UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

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Abstract

Presence of *Cryptosporidium baileyi* in different Red Grouse (*Lagopus lagopus scoticus*) moors and its relation with agents of other avian diseases

Respiratory cryptosporidiosis was first diagnosed in wild Red Grouse (*Lagopus lagopus scoticus*) in autumn 2010 from a grouse moor in the North Pennine Dales in the UK. Three years after, 48% of grouse moors in Northern England had Red Grouse with severe lesions.

The present survey was projected by St David's Game Bird Services, it was performed with the purpose of gathering information regarding *Cryptosporidium baileyi,* concomitant parasites (*Trichostrongylus tenuis* and *Eimeria* spp.) and other different avian disease agents (*Mycoplasma synoviae*, *Mycoplasma gallisepticum*, *Ornithobacterium rhinotracheale*, Infectious Bronchitis Virus and Avian Rhinotracheitis Virus) that may also be implicated in cryptosporidiosis outbreaks. Age, gender and weight were additionally recorded to better comprehend the distribution of this disease through different groups (age and gender) and its relation with weight.

Different agents were evaluated by using: head histopathology and corneal impression smear (stained by the modified Ziehl Neelsen technique) for *C. baileyi* and modified McMaster technique for counting *Eimeria* spp. oocysts and *T. tenuis* eggs per gram of faeces. Pooled samples (ocular swabs) were used to assess, through PCR technique, the presence of other different avian disease agents. Regarding statistical analyses, two-sample Wilcoxon test, linear regression analysis and Fisher's exact test were used to test statistical significance differences among variables.

Positive results for *C. baileyi* were composed by 18.42% (7 out of 38) adults and 30.77% (16 out of 36) young birds. Statistically significant differences between age and gender groups were not found (p>0.05; OR=0.51). 70.00% (21 out of 30) of birds with clinical signs were positive for *C. baileyi*, compared with 3.33% (2 out of 60) that were positive without clinical signs, which differences were statistically significant (p<0.001; OR= 0.02). The median weight of positive animals for *C. baileyi* was 604g and 629g for negative birds, however no statistical significance was found between weight and the presence of *C. baileyi* (W=601; p>0.05). No statistically significant relationships were found between the presence of *C. baileyi* and *Eimeria* spp. or *T. tenuis* intensities (p>0.05). A statistically significant relation was found between grouse weight and *Eimeria* spp. intensity (p<0.01).

Additionaly, three positive results (pools composed by 5 birds) were obtained for Infectious Bronchitis Virus, two of them from birds with evident clinical signs and one from aparently healthy birds.

This study did not find any statistically significant relation between the presence of *C. baileyi* and other parasites, viruses or bacteria. However a worthy description of those agents and how they can be implicated in the Red Grouse life span was made, also aiming to raise the awareness and potentially encouraging new perspectives on cryptosporidiosis prevention and control.

Key-words: Red Grouse, *Cryptosporidium baileyi*, "bulgy eye", intestinal parasites, infectious agents, GB, Scotland.

Resumo

Presença de *Cryptosporidium baileyi* em diferentes reservas de Lagópodes-escoceses (*Lagopus lagopus scoticus*) e a sua relação com outros agentes de doença aviária

O primeiro caso de criptosporidiose respiratória em Lagópodes-escoceses (*Lagopus lagopus scoticus*) selvagens foi diagnosticado em Outono de 2010, numa reserva de caça em North Pennine Dales, Reino Unido. Após três anos, cerca de 48% das reservas de caça do Norte do Reino Unido já tinham reportado a presença de sinais clínicos em Lagópodes-escoceses.

O presente estudo foi projetado pela equipa St David's Game Bird Services, este procurou reunir informações acerca do *Cryptosporidium baileyi*, parasitas concomitantes (*T. tenuis* e *Eimeria* spp.) e outros agentes patogénicos (*M. synoviae*, *M. gallisepticum*, *O. rhinotracheale*, vírus da bronquite Infecciosa e de vírus da rinotraqueíte aviária) que possam estar implicados em surtos da doença. A idade, o sexo e o peso foram adicionalmente registados visando a compreensão da distribuição desta doença nos diferentes grupos (idade e sexo) e qual a sua relação com o peso das aves.

Os diferentes agentes etiológicos foram avaliados recorrendo a: histopatologia da cabeça e citologia de impressão da superfície ocular (colorada pela técnica de Ziehl Neelsen modificada) para *C. baileyi* e técnica de McMaster modificada para a pesquisa e contagem de oocistos de *Eimeria* spp. e ovos de *T. Tenuis.* Foram utilizados *pools* de amostras (zaragatoas oculares) para avaliar a presença de outros agentes patogénicos por PCR. Os testes estatísticos utilizados para testar diferenças estatisticamente significativas entre grupos foram: two-sample Wilcoxon test, análise de regressão linear e o teste exato de Fisher.

Os resultados positivos para *C. baileyi* foram de 18,42% (7 em 38) em adultos e 30,77% (16 de 36) em aves jovens. Não foram encontradas diferenças estatisticamente significativas entre idade e sexo (p> 0,05; OR = 0,51). Em 70,00% (21 em 30) das aves com sinais clínicos foi diagnosticada infecção por *C. baileyi*, comparativamente com 3,33% (2 de 60) onde foi isolado *C. Baileyi* em aves sem sinas clínicos, diferença estatisticamente significativa (p <0,001; OR = 0,02). A mediana do peso das aves positivas e negativas para *C. baileyi* foi de 604g e 629g, respectivamente, porém a diferença encontrada não foi estatisticamente significativa (W = 601; p> 0,05). É também relevante referir que não foram encontradas diferenças significativas entre a presença de *C. baileyi* e as cargas parasitárias de *Eimeria* spp. ou *T. tenuis* (p> 0,05), no entanto foi encontrada uma relação estatisticamente significativa entre o peso e a contagem de oocistos de *Eimeria* spp. por grama de fezes (p <0,01).

Foram obtidos três resultados positivos (*pools* compostos por 5 aves) para o vírus da bronquite infecciosa, dois provenientes de grupos com sinais clínicos e o restante de um grupo aparentemente saudável.

Este estudo não encontrou qualquer relação estatisticamente significativa entre a presença de *C. baileyi* e outros parasitas, vírus ou bactérias. Foi efectuada uma descrição aprofundada dos agentes, bem como da forma como podem estar implicados no ciclo de vida dos Lagópodes-escoceses, visando a consciencialização e o incentivo de práticas que objectivem a prevenção e o controlo da criptosporidiose.

Palavras-chave: Lagópode-escocês, *Cryptosporidium baileyi,* conjuntivite*, criptosporidiose respiratória, parasitas, GB, Escócia.

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List of Abbreviations and Symbols

% - Percentage

£ - Pound Sterling

aMPV – Avian metapneumovirus

ANOVA – Analysis of Variance between groups

ART – Avian rhinotracheitis Virus

bp – Base pairs

CI95%- Confidence interval, stated at the *95*% confidence level

DNA - Deoxyribonucleic acid

e.g. – *exempli gratia*

FVM – UL – Faculty of Veterinary Medicine – University of Lisbon

g – Gram

GDP - Gross Domestic Product

GPS – Global positioning system

GWCT – Game and Wildlife Conservation Trust

IB – Infectious bronchitis

IBV – Infectious bronchitis virus

l – Litre

Log_{10} – Logarithm (base 10)

Max – Maximum

MG – *Mycoplasma gallisepticum*

Min – Minimum

ml – Millilitre

mm – Millimetre

MS – *Mycoplasma synoviae*

N – Number

ºC – Degree Celsius

OR – Odds ratio

ORT – *Ornithobacterium rhinotracheale*

p – p-value

PCR – Polymerase chain reaction

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

RT-PCR – Reverse transcription polymerase chain reaction

SE – Standard error

SHS – Swollen head syndrome

TRT- Turkey rhinotracheitis

UK – United Kingdom

USA – United States of America

W – Value of W (Wilcoxon test)

µm – Micrometer

σ - Standard deviation

Introduction
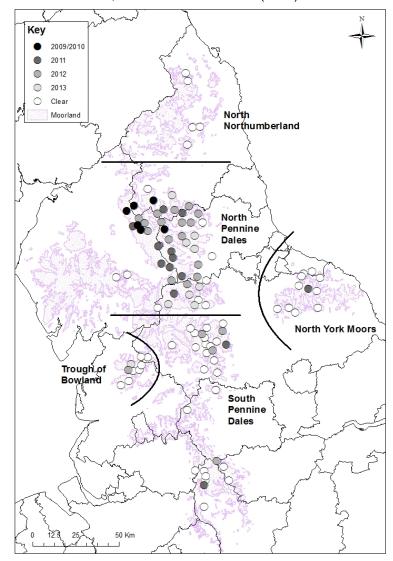
Red Grouse (*Lagopus lagopus scoticus*) is a game bird of great social and economic significance in numerous upland parts of the United Kingdom (UK) (Figure 1) (McGilvray, 1995). In order to increase Red Grouse densities for shooting, moorland estates manage habitat, control predators and parasites, mainly the nematode *Trichostrongylus tenuis* (Hudson & Newborn, 1995). According to the review of 2012 by Game and Wildlife Conservation Trust (GWCT), spring densities averaged 101 Red Grouse per 100 hectares in northern England and 69 per 100 hectares in Scotland (Baines, Howarth & Newborn, 2013); these densities are only possible due to such committed management. The Economic Study of Grouse Moors by GWCT (2010) concluded that grouse shooting might sustain up to 1072 jobs and contribute with £23.3 million to Scottish gross domestic product (GDP). The same study refers that in 2009 the price per driven brace (pair) averaged £131.6, price that was expected to increase in the future.

Cryptosporidium baileyi was first diagnosed in Red Grouse in 2010, following several observations on grouse moors in 2009 (Baines, Newborn & Richardson, 2014) declaring what is sometimes called "bulgy eye" (Coldwell, Caldow, Holliman, Mearns, Errington, Giles, Willoughby & Wood, 2012). These clinical signs refer to swollen eyelids and mucous discharge from the infra-orbital sinuses and nasal passage. Only three years after the first diagnosis, 48% of grouse moors in northern England had Red Grouse with lesions of respiratory cryptosporidiosis, representing almost half of grouse moors (Figure 2) (Baines *et al.*, 2014).

Figure 1 - A watchful and healthy Red Grouse surrounded by heather (*Calluna vulgaris*). (Original)

Figure 2 - The geographical position of grouse moors reporting "bulgy eye" symptoms and the year when first reported, stated on the work of Baines, Newborn & Richardson (2014).

In Scotland, the first bird confirmed with *C. bailey* was reported in 2013 from the Lammermuir Hills in southern Scotland (Baines *et al*., 2014).

Encouraged by a dedicated group of Scottish estates, "St David's Game Bird Services" (a division of "St David's Poultry Team") decided to support a research in Red Grouse aiming to gain better knowledge of respiratory cryptosporidiosis and its etiopathogenesis, targeting the avoidance of an equally fast spread through Scottish grouse moors. In addition to *C. baileyi* other parasites intensities (e.g., *Eimeria* spp. and *Trichostrongylus tenuis*) and several avian pathogens were assessed, highlighting the chance of having an agent that could be predisposing to this disease. A delicate study was performed and individual parameters such as age, gender and weight were also taken into count.

For a good balance on grouse shooting income, as an activity that supports economic activity in remote areas, tourism, biodiversity and as an increasingly profitable one, efforts should be considered to engaging means for performing studies and research that will benefit the preservation and improvement of such a unique ecosystem.

1 Literature Review

1.1 Red Grouse (*Lagopus lagopus scoticus*)

Red Grouse (*Lagopus lagopus scoticus*) has been renowned for centuries as a game bird; it has an economic value that has led to ample research and is well known in the UK. Red Grouse have a reddish-brown plumage across all seasons; their legs are bordered by small scales or feathers, which allow them to walk on snow. In west Scotland, Wales and Ireland birds are lighter coloured than those in the eastern parts of UK, their yellowish plumage matching the paler background of the grassier moorland present in such areas. Red Grouse are smaller than a Ring-necked Pheasant (*Phasianus colchicus*), as long as a Wood Pigeon (*Columba palumbus*), but with a heavier body. The males are 5% longer than females. Adult males weigh 600-690g, their lean period is in March, coinciding with strong territorial behaviour and courtship. Males outweigh females in all months except March through to May, when females exceed them reaching 670g in April, having put on weight before laying eggs. In June and July, females are thinner due to the incubation period, weighing 560g while males weigh 660g (Watson & Moss, 2008).

Red Grouse are well camouflaged on heather ground, often staying motionless when threatened and are easily overlooked by man or predators; they are considered the fastest British game bird as they are adept at using wind to increase speed, moving at the same contour rather than up and down, repeatedly swerving or tilting rapidly, this pattern makes them very hard to shoot.

Eggs are bigger than those of a wood pigeon, they are off-white with a tinge of pale brick red when recently laid and are marked by many brownish spots. Down (layer of fine feathers found under the tougher exterior feathers) covers day-old chicks to their toenails. Brood mates have a similar colour; most of the time it is the same colour of their parents down as chicks, so the colour is inherited. Chicks leave the nest when they are a day old; they run to hide under vegetation if frightened, and peck enthusiastically at small insects or heather (*Calluna vulgaris*). In Scotland, the majority of chicks hatch in late May and appear full grown at 12 weeks, however old birds are still heavier than them in August and September (Watson & Moss, 2008).

A "Grouse moor" is moorland where grouse shooting is the main land use, whereas "Heather moorland" (where heather predominates) includes grouse moor and also land where Red Grouse are seldom or never shot. They are treasured for their landscape and wildlife.

1.1.1 Taxonomy

Red Grouse, *Lagopus lagopus scoticus* Latham, 1787, also known as the Moorcock or Moorbird, taxonomically belongs to Kingdom Animalia, Phylum Chordata, Class Aves, Order Galliformes, Family Phasianidae, Subfamily Tetraoninae, Genus *Lagopus*, Species *Lagopus lagopus* and subspecies *L. lagopus scoticus* (Lovat, 1911).

According to the 'Grouse Status Survey and Conservation Action Plan 2006-2010' made by International Union for Conservation of Nature (2007), there are 18 species of grouse worldwide, 4 of these are present in UK.

Red Grouse are currently classified as a subspecies of Willow Ptarmigan (*Lagopus lagopus*) also known as the Willow Grouse. This species has a circumpolar distribution that encompasses northern Canada, Alaska, Britain, Ireland, Northern Scandinavia and Russia. They live in forests and moorlands in Northern Europe and tundra of Siberia, Scandinavia, Alaska and Northern Canada. Willow Grouse present a dappled brown plumage in the summer and largely white during winter (Freeland, Aderson, Allen & Looney, 2006).

Irish Red Grouse (*Lagopus lagopus hibernicus*) was listed as a distinctive subspecies on Annex II of the Birds Directive, whilst these birds are paler and more yellowish than British birds, a factor that has been assumed to be an adaptation to a different habitat, however today they are considered the same subspecies, recent studies have come to validate it (Murray, Clotworthy & Bleasdale, 2013). Freeland *et al.* (2006) presented no support for the proposal that Irish Red Grouse compose an exclusive subspecies, that study was based on the analyses of short regions of mitochondrial DNA differentiated (ca 300 bp) and found no clear genetic differentiation between British and Irish birds whereas a recent study, where Irish and Scottish grouse were compared, based on genetic variation (19 different microsatellite markers), revealed significantly genetically differences (Murray *et al*, 2013). Irish Red Grouse have a low genetic variability, because of the low population size and fragmented habitat structure. It is critical that remaining habitats and populations are protected, conserved and managed in order to keep the species viable (McMahon, Johansson, Piertney, Buckley & Höglund, 2012).

1.1.2 Distribution and Numbers

Red Grouse are present on moorland over much of England, Ireland, Scotland and Wales, from the coast to the upper limit of this vegetation type. An estimate for Scotland in 2003 was 130 000 pairs on an estimated 1.2 million ha of heather moorland; there are other studies that attribute 250 000 pairs to the entire UK (Watson & Moss, 2008).

1.1.3 Habitat

Red Grouse live on drained moorland and rarely on wet heath. Initial habitat resulted from deforestation by prehistoric man to create cultivated fields or pasture, a persistence of burning and grazing has avoided most of it from returning to forest. Wet heath resulted mainly from a wetter climate, which led to peat growth and with it deforestation, and it too has been burned and grazed since (Figure 3) (Watson & Moss, 2008).

Red Grouse are in high numbers where their main food is present. It favours where short heather grows in a mixture with fairly tall heather as cover. The highest densities have consistently been on moors with both dry heather ground and blanket bog, where heather shares dominance with cotton-grass (*Eriophorum* spp.). Cotton grass has nutritious shoots in spring, while blanket bog supports abundant insects that are food for grouse chicks. Birds can also be abundant where blanked bog has eroded into peat hags, and bare peat covers much of the ground (Watson & Moss, 2008).

Where food abounds without cover, like on short heather after a wide fire, only unmated cocks take territories and even they avoid the centre of previously burned zones. When birds are eating short heather, they tend to keep close to the tall heather, to which they run if a predator approaches. Nevertheless, they avoid dense and tall heather, because it blocks movement and do not offers reachable food. There are some individuals that occupy open covers in woodland. Their densities increase in the early years of planting on moorland (Watson & Moss, 2008).

Figure 3 - Heather moorland, Red Grouse's habitat. (Original)

1.1.4 Diet

1.1.4.1 Summer

Apart from eating heather and some blueberrys (Red Grouse like to eat berries). In summer, birds eat the leaves and flowers of herbs, moss capsules, flowers of bell heather or cross-leaved heath, and seeds of grass, rush and sedge. From a day old, chicks eat newly growing heather shoots as these are tender in early summer (Figure 4). Moss capsules come next in favour, they are rich in protein and phosphorus, being practically as nutritious as insects. Chicks in their first two weeks eat mainly invertebrates while after three weeks they become mostly herbivorous. During the first weeks they frequent flushes, where insects abound and the tall grasses, sedges and bog myrtle give good cover (Watson & Moss, 2008).

Figure 4 - Dead Red Grouse, found during radio tracking procedure. Notice the big amount of heather present in its ruptured crop. That bird presented "bulgy eye" clinical signs and had probably died due to a collision with a fence, as it was found next to one with its ruptured crop. (Original)

1.1.4.2 Autumn

In autumn besides taking smaller amounts of other items as in summer, the birds eat mostly heather shoots and flowers, frequently picking both with each peck (Watson & Moss, 2008).

1.1.4.3 Winter

During this season herbs die back or are covered by snow, so instead the birds must subsist on trees or woody shrubs. Red Grouse sometimes stay on tree branches to eat shoots and buds. They also feed on the leafy shoots of heather or the leafless stalk tips of blueberry. Green heather can turn brown, even without grazing, through desiccation within

a few days if shoots lose humidity in cold weather and then cannot replace it because the soil water is frozen. Blueberry and other heath species are less likely to suffer, so a moor with a monoculture of heather offers much more food in most years but brings with it a risk of starvation during winter (Watson & Moss, 2008).

1.1.4.4 Spring

Birds feed mainly on heather, however in spring they complement it with plants that begin to grow earlier. Red Grouse eat shoots of cotton-grass when available; these have a high content of essential nutrients (as mentioned earlier). Cotton-grasses do not grow on freely drained moorland, so birds with territories in such areas must feed mainly on heather in spring, which makes for a poor diet (Watson & Moss, 2008).

1.1.5 Territories

Red grouse males are territorial, they establish their territories between the end of winter and beginning of spring. Displays between neighbouring males are common, mainly after dawn at the corner boundaries to these territories. Calls can be heard between neighbouring males. After pairing with the males in spring, the female also becomes territorial. The size of each territory may depend on population density and source of each habitat. Those that are wetter and with less heather cover or dominated by bracken and grass are less suitable, holding fewer birds (National Red Grouse Steering Committee, 2013).

1.1.6 Fluctuations in density

In every area, the number of birds changes from year to year. These fluctuations in numbers were studied from bags (number of birds shot in each shooting day) or counts, a wide used method that has been used for decades. Records usually cover many years, but the often inflate the size of fluctuations usually confuse adult density with breeding success. Numbers vary on some areas in equally regular cycles, whereas on others they do not. A study that assessed 289 moors, 63% cited evidence of cycles, with an average period of about eight years. The period rose from south to north, from around seven to about nine years (Watson & Moss, 2008).

Long-term records sometimes show runs of clear cycles, interspersed with less regular fluctuations. Sometimes, cycles in an area stop altogether.

