trypanosome infection

Nine horses died during the study period (22%) and seven of these were attributed to trypanosomiasis (78%). Five of these horses were positive by PCR detection of *T. brucei* on either brain or cerebrospinal fluid (CSF) and two were based on clinical history consistent with chronic trypanosome infection, including anemia, ataxia and weakness. All CSF and brain samples were PCR negative for *T. congolense* and *T. vivax* where tested. Tissues were available for histopathology in two of these animals. Generalized non-suppurative meningoencephalitis was seen in both horses, characterized by expansion of the meninges and perivascular cuffing with moderate numbers of lymphocytes, plasma cells.

1.3.4 Multilocus genotype similarity analysis

A similarity analysis was carried out using MLGs from 5 horse samples which amplified consistently at all five microsatellite loci, and these were compared with MLGs from pre-existing wildlife and human samples collected from various locations in Africa. The dendrogram generated from this analysis (Figure 1.3) shows that the horse samples all cluster within a group which contains Tanzanian wildlife samples (*T. brucei brucei*) as well as human samples from Tanzania, Zambia, Uganda and Malawi (*T. brucei rhodesiense*), but are distinct from a separate group of human samples from Cameroon and the Democratic Republic of the Congo (*T. brucei gambiense*).

1.3.5 Incidence rate estimate

The incidence rate over the whole study period, 2005 to 2010, was 0.11 cases/month at risk (C.I. 0.075-0.16). Inclusion of the date of a positive test result by PCR, if earlier than by microscopy, from 2005-2007, significantly increased the incidence rate relative to microscopic diagnosis alone (Table 1.3).

Reason for sampling	Microscopy positive	*Trypansoma brucei* PCR positive	*T. congolense* positive	*T. brucei* and *T. congolense* PCR positive	*T. vivax* PCR positive	Total
Routine	0	13	10	1	0	132
Sick	11	10	3	3	0	35
Recovery period	0	4	2	1	0	14
Unknown	0	1	0	0	0	1
Total	11	28	15	5	0	182

Table 1.1: Results of microscopy and PCR testing by reason for sample collection; routine monthly sampling (n=132), sampling of horses which were unwell (sick) (n=35), or sampling post treatment for trypanosomes (recovery period) (n=14) or reason for sampling type unknown (n=1).

PCR (*T. brucei* or *T. congolense*)	Microscopy positive	Microscopy negative
Positive	1	16
Negative	0	7

Table 1.2: Results of diagnosis of trypanosome infection by microscopy of Giemsa stained buffy coat smear compared with PCR diagnosis of trypanosome infection, (first positive sample per horse or first negative if no positive test recorded), Sasakawa Lodge, Tanzania.

Time period	Incidence, Microscopy	Incidence, Microscopy and PCR
2005-2007 (n=20)	0.07 (0.038-0.12)	0.14 (0.081-0.21)
2005-2010 (n=41)	0.11 (0.075-0.16)	N/A

Table 1.3: Incidence rate for trypanosomiasis in horses at Sasakawa Lodge, Tanzania (95% C.I)

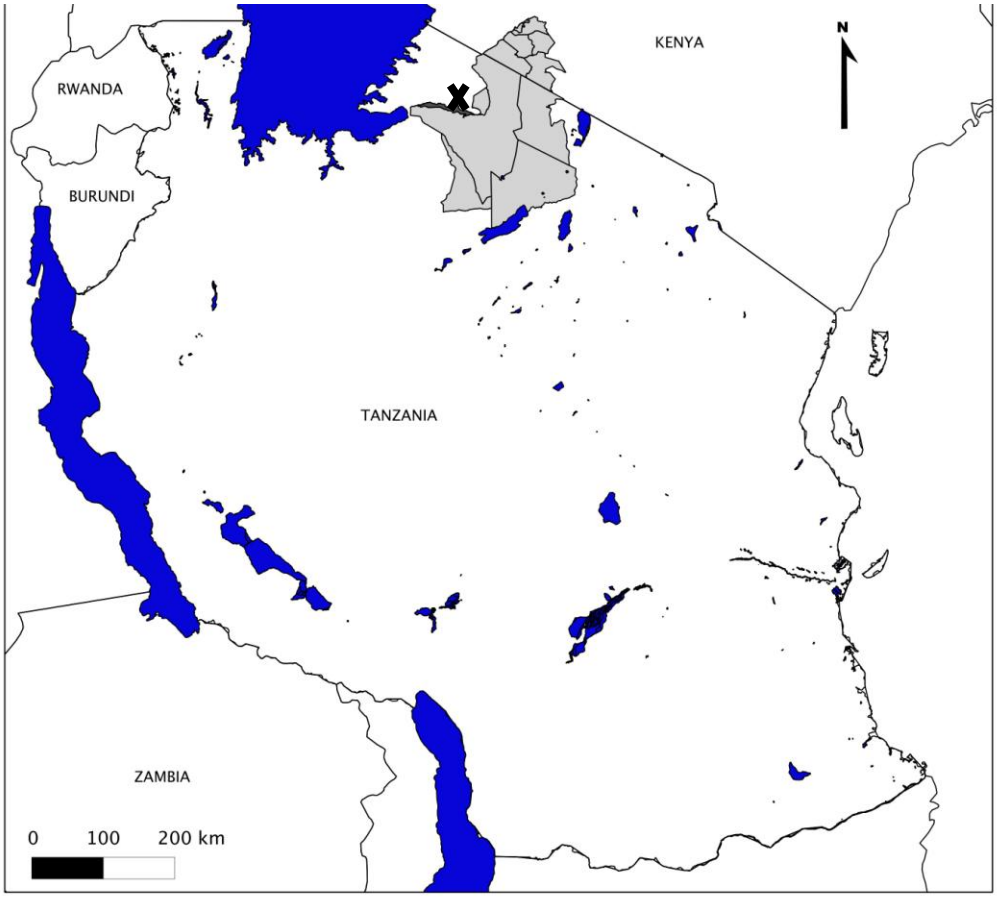

Figure 1.1: Map of Tanzania with the Serengeti-Mara Ecosystem shaded in pale grey, the Grumeti Wildlife Reserve shaded dark grey and Sasakawa Lodge marked **X**.

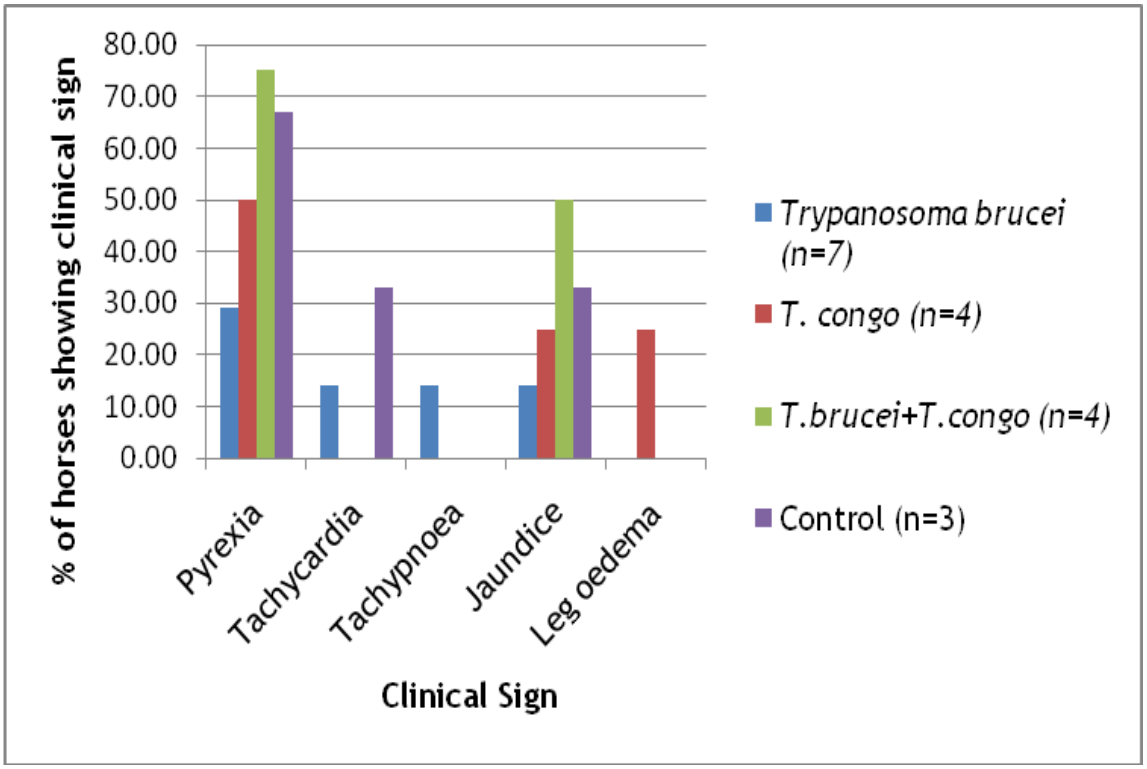

Figure 1.2: Horses infected with different trypanosome species recorded as showing different clinical signs, two weeks prior or post PCR testing, Sasakawa Lodge, Tanzania.