1.1.7 Grouse Moor Management

Management for the sport of shooting Red Grouse, sometimes called grouse moor management, is one of the main land uses on British heather moorlands, the Red
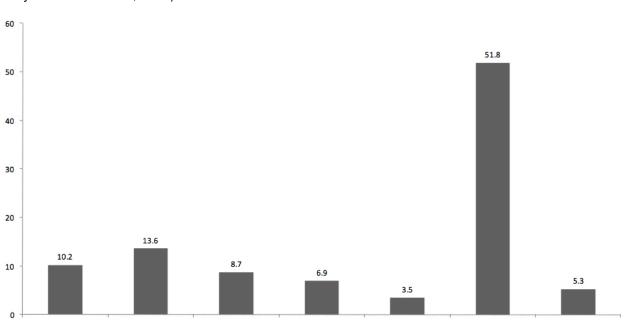
Grouse's main habitat (Murray *et al.*, 2012). From 1500s to 1800s, moorland management was focused mainly on cattle (*Bos taurus*), sheep (*Ovis aries*) and deer (*Cervus elaphus*) grazing, the management specifically for Red Grouse began when 'driven shooting' had become fashionable, from the early 1880s, with sizable areas being dedicated to this land use, yielding a harvestable surplus of birds (Lovat, 1911).

Unfortunately, Scottish moorland has been decreasing since the 1940s, making Red Grouse numbers decrease in the same way. In the 1940s, 49% of the upland states were being managed, a percentage which had reduced to 20% by the 1980s (Robertson, Park & Barton, 2001).

Considering its sustainability, grouse shooting is usually the main source of income and principal attraction of ownership on many grouse moor estates (McGilvray, 1995), grouse moor management has crucial importance in preventing further decreases of heather uplands, with losses to forestry (with exotic conifers) or grassland (in consequence of government agriculture and forestry policies), as profitable grouse shooting reduces the attractiveness of governmental subsidies (Robertson *et al.* 2001).

A wide variety of managements are developed in grouse moors, which directly affect the moorland habitat and its flora and fauna. The most common amongst these are the control of predators and rotational burnings. The control of predators is focused mainly on red foxes (*Vulpes vulpes*), mustelids, and corvids (*Corvus corone* and *C. cornix*).

Practically one-third of annual operating and maintenance spend goes on predator and pest control and heather and bracken management, Figure 5 below demonstrates how 2009 total operating and maintenance expenditure was distributed by a more detailed categorization of expenditure.

Figure 5 - Operating/maintenance expenditure distribution 2009 (%). (Adapted from GWCT- An Economic Study of Grouse Moors, 2010)

1.1.7.1 Rotational Burning

Muirburn is the Scottish term for moorburn, the rotational burning that is performed by setting controlled fire to small parcels of old heather, aiming for the formation of different aged stripes. It is imperious for Red Grouse and sheep as it creates a mixture of young and old heather, providing nutritious food and adequate physical cover (Figure 6) (Watson & Moss, 2008).

Fire promotes the development of young heather, increasing food accessibility of Red Grouse as small shoots are easier to ingest and more nutritive that older plants while grown-up stripes of heather provide cover (Murray *et al*, 2012).

Figure 6 – A burnt parcel of heather after rotational burning. (Original)

1.1.7.2 Grazing

Cattle or sheep at low density can benefit Red Grouse by creating trails that allow birds to enter into tall heather and their manure introduces agricultural plant species that are good food resources for Red Grouse during spring. To be beneficial, sheep or cattle have to be present in a delicate balance. Overgrazing along with overburning also impoverishes grazing for sheep, as well as reducing heather, cover for Red Grouse and causing soil erosion. The excessive deposition of manure by livestock can cause faster loss of heather than frequent burning (Watson & Moss, 2008). Sheep are also important as "tick mops", removing ticks from grouse habitat, when treated with acaricide. Since they can be vaccinated and dipped in acaricide to kill ticks which try to attach, that is a wide used method to control louping ill virus in Red Grouse. Sheep are released onto heather moorlands. Ticks swarm to the sheep and once they come in contact with the acaricide, die off. Louping ill virus is a tick borne disease of livestock, which cause variable mortality in sheep and up to 80% mortality in infected Red Grouse. (Porter, Norman & Gilbert, 2009).

1.1.7.3 *Shooting sport*

When birds are driven, beaters (people who drives game birds out of areas of cover) wave large flags and shout to scare birds, making them flush and fly towards a line of distant "butts" (camouflaged places where hunters hide). The beaters start walking in a straight line, but the outer beaters walk faster so that the line closes as it nears the "butts", each drive usually covers 80-400 ha. When birds are at lower density, hunters "walk up" in line abreast of one another, with dogs ranging close, and at yet lower densities pointing dogs are used, to range widely and find birds (Figures 7, 8 and 9).
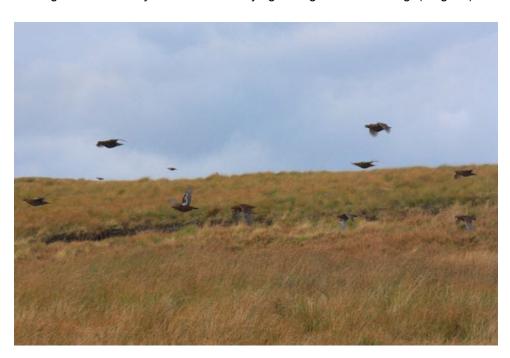
Figure 7 – A covey of Red Grouse flying during driven shooting. (Original)

Some studies, using different statistical analyses, refer that bags (amount of birds shot) are a good "proxy" for assessing Red Grouse densities. Bags are said to confound breeding stocks with breeding success unless they record the proportion of young in the bag. In field trials, dogs compete to find Red Grouse and then await their handlers without flushing the birds. Some people love and pursue this sport even though they rarely or never shoot a bird, and are a strong voice for preserving moorland. The breeding of hunting dogs has been beneficial and indispensable for Red Grouse research. Dogs, like setters and pointers, help with counts, find nests, chicks and dead birds better than any person (Watson & Moss, 2008).

Figure 8 – Beaters walking and waving flags, driving birds to "butts". (Original)

After shooting some birds, the remaining ones face less competition and so may survive better or rear more chicks. However, if one cause of death becomes less frequent, another could increase. This process is called "compensation" by biologists. If this compensation is total, better survival, better breeding, less emigration or more immigration makes good all losses, and so the population remains at the same density. In the other hand if there is no compensation, all losses are additive. Partial compensation occurs more commonly. Watson and Moss (2008) also refer that marine biologists have highly contributed for a better understanding of this phenomenon. As the rate of fishing increases, it reaches a "maximum sustainable yield", above which extra effort catches fewer fish and risks making fishing unprofitable or extirpating the stock. Unfortunately, Red Grouse demonstrate unbalanced population fluctuations, usually displayed as moderately regular cycles in both shot and unshot populations. The way to reach the

Figure 9 – "Butts", where guns (hunters) are positioned during driven shooting. (Original)

"maximum sustainable yield" in Red Grouse is to intensively shoot during the increase phase of a cycle, enough to prevent birds achieving a peak and undergoing the consequent decline, however it cannot be too intensive as to decrease the following year's breeding stock overly (Watson & Moss, 2008).

1.2 Transmissible agents of disease

1.2.1 *Cryptosporidium* spp.

Cryptosporidium is a genus of protozoan that belongs to the Phylum Apicomplexa. These parasites develop within the microvillus border of respiratory, gastrointestinal and urinary epithelium and can produce intestinal, respiratory (most prevalent form in chickens (Fayer & Xiao, 2008)) and renal disease, causing occasional morbidity and mortality (Lindsay & Blagburn, 2008).

Despite the genetic diversity of *Cryptosporidium* not being well studied, this genus of parasite has been reported in 30 avian species, belonging to orders Anseriformes, Charadriiformes, Columbiformes, Passeriformes, Psittaciformes, Struthioniformes and Galiformes (Fayer & Xiao, 2008).

1.2.1.1 History and Taxonomy

Tyzzer 1907, was the first author to describe the genus *Cryptosporidium*, with the species *C. muris* in the gastric glands of laboratory mice (*Mus musculus*). He described some of the life cycle stages of *C. parvum* and was the pioneer researcher in cryptosporidial infections in avian species (McDougald, 2008; Lindsay & Blagburn, 2008).

Only three avian *Crypstosporidium* spp. are now considered valid, *Cryptosporidium baileyi, Cryptosporidum meleagridis* and *Cryptosporidium galli* (Fayer & Xiao, 2008). However, many other species were identified from a wide range of hosts due to an assumption of unwarranted degree of host specificity (McDougald, 2008), but these species were not adequately described to conclusively classify the involved pathogens as new species (Fayer & Xiao, 2008; Lindsay & Blagburn, 2008).
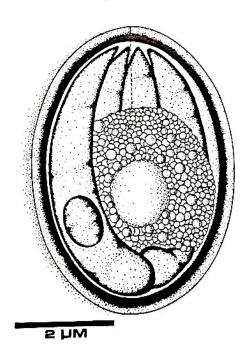
Taxonomy of the coccidian is based on host specificity, site of infection oocyst morphology, similarities in the 18s RNA gene and heat shock gene (HSP-70). Contrary to other coccidia found in poultry, *Cryptosporidium* spp. oocysts do not have sporocysts containing the sporozoites, they just have four naked sporozoites (Figure 10) (McDougald, 2008).

C. meleagridis, was the first species of *Cryptosporidium* classified in avian hosts and, as the name suggests, it was originally described from turkeys (*Meleagris gallopavo*) intestines and is mainly associated with enteritis (Fayer & Xiao, 2008).

C. baileyi Current *et al*, 1986, was the second isolated species of avian *Cryptosporidium*, frequently related with respiratory cryptosporidiosis in chicken (*Gallus gallus domesticus*), it was also described in the bursa, cloaca and respiratory tract of broiler chickens (Fayer & Xiao, 2008).

Both molecular and morphological data have set *C. galli* as a valid species. Their oocysts are bigger than those of *C. baileyi* (McDougald, 2008)*. C. galli* was only found in the proventriculus and to the date is not known enough to ascertain if it can proliferate in other organs (Lindsay & Blagburn, 2008).

Figure 10 - Drawing of an oocyst of *Cryptosporidium baileyi.* Note the 4 sporozoites surrounding the oocyst residuum and the suture in the two-layered oocyst wall. (Adapted from Saif, 2008)

1.2.1.2 Host Range and Clinical Signs
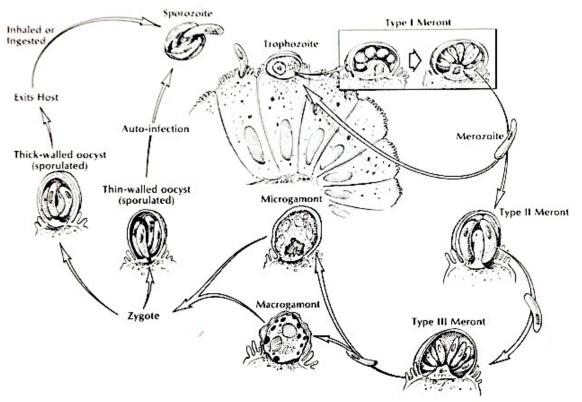
Respiratory disease is the most frequent form of cryptosporidiosis in chicken and has been described in a wide range of species such as: turkeys, common quail (*Coturnix coturnix*)*,* Rig-necked Pheasants (*Phasianus colchicus*) and bugerigars (*Melopsittacus undulatus*)

Faeces of birds were collected in Czech Republic and Western Australia aiming the evaluation of genetic diversity in *Cryptosporidium* spp. oocysts, their results suggested that genetic diversity may be even higher as four non-described genotypes was found, plus *Cryptosporidium muris* (mice), *Cryptosporidium andersoni* (cattle), *C. baileyi, C. galli, C. meleagridis* (Lindsay & Blagburn, 2008). *C. baileyi* have small host specificity among birds (McDougald, 2008).

1.2.1.3 Life Cycle

The life cycles of *C. baileyi* and *C. meleagridis* are known minutely, oocysts are sporulated when released in the faeces (they sporulate within the host cell (McDougald, 2008), infecting the birds whilst they are drinking or eating. After arriving into the digestive tract, a process called excystation, occurs which is the release of infective sporozoites, these penetrate the microvilli of epithelial cells at specific locations, depending from the species of *Cryptosporidium,* within the host cell sporozoites become trophozoites and then merogony, the asexual reproduction occurs inside epithelial cells where trophozoites follow an asexual multiplication, becoming type I meronts. Each type I meront produces 8 merozoites that are released after their development, and then will infect other epithelial cells and moreover initiating extra cycles of asexuated reproduction, reinfecting the host. Gametogony, the sexual reproduction where male and female gametes are formed, come when some type I meronts develop into type II meronts. Type II meronts produce four type II merozoites that invade the microvilli of epithelial host cells, differentiating into gametes. The female stage is the macrogamont while the male stages are the microgamonts that produce nonflagellated microgametes. Fertilization (union of gametes) occurs when microgametes penetrate into macrogametes, forming the zygote. Two types of oocysts are produced, thin-walled and thick-walled; both have four sporozoites and sporulate endogenously. The thick-walled oocysts are excreted in the feces, reaching the environment where they can infect other hosts whereas thin-walled oocysts are mainly autoinfective (Figure 11).

Figure 11- Life cycle of *Cryptosporidium baileyi* as it occurs in the mucosal epithelium of the intestine (bursa of Fabricius and cloaca) and the respiratory tract of broiler chicken. (Adapted from Saif, 2008)

When conjunctival, urinary, or respiratory tracts are in direct contact with oocysts, the development of *Cryptosporidium* can occur, these infections are not due to transportation via blood (Lindsay & Blagburn, 2008).

The life cycle of *Cryptosporidium* spp., identical to other true coccidia belonging to the suborder Eimeriorina may be staged into 6 development events: excystation, merogony, gametogony, fertilization, oocyst wall formation and sporogony (McDougald, 2008).

1.2.1.4 Pathogenesis and Epidemiology

In the poultry industry it is known that birds become infected after picking up oocysts that are present in contaminated litter or cages. *C. baileyi* is also known to invade the epithelium of the cloaca and bursa of Fabricius. The inhalation or aspiration of oocysts present in the environment may cause respiratory disease. It is said that the oral inoculation of 100 oocysts may result in intestinal disease or respiratory disease if given intratracheally, as early as 3 days after inoculation. As referred previously, *Cryptosporidium* spp. are infective when excreted and at the time vectors have not been identified, although the wide variety of *Cryptosporidium* spp. hosts make wild birds carriers. Mammals are not susceptible to *C. baileyi* however they can act as mechanical carriers (mice, rats, dogs and humans included) (McDougald, 2008).

In the chicken, mild to heavy respiratory and intestinal signs can be seen 3 days after oocysts inoculation. Respiratory signs are greater than intestinal ones, which are usually mild.
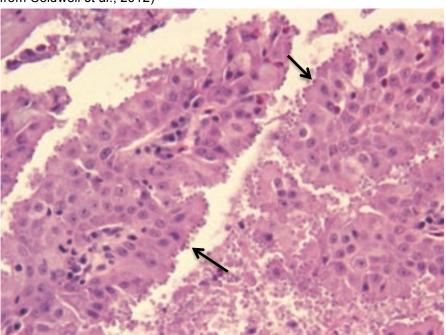
Figure 12 - Large numbers of cryptosporidial (arrows) lining the hyperplastic epithelium from the conjunctiva in a Red Grouse (Adapted from Coldwell *et al*., 2012)

Secondary pathogens (*Escherichia coli*, for instance) exploit *C. baileyi* infection, because it disrupts the mucociliary elevator. Some viruses and bacteria, like infectious bronchitis virus and *E. coli*, can increase the severity of *C. baileyi* in respiratory disease pathogenesis (McDougald, 2008).

1.2.1.5 Pathogenesis

1.2.1.5.1 Respiratory Cryptosporidiosis

Infection of nasal tissues is usually associated with "swollen head syndrome", excess mucus can be present in the nasal cavities and trachea of infected birds, airsaculitis is also present. Microscopic lesions are mainly the reduction or absence of cilia in ciliated locations, hyperplasia and hypertrophy of epithelial surfaces with the presence of heterophils, lymphocytes, macrophages and plasma cells (Lindsay & Blagburn, 2008). These clinical signs are usually referred as "bulgy eye" by producers and gamekeepers.

1.2.1.6 Diagnosis

Respiratory and intestinal infections can both be diagnosed by identifying oocysts from fluids derived from the faeces or from respiratory tract (McDougald, 2008). *Cryptosporidium*'s oocyts are difficult to find due to their small size, in addition it is common to find other parasitic oocysts (*Eimeria, Isospora and Caryospora*) and sporocysts (*Sarcocystis*), especially in wild birds. *Eimeria, Isospora and Caryospora* oocysts are not sporolated when excreted, so they are found as non-sporulated oocyst in samples. Oocysts of *Sarcocystis* oocysts can easily be confused with oocysts of *Cryptosporidium* as they are excreted as sporocysts and their structure is very similar, it is only possible to distinguish by the size and morphology of their residual material (oocysts of *Cryptosporidium* spp. hold a compact residual body and are 4-8 by 5-6 μm whereas sporocysts of *Sarcocystis* spp. hold numerous granular residual granules and are 10-12 by 4-7 μm).

Head histopathology has been used to assess the presence of *C. baileyi* in the conjunctiva of Red Grouse (Figure 12), being considered the most accurate method to assess its presence. For economical reasons many northern UK veterinary laboratories have been using corneal impression smears, stained with the modified Ziehl Neelsen technique for assessing the presence of *C. baileyi* on Red Grouse.

1.2.1.7 Cryptosporidiosis in Red Grouse and Other Wild Birds

Cryptosporidium bailey was first diagnosed in Red Grouse in the autumn of 2010 following several observations of birds with swollen heads and bulging eyes on grouse moors in the north of UK (Figure 13) (Coldwell *et al*., 2012). The findings in those birds included swelling of the eyelids with a sticky mucoid discharge, catarrhal exudate in the nasal

passages and infraorbital sinuses and excess mucus in the trachea and larynx; the source of the cryptosporidial infection in those birds was unknown (Coldwell *et al*., 2012). Only three years after the first diagnosis, 48% of grouse moors in northern England had Red Grouse displaying clinical signs of respiratory cryptosporidiosis, that represents almost half of grouse moors, that may be caused by density-dependent natal dispersal by young birds and driving birds for several kilometres during shooting may have contributed to great rates of cryptosporidiosis spread (Baines *et al*., 2014). In Scotland, the first bird confirmed with *C. bailey* was reported in 2013 from the Lammermuir Hills in southern Scotland (Baines *et al*., 2014). A huge conservation concern is the recent reporting of clinical signs within the fewer Black Grouse (*Tetrao tetrix*), which inhabits with Red Grouse (Baines *et al*., 2014).

Cryptosporidium spp. is widespread in domesticated and wild birds, being reported from 30 different bird species, including game birds. In virtually all cases, respiratory disease associated with *C. baileyi* was among birds kept in captivity. The distribution of reports made worldwide is conditioned by the awareness and proficiency of poultry specialists and biologists as by the capacity of their diagnostic tools (McDougald, 2008).

Figure 13 - On left can be observed a dead Red Grouse with respiratory cryptosporidiosis clinical signs while on right a apparently healthy bird. (Original)

1.2.1.8 Prevention and Control

There is currently no proven treatment for *Cryptosporidium* spp., and even if one were available, successful medication of wild birds would be problematic. At the moment there are not proved vaccines or effective drugs against *Cryptosporidium* spp*.* Other approaches to the control for this disease have been trialled but they are still experimental.

Disinfection and sanitation will reduce drastically the amount of oocysts but there is any proved program or treatment (McDougald, 2008).

Ionophorous anticoccidials, diclazuril and toltrazuril, drugs that are frequently used in the poultry industry are not effective (Lindsay & Blagburn, 2008). In chickens, enrofloxacin is slightly effective and paromomycin reduces oocyst output by 67 to 82% displaying the top efficacy of all drugs tested against avian cryptosporidiosis. However, paromomycin is a very large molecule that only stays in the gut therefore is not effective in respiratory cryptosporidiosis (Sréter, Széll & Varga, 2002 in Lindsay & Blagburn, 2008).

The oocysts of *Cryptosporidium* spp. are extraordinarily resistant to sanitation chemicals that usually destroy the majority of bacteria, virus and fungus. This makes the complete elimination of oocysts from poultry facilities not practical.

In laboratory conditions, oocysts kept their viability for months when stored at 4ºC in a solution of 2.5% potassium dichromate as well as when maintained during 10-15 minutes in 25% commercial bleach (sodium hypochlorite). Tests were made with 9 frequently used disinfectants mixed with water at the maximum concentration recommended, *C. baileyi* was incubated during the 30 minutes at room temperature resulting in little or no effect on oocyst viability. A concentration of 50% of commercial bleach destroyed 95% of oocysts and incubation in 50% ammonia caused the biggest excystation reduction. Temperatures higher than 65ºC should destroy oocysts; thereby steam cleaning is an effective way of disinfecting poultry equipment (Saif, 2008).

Stream options for control of the cryptosporidiosis on grouse moors are limited, but may comprise culling of apparent affected birds and decreasing the chance for disease transmission by increasing the number of gritting areas, steam cleaning grit boxes frequently and stopping to use those where the soil and any adjacent water source may have become contaminated with *C. baileyi* oocysts (Coldwell *et al*., 2012).

1.2.2 *Trichostrongylus* spp*.*

Trichostrongylus is a parasite genus that encompasses fine, tiny and roseate worms usually found in the ceca and small intestine (lesser) of birds. They also occur in the small intestine of ruminants, lagomorphs and rodents.

There are two *Trichostrongylus* species described from birds*, Trichostrongylus tenuis* and *T. cramae.* Reports have been made worldwide from wild birds: Europe, Africa, Asia, USA, and Australasia. These species are considered monoxenous, requiring only one host to close their life cycle. Initially *Trichostrongylus* have been reported in Galliformes, Anseriformes (waterfowl), Gruiformes (cranes) and Otidiformes (bustards) (Tompkins, 2008).

The first avian species to be described was *Trichostrongylus tenuis* Raillet and Henry, 1909, from the Ring-necked Pheasant (*Phasianus colchicus*). *T. tenuis* was afterwards recognized as the trichostrongylid present in the ceca of many "Old-World" birds, although it was first described in Red Grouse as *Trichostrongylus pergracilis* Cobbold, 1873. *T. pergracilis* and many other synonyms for this species have been used during decades until experimental challenges were made, cross-infecting different hosts (Tompkins, 2008).

T. tenuis has been related with losses in populations of Red Grouse in northern England and Scotland for more than 100 years (Lovat, 1911). The first system in which regulation of host population size by parasites was experimentally demonstrated in the field was made with *T. tenuis* in Red Grouse (Hudson, Dobson & Newborn, 1998). Hudson, Newborn & Dobson (1992b) also referred that the prevalence amongst British moorlands is almost 100%, where there impact on both population and individuals have been well characterized (Hudson *et al.*, 1992b; Delahay & Moss, 1996).

1.2.2.1 Taxonomy and Morphological Characterization
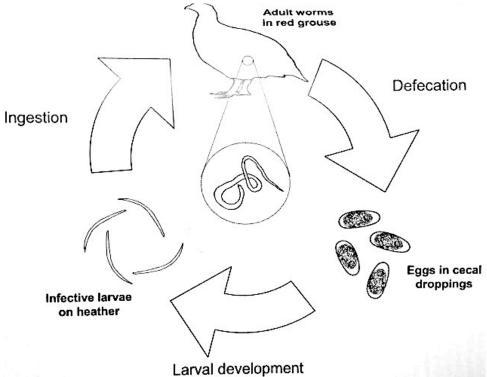
Trichostrongylus spp*.* belongs to the super family Trichostrongyloidea, which makes part of the suborder Strongylida, where bursate nematodes are found.

Adult females are approximately 6-10mm long and adult males are 4-8mm, with width gradually increasing from 10µm at the apical end to 50µm in front of the caudal bursa. After extraction from the intestinal contents (with sieves) avian species of *Trichostrongylus* spp. are easily observed under the microscope. They are small, fine reddish worms. *Trichostrongylus* spp. are unlikely to be confused with other helminths found in the small intestine and ceca of birds, thanks to their morphology, especially the characteristic cuticular striations (in *T. tenuis* transverse striations start from behind the excretory pore of both sexes and cover three to four-fifths of the body surface). These characteristics, plus the configuration of the caudal bursa dorsal array, are useful to distinguish both species of *Trichostrongylus, T. tenuis and T. cramae* (Tompkins, 2008)

1.2.2.2 Life Cycle

Avian species of *Trichostrongylus* have a relatively simple life cycle, as they only require a single host (monoxenous) (Figure 14). Basically, it consists of an egg stage, five larval stages and an adult stage. No paratenic hosts are known for species of *Trichostrongylus.* Eggs, measuring nearly 75 x 46 µm, are excreted in the faecal droppings at the morula stage (64 cells). At incubation temperatures (>5ºC) with humidity, eggs hatch in approximately 36-48h; they are not resistant to extreme cold (-15ºC) or desiccation, with little development occurring at temperatures below freezing point. First stage larvae measure approximately 360µm in length, moult into the second stage within 36-48h and the infective stage (third stage) is reached after 8-16 days. All these periods are dependant on the environmental temperature, like eggs, the larvae are susceptible to extreme cold or desiccation (continuous temperatures of -15ºC are lethal to larvae within

Figure 14 – Life cycle of *Trichostrongylus tenuis* affecting Red Grouse, parasites are not drawn to scale. (Adapted from Tompkins, D.M., 2008)

12 days). Nevertheless, they are able of withstanding cold winter temperatures on grouse moors that can be as low as -10ºC, remaining infective for up to 3 weeks. Ingesting third stage larvae infects birds; these larvae are usually present in the growing tips of moist heather, the main diet of adult Red Grouse. Third stage larvae tend to accumulate in water drops, present at the most likely point of ingestion by an adult bird. Larvae are more available on plants during daylight hours experimentally and on plants collected from the field in the afternoon rather than in the morning, this indicates that larvae migrate back down the plants at night (Saunders, Tompkins & Hudson, 2000). This behaviour most likely evolved to avoid desiccation during night periods, associated with this are time-

series analyses that suggest that some of the year-to-year variation in parasite egg numbers that are passed by Red Grouse is explained by rainfall in the previous summer, likely caused by greater transmission during wetter summers. The process by which a third-stage larvae escapes from the external cuticle (of the second stage), is called exsheathment, it occurs a few days after ingestion. Unsheathed third stage larvae invade the small intestine and cecal mucosa, and then they can undertake a period of "arrest" at this stage within the mucosa or develop directly into fourth stage larvae, afterwards larvae return to the gut lumen where they mature into adults, within a few days (Tompkins, 2008).

Shaw (1988) analysed the larval population present within individual grouse amongst the different seasons, which study provides strong evidence for the *T. tenuis* third larval stage arrestment or hypobiosis. In late summer, most larvae are in the fourth stage but some third stage larvae are sheathed, demonstrating that transmission is still taking place. In winter, ensheathed larvae are not present but exsheathed larvae are present, suggesting that few new larvae are being ingested through this period and that the exsheathed third stage larvae are "arrested" or hypobiotic. Throughout spring, the percentage of third stage larvae decreases considerably in the parasite population and sheathed larvae are absent, indicating the development of overwintering third stage larvae to fourth and adult stages. This development scheme results in a "spring rise" in worm egg production that is evident in grouse faeces (Shaw, 1988). There is a second peak of recruitment into the adult worm population that occurs during late summer, that suggests the existence of increased numbers of larvae on heather in the preceding weeks. These increases may be related to ideal conditions for transmission where mean temperatures are in the range of 10-15 ºC and mean monthly rainfall is in the range of 20-50 mm.

When development of *T. tenuis* is direct, meaning no "arrested" period in the mucosa, the prepatent period between ingestion of infective larvae and the shedding of eggs in feces is 7-8 days. Few host individuals have many parasites, while most host individuals have few parasites (Hudson, Newborn & Dobson, 1992a). Infected host individuals can carry worm intensities of up to 24000 worms (Hudson, 1986).

Eggs are produced at a rate of approximately 5×10^5 eggs per female worm per year (Hudson *et al.*, 1992a) and adult worms can live for more than 2 years, though parasite egg output declines with age of worm population, particularly in winter (Shaw & Moss, 1989a). There is no evidence of density-dependent suppression of parasite establishment or egg production at high worm intensities (Sievwright, Redpath, Mougeot, Watt & Hudson, 2004).

There are remarkable variances between the biology of *T. tenuis* in Red Grouse and the biology of *T. cramae* in Northern Bobwhites (Freehling & Moore, 1993). While *T. tenuis* produces a chronic infection in Red Grouse that increases throughout the life of the bird

(Potts, Tapper & Hudson, 1984), *T. cramae* in Northern Bobwhites in northern Florida is a seasonally occurring parasite. Infections of *T. tenuis* in other host species also look to be more comparable to those of *T. cramae*, with the high burdens and chronic infections seen in Red Grouse likely to be an exception rather than the rule. This may very well be a consequence of the unnaturally high densities of managed Red Grouse populations in the UK moorlands (Tompkins, 2008).

1.2.2.3 Clinical Signs

When birds carry a high intensity of parasites, they show signs of malnutrition with dullness of the plumage, appetite loss, diarrhoea, and emaciation. This signs mirror what sometimes is called "grouse disease". Among Red Grouse, signs are more prevalent during spring, matching with the "spring rise" in worm egg production, and in the autumn in young hatch-year birds (Tompkins, 2008).

1.2.2.4 Pathogenesis

Adult worms are threaded into the caecal mucosa, causing trauma, flattening and atrophy of the epithelial cells. This interferes with the digestive process, namely the normal digestion of heather and other plants. High burdens can cause haemorrhagic typhilitis and when chronic the caecal contents may contain a whitish material (Tompkins, 2008).

The huge ceca of Red Grouse play an important role in the digestion of its fibrous diet and in nutrient recovery. The disruption in normal caecal function by heavy infections with *T. tenuis* leads to a loss of body condition, increased mortality and reduced fecundity with decreases in both clutch dimension and brood size (Hudson *et al.*, 1992a; Delahay & Moss, 1996a).

The redness of males Red Grouse's combs and the concentration of plasma carotenoids are also negatively correlated with the burden of *T. tenuis* (Mougeot, Redpath & Leckie, 2005a; Mougeot, Martinez-Padilla, Perez-Rodriguez & Bortolotti 2007a)*.* The redness of the comb is a secondary sexual characteristic of Red Grouse that functions in both intra and inter-sexual selection (Redpath, Mougeot, Leckie & Evans, 2006a), plasma carotenoids are responsible for the redness (Mougeot *et al.*, 2007a). This has been experimentally confirmed within interpretations of plasma carotenoid concentration and comb redness both increasing following reduction of *T. tenuis* burdens (Martinez-Padilla, Mougeot, Perez-Rodriguez & Bortolotti, 2007) and reducing after challenge with *T. tenuis* (Mougeot, Perez-Rodriguez, Martinez-Padilla, Leckie & Redpath, 2007b).

High intensities of infection have only been reported for *T. tenuis* infecting Red Grouse, the direct effects of such nematode infection is on mortality and fecundity, interacting with other factors to impact individual hosts (Tompkins, 2008). When intensities of *T. tenuis* are high, birds experience increased predation, maybe because there is an increase of scent emission (Hudson *et al.*, 1992b; Dobson & Hudson, 1995). Birds heavily infected are also

less able to keep their territories due to the energetic consequences of parasitism (Delahay, Speakman & Moss, 1995) and its interaction with testosterone (Seivwright *et al*, 2005). When dewormed bird's territorial behaviour increases drastically (Mougeot, Evans & Redpath, 2005b), this shows that *Trichostrongylus* infection also affects mating success of males Red Grouse, in relation to the interactions with carotenoids discussed previously. The effect of nematodes at the scale of host population have been extensively studied and experimentally demonstrated for *T. tenuis* in Red Grouse on British moorland (Tompkins, 2008). Red Grouse populations have characteristic cyclical dynamics, with periods between 4 and 8 years (Tompkins, 2008). The connection between these cycles and *T. tenuis* has long been supposed, thanks to the association between population crashes and high parasite burdens recovered from individuals. There are some studies that experimentally demonstrated that the interaction with *T. tenuis* was a potential cause of these cycles (Dobson & Hudson, 1995), Using anthelmintic at replicated sites, was shown to drastically reduce the extent of population crashes (Hudson, Dobson & Newborn, 1998). That was a pioneering study in the demonstration of parasite regulation of host numbers in any wildlife population; unfortunately it did not prove that the population dynamics were just a function of *T. tenuis* only. Other studies refer that is an interaction between aggressiveness, territoriality and parasitism that motivates such dynamics, differently between different parts of the UK (Mougeot *et al.*, 2005c; Redpath *et al.*, 2006a, b). Red Grouse populations rapidly recover following crashes but the game industry is interested in maintaining high numbers of birds at all times (Tompkins, 2008).

1.2.2.5 Diagnosis

For a reliable diagnosis, the parasites should be recovered from post-mortem examinations. Eggs or adult worms in faeces (infective stages) are also indicative of infection. However, diagnosis based on infective stages should be confirmed by a post-mortem examination to assess the presence of adult *T. tenuis* in the caeca and to establish the degree of infection and harm (Tompkins, 2008). Faecal egg counts are a reliable method for estimation of relative parasite intensity across individuals, only if samples are collected at the same period of year (Seivwright *et al.*, 2004), however

Figure 15 - *T. tenuis* copulatory bursa and spicules, lateral view. (Original)

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Tompkins (2008) consider that they should not be considered reliable enough to measure impacts on populations.

T. tenuis are only likely to cause disease when found in high numbers, namely with thousands of worms per bird (Tompkins, 2008).

1.2.2.6 Immunity

Studies with Red Grouse have found no evidence of "acquired" immunity to *T. tenuis* infection. Wilson (1983) concluded in his studies that the number of worms increases throughout the life of individuals. Similar studies, with other species like Red-legged Partridges (*Alectoris rufa*) observed a similar increase with age (Millan, Gortazar & Villafuerte, 2004).

Susceptibility to infection by this parasite varies throughout seasons in Red Grouse, rates of *T. tenuis* establishment being lower in summer than in autumn, this should be linked with behaviour, as males show higher levels of territoriality in the autumn. The stress from this behaviour increases corticosteroid levels, which reduces immune responses against parasites. There are other experiments that support this fact, Seivwright, Redpath, Mougeot, Leckie & Hudson (2005) showed that high levels of testosterone (which is not corticosteroid) in males birds increases the susceptibility to this nematodes (Tompkins, 2008).

1.2.2.7 Treatment and Control

The oral administration of anthelmintics (levamisole hydrochloride or fenbendazole) has show to be effective in the elimination of adult and developing worms, although these treatments are not efficient against arrested larvae present in the mucosa. Resistance has not been reported for *Trichostrongylus tenuis* species of birds (Tompkins, 2008). Wild populations containing infected birds may work as reservoirs, increasing risks of infection on sympatric populations composed by more susceptible host species (Tompkins, 2008). In Red Grouse moorlands, at UK, a large-scale management of *T.*

Figure 16 - Grit boxes where medicated grit is placed. Notice that there are two compartments with one cover to switch to plain grit before game season, so the anthelmintic withdrawal period can be respected. (Original)

tenuis is consistently done by the indirect application of anthelmintics via "medicated grit" (Figure 16). This treatment decreases nematode burdens, improving breeding success by reducing chick mortality and treating Red Grouse females (Newborn & Foster, 2002).

The rise in bird numbers, result from the use of "medicated grit" is a viable scheme for improving Red Grouse bags, as the cost of treatment is low comparative to the profit from a bigger harvest. This large-scale method using anthelmintics may also be applied to the management of endangered species that are affected by infections with *Trichostrongylus,* since resistance is not known for this genus of nematode in birds (Tompkins, 2008)
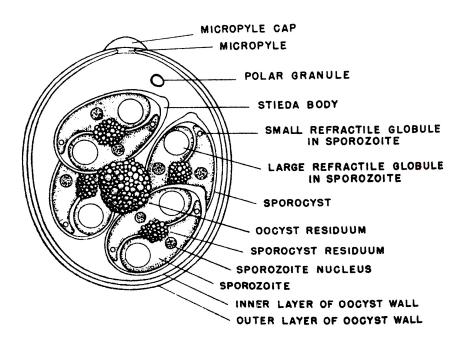
1.2.3 *Eimeria* spp.

Poultry coccidiosis was reported as early as the late 1800s, by Railliet & Lucet (1890) and Salmon (1899) (Saif, 2008). Fantham (1910) described the life cycle of the first intestinal species of avian *Eimeria*; this species, detected in Red Grouse in the UK, was named *Eimeria avium* (Lovat, 1911). Initially it was thought to cause coccidiosis in numerous avian species. Nowadays it is known that members of this genus are mostly species-specific and nearly 200 species of avian *Eimeria* have been officially described. Plenty more have been reported but not described as distinct species (Yabsley, 2008).

The coccidian infect all classes of vertebrates and are a complex and enormous group of obligate intracellular parasites in the phylum Apicomplexa. The majority of the coccidia are significant medical and veterinary pathogens, especially *Eimeria* spp*.,* which infects the kidneys, liver, and intestines of birds.

The classification of coccidia is centred on morphologic characteristics, particularly those of the environmentally resistant sporulated oocyst (Figure 17). This stage is the only one

Figure 17 - Drawing of sporulated oocyst of genus *Eimeria.* Notice the presence of 4 sporocysts, each with two sporozoites, after sporulation. (Adapted from Saif, 2008).

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that is shed in faeces of the host and is consequently the stage most frequently observed. The wide range of coccidia is described exclusively on the morphology of voided oocysts, and in numerous situations nothing more about their development in the host is known.

Most of the avian species of *Eimeria* develop inside intestinal epithelial cells; although, certain species of *Eimeria* develop in extraintestinal sites. Sexual stages, asexual stages and the oocysts progress within the cytoplasm or nucleus of infected cells. Compared with intestinal species of *Eimeria*, little is known about the host specificity and endogenous development of renal coccidia because of the difficulty in getting oocysts to sporulate to the infective stage (Yabsley, 2008).

It is very important to differentiate infection and disease when discussing coccidia. Coccidian infections are often asymptomatic; therefore, the accurate terminology for infection is "infection with" or "coccidiasis". Coccidiosis refers to infections causing clinical disease, but the term is frequently applied unsuitably to designate subclinical infection. Below certain variable conditions, including age of birds, inoculation dose, stress, absence of earlier infection, concurrent disease, or immunosuppression, species of *Eimeria* that usually do not origin disease can produce pathogenic effects and originate coccidiosis. Commonly, species of *Eimeria* rarely cause disease in wild birds. Young birds or adults that are stressed or unhealthy are more likely to develop clinical coccidiosis (Yabsley, 2008).

1.2.3.1 Distribution and Host Range

Avian *Eimeria* spp. have been reported worldwide, it is a common and economically important disease of domestic birds such as turkeys, chickens, however only sporadic cases of intestinal coccidiosis in free-living birds have been reported.

Infection with intestinal coccidia is doubtless ubiquitous among avian species; though, prevalence of infection with *Eimeria* spp. differs through avian orders. Coccidiosis is reported most frequently from these two orders. Species of birds from some orders, such as Passeriformes, are mainly infected with *Isospora* and/or *Atoxoplasma*. Although undescribed species of *Eimeria* have been found in many orders of birds, there are many other orders where *Eimeria* probably occurs but has not been observed since insufficient hosts have been tested. In other orders, like Passeriformes, a substantial amount of hosts have been examined for coccidia, but few *Eimeria* have been found (Yabsley, 2008).

Eimeria is generally host specific although some species infect various hosts. One example is *Eimeria dispersa*, which infects chickens, turkeys, Chukar Partridges (*Alectoris chukar*) and Red-necked Pheasants (Doran, 1978). Genetic studies and/or controlled experimental infections are required to prove the occurrence of a single species of *Eimeria* species in multiple hosts, particularly those in different genera (Yabsley, 2008).

1.2.3.2 Etiology

The genus *Eimeria* is in the family Eimeriidae, order Eucoccidiorida, phylum Apicomplexa and is one of the most common coccidia reported from fowl. It can be differentiated from other genera by its characteristic oocysts containing 4 sporocysts, each of which contains 2 sporozoites (Yabsley, 2008).

Almost 196 species of *Eimeria* have been formally described from 17 avian orders. Nevertheless, uncharacterized species of *Eimeria* have been reported from various other wild avian hosts. Most of the species of *Eimeria* found in wild birds are reported from the orders Anseriformes and Galliformes (Yabsley, 2008).