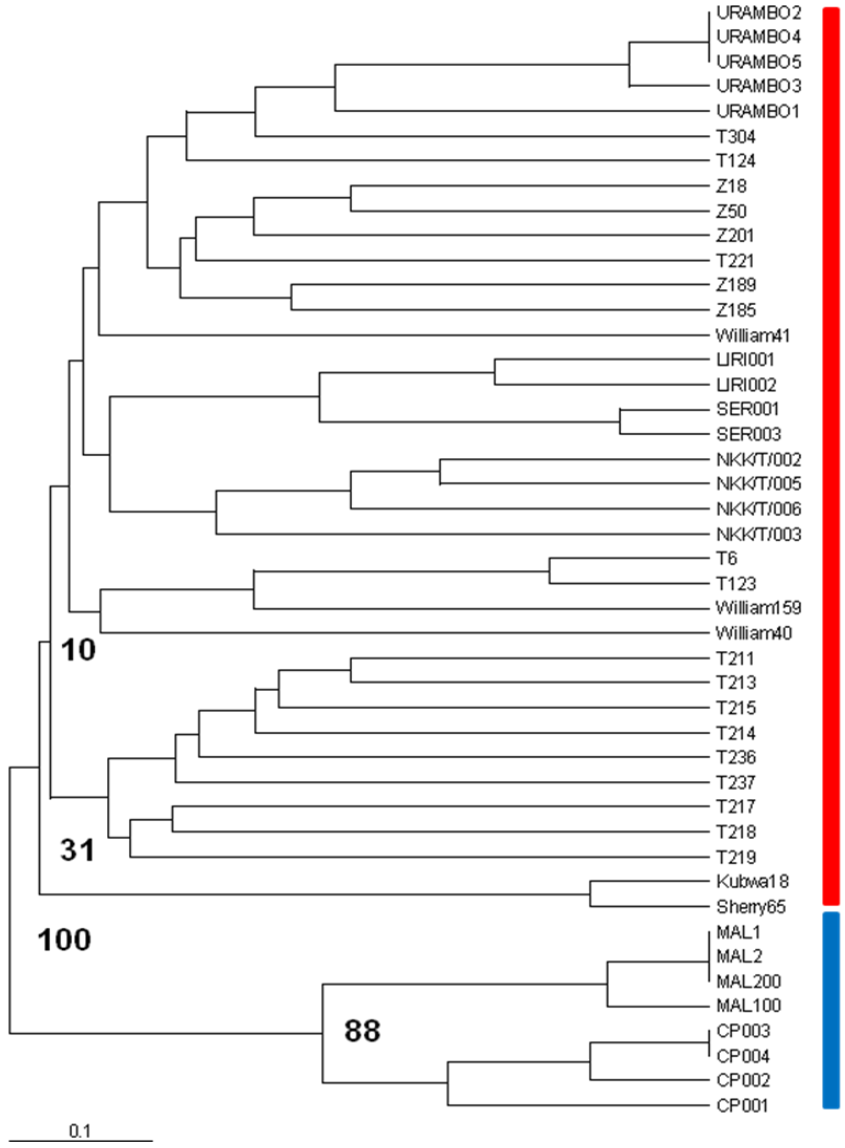

Figure 1.3: Dendrogram of multilocus genotypes (MLGs). Prefix 'URAM' – Tanzanian humans (*T. b. rhodesiense*), 'T'- Tanzanian wildlife (*T. b. brucei*), 'Z' – Zambian humans (*T. b. rhodesiense*), 'LIR' and 'SER' Ugandan humans (*T. b. rhodesiense*), 'NKK'- Malawi humans (*T. b. rhodesiense*), 'MAL' – Democratic Republic of the Congo humans (*T. b. gambiense*), 'CP' – Cameroon humans (*T. b. gambiense*). 'William','Sherry'and 'Kubwa', horses from this study. Colour coding represents the division in the dendrogram between *T. b. brucei* and *T. b. rhodesiense* from *T. b. gambiense*. The red bar incorporates Tanzanian wildlife, the horses from this study as well as Tanzanian, Zambian, Ugandan and Malawi human samples. The blue bar incorporates Cameroon and Democratic Republic of the Congo samples. Bootstraps from 100 iterations.

1.4. Discussion

1.4.1 Clinical signs with trypanosome infections

We were unable to detect a difference in the pattern of clinical signs between horses infected with single and mixed species of trypanosome. This was due to a combination of low sample size in each species category, animals in the control group showing some clinical signs and a relatively high proportion of PCR positive horses not showing clinical signs. Horses in the control group may have been infected with undiagnosed trypanosome infections or with a different pathogen. Clinical signs produced by trypanosome infections are non-specific and there are a number of other diseases in the region which may overlap with one or more of the clinical signs. Of these, biliary fever caused by *Babesia caballi* and *Theileria equi* mimics trypanosome infection most closely, may induce all but the neurological clinical signs, and responds to but is not cured by some trypanocidal medications[35].

A relatively high proportion of horses which tested PCR positive were not recorded as showing clinical signs in the two weeks prior or post diagnosis. It is possible that a degree of observer bias may have affected the results as a number of observers reported clinical signs over an extended time period. Ideally a checklist to record presence or absence of clinical signs as well as heart rate, respiratory rate and temperature would be used in future. Horses infected with *T. brucei* without exhibiting clinical signs is of interest as this species of trypanosome is traditionally thought of as being highly pathogenic to horses[22]. Variation in disease may be a result of parasite genotype, dose, mixed trypanosome infections which may modulate pathogenicity[16,22] and the host response. A spectrum of disease has also been found in human African trypanosomiasis (HAT)[36]. *T. brucei rhodesiense*, the cause of HAT in East Africa, is characterized by presence of the *SRA* gene and can be regarded as a host range variant of *T. brucei brucei*[37]. Encephalitis due to trypanosome invasion of the central nervous system (CNS) is a feature of late stage trypanosomiasis in HAT. It is uncommon for *T. brucei* infections in cattle, sheep or goats to involve the CNS[21]. A murine model has been developed to study the neuropathology of trypanosome infections, generally using laboratory strains of trypanosomes[38].

Further investigations of *T. brucei brucei* genotypes collected from horses with a range of outcomes, from clinically insignificant to cases of mortality, may yield insights into virulence determinants for *T. brucei* in horses and humans.

1.4.2 Trypanosoma vivax infections

No horses were diagnosed with *T. vivax* infections during the study in contrast to studies from The Gambia where *T. vivax* infections are common in the horse and donkey populations[16,18,39]. The current study's result is consistent with another survey from the Serengeti and surrounding villages on livestock and wildlife using the same species specific primer sets[27,28] where a low prevalence of 0.04% in cattle[40] and 0.03% in wildlife were found[40]. However, a high level of genetic diversity has been reported in East African *T. vivax*[41,42] and whether the species specific primers used in this study are capable of detecting all East African *T. vivax* genotypes is not known. This could be investigated by laboratory testing of representative samples of each *T. vivax* genotype isolated from tsetse with species specific primers. It would also be of interest to test samples from three horses which tested positive by microscopy, but consistently PCR negative on all three trypanosome-specific PCRs, with alternate trypanosome primers or alternate methods such as fluorescent fragment length barcoding (FFLB) as used in the tsetse survey[42]. Other trypanosome species not currently known to infect horses but commonly found in tsetse from the area include *T. godfreyi*, *T. simiae* and *T. simiae* Tsavo[43].