Numerous genera of coccidia infect the intestinal epithelium of avian hosts, comprising *Eimeria*, *Isospora, Tyzzeria, Caryospora, Cryptosporidium, Sarcocystis* and *Atoxoplasma.* Members of the genus *Tyzzeria* and *Cryptosporidium* do not have sporocysts and contain 8 and 4 sporozoites respectively, within the oocyst. Species within the genera *Isospora* and *Atoxoplasma* have 2 sporocysts, each with 4 sporozoites. Members of the genus *Caryospora* have oocysts that hold a single sporocyst with 8 sporozoites. *Sarcocystis* are particularly thin and free sporocysts containing 4 sporozoites are usually the only forms found in feces. Molecular classification of avian coccidia has revealed that several of these early classifications have created polyphyletic genera and that morphologic characters are not enough to determine relationships, for instance: avian *Isospora* are more closely related to *Eimeria* than to species of mammalian *Cystoisospora* (Yabsley & Gibbs, 2006).

Ten species of *Eimeria* have been described from pheasants, of which three – *Eimeria colchici*, *Eimeria duodenalis* and *Eimeria phasiani* – are associated with severe disease in captive Ring-necked Pheasants*; E. colchici* is considered the most pathogenic (Jones, 1966 cited in Yabsley, 2008).

1.2.3.3 Epidemiology

These coccidia have a direct life cycle as they are transmitted from one host to another without any vectors or intermediate hosts (Figure 18). Development inside the host comprises both sexual and asexual stages within epithelial cells. Unsporulated, non-infective oocysts shed in the faeces of the host, undergo sporulation outside the host to become infective. The first step of sporulation is the asexual course of sporogony by which sporocysts and sporozoites, the infective stage, are produced from the germ ball within the resistant oocyst. This development is conditioned by different traits such as temperature, oxygen and light; which are species dependent. Commonly, oocysts are exceptionally resistant and can also tolerate freezing temperatures or desiccation (Sathyanarayanan & Ortega, 2006), though long freezes or high temperatures may destroy them (Parker & Jones, 1990). When ingested by a suitable host, physical and chemical issues trigger the rupture of oocysts, releasing sporocysts, which then break and release sporozoites. Sporozoites then enter intestinal epithelial cells and convert into trophozoites. Trophozoites reproduce asexually to produce meronts, which later change into merozoites by a route called merogony. Merozoites cause the cell to rupture and they
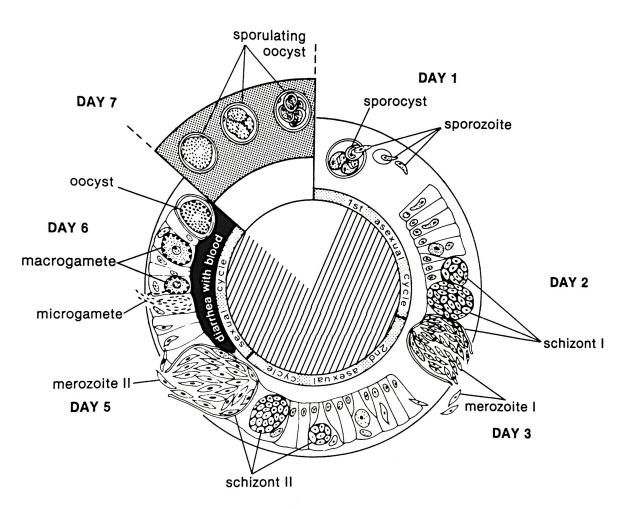
Figure 18 - Life cycle of *Eimeria tenella*. (Adapted from Saif, 2008)

can invade other epithelial cells moreover to complete further rounds of merogony or to start gametogony. The number of cycles of merogony and number of merozoites produced during each cycle varies between species of *Eimeria*. Throughout gametogony, merozoites transform into macrogametocytes, which are the female cells, or microgametocytes, the male cells. A microgametocyte matures into a sole macrogamete while a microgametocyte buds to form numerous flagellated microgametes. These microgametes leave the host cell and invade cells holding macrogametes, where fertilization takes place. A fertilized macrogamete develops an external wall to become an oocyst, which is shed in the faeces (Yabsley, 2008).

Coccidiosis rarely occurs in free-living fowl and is commonly renown as a disease of intensification, captivity and overcrowding. Some species of *Eimeria*, disease level rises with ingestion of higher numbers of oocysts in a dose-dependent way (Williams, 2001). It is more likely for disease to occur in young or naïve birds exposed to elevated numbers of oocysts (Yabsley, 2008).

Coccidiosis is a wide-reaching economically significant disease of poultry, mainly chickens and turkeys. Species of Galliformes are hosts of several species of coccidia (ten in pheasants, seven in turkeys and eight in chickens). To ascertain the economic significance to poultry, numerous exceptional assessments of domestic fowl coccidia have been published. The eight species of Eimeria described from chickens (*Eimeria acervulina, Eimeria brunetti, Eimeria maxima, Eimeria mitis, Eimeria mivati, Eimeria necatrix, Eimeria praecox and Eimeria tenella*) each invade specific parts of the lower gastrointestinal tract and cause different presentations of disease (McDougal & Fitz-Coy, 2008). Similar species of *Eimeria* have been discovered in the free-living ancestors of domestic fowl: the Wild Red Junglefowl (*Gallus gallus*) and Ceylon Junglefowl (*Gallus lafayetii*); nonetheless, more reports of coccidial disease in these hosts are needed (Yabsley, 2008).

1.2.3.4 Clinical Signs

The majority of birds infected with intestinal *Eimeria* do not show any clinical sign as low burdens affect a small number of epithelial cells that can be rapidly supplanted. In Infections of moderate to high burdens, greater numbers of cells are destroyed, resulting in decreased food and water intake, reduced intestinal absorption and haemorrhage. Ill animals occasionally display diarrhoea stained with blood or mucus, anorexia, emaciation, loss of coordination, tiredness, ruffled feathers and decreased egg production (McDougal & Fitz-Coy, 2008; Yabsley, 2008).

Expansion of clinical signs varies according to numerous factors such as species of parasite, intensity of infection and host issues including health and age. For instance, atypical hosts can become infected, although replication and oocyst excretion by the parasite might be reduced, leading to no disease. Intensity of infection might be an

important factor that led to development of disease. In a study, Ring-necked Pheasants which were infected with low numbers (10 000 oocysts) of *E. phasiani* showed only diarrhoea, but pheasants that were inoculated with higher dosages (100 000 oocysts) exhibited mucoid diarrhoea, ruffled feathers, incoordination, and decreased weight gain (Trigg, 1967). Correspondingly, some trial infections of pheasants with different numbers of *E. colchici* produced a dose-dependent disease (Norton, 1967). 8 of 10 birds exposed to low burdens (20 000 oocysts) survived infections; however, none resisted to high intensity inoculations (80 000 and 320 000 oocysts) (Yabsley, 2008).

1.2.3.5 Pathogenesis

Pathological findings differ extensively, namely with the bird species, *Eimeria* species and intensity of infection. Intestines can be filled with mucus, ballooned, haemorrhagic and discoloured. Desquamation of the intestinal mucosa is frequently seen in severe infections. Some species cause the formation of white caseous cores in the ceca. Between Galliformes infections with coccidia produce intestinal harm and modifications in the gut motility that might predispose the intestine to infections with additional pathogens including *Clostridium perfringens* or *Salmonella typhimurium* (McDougal & Fitz-Coy, 2008; Yabsley, 2008).

Various species are harboured in the cytoplasm of infected cells, but few species of *Eimeria* from geese inhabit the nuclei of epithelial cells. Affected birds exhibit wide histopathologic lesions, which are usually evident including host cell damage and lymphocytic infiltration. Nevertheless, depending on species of *Eimeria* and level of infection, lymphocytic infiltration may or may not be evident (Yabsley, 2008).

1.2.3.6 Diagnosis

Oocysts, meronts and gamonts can be seen within intestinal epithelial cells, using a microscope. The diagnosis of coccidiosis is dependent on the detection and identification of oocysts in faecal samples laterally with the existence of characteristic lesions of the disease found in necropsies or clinical signs in live birds. Faeces can be collected from live birds or at necropsy and can be scanned directly for oocysts or processed in the laboratory looking at parasite intensities per gram of faeces. Diurnal periodicity on their shedding has been reported for an *Eimeria* sp. from Red-legged Partridge, which most commonly sheds oocysts in the late afternoon (Villanúa, Pérez-Rodrígez, Gortázar, Hofle & Viñuela 2006). However, this diurnal periodicity has not been reported for species of poultry *Eimeria* (Long, 1982 cited in Yabsley, 2008). It is undetermined whether species of *Eimeria* from free-living fowl demonstrates diurnal periodicity in oocyst excretion, but this should be taken into account when doing assessments in wild hosts (Yabsley, 2008).

To assess species, oocysts must be allowed to sporulate by mixing faeces with 1-3% (w/v) potassium dichromate, at room temperature and observed day-to-day for verification

of sporulation. Placing the faecal/potassium dichromate solution in a covered petri dish facilitates sporulation. To prevent desiccation, adequate potassium dichromate solution should be used. After sporulating, faeces must be kept at 4ºC to preserve morphologic characteristics. Since many infections are not clinical, observing oocysts in a sample does not necessarily indicate that coccidia is the main cause of disease; other causes of the disease must be ruled out in live birds and suggestive pathologic findings must be present at the necropsy (Yabsley, 2008).

1.2.3.7 Impact on Wildlife Population

Infections of birds with *Eimeria* are common, however coccidiosis amongst these birds in their natural habitat is rarely a significant issue. Occurrences can arise when circunstances combine to crowd or stress fowl (breeding and loss of habitat are two examples). Wild birds kept in captivity may succumb to significant illness from *Eimeria* infection (Yabsley, 2008).

Eimeria angusta has been related with caecal coccidiosis in captive and free-living species of Ruffed Grouse (*Bonasa umbellus*) and mortality has not been reported in free-living Greater Sage-Grouse (*Centrocercus urophasianus*) since the 1960s, apparently because numbers of grouse have declined, leading to reduced crowding and/or stress and reduced transmission of the parasite (Yabsley, 2008).

Outbreaks of intestinal coccidiosis are sporadically reported as the cause of mortality of free-living birds, although this situation does not seem to have a relevant influence on wild fowl populations. Decreased egg production and fertility have been reported in assessment studies of coccidiosis and can also happen in free-living birds. Weight loss has not been described in young wild birds with eimerian infections; nevertheless, this occurrence is usual in both poultry and experimental studies and could go unrecognized in wild birds (Ruff, Fagan & Dick, 1984). Field studies of the subclinical effects of coccidian infections are lacking (Yabsley, 2008).

1.2.3.8 Coccidiosis in Red Grouse

Fanthan (1910) first described coccidiosis in Red Grouse in 1910. He was employed to investigate mortality in Red Grouse (*Lagopus lagopus scoticus*) on the grouse moors of Scotland and from these he isolated and described a parasite that he named *Eimeria avium* (Fantham, 1910), which was the first detailed description of the life cycle of an eimerian parasite in an avian host (Chapman, 2014). Fanthan (1910) preliminary studies mentioned the association between weight loss and chick mortality with high burdens of *Eimeria*.

After those studies less was published regarding coccidiosis in Red Grouse being uncertain to recognize the relation between *Eimeria* burdens and subsequent lethal and sub-lethal effects.

1.2.3.9 Treatment and Control

Almost all that is known about treatment or control of fowl coccidiosis has resulted from studies of *Eimeria* of poultry. The use of anticoccidial feed or water supplements such as amprolium or monensin has been the initial approach for controlling coccidiosis for the poultry industry. Recently, resistance has been reported against several of the most common drugs (Martin, Danforth, Barta & Fernando, 1997).

Poultry coccidia encourage a durable immunity; consequently, vaccination has been investigated as a substitute to anticoccidials drugs for controlling coccidiosis. Historically, vaccines were made of wild-type, live or attenuated coccidia, although these vaccines were specific to certain species of *Eimeria* and, in some circumstances, specific to certain parasite strains. Wild-type vaccines act by offering a low-level of exposure; though, uniform exposure through all birds is crucial to preventing progress of coccidiosis and for building protective immunity against future infections with high burdens of parasites (Shirley, Smith & Tomley 2005). Attenuated strains (with limited reproductive ability) are equally immunogenic as wild-type strains but lower the risk of developing disease. Recent vaccines based on recombinant protective antigens are under research and may enable a safer and cheaper vaccination (Shirley, Smith & Blake, 2007). Presently, vaccines are species/strain specific, expensive, hard to produce and would not be viable for use in free-living birds (Yabsley, 2008).

Birds kept in captivity that develop infection usually respond to commercial anticoccidial drugs, although studies on their effectiveness and safety are reduced. Additionally, each *Eimeria* may differ in susceptibility to the most frequently used anticoccidials. For instance, caecal coccidiosis (*Eimeria colchici*) in Ring-necked Pheasants is controlled with medicated feed containing amprolium. Sulfametiosis has also been used effectively to control *Eimeria* infections in Rock Pigeons and Budgerigars kept in captivity, when mixed with drinking water. Raising birds on wire or clean housing are two ways of preventing the build-up of infective oocysts and may reduce the risk of disease (Yabsley, 2008).

Coccidiosis outbreaks in free-living wild birds are hard to control since neither dosage nor dosing intervals can be controlled. Avoiding stress or high densities are more efficient approaches to decreasing or preventing episodes of coccidiosis in wild birds.

1.2.4 *Mycoplasma* spp.

Twenty-three different species of *Mycoplasma* have been described in avian hosts, most of them are found in domestic poultry, mainly in turkeys and chickens, but 17 of those species have also been identified in wild hosts. Only 5 of these species have been isolated form wild birds only and these are: *Mycoplasma corogypsi, M. falconis, M. gypis, M. buteonis and M. sturni.* The three well-known pathogens in commercial poultry are *Mycoplasma gallisepticum, M. synoviae and M. meleagridis.* They cause substantial

economic losses. *M. gallisepticum* has been isolated from Wild Turkeys (*Meleagris gallopavo*) with sinusitis and from wild fringillids with conjunctivitis, especially House Finches (*Carpodacus mexicanus*). *Mycoplasma gallisepticum* is the main focus of this review of mycoplasmal diseases in wild fowl, thanks to its impact in commercial and wildlife birds (Luttrell & Fischer, 2007).

Mycoplasmosis was first described as a respiratory disease in poultry in 1905, although the causative agent, *Mycoplasma gallisepticum*, was not successfully cultivated until nearly 50 years later (Ley, 2008). Well-known as infectious sinusitis in turkeys and chronic respiratory disease in chickens, this disease is characterized by rales, sinus exudate, coughing, airsacculitis and swollen sinuses (in turkeys). This disease has a tremendous economic impact in poultry industry, causing serious losses from carcass condemnations, reduced feed and egg production and retarded growth in young birds. Before an outbreak of mycoplasmal conjunctivitis in finches in the 1990s, *Mycoplasma gallisepticum* rarely had been isolated from free-living birds apart from the incidence of clinical disease in Wild Turkeys (Luttrell & Fischer, 2007).

1.2.4.1 Etiology

Mycoplasma gallisepticum is included in the family Mycoplasmataceae in the class Mollicutes, renowned as the smallest self-replicating prokaryotes. Their distinctive characteristics include the very small genome, the lack of cell walls and the highly variable surface proteins. These bacteria infect a wide range of hosts including insects, plants, humans and animals. Mycoplasmas are usually host and tissue specific and they primarily infect mucosal surfaces of the respiratory, reproductive and urinary tracts. Pathogenic mycoplasmas can cause a variety of conditions from minor to acute symptoms and frequently form a synergistic complex in conjunction with other bacteria, viruses or environmental stresses disturbing the host. Most species exist commensally in their hosts as asymptomatic or mild infections (Luttrell & Fischer, 2008).

1.2.4.2 Epidemiology

The main mode of transmission is horizontal, via direct contact or aerosol droplets between a susceptible bird and a carrier, and vertical, through eggs in chickens and turkeys (Ley, 2008). The infection of the reproductive tract happens with the proximity of the oviduct to abdominal air sacs (Luttrell & Fischer, 2008).

Mycoplasma gallisepticum is kept mainly in avian reservoirs; domestic birds in commercial or garden flocks as well as individuals in free-living populations may serve as foci of infection or be nonclinical carriers. *M. gallisepticum* is a fragile organism, however it can exist for short periods of time on substances such as litter, rubber boots, feathers, dust and clothing, with that mechanical transmission can be possible (Christensen, Yavari, McBain & Bradbury, 1994). Contaminated feathers and litter can pose risks of

transmission to wild birds that may visit poultry farms for nesting or feeding (Luttrell & Fischer, 2008).

Transmission of mycoplasmas is principally supported on contact amongst infected and susceptible hosts and consequently is increased by circumstances in which birds are living in concentrated numbers. With those conditions *M. gallisepticum* can spread rapidly and infect a high proportion of exposed birds (Luttrell & Fischer, 2008).

1.2.4.3 Pathogenesis

M. gallisepticum triggers acute or chronic disease in birds and often acts synergistically with other pathogenic mycoplasmas, viruses or bacteria to create more intense clinical disease. Absence of adequate nutrition, environmental stresses and extreme temperatures also can play a role in increasing the pathogenic effects on the host (Ley, 2008).

The pathogenesis of mycoplasmas is only moderately understood, as it is too complex, but a primary event is the adherence of the organism to the host cell surface. One crucial and specialized feature in some species, including *M. gallisepticum,* is a tip structure that allows attachment to host epithelial cells and causes cell damage. After attachment, mycoplasmas can cause cell injury directly through pathogenic mechanisms, such as production of cytotoxic substances and inhibition of ciliary movement, or indirectly via the host's response to the infection. In the case of *M. gallisepticum*, the pathogen attaches to epithelial cells of the respiratory tract and the subsequent cell damage and host inflammatory response contribute to the progress of clinical disease. Additional mechanisms contributing to the pathogenicity of *M. gallisepticum* include quick antigenic variations in surface proteins and evasion of host immune responses (Luttrell & Fischer, 2008).

1.2.4.4 Clinical Signs

Clinical signs of *M. gallisepticum* in wild birds are usually described in Wild Turkeys and House Finches. Wild Turkeys usually exhibit clinical signs analogous to those of domestic turkeys with *M. gallisepticum* infections (Ley, 2008). Periorbital sinuses on one or both sides of the head become swollen, and vision may be impaired if swelling is severe. Other symptoms involve sneezing, nasal discharge, moist rales and effort in breathing. Weight loss and depression can also be present. During *post mortem* examination, unilateral or bilateral periocular swelling, infraorbital sinusitis with serous, catarrhal, or fibrinous exudate and airsacculitis can be found (Luttrell & Fischer, 2008).

In House Finches, *M. gallisepticum* causes slight to severe eyelid swelling, conjunctivitis and watery discharge from one or both eyes and/or nares. Probably due to dried discharge, crusty lesions are formed on eyelids and nares. Impaired vision or blindness can develop as a consequence of these lesions. Signs such as sneezing, rales and

coughing usually are not present. This makes birds being more susceptible to predation or trauma, as birds become reluctant to fly (Luttrell & Fischer, 2008).

1.2.4.5 Diagnosis

Diagnosis of *M. gallisepticum* in live animals is based on clinical signs, history and detection of the pathogen by culture or molecular techniques. The arrival of molecular techniques has assisted the capacity to identify species of mycoplasmal isolates from domestic and wild birds and to differentiate strains, thereby defining the sources of outbreaks. The detection of *M. gallisepticum* may be accomplished by use of species-specific DNA probes or by polymerase chain reaction (PCR) techniques (Luttrell & Fischer, 2008).

As other mycoplasmas, *M. gallisepticum* is a fastidious organism that necessitates a compound selective medium enriched with 10-15% animal serum, yeast and dextrose source. Growth occurs at an ideal temperature of 37ºC (Luttrell & Fischer, 2008).

Swabs taken from the conjunctiva, trachea, choanal cleft, or sinus, or tissue suspensions made from lung or brain can be used to culture in special media (Ley 2008).

Although highly pleomorphic, *M. gallisepticum* colonies grown on agar plates typically appearing as rounded, smooth and translucent masses around 0.2-0.3 mm in diameter, with dense centres, that sometimes are called "fried eggs" (Ley 2008).

Differential diagnosis should include chlamydiosis, Newcastle disease, avian influenza and infectious bronchitis (Ley 2008).