1.4.3 Mortality due to trypanosome infection

The case specific mortality rate due to trypanosome infection was high in this horse population, at 78% (7/9). Additionally, six horses which arrived at the safari lodge prior to 2005 and were not included in the study died with clinical histories consistent with trypanosomiasis, two of which were laboratory confirmed. Five horses tested PCR positive for *T. brucei* in the brain or on CSF. Although not all these samples were also tested for *T. congolense* and *T. vivax*, where this was done, these tests were negative. Histopathology carried out on two of the horses found a lymphoplasmactic meningoencephalitis, which is consistent with previous reports of *T. brucei* complex infections in horses[13,44].

Differential diagnoses for meningoencephalitis including equine herpes virus and West Nile virus were ruled out in these cases.

1.4.4 Trypanosomes on the move

A number of horses were sold during the study period as they were unsuitable for pleasure riding. Of these one went onto develop trypanosomiasis and died and another left after testing PCR positive for *T. congolense* while microscopy negative, and did not receive any treatment. Movements of infected horses are of concern in the development of new geographic foci of trypanosome infections. Recent studies have shown that frequent emergence of *T. brucei evansi* and *T. brucei equiperdum* is possible due to partial or complete loss of the kinetoplastid DNA of *T. brucei*[19]. These mutant strains of *T. brucei* are not restricted by the range of the tsetse vector and may be transmitted by biting flies or sexual contact.

1.4.5 Multilocus genotype similarity analysis

Analysis of MLGs from five samples which consistently amplified found that the isolates in the horse cases clustered within a clade which contained Tanzanian wildlife samples, and human samples from Malawi, Zambia and Uganda (*T. b. rhodesiense*), but were distinct from *T. b. gambiense* isolates, which fits with expectations. This confirmed the hypothesis that infections in the horse population arose as spill over from the wildlife populations in the Serengeti. Three of the samples were taken from a single horse 'William' at different time points; sample 41 on 4 October 2006, sample 43, 18 October 2006 and 159 on 1 July 2007. A different *T. brucei* genotype was identified at each time point suggestive of reinfection. However, while no evidence of a mixed infection was found in the microsatellite data results, this cannot be ruled out as there is evidence that a single dominant genotype can modulate levels of coinfecting genotypes[45]. Therefore, we cannot formally rule out treatment failure and emergence of an underlying parasite genotype.

1.4.6 Incidence rate estimate

The incidence rate estimate of 0.11 cases per month at risk is robust as an estimate of the lower limit of the incidence in this population. The threshold of detection by buffy coat smear has been recorded as $2.5x10^2$-$5x10^3$ parasites/ml[46] and *T. brucei* species specific PCR testing on a single disc of FTA card as 1×10^3 parasites/ml[47]. Therefore, horses with parasitemias lower than this are unlikely to be detected. Improving the test sensitivity will increase both the numerator (number of cases detected) and the decrease the denominator (time at risk). The first case of trypanosome infection only was taken to calculate the incidence. This was due to difficulty in reliably saying an animal was clear of infection following treatment. Microsatellite multilocus genotyping was carried out to distinguish between new infections and relapses of a previous infection. Unfortunately only a small proportion of the diagnostic positive PCR-positive sample amplified consistently with the microsatellite PCRs, most likely due to low parasitemias in the blood samples. The nested microsatellite PCR target a single copy locus on the trypanosome genome in contrast to thousands of copies of target for the *T. brucei* species-specific PCR. An important assumption made in this estimate was that prophylactic medication was effective for 90 days and horses were not contributing to time at risk during this period. In fact three horses were diagnosed with trypanosome infections 15, 53 and 85 days post prophylactic treatment administration. In the case infected at 85 days post prophylaxis it was noted that the horse had been exposed to a high tsetse challenge two weeks previously. The other two cases may also be examples of 'break through' infections due to high challenge, or problems related to drug dose and administration, or an undiagnosed infection prior to the drug treatment. Studies which measure incidence in equine populations are uncommon; a study from the Gambia[39] found an average monthly incidence of 16%. Whereas in the current study an average of the time to onset of the first trypanosome infection in each horse was made over the five year study period, the Gambian study calculated the average monthly incidence as a proportion of the total number of horses which tested positive on any given month. Horses which were found to be infected were treated and assumed naïve to infection. This is akin to studies in cattle whereby reinfection rates in treated cattle are

known as the 'Berenil Index' and used to give an index of trypanosome challenge[48].

1.4.7 Test sensitivity and suggested improvements

Test sensitivity was a major obstacle in trying to carry out epidemiological and genetic analysis and also in assessing patterns of clinical signs. Parasitemia tends to be higher in the early acute stages of a trypanosome infection, and the duration and size of patent parasitemia will depend on the host and trypanosome strain[21] but relatively susceptible hosts such as the horse can reach 10^8 parasites per ml in the early stages of infection. Clinical signs in the acute stage of infection tend to coincide with the peaks of parasitemia[21]. However, a feature of trypanosome infections is that in between peaks, parasitemias drop to below detectable levels for considerable periods of time. As horses were being monitored daily, the clinical signs associated with acute infection should have been detected and blood samples taken at this time. However, the range of values for peak parasitemias in field infections of horses is unknown and it is possible that they lie around the detection threshold with the current methods of diagnosis. Alternative methods of blood storage to FTA cards such as the use of PAXgene tubes (Qiagen) and subsequent DNA extraction prior to molecular testing offers the possibility to exploit the maximum sensitivity of the PCR methods[24]. As the threshold of detection of PCR based methods is 1/10 trypanosome per ml[49] compared with $1x10^3$ parasites per ml using a single FTA card punch[47], simply altering the methods of blood collection and starting template for the PCR offers an estimated 1,000-10,000 times increased sensitivity. The development of highly sensitive and specific molecular techniques such as loop-mediated isothermal amplification (LAMP)[50] provide means of diagnosing trypanosome infections suitable for field use. Currently molecular testing is carried out retrospectively and does not benefit diagnosis or treatment of these horses. Added value could be gained from combining these more sensitive diagnostic tools with standardized recording of clinical signs and regular sampling of both clinically ill and healthy horses. This would be of interest for the prevention and treatment of trypanosome infections in horses kept in this area of high challenge and also in understanding variation in

outcome of infections with *T. brucei* and how this relates to parasite genotype, host responses and other factors such as drug treatments.

Conclusion

To conclude, a number of infections with *T. brucei*, *T. congolense* and mixed infections were diagnosed in this horse population. Analysis of MLGs from five cases of *T. brucei* confirmed that these likely arose as spill over of infection from the resident wildlife population. No characteristic clinical signs could be associated with the onset of infection with either species of trypanosome, however *T. brucei* was the only trypanosome detected in the brain and CSF of fatal cases. No cases of *T. vivax* infections were found in contrast to studies from the Gambia where this is one of the most common species of trypanosome infecting horses. A proportion of horses found to be infected with *T. brucei* and *T. congolense* had no clinical signs recorded in the two weeks prior and post PCR diagnosis. This is the first report of asymptomatic *T. brucei* infections in horses and while this only assesses a four week window in the course of these infections it suggests that there may be a range of disease associated with this pathogen in horses. A spectrum of clinical signs has also been reported previously in human cases of trypanosomiasis caused by the closely related parasite *T. brucei rhodesiense*. Studies on this population of horses offer the opportunity to evaluate the prevention and treatment of trypanosome infections in an area of high challenge. Further investigations into the host parasite relationship in cases of mild to severe disease with *T. brucei* infections may yield information of value to both animal and human trypanosomiasis.

Chapter 2

Determining Key Hosts for the Transmission of Lyme Borreliosis Pathogens in Scotland

2.1. Introduction

Lyme borreliosis is the most common vector borne disease in the northern hemisphere. In Europe multiple strains of the causative bacteria circulate among several wild reservoir species and the tick vector, *Ixodes ricinus*. Disease in people ranges from a localized skin infection to a range of debilitating diseases which includes carditis, arthritis, neuroborreliosis and chronic skin complaints[51]. Reported cases of lyme disease in Scotland have increased over 15 times in the last decade[4]. A number of causes for the sharp increase in cases have been proposed, these include ecologic factors such as increasing tick populations[52,53], increased human exposure to ticks and increasing public and general practitioner awareness of this disease[54]. In the Highland Health Region in the northwest of Scotland, the annual incidence approaches that seen in endemic areas of central Europe and America[54-56]. The annual cost of lyme borreliosis to Scotland in 2002 was estimated as £331,000[57], however the current cost may be several times higher.

The causative agents of Lyme disease are spirochaete bacteria belonging to the *Borrelia burgdorferi* sensu lato species complex. Of the 15 geno-species in this group, *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. burgdorferi sensu stricto* have been detected in ticks in Scotland[58,59]. *Borrelia afzelii* was the most common geno-species detected (48%) followed by *B. garinii* (36%), *B. valaisiana* (8%) and *B. burgdorferi sensu stricto* (7%)[59]. *Borrelia afzelii* and *garinii* are the dominant genospecies elsewhere in Europe[60] whereas other studies from the UK mainly based in southern England *B. garinii* and *B. valaisiana* dominate[61]. *B. afzelii*, *B. garinii*, and *B. burgdorferi sensu stricto* all cause disease in humans and are associated with different clinical manifestations[62]. *Borrelia valaisiana* has been detected in human skin biopsies with erythema migrans lesions[63] but the pathogenicity is currently uncertain. All genospecies are transmitted by the same vector, *Ixodes ricinus*, and selection is thought to be mediated by the host immune system. Each genospecies has a different set of reservoir hosts due to variable resistance to host complement[64]. *B. garinii* and *B. valaisiana* are associated with bird reservoir hosts, while *B. afzelii* is a rodent associated geno-species and *B. burgdorferi sensu stricto* has both mammalian and avian reservoir hosts[62].

Transmission of lyme disease to humans in Scotland is by the bite of an infected *Ixodes ricinus* tick, most commonly an infected nymph. Nymph infection prevalence is an important risk factor for human lyme disease[65]. Ticks may become infected with *B. burgdorferi* s.l. by three potential routes, transtadial, transovarial and co-feeding transmission. Transtadial infection is thought to be the most significant ecologically[65] and occurs when a larvae or nymph become infected after feeding on a reservoir host and maintains this infection through to the next lifestage. Knowledge of the reservoir hosts in a community, how often they are fed on and by which tick lifestages will affect *B. burgdorferi* s.l. prevalence in the tick population and human risk of infection. Previously large scale field trapping studies were carried out to establish host abundance, tick burdens and borrelia infection rates. An alternate approach is to use molecular tools to identify host blood meal remnants in the gut of questing ticks, and then correlate this with *B. burgdorferi* s.l. infection data[66]. The current study aims to identify potential reservoir hosts for *B. burgdorferi* s.l. in Scotland by a review of the literature and secondly, by adapting the host blood meal analysis using the 12S rDNA mitochondrial marker[66], followed by sequencing of the PCR product.

2.2. Materials and Methods

2.2.1 Literature Review

A search of the literature was carried out to identify information on known reservoir host species in Scotland. Studies which included species present in Scotland, but conducted in another European country were included. Host species of unknown reservoir potential were also identified. Both PubMed and Biosis search engines were used to search the literature, using the key words “lyme disease, Scotland and reservoir host”. References from the articles these searches produced were also included in the review.

2.2.2 Collection of Field-Derived Feeding and Questing ticks

Feeding ticks were collected from bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) trapped as part of a separate study in 2010

and kept in 70% alcohol. Questing nymphs and adult ticks were collected by blanket drags across ground vegetation in mixed woodland, Loch Lomond National Park in July 2010 and April 2011, known to have both bank voles and wood mice present. As previous studies had shown greater than 99% of questing ticks (R. Biek pers.comm.) to be *Ixodes ricinus* no species identification was carried out. Ticks were removed from the blanket with forceps and placed in 70% alcohol.

2.2.3 DNA Extraction

Nymphs and adults were removed from 70% alcohol, allowed to air dry, then placed in an eppendorf tube with 180µl of lysis buffer (QIAGEN) and 20µl of protease K (QIAGEN) and cut up into fragments with scissors. Eppendorfs were placed on a rotating incubator at 56ºC overnight to allow digestion. DNA extraction was then carried out using DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions in a biosafety cabinet with built in UV lamps. One negative control for every two samples was included with each DNA extraction. Tick lysates were stored at -20ºC prior to PCR analysis.

2.2.4 PCR Amplification

Forward and reverse primers (12S-6F CAAACTGGGATTAGATACC, and B-12S-9R 5'biotin-AGAACAGGCTCCTCTAG) were used to amplify the ~145bp 12S rDNA fragment to distinguish between vertebrate host species[66]. Briefly, a 50µl reaction mix was prepared containing 3.0mM $MgCl_2$, 0.2mM dNTPs, 0.8µl of each primer, 1 x Taq buffer, 1.25U Taq polymerase (Invitrogen) and water. 20µl of tick lysates was added as template. 2µl of DNA extract from blood fed ticks and 18µl of water was used as a positive control, 20µl of water was used in negative controls. DNA extraction controls were included in the PCR as well as one PCR negative control per 3 samples. Touchdown amplification conditions were as previously described[66]. PCR products were run on a 2% agarose gel stained with ethidium bromide. As cross contamination was a risk during this protocol, DNA extractions, PCR set up and loading of gels were all carried out in separate rooms.

2.2.5 Sequencing

The PCR product was purified by adding 5µl of PCR product to 2µl of Exo-SAP-IT® (Affymetrix) and incubating for 15 minutes at 37ºC and 15 minutes at 80ºC. Then, 4µl of purified product was added to 26µl of water and sent to The Sequencing Service, University of Dundee. The 12S-6F ext 1 primer was used to amplify the 145bp product. The cycle sequencing products were run in an ABI 3730 capillary DNA sequencer (Applied Biosystems). Sequences were aligned in Geneious software and then compared to sequences in Genbank using the nucleotide BLAST program (NCBI) to identify the host species.

2.3. Results

Reservoir hosts for *B. afzelii* are shown in Table 1.1, incompetent host species and species of unknown reservoir status for *B. afzelii* are listed in Table 2.2, reservoir hosts for *B. garinii* and *B. valaisiana* are listed in Table 2.3 and species of unknown reservoir status for *B. garinii* and *B. valaisiana* are listed in Table 2.4.

Four engorged ticks, 3 removed from wood mice and 1 removed from a bank vole were correctly identified using host blood meal analysis. Problems with contamination of DNA extracts with human DNA prevented amplification of host DNA from questing nymphs and adults.

Table 2.1: Vertebrate Reservoir hosts for *Borrelia afzelii* in Scotland. List produced from review of the scientific literature. Xenodiagnosis is the gold standard for identifying a reservoir host species.

Host species	Common name	Method used to determine reservoir status	Reference
Clethrionomys glareolus	Bank vole	Xenodiagnosis	[67,68]
Apodemus sylvaticus	Wood mouse	Xenodiagnosis Infected larvae	[68,69]
Microtus agrestis	Field vole	Infected larvae	[70,71]
Sorex araneus	Common shrew	Infected larvae	[69]
Neomys fodiens	Water shrew	Infected larvae	[71]
Sorex minutus	Pygmy shrew	Infected larvae	[69]
Rattus norvegicus	Norway rat	Xenodiagnosis	[72]
Sciurus vulgaris	Red squirrel	Infected larvae	[70]
Sciurus carolinensis	Grey squirrel	Xenodiagnosis	[70]
Erinaceus europaeus	Hedgehog	Xenodiagnosis	[73]
Vulpes vulpes	Fox	Xenodiagnosis	[70]

Table 2.2: Incompetent/ unknown reservoir hosts, *Borrelia afzelii* in Scotland. List produced from review of the scientific literature.

Host species	Common name	Unknown reservoir status / incompetent reservoir host	Reference
Cervus elaphus	Red deer	Incompetent	[70]
Capreolus capreolus	Roe deer	Incompetent	[71,74]
Cervus nippon	Sikka deer	Incompetent	[70]
Dama dama	Fallow deer	Incompetent	[70]
Mustela nivalis	Weasel	Unknown	N/A
Mustela erminea	Stoat	Unknown	N/A
Meles meles	Badger	Unknown	N/A
Talpa europaea	Mole	Unknown	N/A
Oryctolagus cuniculus	Rabbit	Unknown	N/A
Neovison vison	American mink	Unknown	N/A
Lutra lutra	Otter	Unknown	N/A
Martes martes	Pine marten	Unknown	N/A

Table 2.3: Reservoir hosts for *Borrelia garinii* / *B. valaisiana* in Scotland. List produced from review of the scientific literature. Xenodiagnosis is the gold standard for identifying a reservoir host species.