1.2.4.6 Birds reared outdoors

Mycoplasma gallisepticum have been related with various disease outbreaks in numerous species of captive or pen-raised wild birds. These birds are kept in close confinement, whether they are domestic or wild, are at increased risk for contagious diseases, particularly mycoplasmosis (Luttrell & Fischer, 2008).

M. gallisepticum has been isolated from captive gamebirds such as Northern Bobwhites (*Colinus virginianus*), Wild Turkeys, Chukar Partridges and Ring-necked Pheasants. Obviously, the release of birds that are antibody positive for *M. gallisepticum* is not advocated, due to the chance of a carrier state, released captive birds represent potential reservoirs for the transmission of *M. gallisepticum* to those of the same species or to other free-living wild birds and commercial poultry (Luttrell & Fischer, 2008).

1.2.4.7 Impact on Wildlife Population

Mycoplasma gallisepticum primarily occurs in gallinaceous birds, especially chicken, wild and domestic turkeys and pen-raised gamebirds (Ley, 2008). *Mycoplasma gallisepticum* was isolated from Wild Turkeys in North America with infectious sinusitis in the early 1980s; outbreaks were registered in Georgia, California and Colorado states (Luttrell &

Fischer, 2008). Those cases were attributed to domestic sources that involved close contact between Wild Turkeys and domestic poultry (Luttrell & Fischer, 2008). Some studies conducted in USA occasionally detected *M. gallisepticum* antibodies in clinically normal Wild Turkeys (Hoffman, Luttrell, Davidson & Ley, 1997). Occurrences of overt disease and seropositive turkeys led to an increased consciousness of mycoplasmosis in wild populations (Luttrell & Fischer, 2008).

1.2.4.8 Treatment and Control

Prevention strategies include medication, live and inactive vaccines and farm biosecurity and hygiene. The absence of cell walls in mycoplasmas renders many antibiotics, like penicillin, ineffective in treatment of avian mycoplasmosis. Tetracycline and tylosin have been used effectively for treating clinical disease in domestic poultry (Ley, 2008). There is risk associated with releasing wild birds that have been treated for mycoplasmas, birds that recover from acute disease may convert to asymptomatic carriers in spite of antibiotic therapy and most wildlife caretakers will be incapable to attest that the birds they release are no longer carrying the infectious agent (Ley, 2008).

1.2.5 Infectious Bronchitis Virus

Infectious bronchitis is caused by a Coronavirus, the Infectious Bronchitis Virus (IBV), which is a common, acute, highly contagious and economically important viral disease in poultry. IBV is acquired after inhalation and/or direct contact with infected birds, equipment, debris, litter or other fomites. The virus is also transmitted vertically through contact with contaminated shell surfaces which were infected via shedding from the oviduct or alimentary tract. This disease is wide spread through all poultry producing countries (Cavanagh & Gelb, 2008).

Coronaviruses with similar protein sequences of those of IBV have been isolated from partridges (*Alectoris* sp.), Ring-necked Pheasants, turkeys, Guinea Fowl (*Numida meleagridis*), Peafowl (*Pavo cristato*) and the non-gallinaceous Teal (*Anas* sp.). There are cumulative evidences that IBV has a larger host range than was previously believed (Cavanagh & Gelb, 2008).

1.2.5.1 Etiology

IBV is a member of the *Coronaviridae*, which comprises 2 genera, *Coronavirus* and *Torovirus*. Infectious bronchitis virus is in the group 3 of the Coronavirus genus, together with coronavirus from other fowl species. Groups 1 and 2 include mammalian coronavirus that are extensively different from IBV, particularly in genome organization and gene sequences (Cavanagh & Gelb, 2008).

1.2.5.2 Pathogenesis and Epizootiology

Actually it is not considered that the chicken is the single host for IBV; however, it is probable that it is only in the chicken that IBV may cause disease (Cavanagh & Gelb, 2008).

1.2.5.2.1 Transmission and Incubation Period

IBV spreads quickly through birds in a flock. This viral disease is extremely contagious and has a short incubation period. Vulnerable chickens retained with infected birds frequently acquire clinical signs within 24-48 hours. IBV was isolated consistently from the trachea, kidneys, lungs and bursa of birds at 24 hours and throughout the seventh day follow aerosol contact. Virus isolations declined with time and differed with the strain, although IBV was isolated from faeces 20 weeks and from the caecal tonsils at 14 weeks after infection. The occurrence of airborne spread between flocks in undetermined, although it is considered that IBV spreads readily. Taking in count recent studies of IBV in different species, it should be taken into account that other species may act as vectors of IBV (Cavanagh & Gelb, 2008).

The incubation period of IBV is dose-dependent and is as small as 36 hours for ocular application and 18 hours for intratracheal inoculation (Cavanagh & Gelb, 2008).

1.2.5.2.2 Clinical Signs

In young chicks IBV infections may cause respiratory disease. Reduced weight gain and feed efficiency are observed in broiler chicken. IBV also predisposes broilers to pericarditis, bacterial airsacculitis and perihepatitis. Mortality is usually up to 30% which peak often occurs at 5 to 6 weeks of age. Mortality may be reduced when mild strains of IBV are present (Cavanagh & Gelb, 2008).

Egg production decreases are present in breeder chickens and layers. IBV may replicate in the oviduct and cause permanent damage in immature females, causing limited egg production later in life. Depending on the immune status of the flock infection of hens during lay, may decrease egg production up to 10% or more. Egg shells are usually deformed and weaker due to thinning of the shell (Cavanagh & Gelb, 2008).

Coronavirus infections of laying pheasants has been linked with low hatchability rates, small size and variable colour. In a study, comprising young pheasants, no respiratory signs were described, although mortality had reached 45% by 10 weeks of age. Diseased birds were reported as having ruffled feathers as well as drooping wings (Lister *et al.*, 1985).

1.2.5.3 Diagnosis

Infectious bronchitis diagnosis is based on lesions, clinical signs, seroconversion or increasing of IBV titres, antigen detection by a number of antibody-based capture assays,

virus isolation and by detection of IBV RNA. No technique, whether based on nucleic acid technology or antibodies, is absolutely adequate for confirmation of infection by a specific IBV serotype in the field (Cavanagh & Gelb, 2008).

1.2.5.4 Treatment

There are no specific treatment for IBV, appropriate antimicrobials can only aid in preventing secondary infections (Cavanagh & Gelb, 2008).

1.2.6 *Ornithobacterium rhinotracheale* virus

1.2.6.1 Etiology

Ornithobacterium rhinotracheale (ORT) belongs to the rRNA superfamily V within the Cythophaga-Flavobacterium-Bacteroides phylum and is closely related to 2 other poultry agents, *Riemerella anatipestifer* and *Coenonia anatina*. Historically, ORT was designated as *Pasteurella*-like or pleomorphic gram-negative rod before the name *Ornithobacterium rhinotracheale* was suggested. ORT is a nonmotile, gram-negative, highly pleomorphic, rod shaped and nonsporulating bacterium. ORT can grow in anaerobic, microaerobic or aerobic media, being 37ºC the optimal growth temperature (Chin, Empel & Hafez, 2008).

1.2.6.2 Epidemiology and Pathogenesis

ORT has been reported worldwide from a vast number of avian species such as ducks, geese, guinea fowl, ostriches, partridges, pheasants, pigeons and turkeys. In chicken all ages appear to be susceptible to these bacteria, however pathogenicity appears more significant in old birds (Chin *et al.*, 2008).

Assessments have concluded that, when experimentally inoculated, ORT causes small impact in poultry and that the severity of lesions is enhanced by the presence of concurrent infection with other respiratory viruses or bacteria. Examples of concomitant respiratory pathogens are: *Escherichia coli*, Newcastle disease, infectious bronchitis virus, *Mycoplasma synoviae* and avian metapneumovirus (Chin *et al.*, 2008).

1.2.6.2.1 Transmission and Incubation Period

ORT infects horizontally by direct and indirect contact via aerosols or water. It survives easily on lower temperatures, this may be associated with the higher incidence of infections during cold seasons (winter mainly); however, ORT does not survive 24 hours at 42ºC. Some studies suggest the possibility of a vertical transmission of ORT (Chin *et al.*, 2008).

An experimental study concluded that 2 days after inoculation, 5 weeks old chickens had infected respiratory organs and clinical signs where shown after 4 days (Chin *et al.*, 2008).

1.2.6.2.2 Clinical Signs

Ornithobacterium rhinotracheale infection is a contagious disease of fowl that causes mortality, reduced growth and respiratory distress. Initial symptoms are coughing, sneezing and nasal discharge followed by respiratory distress, dyspnea, prostration and sinusitis. Environmental factors comprising inadequate management, high densities, poor ventilation, poor hygiene and poor litter conditions make highly variable the severity of clinical signs, duration of disease and mortality. All these issues make ORT a cause of high economic losses in poultry industry (Chin *et al.*, 2008).

1.2.6.3 Diagnosis

A presumptive diagnosis is hard to make based on necropsy finding or clinical signs. A proper diagnosis must be supported by the isolation of ORT and/or detection of antibodies (Chin *et al.*, 2008).

1.2.6.4 Treatment

ORT infection is difficult to treat since different strains have different susceptibilities, although 68 ORT isolates from USA were found susceptible to ampicillin, penicillin, spectinomycin, tylosin and erythromycin; 54 of the 68 were susceptible to neomycin, tetracycline and sarafloxacin (Chin *et al.*, 2008).

1.2.7 Avian Metapneumovirus

Avian rhinotracheitis (ART), Turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) are terms that have been used to describe clinical diseases resulting from avian metapneumovirus (aMPV) infections, based on clinical signs and lesions. Nevertheless, these clinical signs and lesions are not specific for aMPV infections and may be confused with other diseases resulting from the infection with other pathogens such as infectious bronchitis virus (IBV), *Mycoplasma* sp., *Ornithobacterium rhinotracheale* (ORT) and other respiratory organisms. However, actually it is unanimously accepted that the conditions referred to as ART, TRT and SHS may occur as a result of aMPV infection (Gough & Jones, 2008).

This virus affection is recognized as unimportant economic and animal welfare issue, especially in turkey industry. In chickens this disease has a lower economic impact, although in sites where it is associated with SHS and egg production losses aMPV can have a huge economic impact (Gough & Jones, 2008).

1.2.7.1 Etiology

Avian metapneumoviruses make part of the subfamily *Pneumovirinae,* which belongs to the family *Paramyxoviridae.* These viruses have been further classified into four subtypes

(A, B, C and D) centred on deduced amino acid sequence data and nucleotide sequencing (Gough & Jones, 2008).

1.2.7.2 Epidemiology and Pathogenesis

Avian metapneumoviruses have been reported worldwide, except in Australasia. The presence of aMPV is usually estimated based on serological evidence. The number of countries that have reported this disease is relatively small due to difficulties in identifying or detecting aMPV (Gough & Jones, 2008).

Chickens and turkeys are believed to be natural hosts of aMPV. However, aMPV antibodies have been found in numerous other species such as guinea fowl and Ring-Necked Pheasants which shown swollen head syndrome. In Britain aMPV has been detected in Ring-Necked Pheasants with respiratory signs and serological assessments suggest that aMPV is widespread through gamebirds (Gough, Drury, Aldous & Laing, 2001).

1.2.7.2.1 Transmission

In countries where aMPV has appeared as a new disease, it has spread rapidly. Within 9 days of the first outbreak in UK, disease was reported from most of the turkey-producing areas. The ways of spread are still unclear and even on a single site, spread is unpredictable. Movement of affected or recovered animals, contaminated water, personnel, equipment, feed trucks etc. have been implicated in various outbreaks, whereas vertical transmission or airborne spread also have been put forward as possibilities. Actually, only direct contact spread has been proven. Wild fowl are suggested to have an active role in aMPV transmission (Gough & Jones, 2008).

1.2.7.2.2 Clinical Signs

The main clinical signs in young turkeys include swollen infraorbital sinus, rales, sneezing, snicking, conjunctivitis, nasal discharge and submandibular oedema. When disease is seen, morbidity in birds is commonly described as up to 100%, while mortality may range between 0.4% and 50%. In uncomplicated infections birds recover in 10 to 14 days. In chickens aMPV infection is less clearly defined and may not constantly be related with clinical signs. The aMPV has also been connected with swollen head syndrome (SHS) in chickens, associated with a secondary *E. coli* infection. In broilers, mortality rarely surpasses 2% (Gough & Jones, 2008).

1.2.7.3 Diagnosis

Nowadays the most accurate and feasible way of assessing the presence of aMPV is by using molecular techniques, particularly RT-PCR tests, which have been developed to detect the virus (Gough & Jones, 2008).

1.2.7.4 Prevention

In the poultry industry prevention is based in biosecurity, good management practices such as good ventilation, temperature control, low stocking densities, suitable litter quality and good hygiene. Multi-age stock and the presence of secondary pathogens may exacerbate aMPV infection. Both live attenuated and inactivated vaccines are available commercially for use in poultry (Gough & Jones, 2008).

2 Presence of *Cryptosporidium baileyi* in different Red Grouse moors and its relation with other avian disease agents

2.1 Study objectives

In order to meet the needs of some Scottish Red Grouse moors, which have demanded help regarding the onset of cryptosporidiosis outbreaks, "St David's Game Bird Services" (a division of "St David's Poultry Team") decided to support a research through Red Grouse moors, aiming a better knowledge of eye and respiratory cryptosporidiosis and its etiopathogenesis, targeting the following objectives:

- Search for *Cryptosporidium baileyi* infection in different grouse moors amongst northern England and Scotland;
- Evaluate the presence and burden of other parasites in the study population and its potential association with *C. baileyi* infection;
- Assessing the presence of other avian disease agents, namely bacterial and viral, and its possible relation with the presence of *C. baileyi*;
- Measuring weight, age and gender effect in collected birds from different groups, whether infected or not with *C. baileyi*.

This study does not have proper control groups as it was mainly supported by grouse moors and for this reason, birds with clinical signs were preferred as samples. When diseased or apparently healthy birds were present in small numbers or absent, these animals were also selected as samples.

2.2 Material and Methods
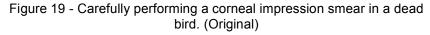
2.2.1 Study areas, sample collection and storage

This study was performed between the 1st of September and 17th of November 2014 on ten sporting estates in northern England and Southern Scotland. For the development of this study, St. David's Game Services provided a 4x4 truck and all the necessary equipment for sample collection (Figure 20). Visits to estates were previously scheduled, generally 24h (maximum) after sporting activities. After shooting drives, Red Grouse carcases were preserved in refrigerated chambers and kept between 5 and 8ºC, prior to sample collection. One hundred and ten birds were selected in 11 different estates, when clinical signs were evident those affected birds were preferred as samples. That preference was raised by estates (which financially supported the project) desire in

understanding the cause of such disease. After a quick briefing with gamekeepers, each bird was numbered, weighed, photographed and aged. Age was always attributed by experienced gamekeepers and when in doubt they adopted various methods for assessing age; not only feathers but also skull strength and the point of detachment of the old nail (Lovat, 1911).

Generally, young birds (born in that year) are distinguished from adults by their fresh, unmoulted plumage and an absence of toe nail scars (Cramp & Simmons, 1980). Birds under one year of age were defined as 'young', all the others were classified as 'old' (more than one year). After this initial procedure the sample collection took place.

A microscope slide was used to obtain impression smears from both corneas (side by side). The smear was obtained by pushing a clean slide firmly against the cornea, avoiding the contact with eyelids by spreading them carefully with two fingers (Figure 19). Afterwards, slides were numbered and stored without touching, in 5-capacity slide boxes. Those samples were delivered (within 12 hours) at Eden Valley Vet Lab (a specialized veterinary laboratory) in Penrith, Cumbria. There, specialists performed modified Ziehl Neelsen technique aiming the search of *C. baileyi* (Ortolani, 2000).

Figure 19 - Carefully performing a corneal impression smear in a dead bird. (Original)

Additionally, both cornea and conjunctivae were carefully swabbed using sterile swabs (Sarstedt[®]) with the purpose of testing the following avian disease agents: infectious bronchitis virus (IB), avian rhinotracheitis virus (ART), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS) and *Ornitobacterium rhinotracheale* (ORT), that may play a role in the disease ethiopathogenesis. Swabs were stored in the refrigerator and sent (within 24 hours) to Sci-Tech Laboratories (poultry specialized and accredited laboratories), for molecular diagnostic testing (PCR). Swabs were pooled (one pool for each separate grouse moor) for economical reasons.

Figure 20 - Dead Red Grouse and sample collection equipment (left), specimen container containing a head immersed in a 10% formalin solution. (Original)
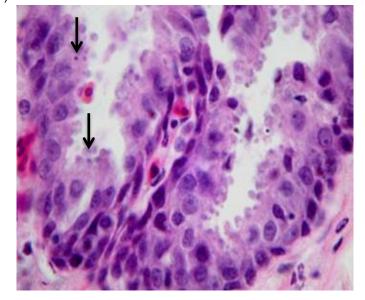
Thereafter, grouse heads were collected by severing them through the neck with scissors, and then stored in numbered 100ml specimen containers, containing 10% formalin solution. Samples were carefully stored and some were sent to "FINN Pathologists" (pathology specialized and accredited laboratory).

Only 6 out of 11 grouse moors made part of a private project, which aimed the research of *Cryptosporidium baileyi* and other avian disease agents that might be implicated the disease ethiopathogenesis that is causing "bulgy eye" in Red Grouse. A complete histopathology report was made for each bird belonging to those 6 grouse moors.

Part of grouse moors which were out of the Scottish project opted for performing corneal impression smears, as it is also an accurate, but cheaper method. 35 slides were lately delivered at Eden Valley Vet Lab (specialized veterinary laboratory). There, samples were stained using the modified Ziehl Neelsen technique (Ortolani, 2000), has mentioned before (Figure 21).

Figure 21 - *Cryptosporidium baileyi* (arrows) stained using the modified Ziehl Neelsen technique in the conjunctiva of a Red Grouse. (Courtesy of Helen Errington)

Finally, a basic *post mortem* examination was performed on each individual. However, it has shown to be inadequate to relate findings with pathological processes, due to the fact that animals have been shot. Nevertheless all findings have been recorded, the digestive tract was examined and the reproductive tract observed, in order to properly classify the gender (avoiding errors associated with dubious sexual dimorphism). Subsequently both caeca were collected and then stored in the fridge (5ºC) using numbered specimen containers, with the purpose of evaluating caecal parasitic burdens, namely nematodes and protozoa. Faecal worm egg and *Eimeria* spp. oocyst counts must be conducted as soon as possible after collection, within a period of three weeks, maximum (stored on the fridge with temperatures averaging 5ºC) (Seivwright *et al.*, 2004).

2.2.2 Laboratory analyses for detection of *T. tenuis* and *Eimeria* spp. performed by the student

2.2.2.1 *T. tenuis* egg counts and *Eimeria* spp. oocyst counts

T. tenuis egg counts and *Eimeria* spp. oocyst counts were assessed by using the modified McMaster egg counting technique (LaMann, 2010), within one week after collection.

As *T. tenuis* eggs are not equally distributed inside the caeca (Seivwright *et al.*, 2014), faecal contents were thoroughly extracted and mixed by squeezing it out carefully from distal to proximal end, directly to a clean Petri dish where it was homogenized. Then, 3g of this caecal content was weighed and put into a 100ml specimen container where 42ml of saturated salt solution were added. With the aid of 10 glass balls (Seivwright *et al.*, 2014), within the solution, faecal content and water were completely mixed by cautiously shaking the container (avoiding bubble formation that may harm oocyst/egg count); the resulting suspension was passed thoroughly over a 150µm sieve (Endecotts Ltd., Moreden, London) obtaining a homogenised suspension free of debris.

Disposable Pasteur pipettes were used to extract samples from the faecal suspension and to place them into both chambers of a McMaster slide.
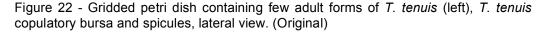
The McMaster slides were then left on the laboratory bench for 3-6 minutes, permitting parasite eggs and oocysts to float and attach to the top glass of the counting chamber. After carefully placing the McMaster slide on the compound microscope, eggs and oocysts inside each chamber grid were counted, using a 100x magnification.

Each egg counted represents 100 eggs per gram of faeces. This calculation is based on the fact that the depth of chamber is 1.5mm (10 mm squares) and consequently the volume of fluid examined is 0.15 ml, which is 1/300 of the original volume of 45 ml, made up of 42 ml of saturated salt solution and 3 g of faeces. Therefore each egg counted represents 300 per 3 grams, which is equivalent to 100 per gram. Two chambers were

employed so the total count was multiplied by 100 and divided by 2. Caecal egg counts provide reliable estimation of worm burdens (Seivwright *et al.*, 2004).