Host species	Common name	Method used to determine reservoir status	Reference
Turdus merula	Blackbird	Xenodiagnosis	[70]
Turdus philomelos	Song thrush	Infected larvae	[70]
Anthus trivialis	Tree pipit	Infected larvae	[70]
Erithacus rubecula	Robin	Infected larvae	[70]
Fringilla coelebs	Chaffinch	Infected larvae	[70]
Parus major	Great tit	Infected larvae	[70]
Phasianus colchicus	Pheasant	Xenodiagnosis	[75]
Phoenicurus phoenicurus	Redstart	Infected larvae	[70]
Sylvia atricapilla	Blackcap	Infected larvae	[70]
Prunella modularis	Dunnock	Infected larvae	[76]
Carduelis chloris	Common greenfinch	Infected larvae	[59]

Table 2.4: Species of unknown reservoir status for *B. garinii*/ *B. valaisiana* in Scotland. List produced from review of the scientific literature.

Host species	Common name	Incompetent/ Unknown reservoir status
Carduelis flammea	Redpoll	Unknown
Carduelis spinus	Eurasian siskin	Unknown
Turdus viscivorus	Mistle thrush	Unknown
Phylloscopus trochilus	Willow warbler	Unknown
Regulus regulus	Goldcrest	Unknown
Periparus ater	Coal tit	Unknown
Cyanistes caeruleus	Blue tit	Unknown
Pyrrhula pyrrhula	Bullfinch	Unknown
Certhia familiaris	Treecreeper	Unknown
Lagopus lagopus scotica	Red grouse	Unknown
Numenius arquata	Curlew	Unknown

2.4. Discussion

2.4.1 Reservoir hosts

A number of reservoir hosts for lyme disease in Scotland were identified by reviewing the literature. As well, a number of vertebrate hosts considered incompetent or of unknown reservoir status were also identified. Criteria used to identify reservoir hosts varied widely in the literature and made comparison of relative reservoir competence difficult. Larval xenodiagnosis is considered to be the gold standard for identifying reservoir hosts[70]. This method involves keeping a potential reservoir host in captivity, experimentally infecting the host with *B. burgdorferi*, then allowing laboratory reared uninfected larval ticks to feed on the animal, moult into nymphs, and testing the nymphs for infection.

Xenodiagnostic studies varied in the method of infection, needle versus tick inoculation and the strain of *B. burgdorferi* used. Care must be taken in extrapolating these data to the field as reservoir competence is not constant and will vary following infection according to a number of intrinsic and extrinsic factors[68]. An alternative method to establish reservoir status is to collect engorged larvae from field trapped animals and measure infection prevalence in the larvae. The prevalence in the host-fed larvae is then compared to the prevalence in questing larvae to allow for transovarial transmission of *B. burgdorferi*. Challenges associated with this approach are that it is difficult to trap all members of a community which may be hosting ticks. Rodents are frequently trapped and avian species that are easily mist netted are most commonly sampled. Larval ticks can be hard to identify and remove from hosts and so infected larvae may be missed.

Blood meal analysis offers an alternative method to identify reservoir hosts which accounts for natural variation in reservoir status and host associated nymph survival. It also allows the opportunity to evaluate alternate mechanisms of *B. burgdorferi* transmission. For example co-feeding transmission, demonstrated to occur experimentally[77] has been found to be important in some locations[78,79]. Although deer are considered incompetent reservoir hosts[70] finding spirochaetes in the skin of Sikka deer[80], together with the presence of *B. afzelii*,

B. valaisiana and *B. burgdorferi* s.s with artiodactyl blood remnants[66] suggest that it may be possible. The disadvantages of blood meal analysis must also be born in mind, detection rates as discussed above can be disappointingly low and blood meal analysis does not provide any measure of host associated tick mortality associated with unsuccessful feeding attempts.

2.4.2 Host blood meal analysis

Ixodes ricinus are generalist feeders and may have fed several weeks to months prior to questing so host blood meal analysis has been designed to be extremely sensitive and also non-specific, capable of detecting a wide variety of vertebrate host species. Both of these design features have the undesirable effect of making the protocol very susceptible to contamination. The current study faced challenges associated with contamination with human DNA which was found to have been introduced during the DNA extraction procedure. This was eventually controlled by stringent laboratory protocols, performing the extractions in a UV cabinet in a separate room to the PCR set up and running of gels, using new reagents and allowing one control for every two samples. Carrier RNA (QIAGEN) which increases the yield of DNA using the DNeasy blood and tissue kits (QIAGEN) was found to amplify in the PCR reaction resulting in false positives, and thus was left out of the extraction protocol. Whether this will significantly affect the yield of host DNA from questing ticks is not known and needs to be investigated further.

Reverse line blotting (RLB) has been used previously as an alternate method to sequencing of the PCR product to identify the host species[66]. This technique runs a number of genus and species specific probes against the PCR product on a miniblotter. Contamination with human DNA was reported as a major problem using this technique as well, and general mammalian and human probes were omitted from the RLB protocol[66].

Previous host blood meal analysis studies have successfully amplified DNA from between 30-48% of questing *I. ricinus* nymphs and adults[66,81,82] The relatively low detection rate in the field is despite laboratory studies which have found 100% detection over 280 days post feeding[83]. Causes for the low detection rate have

not been investigated, and need to be established before the data is used in ecological models. Potential reasons include increased DNA degradation with increasing time from the host blood meal and differential sensitivity of the PCR between host species. As a first step the time since feeding could be estimated by measuring the fat content of questing ticks. Fat is gradually depleted after the tick moults according to tick activity and has previously been used as a marker of physiological aging in studies of *I. ricinus* seasonal dynamics[84].

Conclusion

The review of the literature identifies which hosts are potentially important as reservoir for *B. burgdorferi s.l.* in Scotland. By combining this data with information on host abundance and distribution the relative importance of different wildlife species can be established. Blood meal analysis offers an elegant way to find out the community of hosts *I. ricinus* are feeding on, which species are the important reservoir hosts, a means of identifying new reservoir hosts and to address whether alternate means of transmission are significant. Low detection rates in questing ticks and laboratory contamination are the main challenges with this technique which require further exploration and refinement. Knowledge of which hosts are important in maintaining tick populations and as reservoirs for *B. burgdorferi* will allow a greater understanding of lyme disease dynamics and human risk of infection in Scotland.

Chapter 3

Analysis of Suspected Wildlife Crime Cases submitted for Forensic Examination in Scotland, 2010

3.1. Introduction

Wildlife crimes have significant impacts on conservation, animal welfare, domestic animal and human health and the economy. The abuse and misuse of pesticides as well as unlicensed and illegal use of firearms in the countryside are risks to domestic animal and human health[85]. In Scotland several high profile wildlife crime cases have contributed to increased public and political will to prevent and prosecute those responsible. In 2006, the National Wildlife Crime Unit (NWCU) was established to combat wildlife crime in the United Kingdom. At the launch of the NWCU, Paddy Tomkins the former Chief Constable of Lothian and Borders Police and ACPOS lead on wildlife and environmental crime at the time said[86]:

> "The significance of wildlife crime cannot and should not be under-estimated. It has a direct impact on the economic, environmental and cultural lives of communities and that is especially true in Scotland where we have some very diverse and critically important wildlife. Wildlife crime is not victimless, it impoverishes us all."

Wildlife crime has been defined by the Partnership for Wildlife Crime Scotland (PAWS) as any unlawful act or omission, which affects any wild creature, plant or habitat, in Scotland [87]. Unlawful acts are described in a number of different primary and secondary legislation[87]. As well as the establishment of the NWCU there have been a number of specialist positions for wildlife crime police officers and wildlife crime coordinators created within Scottish Police Forces and specialist wildlife crime prosecutors appointed within the Crown Office and Procurator Fiscal Service (COPFS). Changes have been made to legislation to improve powers of enforcement[88], however, despite these advances, figures on wildlife poisoning in Scotland in 2007 were found to rise together with low numbers of prosecutions[88]. A parliamentary debate on wildlife crime the same year achieved cross-party support in acknowledging the value of Scotland's wildlife and natural heritage and in finding ways to improve the existing system of wildlife crime prevention, investigation and prosecution. As a result of this, a

major review of wildlife crime investigation and prosecution was initiated and completed in 2008[88] which made a number of recommendations on how to improve the current system.

The contribution of forensic pathology to the investigation and prosecution of wildlife crimes has not yet been evaluated in Scotland. Forensic veterinary pathology is the application of knowledge of veterinary pathology to provide evidence for the Courts[89] and is often an integral part of a wildlife crime investigation. It includes recording descriptions of injuries or disease and interpreting these findings appropriately so they may be used as evidence in a Court of Law. For the purpose of this study the definition of wildlife crime was restricted to animal cases in which post-mortem examinations and/or toxicology screening was carried out. Of interest was the types of crime committed, which species were targeted, and the outcome of investigations.

3.2. Methods

3.2.1 Case Selection

Forensic pathology reports from cases submitted as suspected wildlife crime cases were requested from all eight of the Scottish Agricultural College (SAC) Consulting: Veterinary Services Veterinary Investigation Centres and the University of Glasgow, and toxicology reports were requested from Science and Advice for Scottish Agriculture (SASA), from January 1 - December 31 2010.

3.2.2 Data Collection

Information on the species, type of crime, location, details of ancillary testing (e.g toxicology and bacteriology), and submitting agency were collected from the reports and summarized. A case was classified as a suspected crime, a possible crime or not suspicious based on the post-mortem evidence. Possible crimes were classified as those cases in which the history and results of forensic examination were suspicious for criminal activities, but could not be confirmed definitively. Cases were classified as not suspicious if an alternate, non-criminal cause of death for the animal was found, or the cause of death was not able to

be determined in cases found dead with no other suspicious circumstances. The submitting agency or the National Wildlife Crime Unit were contacted in cases where crimes were suspected to determine whether a report was submitted to the Fiscal, if the case was prosecuted and what the outcome of the prosecution was.

3.3. Results

3.3.1 Cases of suspected wildlife crime

A total of 188 cases of suspected wildlife crime were submitted for forensic examination. Cases submitted to the SAC (n=142) and University of Glasgow (n=5) received full postmortem examinations, cases submitted to SASA (n=41) were sampled for toxicology testing only. Additional testing including toxicology, bacteriology, histology and DNA analysis was carried out on post-mortem cases where required. The majority of cases were from avian species which composed 80% of submissions (n=150) (Figure 3.1). Raptors were the most frequently submitted category of birds (n=114). After adjusting for single submissions that included multiple raptors, the total number of birds submitted increased to n=130 (Figure 3.2). Buzzards (*Buteo buteo*) were the most common type of raptor submitted and composed 55% of raptor species (n=71). A number of endangered* (International Union for the Conservation of Nature (IUCN) Red List) or UK bird species of conservation concern[90] (Royal Society for the Protection of Birds (RSPB) red or amber status but not IUCN listed) were also submitted including Red Kites* (*Milvus milvus*) (n=18), Golden Eagles (*Aquila chrysaetos*) (n=8), White Tailed Eagles (*Haliaeetus albicilla*) (n=3), Kestrel (*Falco tinnunculus*) (n=1) and the Hen Harrier (*Circus cyaneus*) (n=1).

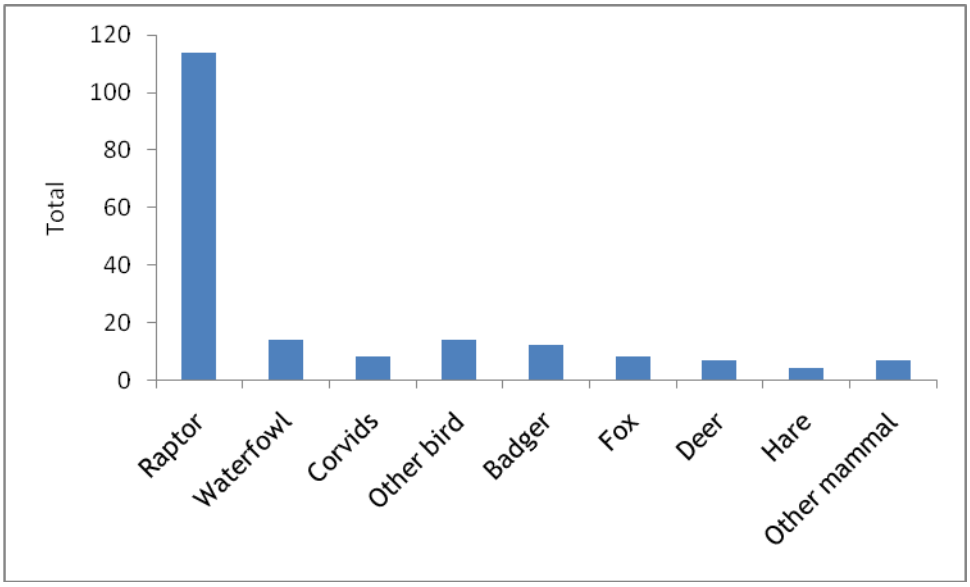

Figure 3.1: Wildlife submitted to SAC Consulting: Veterinary Services Veterinary Investigation Centres, Science and Advice for Scottish Agriculture (SASA), and the University of Glasgow for forensic post-mortem examination, January 1 – December 31, 2010 (n=188).

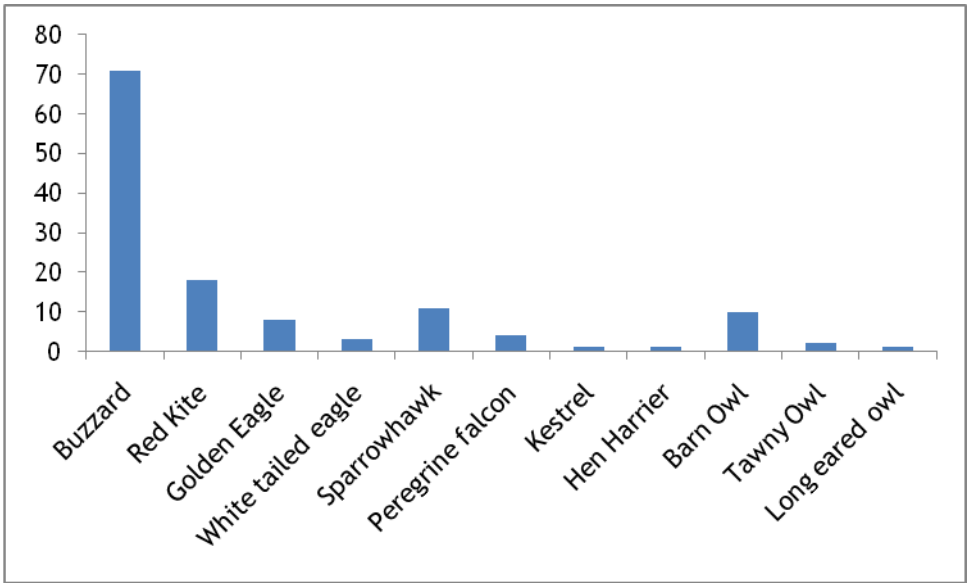

Figure 3.2: Species of raptor submitted to SAC Consulting: Veterinary Services Veterinary Investigation Centres, Science and Advice for Scottish Agriculture (SASA), and the University of Glasgow for forensic post-mortem examination, January 1 – December 31 2010, for forensic post-mortem examination. Includes raptors listed under multiple carcass submissions (n=130)

3.3.2 Cause of death

The cause of death was established in 66% of cases (Figure 3.3) (n=124), trauma from either gunshot, predator attack or malicious and non malicious trauma was the most common cause of death, accounting for 38% of cases (n=72). The cause of death was undetermined in 34% of cases (n=64). Poisoning accounted for 18% of cases (n=33).

A suspected cause of death was recorded in 91 reports, poisoning was suspected in 58 of these. Poisoning was confirmed in 31% of suspected cases (n=18). In the 69% of suspected poisoning cases where no poisons were detected, other causes of death, including suspected crimes such as trauma from gunshot and leg traps were diagnosed. Cases of infectious disease and starvation were also diagnosed.

The post-mortem evidence supported the suspicion that a crime had been committed in 28% of cases (n=53), was supportive but not conclusive in a further 16% (n=31) of cases and no evidence was found to support a crime in 55% of cases (n=104). This figure included cases of non-suspicious causes of death (n=51), and cases where the cause of death was undetermined (n=53).