2.2.2.2 *T. tenuis* adult worm counts

For assessing *T. tenuis* intensities, not only worm egg counts were conducted (explained before) but also total adult worm counts. Total worm counts were performed in the second caecum from each bird. Caecum was carefully cut lengthways and all its content was thoroughly extracted into 2 litres of warm water; then 40ml of this properly mixed solution was extracted using a 20ml syringe (20ml were taken twice, 40ml in total), and passed over a 38µm sieve (Endecotts Ltd., Moreden, London) to collect worms. The contents of the sieve were flushed with 40ml of tap water (using one syringe) to a gridded large Petri dish, which was placed on a black surface aiding the count of nematodes (Figure 22). The total worms in the 40ml sample were counted and that number was multiplied by 100 to obtain the total number of worms per bird. The worms present in 40ml represent 2% of the worms found in one caecum if its content is diluted in 2l of water. *T. tenuis* burdens do not significantly differ between the two caeca (Wilson, 1979), that number multiplied by 100 will approximately characterize the totality of nematodes found in both caeca.

Figure 22 - Gridded petri dish containing few adult forms of *T. tenuis* (left), *T. tenuis* copulatory bursa and spicules, lateral view. (Original)

2.2.3 Statistical analyses

The results were recorded in a Microsoft Excel 2010[®] file and statistical analyses were performed using R software (version 3.1.1) with the extension R Commander (R Foundation for Statistical Computing, 2014). In order to test if our data were normally distributed, Shapiro-Wilk test was performed. When data do not exhibit a normal distribution, data was transformed into logarithmic functions. Some of the measures (weight, worm egg counts and worm intensity) were not normally distributed. Therefore non-parametric statistical hypothesis test was used; two-sample Wilcoxon test was used to evaluate means differences between groups. The relation between worm egg counts and worm intensity, worm egg counts and oocyst counts, worm egg counts and weight and oocyst counts and weight were tested using linear regression analysis. Contingency tables were used for displaying and comparing the frequency distribution of some variables (age, gender, presence of clinical signs and presence of *C. baileyi*), Fisher's exact test was the statistical significance test used to evaluate the association (contingency) between categorical and binomial variables. The 5% significance level was used, meaning p<0,05.

3 Results

3.1 General Distributions

3.1.1 Age and Gender

Regarding age, approximately 44% (48 out of 110) of collected birds were classified as adults (> 1 year), while 56% (62 out of 110) as young (< 1 year); nearly 48% (53 out of 110) of Red Grouse were females and 52% (57 out of 110) were males. 22% (24 out of 110) were adult females, 22% (24 out of 110) were adult males whereas 23% (29 out of 110) were young females and 30% (33 out of 110) were young males (Table 1).

3.1.2 Weight

The mean weight of the totality of collected Red Grouse was 623.66g (σ=84.61; SE=8.07). The weight mean of adult females was 614.75g (σ=47.95; SE=9.79) and 684.71g (σ=77.03; SE=15.72) for adult males. Whilst the mean of young females were 550.17g (σ=67.93; SE=12.61) and 647.00g (σ=78.27; SE=13.62) for young males (Table 1).

Table 1 – Distribution of samples and weights among age and gender groups. N - number of birds, [CI95%] - 95% confidence interval, σ – standard deviation and SE – standard error

	Age			
	Adult (> 1 year)		Young (< 1 year)	
	Gender		Gender	
	Females	Males	Females	Males
N (% of total)	24 (21.82%)	24 (21.82%)	29 (26.36%)	33 (30.00%)
[CI195%]	[14.10%-29.54%]	[14.10%-29.54%]	[18.13%-36.60%]	[21.44%-38.56%]
Weight Mean (g)	614.75	684.71	550.17	647
Weight Min (g)	479	533	346	325
Weight Max (g)	702	839	632	755
σ	47.95	77.03	67.93	78.27
Variance	2299.15	5933.43	4614	6125.63
SE	9.79	15.72	12.61	13.62

3.2 *Cryptosporidium baileyi* results

3.2.1 *Post-mortem* findings

35 out of 110 (32%) of collected birds presented lesions, namely what is called "bulgy eye" among guns and gamekeepers. The percentage of affected birds distributed into age and gender groups were: 2.73% (3 out of 110) for adult females, 4.55% (5 out of 110) for adult males, 12.73% (14 out of 110) for young females and 11.82% (13 out of 110) for young males (Table 2).

Table 2 – Contingency table concerning the presence of lesions among age and gender groups. N - number of birds, [CI95%] - 95% confidence interval.

		Age			
		Adult (> 1 year)		Young (< 1 year)	
		Gender		Gender	
Evident Clinical Signs		Females	Males	Females	Males
No	N (% of total)	21 (19.09%)	19 (17.27%)	15 (13.64%)	20 (18.18%)
	[CI95%]	[11.75%-26.44%]	[10.21%-24.34%]	[7.22%-20.05%]	[10.97%-25.39%]
Yes	N (% of total)	3 (2.73%)	5 (4.55%)	14 (12.73%)	13 (11.82%)
	[CI95%]	[0%-5.77%]	[0.65%-18.96%]	[6.50%-18.96%]	[5.79%-17.85%]

3.2.2 Laboratory Results

3.2.2.1 Histopathology

In total, 56 samples were sent to FINN Pathologists targeting the search of *C. baileyi* and related lesions*.* 55 out of 56 samples belonged to the Scottish project (grouse moors which requested the extent study about the presence of *C. baileyi*) none of those presented evident clinical signs. The isolate sample (1 out of 56) belongs to a confirmation of a negative result from the corneal impression smear. That bird had evident clinical signs and was a sample from a grouse moor which was out of the private project. Only three out of 56 results (5.36%) came with a positive result in their report, one presented evident clinical signs and was the confirmation case previously mentioned and the other two belonged to birds without manifested clinical signs.

3.2.2.2 Corneal Impression Smears (modified Ziehl Neelsen technique)

30 out of those 35 slides belonged to birds with evident clinical signs, while 5 were corneal impression smears taken from birds where marked clinical signs were absent.

57.14% (20 out of 35) of samples had a positive result, all birds with marked clinical signs. In 42.86% of samples (15 out of 35), *C. baileyi* were not seen, 10 from birds with clinical signs and 5 from birds without.

3.2.2.3 Combined Results (Histopathology and Corneal Impression Smear)

From 90 tested samples (histopathology and corneal impression smear), 25.56% (23 out of 90) obtained a positive result and 74.44% (67 out of 90) a negative result.

18.42% (7 out of 38) of adult birds and 30.77% (16 out of 52) of young birds showed positive results for *C. baileyi* in histopathology or corneal impression smear results. Difference between ages was not statistically significant ($p > 0.05$; OR=0.51) (Table 3).

17.50% (7 out of 40) of females and 32.00% (16 out of 50) of males were positive for *C. baileyi* in histopathology or in corneal impression smears. The presence of *C. baileyi* between genders is not statistically significant ($p > 0.05$; OR=0.45).

When comparing the presence of positive results with the presence of clinical signs we find that 70.00% (21 out of 30) of birds with clinical signs had positive results, compared with 3.33% (2 out of 60) that had positive results without clinical signs. We found these differences statistically significant ($p < 0.001$; OR= 0.02).

Positive results were mainly in northern England (appendices).
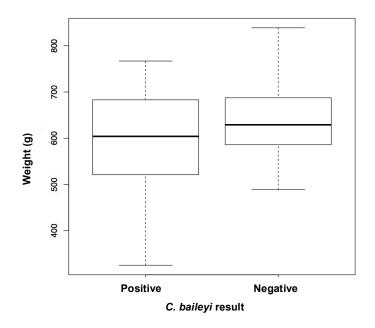
Table 3 – Distribution (number and % of total) of *C. baileyi* among age groups.

Age	*C. baileyi* positive	*C. baileyi* negative
Adult	7 (18.4%)	31 (81.6%)
Young	16 (30.8%)	36 (69.2%)

3.2.2.4 **Relation with weight**

The median of weight of the positive birds for *C. baileyi* was 604.00g whereas in negative birds the median was 629g. No statistical significance was found between weight and the presence of *C. baileyi* (W=601; p>0.05) (Figure 23).

Figure 23 - Box plot of data regarding weight among groups were *C. baileyi* was found compared to those where it was absent.

3.3 *Eimeria* spp.

The mean of oocysts per gram of mixed caecal feces was 9686.11 (σ=20776.48). Adult birds shown a mean of 7819.15 oocysts/g (σ=17418.56) while in young birds the mean was 11124.59 oocysts/g (σ=23072.76). Females shown a higher mean compared with males, respectively 10532.69 oocysts/g (σ=23019.14) and 8900.00 oocysts/g (σ=18631.39). All samples (caecal feces) presented similar texture.

Figure 24 - *Eimeiria* spp. oocysts, 100x magnification. (Original)
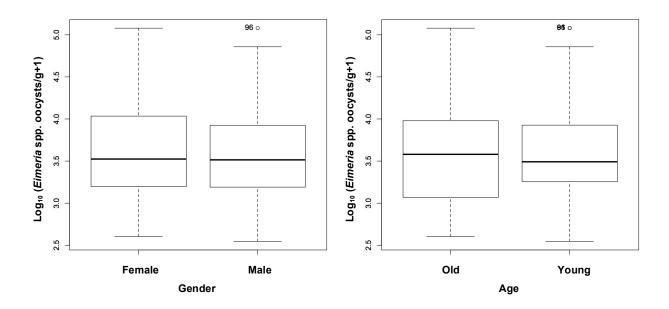
3.3.1 Relation with gender and age

The median of Log_{10} of *Eimeria* oocysts per gram was 3.52 for females and 3.51 for males. Differences between age and oocyst intensity were not statistically significant (W=1531; p>0.05) (Figure 25).

The median of Log_{10} of *Eimeria* spp. oocysts per gram was 3.58 in adult birds and 3.49 in young ones. No statistically significant differences were also found (W=1358.5; p>0.05) (Figure 25).

Figure 25 - Box plots of data referring the function Log_{10} (*Eimeria* spp. oocysts/g+1) amongst gender groups (left) and age groups (right).
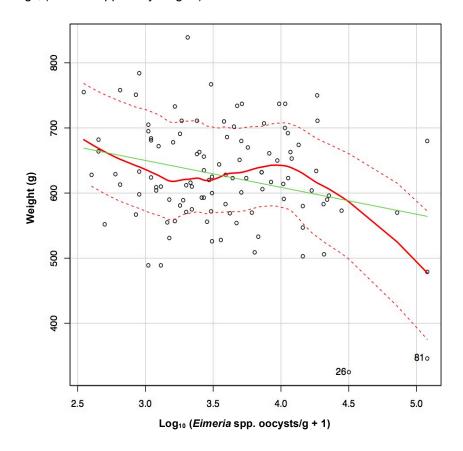
3.3.2 Relation with weight

A linear regression was created to test the relation between weight and the logarithmic function of *Eimeria* spp. oocysts per gram (Table 4). That allowed to understand how coccidial burdens influences the weight of birds and *vice versa*. Figure 26 illustrates a scatterplot where the relation between two variables can easily be perceived. A statistically significant relation was found between weight and *Eimeria* intensity (p<0.01).

Table 4 – Linear regression model of weight in function of Log_{10} (*Eimeria* spp. oocysts/g+1).

Coefficients	Estimate	SE	*t* value	*p* value
(Intercept)	774.06	50.60	15.30	< 2e-16 ***
Log_{10} (*Eimeria* spp. oocysts/g+1)	-41.31	13.95	-2.96	0.00377 **
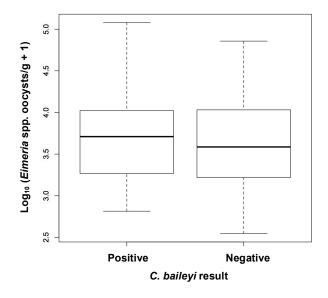
Figure 26 - Scatter plot and linear regression with values of weight and Log$_{10}$ (*Eimeria* spp. oocysts/g+1).

3.3.3 Relation with the presence of *Cryptosporidium baileyi*

A comparison between the logarithmic function of *Eimeria* spp. oocysts per gram and the presence of a positive result for *C. baileyi* was also made. The median of Log$_{10}$ of *Eimeria* spp. oocysts per gram for positive birds was 3.71 and 3.59 for negative birds. That difference does not support a statistically significant result (W=790.50; p>0.05) (Figure 27).

Figure 27 - Box plot of data regarding the Log$_{10}$ (*Eimeria* spp. oocysts/g+1) amongst groups were *C. baileyi* was found compared to those where it was absent.

3.4 Trichostrongylus tenuis

As for *Eimeria* spp. 108 out of 110 samples were analysed. Caecal feces shown similar texture throughout all samples.

Regarding worm egg counts (Figure 28) the mean of eggs per gram of mixed caecal feces was 785.65 (σ=2825.91). Adult birds presented a mean of 1481.91 eggs/g (σ=4121.4896) while in young birds the mean was 249.18 eggs/g (σ=738.78). Females displayed a higher mean relatively to males, correspondingly 919.23 eggs/g (σ=2659.01) and 661.61 eggs/g (σ=2991.20).

Concerning total adult worm counts the mean of worms per bird was 940.74 (σ=2776.88). Equally to worm egg counts, old birds presented a higher mean for adult worm burden than young ones, respectively 1748.94 worms/bird (σ=4024.59) and 318.03 worms/bird (σ=653.84).

Figure 28 - *T. tenuis* eggs, 100x magnification. (Original)
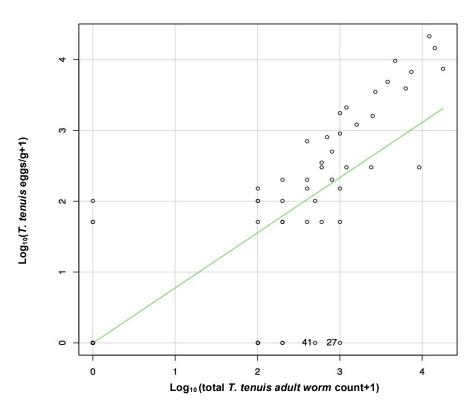
3.4.1 Relation between adult worm burdens and worm egg counts

The values regarding the linear regression between *T. tenuis* adult worm intensity and eggs per gram of feces are shown on table 5. Figure 29 illustrates a scatter plot where the linear regression can easily be perceived, notice the close connexion between both variables. A statistically significant relation ($p<0.001$) among variables was found.

Table 5 - Linear regression model of Log_{10} (total *T. tenuis* adult worms/g+1) in function of Log_{10} (*T. tenuis* eggs/g+1).

Coefficients	Estimate	SE	*t* value	*P* value
(Intercept)	-0.003158	0.093535	-0.034	0.973
Log_{10} (Total *T. tenuis* adult worms+1)	0.778875	0.048309	16.123	<2e-16 ***
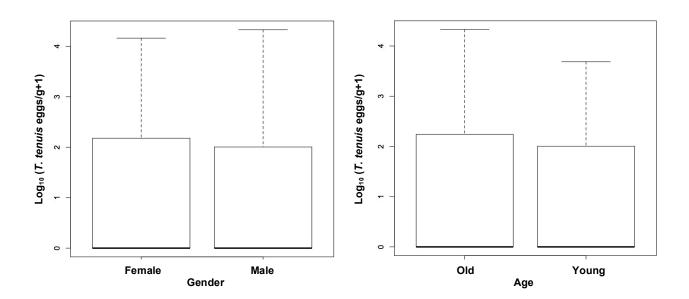
Figure 29 - Scatter plot with values of Log_{10} (total *T. tenuis* adult counts/g+1) and Log_{10} (*T. tenuis* eggs/g+1).

3.4.2 Relation with age and gender

The median of both (total *T. tenuis* adult worms per bird and *T. tenuis* egg counts) logarithmic functions for age and gender groups was 0 for all classes (adult, young, females and males). Therefore, differences amongst groups were not statistical significant for age (W=1497; p>0.05) nor for gender (W=1584; p>0.05) (Figure 30).

Figure 30 - Box plots of data referring the function Log_{10} (*T. tenuis* eggs/g+1) amongst gender groups (left) and age groups (right).

3.4.3 Relation with Weight

Linear regression comprising Log_{10} of *T. tenuis* egg counts and weight (g) of birds (table 5 and 6). Figure 31 shows a scatter plot where the linear regression for the two variables can simply be observed. A statistically significant relation was not found between weight and the logarithmic function of *T. tenuis* egg counts (p>0.05).

Table 6 - Linear regression model of weight in function of Log_{10} (*T. tenuis* eggs/g+1).

Coefficients	Estimate	SE	*t* value	*P* value
(Intercept)	2.521673	1.012996	2.489	0.0144*
Weight (g)	-0.002453	0.001605	-1.528	0.1295
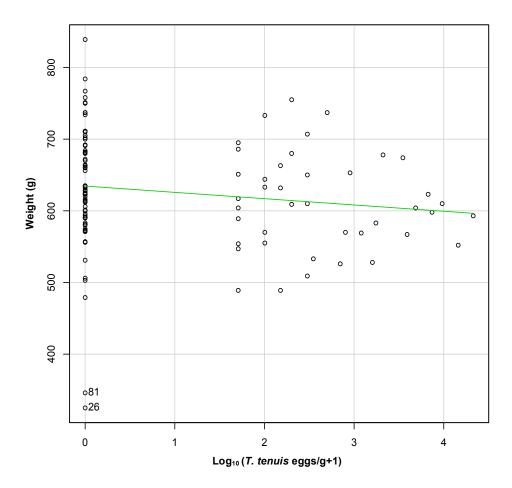
Figure 31 - Scatter plot with values of Log_{10} (*T. tenuis* eggs/g+1) and weight (g).
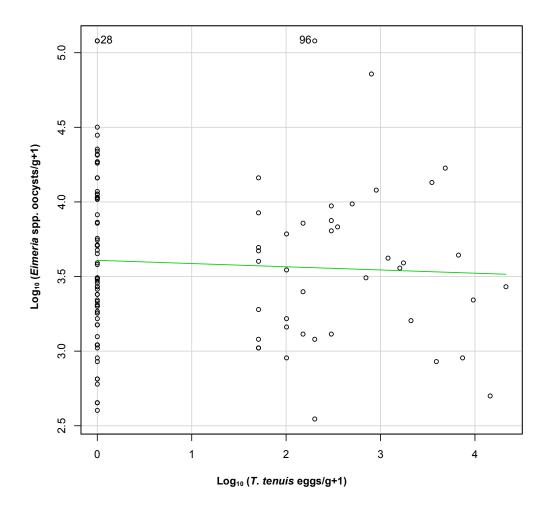
3.4.4 Relation with *Eimeria* spp.

Linear regression between the Log_{10} of *Eimeria* spp. oocyst counts per gram of feces and the Log_{10} of *T. tenuis* adult worm counts per bird (table 6) shows that there was no statistically significant relation between variables (p>0.05). Figure 32 allows to recognize that there was a slightly tendency to have lower *Eimeria* spp. burdens as *T. tenuis* adult worms per bird increases.

Table 7 - Linear regression model of Log_{10} (*T. tenuis* eggs/g+1) in function of Log_{10} (*Eimeria* spp. oocysts/g+1)

Coefficients	Estimate	SE	*t* value	*P* value
(Intercept)	3.60868	0.06496	55.553	<2e-16 ***
Log_{10} (*T. tenuis* eggs/g+1)	-0.02159	0.03885	-0.556	0.58

Figure 32 - Scatter plot with values of Log_{10} (*T. tenuis* eggs/g+1) and Log_{10} (*Eimeria* spp. oocysts/g+1).

3.4.5 Relation with the presence of *Cryptosporidium baileyi*

Medians of logarithmic functions of *T. tenuis* eggs per gram of feces belonging to clusters with and without a positive result for *C.baileyi* were 0, a non statistically significant result was obtained (W=734.5; p>0.05), thus we cannot support the hypothesis of having a possible relation between *T. tenuis* burdens and the presence of *C.baileyi* (Figure 33).
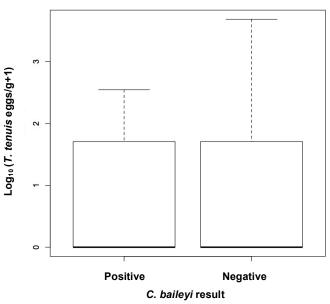
Figure 33 - Box plots of data regarding the function Log_{10} (*T. tenuis* eggs/g+1) amongst *C. baileyi* positive and negative birds.