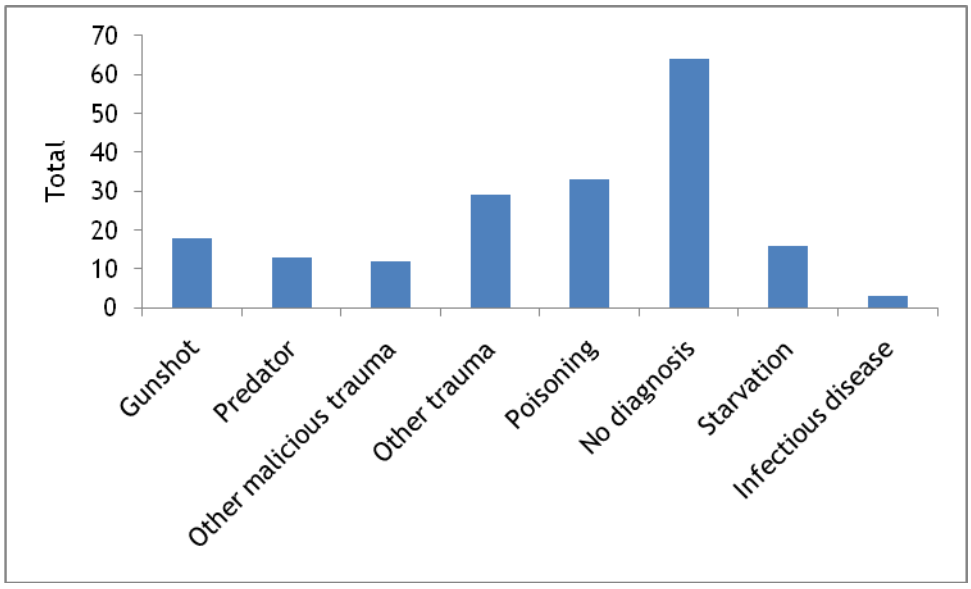

Figure 3.3: Cause of death for wildlife forensic pathology cases submitted to SAC Consulting: Veterinary Services Veterinary Investigation Centres, Science and Advice for Scottish Agriculture (SASA), and the University of Glasgow from January 1 – December 31 2010.

3.3.3 Ancillary testing

Toxicology was the most frequent ancillary diagnostic test carried out on forensic cases, being completed in 67% of cases (n=126) (Figure 3.4). Poisoning was the cause of death in 18% of cases (n=33), the most common poison detected was the carbamate carbofuran, which was responsible for 42% of deaths (n=14) (Figure 3.5). Poisoning due to diazinon, cyanide and rodenticides were not considered to be crimes. The source of diazinon was thought to be a licenced veterinary ectoparasite product applied to lambs, crows became intoxicated after eating lambs tails following tail docking. Cyanide poisoning was suspected in birds in which toxic berries were found in their stomachs. Rodenticide poisoning is likely to be secondary, following consumption of rodents poisoned with licensed products.

Sublethal poisoning with rodenticide products was found in 32% of raptors (n=37). This was most commonly with bromodiolone alone or combinations of bromodiolone and difenacoum or brodifacoum. Dichlorodiphenyldichloroethylene (DDE), a break down product of organochlorine insecticide dichlorodiphenyltrichloroethane (DDT) was detected in three raptors and the chlorinate hydrocarbon insecticide dieldrin in one bird. Bromodiolone was also detected in one crow (*Corvus corone*) and a grey heron (*Ardea cinerea*).

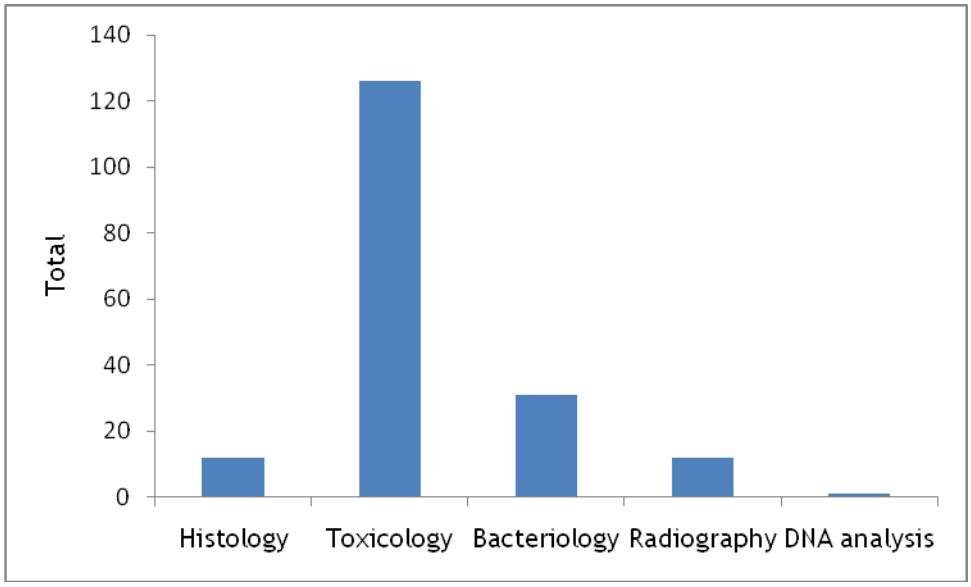

Figure 3.4: Ancillary testing carried out on wildlife forensic pathology cases submitted to SAC Consulting: Veterinary Services Veterinary Investigation Centres, Science and Advice for Scottish Agriculture (SASA), and the University of Glasgow from January 1 – December 31 2010. Each category is not mutually exclusive.

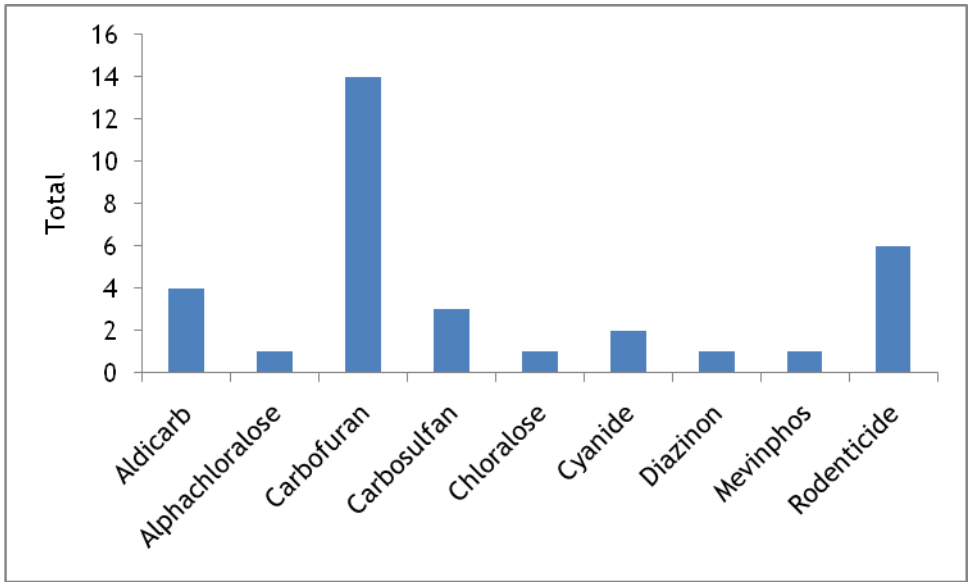

Figure 3.5: Toxins detected at lethal levels in wildlife forensic pathology cases submitted to SAC Consulting: Veterinary Services Veterinary Investigation Centres, Science and Advice for Scottish Agriculture (SASA), and the University of Glasgow, January 1 – December 31 2010.

3.3.5 Outcomes of wildlife crime investigations

The police incident number was not always available from the post mortem report to track investigations. Each of the eight police forces in Scotland has a separate computer database and outcomes of investigations and prosecutions are not reported back to either the submitter or a central agency such as the NWCU. Further information was available on a proportion of suspected crime cases (n=18), found by contacting individuals involved in the investigation. Of these, 13 cases were submitted to the Fiscal, and five of these were successfully prosecuted. The remaining cases submitted to the Fiscal are ongoing or the outcome is currently unknown. One case was time barred due to not being prosecuted within 6 months. At least one case was prosecuted for offences not connected to the original incident. One case was given a warning and no report was submitted to the Fiscal.

3.4. Discussion

3.4.1 Cases of suspected wildlife crime

Eighty percent of the cases submitted for forensic post-mortem examination or toxicology sampling were avian species and raptors made up the majority of these cases. Various potential sources of bias will affect which cases are submitted. These include detection bias, relating to the size of the carcass, rate of predation and decomposition[91], public awareness of wildlife crime, and the species concerned. Many cases in rural areas will be detected by chance by the public during other recreational pursuits. Their awareness of the range of potential wildlife crimes, and the responsiveness of the agencies contacted including the local police force, RSPB or SSPCA, will determine the likelihood of a submission.

There is good evidence that police forces which have a wildlife crime coordinator are more likely to investigate and submit potential cases[88] . The national and local wildlife crime priorities for investigating wildlife crime are also likely to affect the cases received. These priorities agreed by the Wildlife

Law Enforcement Working Group (WLEWG)[92] include combating badger, bat and raptor persecution and deer, hare and fish poaching[92]. Certain raptor populations of conservation concern such as Red Kites, Golden Eagles and Sea Eagles have higher levels of monitoring, hence mortalities are more likely to be detected and investigated.

Species that were submitted for forensic post-mortem examination less frequently than expected include certain mammal species and fish. Mammalian cases made up 20% of submissions, badger (*Meles meles*) cases represented 6%, deer cases 4% and hare cases 2%. According to NWCU incident data hare coursing represents 20% of wildlife crime, badger persecution 14% and deer poaching 8%. These data are compiled for the United Kingdom from incidents reported to the police and categorized according to defined criteria. Scotland is overrepresented for all of the above categories of wildlife crime incidents and also reports over a third of all reports of fish poaching[92]. Reasons as to why these species are not commonly submitted for post-mortem examination should be explored with both police wildlife crime investigators and with the COPFS to see if forensic evidence would be of benefit in prosecuting these crimes. Possible reasons for low numbers of submissions include lack of awareness for the need or usefulness of forensic examination and logistical difficulties in transporting larger carcasses to a laboratory.

3.4.2 Forensic examination

The post-mortem examination is critical to describe the nature of the injuries causing death, to recover evidence such as bullets or shot from the carcass to be used as evidence and to rule out non criminal reasons for the animal's death[93]. The most common cause of death was due to trauma of various causes and included a number of different types of crimes including gunshot, snares and predator attacks and cases of abuse. Time to discovery of a carcass can be critical in order to diagnose certain types of malicious trauma, especially those involving soft tissue injuries only, due decomposition of the carcass. Under the Snares Scotland Order 2010, it is necessary to that snares are free running and checked at least once in 24 hours and under the Wildlife and Countryside Act 1981, as amended by the Nature Conservation (Scotland) Act 2004, any dead or

live animals should be then removed from the snare. Establishing a crime had occurred often depended on being able to confirm an animal had been in snare for longer than 24 hours. This was found to be challenging in a number of reports due to the time from death to examination, as well as continual friction from the snare and potential infection affecting the usual processes taken as measures of wound age.

There were 13 cases where the cause of death was due to a suspected predator attack. None of these had bite wounds on the carcass swabbed for DNA evidence. DNA profiling to identify individual dogs is possible[94], testing is available within the PAWS framework and it is a simple procedure to incorporate into the post mortem examination. A number of cases of suspected poisonings were submitted directly for toxicology sampling and testing. As no post mortem examination was carried out, alternate causes of death including potential crimes were missed by this process. From reports where poisoning was listed as the suspected cause of death, 69% died due to an alternate cause, including some which were a result of criminal behaviour.

Poisoning was established as the cause of death in 18% of cases and the most common poison detected was the carbamate carbofuran, which was responsible for 42% of deaths. Carbofuran was withdrawn from use in the UK as a pesticide in 2001 and it is an offence to be found in possession of this chemical. This is consistent with the trend noted by the RSPB that carbofuran is dominated as the poison of choice in the majority of recorded incidents in recent years[85]. The illegal and indiscriminate use of this pesticide and others including aldicarb in baits placed out in the countryside have resulted in the death of many raptor species, and also of domestic species. Three separate incidents involving poisoning of cats were recorded in 2009[85].

A high level of exposure of raptors to secondary rodenticide products was found with 32% of cases having sublethal levels of one or more products. This was most commonly with bromodiolone alone, or combinations of bromodiolone and difenacoum or brodifacoum. These products are used legally for the control of common rats (*Rattus norvegicus* Berkenhout) around farm buildings and in fields

and hedgerows to protect crops from various rodent species[95]. Widespread exposure of raptor species to rodenticide products in the UK and elsewhere has been documented previously[96] and the significance of this is currently unknown. Sublethal levels of exposure may for example contribute to raptor deaths in association with trauma, if birds are more likely to haemorrhage. The range of rodenticide compounds and exposures encountered in natural environments, large number of wildlife species involved, and protected status of many of these species make laboratory toxicological testing for adverse effects unlikely. Without these studies interpretation of levels found in carcasses resulting from natural exposures is unclear.

3.4.3 Outcomes of suspected wildlife crime investigation

Tracking the results of suspected wildlife crimes submitted for forensic examination was challenging due to many reports not containing police incident numbers and no central reporting system for recording of crimes. Currently the NWCU records incidents of possible wildlife crimes, and is considering ways to record outcomes of investigations. Each of the eight police forces within Scotland has a separate computer database, and it was necessary to contact each force and sometimes the individual responsible for the case to find out the result of an investigation. Records of outcomes are important for the submitting agencies, to improve collection and presentation of evidence and also in the allocation of resources to tackle these types of crimes. Of the five cases successfully prosecuted, one was convicted of offences unrelated to the original crime.

Conclusion

To conclude, a variety of wildlife species were submitted for forensic post-mortem examination for suspected crimes in 2010. The majority of submissions were avian species, particularly raptors. Mammals were seen less frequently than might be expected based on NWCU incident reports. Post-mortem examinations were available as required during wildlife crime investigations.

Many suspect poisoning cases died due to other causes, underlining the importance of a full forensic post-mortem examination, with samples being submitted for toxicology as part of this examination. Swabbing bite wounds in suspected cases of predator attack and submitting for DNA analysis would aid in investigation and prosecution of suspected deer and hare coursing cases. Poisoning is a frequent cause of death, and is most likely to be due to abuse of illegal compounds such as carbofuran. Outcomes of wildlife crime investigations were challenging to follow up due to lack of a central recording system for cases. At least five of the cases submitted for forensic examination were successfully prosecuted.

Conclusion

Conclusion

The papers presented here represent an interdisciplinary approach to the investigation of disease, including the integration of molecular approaches, epidemiology and pathology. The first project used molecular tools to diagnose different trypanosome infections in a series of equine blood samples from a safari lodge near the Serengeti-Mara ecosystem, Tanzania. Microsatellite PCR's and multilocus genotyping was used to analyze the most likely source of infections. Equine infections were found to originate from the resident wildlife population, as suspected. As relatively new introductions to this ecosystem, horses have relatively low susceptibility to infection and despite the preventative measures employed by the owners of the safari lodge, still suffered a high mortality rate. The study also highlighted the difficulty in carrying out epidemiological studies using blood stored on filter paper such as the FTA cards used in the study. Samples were collected in this way due to ease of storage and transport to the UK, however as trypanosomiasis has a varying and often low parasitemia, sensitivity using this method can be disappointingly low. Horses, in contrast to other domestic species develop central nervous system infections with *Trypanosoma brucei brucei* which is similar to the disease seen in humans infected with the closely related *T. brucei rhodesiense*, and these natural infections may be a useful model of the disease in people.

The second project also highlighted some of the challenges associated with laboratory work, in this case contamination being a major issue relating to the use of non-specific primers in host blood meal analysis. Using stringent laboratory procedures we were able to correctly identify blood meals from ticks which had received a recent blood meal and removed from a known host. The literature review to identify potential reservoir hosts for Borrelia in Scotland is the first step in understanding the ecology of this disease in this environment. This will be followed by tick collections and host blood meal analysis in a range of habitats to understand which vertebrate hosts are important for maintaining tick and Borrelia populations and which habitats pose the greatest risk of infection to humans.

The third project to survey wildlife crime in Scotland involved an extensive collaborative effort with a number of agencies including police forces, the SAC, SASA, SSPCA and RSPB. A number of cases of wildlife crime were documented and the species targeted, types of crimes and locations documented. The outcomes of wildlife crime investigations were challenging to follow up due to the lack of a centralized system for recording crimes. A proportion of cases that were successfully traced were successfully prosecuted.

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