3.5 *Mycoplasma synoviae*, *Mycoplasma gallisepticum*, *Ornithobacterium rhinotracheale*, Infectious Bronchitis Virus and Avian Rhinotracheitis Virus results

Only three PCR pools were positive for Infectious Bronchitis Virus, two belonged to the same grouse moor where evident "bulgy eye" lesions were present. The last positive pool belonged to a grouse moor where lesions were not present. *Ornithobacterium rhinotracheale* have also been identified in one pool of birds previously to this study. Unfortunately fewer data were collected from those birds, causing the omission of them from our study population.

In the present study, *Mycoplasma synoviae*, *Mycoplasma gallisepticum* as well as Avian Rhinotracheitis Virus have not been identified in pooled swabs from Red Grouse, tested by PCR.

4   Discussion

It is essential to note that our study population was not randomly selected, not having appropriate control groups, thus, data should be carefully analysed with critical sense. Additionally, there are various parameters that are being compared at the same time, fact that my cause biases to the study.

4.1   General Results (Age, Gender and Weight)

After analysing our data regarding the weight of collected Red Grouse we noticed that its means for each group of birds, namely adult females (614.75g; σ=47.95), adult males (684.71g; σ=77.03), young females (550.17g; σ=67.93) and young males (647g; σ=78.27), are concordant with reported average weights (Watson & Moss, 2008). Unfortunately these data are not normally distributed, fact that might be associated with some outliers (very thin or heavy birds were collected occasionally).

Occurrences for age and gender were very similar: adult females (21.54%), adult males (23.99%), young females (23.29%) and young males (31.17%) not existing discrepancies with those parameters.

4.2   *Cryptosporidium baileyi* ('bulgy eye')

4.2.1   *Post-mortem* findings and Laboratory Results

Birds collected in 11 different grouse moors, located in northern England and Scotland, compose the study population used in this study. Estates information (name and location) is confidential. A Scottish private project, with the aim of better understanding the disease complex that might be associated with cryptosporidiosis in Red Grouse was the great propelling for the present study. Despite head histopathology, they supported the search of various pathogens that might be associated with *C. baileyi.* Regardless of having less lesions, which are mostly absent, that group does not regret the investment in the study of this disease.

As St David's Game Bird Services was moving funds to support that initiative, it was decided to support other grouse moors that would be interested in investigating the causative agents for "bulgy eye" in their estates. With that, 4 more grouse moors, which were highly interested in understanding that concerning situation, were added to this study. Those decided to direct the collection of samples to birds presenting apparent lesions, in order to confirm the presence of *C. baileyi* and try to relate cryptosporidiosis

with other causative agents that could be associated. Cheaper diagnosis methods (but also accurate) were used by them.

35 out of 110 collected birds (32%), presented evident clinical signs, namely what is called "bulgy eye" between hunters and gamekeepers. The percentage of affected birds distributed into age and gender groups were: 2.73% (3 out of 110) for adult females, 4.55% (5 out of 110) for adult males, 12.73% (14 out of 110) for young females and 11.82% (13 out of 110) for young males. Differences between groups was not statistically significant ($p > 0.05$). However, biases could be present at the time of collection, by the fact that when "diseased" birds were numerically superior to the predicted sample size (for each estate), same number of adult and young birds was selected. Baines *et al.* (2014) also obtained a higher prevalence in young birds than in adults, respectively 4.1% (80 out of 1942) and 3.2% (32 out of 987).

Cryptosporidium baileyi has shown to cause intense impacts in Red Grouse population, as only three years after the first diagnosis, almost half of grouse moors had birds exhibiting clinical signs (Baines *et al,* 2014).

The weight median for *C. baileyi* negative birds was 629g, in positive birds the median was 604g. The difference has not shown to be statistically significant ($p > 0.05$). In Baines (2014) studies, is mentioned that infected males and females Red Grouse were on average 5% and 7% lighter correspondingly than healthy birds. In the present study, the diseased animals were 4% (3.97%) lighter than the healthy ones, but differences were not statistically significant ($p > 0.05$).

Positive results were mainly in northern England (appendices), where first disease reports were made.

4.3 *Eimeria* spp.

Interestingly the species of *Eimeria* spp. present in Red Grouse was one of the first species reported in avian species. Eimeriosis is a concerning parasitic disease throughout the world, although there are little research about its impact neither on Red Grouse's life cycle nor on its cyclic dynamics. Frequently it is considered as a disease of intensification and the current data reflects that it should correspond to several years of high Red Grouse numbers on the moors.

Death in Red Grouse due to *Eimeria* spp. parasitism has been seen at practice since a century ago (Lovat, 1911), but sometimes neglected. Researches regarding coccidiosis in Red Grouse are lacking, sub-lethal consequences are unknown and reveal to be harder to quantify.

With the use of medicated grit, birds are attracted to the areas where boxes are placed, originating contamination around them, which might increase the incidence of disease.

Infection of young birds can be associated with poor development due to damage to the gut lining, which is likely but less obvious than the effect *Eimeria* spp. will have at lower levels on a bird's performance.

The general mean of oocysts per gram of mixed caecal feces was 9686.11 (σ=20776.48). Adult birds shown a mean of 7819.15 oocysts/g (σ=17418.56) while in young birds the mean was 11124.59 oocysts/g (σ=23072.76). Females shown a higher mean compared with males, respectively 10532.69 oocysts/g (σ=23019.14) and 8900.00 oocysts/g (σ=18631.39).

Differences between age and gender groups and the Log_{10} of *Eimeria* spp. oocysts per gram were not statistically significant ($p>0.05$).

Contrary to what was expected, adult birds presented a slightly higher mean of oocysts per gram of faeces than young ones. According to poultry literature young birds use to be more susceptible, with less immunity against *Eimeria* spp., consequently having higher burdens and suffering more consequences from the disease than adult birds (Yabsley, 2008). Curiously, Red Legged Partridges did not seem to build up a competent mucosa immune response even after severe infection. That fact may explain recurrent outbreaks of coccidiosis in Red Legged Partridges reared in captivity or appearing after a stress such as going out to grass (Naciri, Fort, Briant, Duperray & Benzoni, 2014).

The association between *Eimeria* spp. intensity and its effect on the health of Red Grouse is not well studied, being inaccurate to assume a level of intensity that could be harmful for Red Grouse. However we obtained 100% prevalence in our study population (108 out of 108), averaging 9686 oocysts/g, and we believe that this level of burden should not be disregarded.

A linear regression model between weight and Log_{10} of *Eimeria* spp. oocysts per gram revealed a statistical significant relation ($p<0.05$). In short, as Log_{10} of oocysts/g increases, weight decreases. The relation between oocysts/g and weight found in the present study is a strong argument for considering Red Grouse coccidiosis an important factor in its body condition, thereafter in its health and predisposition to disease. Historically, little significance has been given to coccidiosis in Red Grouse, being only associated with chick mortality; our results suggest that more should be known about coccidiosis in Red Grouse. Studies and assessments regarding *Eimeria* spp. impacts in Red Grouse populations are lacking and should be developed in the future.

4.4 *Trichostrongylus tenuis*

Trichostrongylus tenuis, sometimes known as caecal threadworm, is recognized for having a intense effect on the population dynamics of Red Grouse. Losses from the population were associated with intensity of parasite infection and experimental evidence demonstrates that these associations were one of cause and effect (Hudson *et al.*, 1992a). The use of medicated grit using anthelmintic molecules is widely spread through estates, all study sites declared that make use of medicated grit to control worm burdens through birds. Grit boxes were switch to plain grit before the beginning of game season (August the 12th) respecting withdrawal period.

Egg counts are shown to be reliable for assessing worm burdens for three weeks using McMaster's technique if refrigerated samples are used (Seiwright *et al.*, 2004).

Adult *T. tenuis* worms are found only in the caeca of Red Grouse, which is thought to play an important role in the absorption of water and proteins and the digestion of cellulose (Moss & Parkinson, 1972).

Adult birds presented a mean of 1481.91 eggs/g (σ=4121.4896) while in young birds the mean was 249.18 eggs/g (σ=738.78). Females displayed a higher mean relatively to males, correspondingly 919.23 eggs/g (σ=2659.01) and 661.61 eggs/g (σ=2991.20).

Regarding total adult worm counts the mean of worms per bird was 940.74 (σ=2776.88). Equally to worm egg counts, old birds presented a higher mean for adult worm burden than young ones, respectively 1748.94 worms/bird (σ=4024.59) and 318.03 worms/bird (σ=653.84).

No statistically significant differences ($p > 0.05$) were found between age or gender groups regarding *T. tenuis* intensities. Previous studies mentioned that the number of worms present in the caeca of Red Grouse increases throughout the life of the bird (Wilson, 1983), as evidences suggest that there is little or no effective acquired immunity in Red Grouse to infections of *T. tenuis* (Shaw & Moss, 1989b).

In the present study was obtained a statistically significant relation ($p < 0.05$) between Log_{10} of worm egg counts and Log_{10} of total worm counts. Previous studies have shown that the reliability of using worm egg counts to indirectly estimate the worm intensity within a living host may be influenced by both seasonal variation in worm egg production (Shaw & Moss, 1989a; Moss, Watson, Trenholm & Parr, 1993) and by density-dependent constrains in worm fecundity (Tompkins & Hudson, 1999). However, Seivwright *et al.* (2004) found that the strong relationship between faecal worm egg count and host worm intensity derived from samples collected in the autumn is similar to that derived from samples collected during spring. They also found no evidence for a decline in egg count reliability with increasing worm intensity.

Additionally we could not support the fact of having a connection between *T. tenuis* intensity and the presence of *C. baileyi,* since mean differences between groups were not

statistically significant (p>0.05). That absence of a statistically significant result could be caused by the small sample size, so future studies would be of much value for better understanding the interactions between these agents.

4.5 *Mycoplasma synoviae, Mycoplasma gallisepticum, Ornithobacterium rhinotracheale*, Infectious Bronchitis Virus and Avian Rhinotracheitis Virus results

In the present study, MS, MG as well as ART virus have not been identified in pooled swabs from Red Grouse, tested by PCR. Nevertheless these agents can eventually play a role predisposing disease as they have previously been implicated in other wild bird's diseases, especially *Mycoplasma*, which has been identified previously in 3 samples out of 13, from Red Grouse presenting "bulgy eye" lesions (Baines, Newborn & Richardson, 2014).

Only three PCR pools came positive for IBV, two belonged to the same grouse moor where evident "bulgy eye" clinical signs were present. The last positive pool belonged to a grouse moor where clinical signs were not present. Positive PCR pools for IBV made us rethink the etiology of "bulgy eye" in Red Grouse, as some other pathogens can be predisposing the infection with *C. baileyi,* or *vice versa*. Nowadays it is not considered that the chicken is the single host for IBV; however, it is probable that it is only the chicken that IBV may cause disease (Cavanagh & Gelb, 2008). Whether or not, the question still remains, future studies regarding the prevalence and effect of IBV are suggested, since Red Grouse can only be a reservoir.

ORT has also been identified in one pool of birds previously to this study. Unfortunately fewer data were collected from those birds, causing the exclusion of them from our study population. Equally for ORT, future research aiming the assessment of presence and effects of ORT is needed to better understand its role in Red Grouse populations.

5   Conclusion

Grouse cycles are broadly acknowledged to be caused by density-dependent regulatory effects, acting with time delays. Along the years there has been great debate about whether these effects derive from the Red Grouse population interactions with parasite infections, vertebrate predators, food supplies, territory size or other factors.

Cryptosporidium baileyi has ultimately shown to cause great impacts in Red Grouse populations, as only three years after the first diagnosis, almost half of grouse moors had birds with lesions. *Cryptosporidium* is widely known as a pathogen associated with intensification and can possibly be associated with high densities reached in the last few years due to committed grouse moor management.

Over all acceptance of this study by the grouse moor community as very good, revealing interest in the better comprehension of respiratory cryptosporidiosis in Red Grouse.

Despite having a greater positivity percentage for juveniles, no statistically difference was found. The same situation occurred with weight, aside having a greater median for healthy birds (629g) than for those that were positive to *C. baileyi* (604g), the difference has not shown to be statistically significant. Positive results were mainly in northern England, where the majority of reports have been made in the last few years.

Statistically significant differences were found between groups with and without clinical signs and the presence of *C. baileyi.* 70% (21 out of 30) of birds with evident "bulgy eye" clinical signs were positive for *C. baileyi.* This showed that lesions are highly associated with disease, being virtually ensure to accept that *C. baileyi* is present when "bulgy eye" clinical signs are present in Red Grouse populations.

As previously referred, different methodologies were used to identify *C. baileyi* in samples (histopathology and corneal impression smear) and despite being preferable to use histopathology for assessing the presence of *Cryptosporidium*, corneal impression smear, using modified Ziehl Neelsen staining technique, showed to be an affordable and still accurate way for testing the presence of *C. baileyi*.

Additionally, our analysis regarding *Eimeria* spp. and *Trichostrongylus tenuis* were not correlated significantly with the presence of *C. baileyi*, however both parasite intensity medians were higher for positive birds. At the same time, there was not a statistically significant relation between *T. tenuis* burdens and weight, however a statistically significant relation was found between *Eimeria* spp*.* burdens and weight.

The study failed to demonstrate the relation between the presence of *C. baileyi* and other avian pathogens (IBV, ORT virus, ART virus, MG and MS). Only three PCR pools were positive for IBV, being impossible to make any kind of association. Nonetheless we think that those pathogens should not be set aside in future studies since they can eventually

play a role in this disease complex, furthermore they have previously been implicated in other wild bird's diseases with similar clinical signs.

6 Recommendations and future perspectives

Concerning *Cryptosporidium*, prevention and control measures are harder to establish due to wide habitats and wild nature of this species. A biosecurity scheme is also difficult to implement, knowing that guns (hunters), beaters, dogs and gamekeepers are a community that circulates throughout estates during the game season, without restrictions or precautions.

To avoid the spread of cryptosporidiosis, grouse moors should develop a biosecurity scheme, monitoring is highly recommended for all grouse moors, as well as registering all data about clinical signs. Each estate should have its own beaters and dogs, hunters must also be aware that their equipment might be fomite for this disease. Wellington boots should be washed and disinfected using commercial bleach in 50% concentration (5,25% sodium hypochlorite) as it is described to be effective.

Since affected birds may not be reaching the shooting line due to insufficient capability for flying, increasing shooting pressure may not be an efficient way for controlling disease. The use of dogs along beaters line could be a thinkable approach for capturing diseased birds which refrain to fly or to make them flush and reach the shooting line.

High densities are reached around grit boxes, making those places a common communal area for large number of birds, thus allowing easy spread of *C. baileyi* through birds. We strongly recommend an increase in the number of grit boxes (in order to reduce birds per box), an annual position change for each box and a thoroughly disinfection between seasons targeting the reduction of *C. baileyi* spread.

Moreover we think that the effect of *Eimeria* spp. burdens on Red Grouse health is not well studied, since literature is lacking. It is inaccurate to assume a level of intensity that could be harmful for Red Grouse, nevertheless we found 100% prevalence in our study population, averaging 9686 oocysts/g and we believe that this level of parasite intensity should also not be disregarded and further studies should be performed.

From all of the above, the pursuing of this applied research in Red Grouse diseases is of the utmost importance for all parts involved. We must bear in mind that Red Grouse is not only the iconic symbol of the natural environments in Northern England and Scotland, but also an important source of financial input for those areas and therefore the better knowledge on its pathologies will improve both animal health/welfare and the local economy.

7   Literature Cited

Baines, D., Howarth, D. & Newborn, D. (2013) Uplands monitoring in 2012. In review of 2012 by Game and Wildlife Conservation Trust (pp. 38-41). UK: GWCT

Baines, D., Newborn, D. & Richardson, M. (2014) A spread of *Cryptosporidium baileyi* in wild red grouse Lagopus lagopus scoticus in northern England. *Veterinary Record* 175: 149

Cavanagh, D., & Gelb, J. (2008) Infectious Bronchitis. In Saif, Y.M., *Diseases of poultry*. (pp. 117-135). UK: Blackwell Publishing Ltd.

Chapman, H.D. (2014) Milestones in avian coccidiosis research: A review. Fayetteville: Department of Poultry Science – University of Arkansas

Chin, R.P., Empel, P.C.M. & Hafez, H.M. (2008) *Ornithobacterium rhinotracheale infection*. In Saif, Y.M., Diseases of poultry. (pp. 765-774). UK: Blackwell Publishing Ltd.

Christensen, N.H., Yavari, C.A., McBain, A.J., & Bradbury, J.M. (1994) Investigations into the survival of *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Mycoplasma iowae* on materials found in the poultry house environment. Avian Pathology 23:127–143.

Coldwell, L., Caldow, G., Holliman, A., Mearns, R., Errington, H., Giles, M., Willoughby, K. & Wood, A. (2012) *Cryptosporidium bailey* in wild red grouse with 'bulgy eye'. *Veterinary Record* 170: 603-604

Cramp, S. & Simmons, K.E.L. (1980). The Birds of the Western Palearctic, Vol. 2. Oxford University Press, Oxford.

Delahay, R.J., Speakman, J.R. and Moss, R. (1995) The energetic consequences of parasitism - Effects of a developing infection of *Trichostrongylus tenuis* (nematode) on red grouse (*Lagopus lagopus scoticus*) energy-balance, body-weight, and condition. *Parasitology* 110: 473-482

Delahay, R. & Moss, R. (1996) Food intake, weight changes and egg production in captive red grouse before and during laying: Effects of the parasitic nematode *Trichostrongylus tenuis. Condor* 98(3): 501-511.

Dobson, A.P. & Hudson, P.J. (1995) The interaction between the parasites and predators of red grouse *Lagopus lagopus scoticus*. *Ibis* 137: 87-96.

Doran, D.J. (1978) The life cycle of *Eimeria dispersa* Tyzzer 1929 from the turkey in gallinaceous birds. *Journal of Parasitology* 64:882-885.

Fantham, H.B. (1910). The morphology and life-history of *Eimeria* (coccidium) *avium*: A sporozoon causing a fatal disease among young grouse. *Prod. Zool. Soc. Lond*. 3:672-691

Fayer, R. & Xiao, L. (2008) Cryptosporidium and cryptosporidiosis. (2nd ed.). USA: CRC Press.

Freehling, M. & Moore, J. (1993) Host-specificity of Trichostrongylus tenuis from red grouse and northern bobwhites in experimental infections of northern bobwhites. Journal of Parasitology 79(4):538-541.

Freeland, J.R., Anderson, S., Allen, D. & Looney, D. (2006) Museum samples provide novel insights into the taxonomyand genetic diversity of Irish red grouse. *Conservation Genetics* 8: 695–703.

Gough, R.E. & Jones, R.C. (2008) Avian Metapneumovirus. In Saif, Y.M., *Diseases of poultry*. (pp. 100-110). UK: Blackwell Publishing Ltd.

Gough, R.E., Drury, S.E., Aldous, E. & Laing, P.W. (2001) Isolation and identification of an avian pneumovirus from pheasants. *Veterinary Record* 149.

Hoffman, R.W., Luttrell, M.P., Davidson, W.R. & Ley, D.H. (1997) Mycoplasmas in wild turkeys living in association with domestic fowl. *Journal of Wildlife Diseases* 33:526–535.

Hudson, P.J., Dobson, A.P. & Newborn, D. (1992b) Do parasites make prey vulnerable to predation? Red grouse and parasites. *Journal of Animal Ecology* 61: 681-692.

Hudson, P.J., Dobson, A.P. & Newborn, D. (1998) Prevention of population cycles by parasite removal. *Science* 282(5397): 2256-2258.

Hudson, P.J., Newborn, D & Dobson, A.P. (1992a). Regulation and stability of a free-living host parasite system - *Trichostrongylus tenuis* in red grouse. 1. Monitoring and parasite-reduction experiments. *Journal of Animal Ecology* 61(2): 477-486.

Hudson, P.J. & Newborn, D. (1995) A manual of red grouse and moorland management. Fordingbridge, UK: The Game Conservancy Trust.

Hudson, P.J. (1986) *Red Grouse: The biology and management of a wild gamebird*. Fordingbridge, UK: The Game Conservancy Trust.

University of Strathclyde, Glasgow & GWCT (2010) *An economic study of Scottish grouse moors: An update (2010)*. UK: Game & Wildlife Conservation Trust.

International Union for Conservation of Nature (2007) *Grouse: Status survey and Conservation Action Plan 2006-2010*. United Kingdom: IUCN Publication Services.

Jones, M.B. (1966) Survey of game bird diseases. Report of the Game Research Association 5:34.

LaMann, G.V. (2010) Veterinary Parasitology. New York: Nova Biomedical Press, Inc.

Ley, D.H. (2008) Mycoplasma gallisepticum infection. In Saif, Y.M., *Diseases of poultry*. (pp. 807-834). UK: Blackwell Publishing Ltd.

Lindsay, D.S. & Blagburn, B.L. (2008) Cryptosporidium. In Atkinson, C.T., Thomas, N.J. & Hunter, D.B., *Parasitic diseases of wild birds.* (pp.195-203). USA: Willey-Blackwell.

Lister, S. A., Beer, J.V., Gough, R.E., Holmes, R.G., Jones, J.M.W. & Orton, R.G. (1985) Outbreaks of nephritis in pheasants (*Phasianus colchicus*) with a possible coronavirus aetiology. *Veterinary Record* 117:612–613.

Long, P.L. (1982) *The Biology of the Coccidia*. Baltimore: University Park Press.

Lovat, L. (1911) *The Grouse in Health and Disease. Report of the Committee of Inquiry on Grouse Disease*. London, UK: Smith, Elder and Co.

Luttrell, P. & Fischer, J.R. (2007) Mycoplasmosis. In Thomas, N.J., Hunter, D.B. & Atkinson, C.T., *Infectious diseases of wild birds*. (pp.317-331). USA: Willey-Blackwell.

Martin, A.G., Danforth, H.D., Barta, J.R. & Fernando, M.A. (1997) Analysis of immunological cross-protection and sensitivities to coccidial drugs amongst five geographical and temporal strains of Eimeria maxima. *International Journal for Parasitology* 27:527-533.

Martinez-Padilla, J., Mougeot, F., Perez-Rodriguez, L. & Bortolotti, J.R. (2007) Nematode parasites reduce carotenoid-based signalling in males red grouse. *Biology Letters* 3: 161-164.

McDougald, L.R. (2008) Cryptosporidiosis. In Saif, Y.M., *Diseases of poultry*. (pp. 1085-1091). UK: Blackwell Publishing Ltd.

McDougald, L.R. & Fitz-Coy, S.H. (2008) Coccidiosis. In Saif, Y.M., *Diseases of poultry*. (pp. 1068-1085). UK: Blackwell Publishing Ltd.

McGilvray, J. (1995) An economic study of grouse moors. Fordingbridge, UK: The Game Conservancy.

McMahon, B.J., Johansson, M.P., Piertney, S. B., Buckley, K. & Höglund, J. (2012). Genetic variation among endangered Irish red grouse (*Lagopus lagopus hibernicus*) populations: Implications for conservation and management. Ireland: Springer.

Millan, J., Gortaz, C. & Villafuerte, R. (2004) Ecology of nematode parasitism in red-legged partridges (*Alectoris rufa*) in Spain. *Helminthologia* 41: 33-37.

Moss, R. & Parkinson, J.A. (1972) The digestion of heather (*Calluna vulgaris*) by red grouse (*Lagopus lagopus scoticus*). *British Journal of Nutrition* 27: 285-298.

Moss, R., Watson, A., Trenholm, I.B. & Parr, R. (1993) Caecal threadworms *Trichostrongylus tenuis* in red grouse *Lagopus lagopus scoticus*: effects of weather and host density upon estimated worm burdens. *Parasitology* 107: 119-209

Mougeot, F., Martinez-Padilla, J., Perez-Rodriguez, L. & Bortolotti G.R. (2007a). Carotenoid-based coloration and ultraviolet reflectance of the sexual ornaments of grouse. *Behavioural Ecology and Sociobiology* 61: 741-751.

Mougeot, F., Perez-Rodriguez, L., Martinez-Padilla, J., Leckie, F. & Redpath, S.M. (2007b). Parasites, testosterone and honest carotenoid-based signalling of health. *Functional Ecology* 2007: 886-898.

Mougeot, F., Evans, S.A. & Redpath, S.M. (2005b). Interactions between population processes in a cyclic species: Parasites reduce autumn territorial behaviour of males red grouse. *Oecologia* 144: 289-298.

Mougeot, F., Piertney, S.B., Leckie, F., Evans, S., Moss, R., Redpath, S.M. & Hudson, P.J. (2005c) Experimentally increased aggressiveness reduces population kid structure and subsequent recruitment in red grouse *Lagopus lagopus scoticus*. *Journal of Animal Ecology* 74: 488-497.

Mougeot, F., Redpath, S.M. & Leckie, F. (2005a) Ultra-violet reflectance of males and females red grouse, *Lagopus lagopus scoticus*: Sexual ornaments reflect nematode parasite intensity. *Journal of Avian Biology* 36: 203-209.

Murray, T., Clotworthy, C. & Bleasdale, A. (2013). A survey of red grouse (*Lagopus lagopus scoticus*) in the Owenduff/Nephin Complex Special Protection Area, County Mayo. Ireland: National Parks & Wildlife Service.

Naciri, M., Fort, G., Briant, J., Duperray, J. & Benzoni, G. (2014) Incidence of single and mixed infections with *Eimeria Kofoidi*, *E. caucasica* and *E. legionensis* on the health of experimentally infected red-legged partridges (*Alectoris rufa*). *Veterinary Parasitology* 205(1-2): 77-84.

National Red Grouse Steering Committee (2013). *Red Grouse Species Action Plan*. Ireland: National Red Grouse Steering Committee.

Newborn, D., and Foster, R. (2002) Control of parasite burdens in wild red grouse *Lagopus lagopus scoticus* through the indirect application of anthelmintics. *Journal of Applied Ecology* 39: 909-914.

Norton, C.C. (1967) *Eimeria cochici* sp. Nov. (Protoza: Eimeriidae), the cause of cecal coccidiosis in english covert pheasants. *Journal of Protozoology* 14:772.

Ortolani, E.L. (2008) Standardization of the modified Ziehl-Neelsen Technique to stain oocysts of *Cryptosporidium* SP. *Revista Brasileira de Parasitologia Veterinária* 9, 1:29-31

Parker, R.J. & Jones, G.W. (1990) Destruction of bovine coccidial oocysts in simulated cattle yards by dry tropical winter weather. *Veterinary Parasitology* 35:269-272.

Porter, R, Norman, R & Gilbert, L, (2009) Predicting the effectiveness of tick control methods using mathematical modelling. Aberdeen: University of Stirling

Potts, G.R., Tapper, S.C. & Hudson, P.J. (1984) Population fluctuations in red grouse: Analysis of bag records and a simulation model. *Journal of Animal Ecology* 53:21-36.

Redpath, S. M., Mougeot, F., Leckie, F.M. & Evans, S.A. (2006a) The effects of autumn testosterone on survival and productivity in red grouse *Lagopus lagopus scoticus. Animal behaviour* 71: 1297-1305.

Redpath, S. M., Mougeot, F., Leckie, F.M., Elston, D.A. & Hudson, P.J. (2006b). Testing the role of parasites in driving the cyclic population dynamics of a gamebird. *Ecology Letters* 9: 410-418.

Robertson, P.A., Park, K.J. & Barton, A.F. (2001). Loss of heather moorland in the Scottish uplands: The role of red grouse management. Aberdeen: Department of Biological Sciences – University of Stirling.

Ruff, M.D., Fagan, J.M. & Dick, J.W. (1984) Pathogenicity of coccidia in japanese quail (*Coturnix coturnix japonica*). *Poultry Science* 63:55-60.

Saif, Y.M. (2008) *Diseases of poultry*. UK: Blackwell Publishing Ltd.

Sathyanarayanan, L. & Ortega, Y. (2006) Effects of temperature and different food matrices on *Cyclospora cayetanensis* oocyst sporulation. *Journal of Parasitology* 92:218-222.

Saunders, L.M., Tompkins, D.M. & Hudson, P.J. (2000) Spatial aggregation and temporal migration of free-living stages of the parasitic nematode *Trichostrongylus tenuis*. *Functional Ecology* 14(4): 468-473.

Shaw, J.L. (1988) Arrested development of Trichostrongylus tenuis as thrid-stage larvae in red grouse. *Research in Veterinary Science* 48:59-63.

Shaw, J.L. & Moss, R. (1989a) The role of parasite fecundity and longevity in the success of *Trichostrongylus tenuis* in low density red grouse populations. *Parasitology* 99:253-258.

Shaw, J.L. & Moss, R. (1989b) Factors affecting the establishment threadworm *Trichostrongylus tenuis* in red grouse (*Lagopus lagopus scoticus*). *Parasitology* 99:259-264.

Shirley, M.W., Smith, A.L. & Blake, D.P. (2007) Challenges in the successful control of avian coccidia. *Vaccine* 25:5540-5547.

Shirley, M.W., Smith, A.L. & Tomley, F.M. (2005) The biology of avian *Eimeria* with an emphasis on their control by vaccination. *Advances in Parasitology* 60:285-330.

Sievwright, L. J., Redpath, S.M., Mougeot, F., Leckie, F. & Hudson, P.J. (2005) Interaction between intrinsic and extrinsic mechanisms in a cyclic species: Testosterone increases parasite infection in red grouse. *Proceedings of the Royal Society B-Biological Sciences* 272: 2299-2304.

Sievwright, L. J., Redpath, S.M., Mougeot, F., Watt, L. & Hudson, P.J. (2004) Faecal egg counts provide a reliable measure of *Trichostrongylus tenuis* intensities in free-living red grouse *Lagopus lagopus scoticus*. *Journal of Helminthology* 78:69-76.

Sréter, T, Széll, Z & Varga, I (2002) Anticryptosporidial prophylactic efficacy of enrofloxacin and paromomycin in chickens. *Journal of Parasitology* 88:209-211

Tompkins, D.M. & Hudson, P.J. (1999) Regulation of nematode fecundity in the ring-necked pheasant (*Phasianus colchicus*): not just density dependence. *Parasitology* 97: 89-99.

Tompkins, D.M. (2008) *Trichostrongylus*. In Atkinson, C.T., Thomas, N.J. & Hunter, D.B., Parasitic diseases of wild birds. (pp.316-325). USA: Willey-Blackwell.

Trigg, P.I. (1967) *Eimeria phasiani* Tyzzer, 1929 – A coccidium form the pheasant (*Phasianus colchicus*). II. Pathogenicity and drug action. *Parasitology* 57:147.

Villanúa, D., Pérez-Rodríguez, L., Gortázar, C., Hofle, U. & Viñuela, J. (2006) Avoiding bias in parasite excretion estimates: The effect of sampling time and type of faeces. *Parasitology* 133:251-259.

Watson, A. & Moss, R. (2008). The natural history of british and irish species: Grouse. London: Collins.

Williams, R.B. (2001) Quantification of the crowding effect during infections with the seven *Eimeria* species of the domesticated fowl: its importance for experimental designs and the production of oocyst stocks. *International Journal for Parasitology* 31:1056-1069.

Wilson, G.R. (1979) Effects of the caecal threadworm *Trichostrongylus tenuis* on red grouse. PhD Thesis. University of Aberdeen.

Wilson, G.R. (1983) The prevalence of caecal threadworms (*Trichostrongylus tenuis*) in red grouse (*Lagopus lagopus scoticus*). *Oecologica* 58: 265-268.

Yabsley, M.J. (2008) *Eimeria*. In Atkinson, C.T., Thomas, N.J. & Hunter, D.B., *Parasitic diseases of wild birds.* (pp.162-180). USA: Willey-Blackwell.

Yabsley, M. J. & Gibbs, S. E. J. (2006) Description and phylogeny of a new species of *Eimeria* from double-crested cormorants (*Phalacrocorax auritus*) near Fort Gaines, Georgia. *Journal of Parasitology* 88:1230-1233.

8   Annex – Complete data table

ID	GENDER	AGE	WEIGHT (g)	Cryp CLINICAL SIGNS	HEAD HISTOPATHOLOGY (Aiming the search of Cryptosporidium baileyi)	EYE SMEAR (Aiming the search of C. bailey)	C. bailey "CONCLUSION" (HISTOPATH/EYE SMEAR)	PCR M. synoviae M. galisepticum	PCR O. rhinotracheale	PCR Infectious Bronchitis virus	PCR Avian rhinotracheitis virus	Eimeria OOCYSTS/g	T. tenuis EGGs/g	T. tenuis Adult WORMS	LOCATION
1	Male	Old	758	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	650	0	0	Northern UK 1
2	Female	Old	692	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	11250	0	0	Northern UK 1
3	Male	Old	839	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	2050	0	0	Northern UK 1
4	Male	Old	682	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	450	0	0	Northern UK 1
5	Male	Old	767	NO	WITH Cryptosporidial infection	N A	CRYPTOSPORIDIA FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	3050	0	0	Northern UK 1
6	Male	Old	705	NO	WITH Cryptosporidial infection	N A	CRYPTOSPORIDIA FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	1050	0	0	Northern UK 1
7	Male	Young	620	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	2950	0	0	Northern UK 1
8	Female	Young	610	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	1300	300	1200	Northern UK 1
9	Male	Old	737	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	10750	0	0	Northern UK 1
10	Male	Old	650	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	9400	300	9200	Scotland 1
11	Male	Old	707	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	7500	300	2400	Scotland 1
12	Female	Old	589	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1900	50	1000	Scotland 1
13	Male	Young	751	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	850	0	0	Scotland 1
14	Male	Young	711	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	18600	0	0	Scotland 1
15	Female	Young	547	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	14500	50	600	Scotland 1
16	Male	Old	784	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	900	0	0	Scotland 2
17	Female	Old	628	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	400	0	0	Scotland 2
18	Male	Young	734	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	4750	0	0	Scotland 2
19	Male	Young	660	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2400	0	0	Scotland 2
20	Male	Young	700	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	10700	0	0	Scotland 2
21	Male	Young	663	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2500	150	1000	Scotland 2
22	Female	Old	623	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	5550	0	200	Scotland 2
23	Female	Young	557	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1650	0	0	Scotland 2
24	Female	Old	634	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	18200	0	0	Scotland 2
25	Female	Old	554	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	4700	50	400	Scotland 2
26	Male	Young	325	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	31700	0	0	Northern UK 2
27	Male	Young	601	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	5100	0	1000	Northern UK 2
28	Female	Old	479	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	120 000	0	0	Northern UK 2
29	Male	Young	613	YES	WITH Cryptosporidial infection	No cryptosporidia seen	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	650	0	0	Northern UK 2
30	Female	Young	632	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	7200	150	400	Northern UK 2
31	Male	Old	533	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	6800	350	600	Northern UK 2
32	Male	Old	695	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	1050	50	0	Northern UK 2
33	Male	Old	686	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	4000	50	0	Northern UK 2
34	Male	Old	591	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	10500	0	0	Northern UK 2
35	Male	Young	711	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	1850	0	0	Northern UK 2
36	Female	Young	583	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	3900	1750	1000	Scotland 3
37	Male	Young	663	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	11750	0	0	Scotland 3
38	Female	Young	604	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	16850	4850	3800	Scotland 3
39	Female	Young	617	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	8450	50	200	Scotland 3
40	Male	Young	678	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	1600	2100	1200	Scotland 3
41	Female	Old	702	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	4500	0	500	Scotland 3
42	Female	Old	661	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	8200	0	0	Scotland 3
43	Male	Old	670	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	5700	0	0	Scotland 3

#	Sex	Age													Location
44	Male	Old	590	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1500	0	0	Scotland 3
45	Female	Old	653	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	12000	900	1000	Scotland 3
46	Female	Young	612	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2000	0	100	Scotland 4
47	Female	Young	581	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1800	0	0	Scotland 4
48	Female	Young	628	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	3900	0	0	Scotland 4
49	Male	Young	680	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	5100	0	0	Scotland 4
50	Male	Young	570	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	72000	800	700	Scotland 4
51	Female	Old	625	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	3100	0	0	Scotland 4
52	Male	Old	750	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	18500	0	0	Scotland 4
53	Male	Old	684	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1100	0	0	Scotland 4
54	Female	Old	614	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	10400	0	0	Scotland 4
55	Female	Old	606	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	7300	0	0	Scotland 4
56	Female	Young	573	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	28000	0	0	Scotland 5
57	Male	Young	691	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1800	0	0	Scotland 5
58	Female	Young	575	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2200	0	0	Scotland 5
59	Male	Young	656	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2700	0	0	Scotland 5
60	Female	Young	571	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2000	0	100	Scotland 5
61	Male	Old	737	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	9700	500	800	Scotland 5
62	Female	Old	600	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	3100	0	0	Scotland 5
63	Male	Old	674	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	13500	3500	2700	Scotland 5
64	Male	Old	710	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	3800	0	0	Scotland 5
65	Female	Old	623	NO	No evidence of cryptosporidia	N A	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	11300	0	0	Scotland 5
66	Male	Old	623	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	4400	6700	7400	Northern UK 3
67	Female	Old	610	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2200	9600	4700	Northern UK 3
68	Male	Old	593	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2700	21300	12200	Northern UK 3
69	Female	Old	598	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	900	7400	17900	Northern UK 3
70	Female	Old	633	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	900	100	100	Northern UK 3
71	Male	Young	644	YES	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	3500	100	200	Northern UK 3
72	Male	Young	570	YES	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	6100	100	0	Northern UK 3
73	Female	Young	569	YES	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	4200	1200	1600	Northern UK 3
74	Female	Young	531	YES	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1500	0	0	Northern UK 3
75	Female	Young	528	YES	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	3600	1600	2500	Northern UK 3
76	Male	Young	737	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	5150	0	0	Northern UK 4
77	Female	Young	503	YES	N A	No cryptosporidia seen	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	14500	0	0	Northern UK 4
78	Male	Young	616	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2150	0	200	Northern UK 4
79	Male	Young	590	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	21900	0	0	Northern UK 4
80	Male	Young	609	YES	N A	No cryptosporidia seen	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1200	200	800	Northern UK 4
81	Female	Young	346	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	120 000	0	0	Northern UK 4
82	Female	Young	506	YES	N A	No cryptosporidia seen	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	20800	0	200	Northern UK 4
83	Female	Old	580	YES	N A	No cryptosporidia seen	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	14500	0	0	Northern UK 4
84	Male	Old	535	YES	N A	No cryptosporidia seen	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	N A	N A	N A	Northern UK 4
85	Female	Old	672	YES	N A	No cryptosporidia seen	NOT FOUND	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1250	0	0	Northern UK 4
86	Male	Young	651	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	4950	50	100	Scotland 6
87	Male	Young	681	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1100	0	0	Scotland 6
88	Female	Young	556	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2850	0	0	Scotland 6
89	Female	Young	596	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	22600	0	0	Scotland 6
90	Female	Young	593	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2600	0	0	Scotland 6

91	Female	Old	635	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	2700	0	0	Scotland 6
92	Female	Old	567	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	850	3900	6300	Scotland 6
93	Female	Old	624	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1100	0	100	Scotland 6
94	Male	Old	733	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	1650	100	500	Scotland 6
95	Female	Old	552	NO	N A	N A	N A	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	500	14500	14200	Scotland 6
96	Male	Young	680	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	120000	200	400	Northern UK 4
97	Male	Young	632	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	7200	0	100	Northern UK 4
98	Female	Young	555	YES	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	N A	1450	100	100	Northern UK 4
99	Female	Young	604	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	1200	50	200	Northern UK 4
100	Female	Young	365	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	N A	N A	N A	Northern UK 4
101	Male	Young	572	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	3050	0	0	Northern UK 4
102	Female	Young	509	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	6400	300	600	Northern UK 4
103	Female	Young	526	YES	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	f	3100	700	400	Northern UK 4
104	Female	Young	489	YES	N A	WITH Cryptosporidial infection	CRYPTOSPORIDIA FOUND	N A	N A	N A	N A	1050	50	200	Northern UK 4
105	Female	Young	489	YES	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	N A	1300	150	100	Northern UK 4
106	Male	Young	755	NO	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	N A	350	200	200	Northern UK 4
107	Male	Young	711	NO	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	N A	2400	0	0	Northern UK 4
108	Male	Young	664	NO	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	N A	450	0	100	Northern UK 4
109	Male	Young	629	NO	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	N A	600	0	0	Northern UK 4
110	Male	Young	583	NO	N A	No cryptosporidia seen	NOT FOUND	N A	N A	N A	N A	20600	0	100	Northern UK 4