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Development of molecular tools for the rapid assessment of benzimidazole resistance and investigation of possible risk factors in resistance development in *Nematodirus battus* populations

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1. Abstract

Nematodirus battus is an economically important roundworm which threatens lamb health and the sustainability of UK farming. In recent years, apparent changes in hatching and the timing of infection have occcured in conjunction with the first reports of fenbendazole resistance. The aims of this project were to develop molecular tools to study the frequency of mutations associated with white drench resistance in UK *N. battus* populations; assess farmers' perceptions of *N. battus* infection and to gather farm management data to assess current control methods. Finally, to explore possible associations between management decisions and the development of anthelmintic resistance (AR) using mathematical modelling (generalised linear mixed effect models; GLMM). Pyrosequencing and deep-amplicon sequencing by Illumina MiSeq were evaluated within the current project to detect and quantify the mutations associated with white drench-resistance; namely F176Y, E198A and F200Y. Pyrosequencing and MiSeq tests were developed, evaluated and used to detect and quantify mutations in *N. battus* populations from UK commercial farms. Results were comparable between the two tests, indicating that either method would be suitable as a laboratory diagnostic tool. A total of 282 UK *N. battus* populations were tested for the presence and frequency of mutations associated with white drench-resistance. The main mutation, F200Y, was identified in 26% of the populations tested, albeit at low overall frequency (~2%) with a focal region of high frequency identified in North West England. The F167Y mutation was identified for the first time in *N. battus*, at low frequency on a small number of farms and no mutations were identified at codon 198. Risk factor analysis identified the administration of quarantine as a protective factor in the development of white drench-resistance. The farm management questionnaire highlighted significant regional variation in the observation of *N. battus*. The perception of increasing severity of symptoms were reported from respondents in the North whilst the time at which *N. battus* is becoming more apparent to respondents has changed in the South. Diagnostic methods also varied regionally, with greater uptake of faecal egg counting and online risk maps to determine anthelmintic treatments in the South. This project represents the largest survey of UK *N. battus* populations ever conducted and has provided a valuable insight into how this species has changed in recent years. Differences in disease timings observed North to South questions the suitability of the current 'one fits all' approach to advice. Validation of the risk factors identified, could provide the basis for novel control strategies to minimise production losses and the associated economic cost of *N. battus* infection.

2. Introduction

Nematodirus battus: lifecycle, pathology and epidemiology

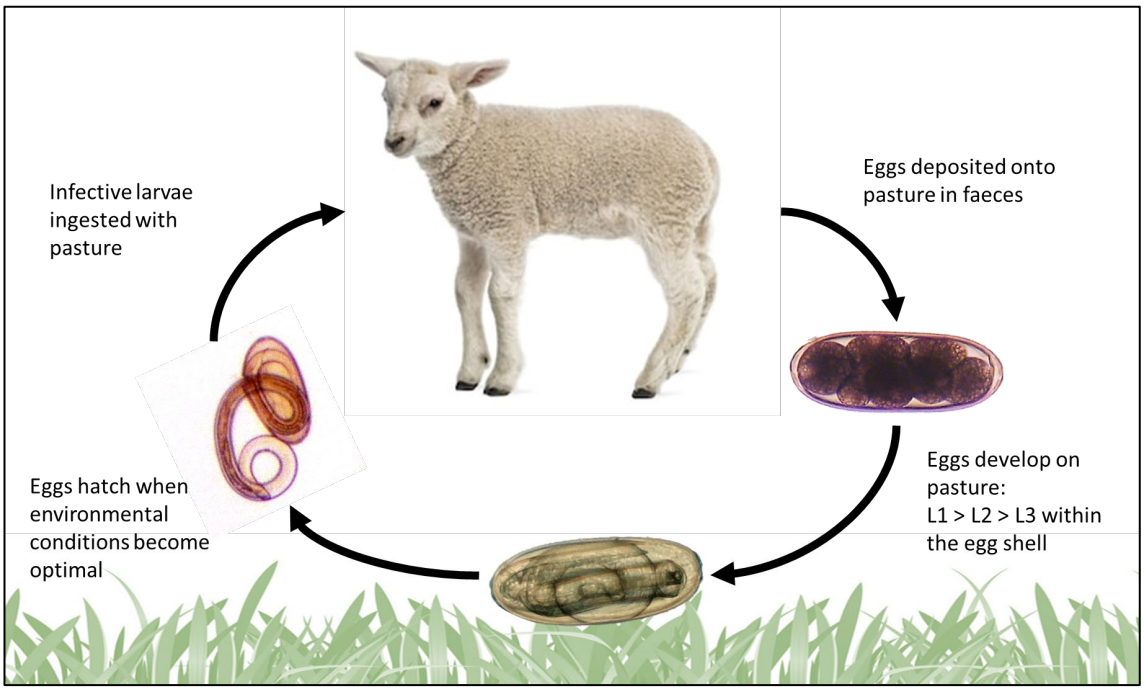

Figure 1. Typical N. battus lifecycle.

Eggs passed out onto pasture in faeces, develop to L3 stage within the egg shell. Developed eggs remain intact on pasture until environmental conditions are optimal for hatching then L3 emerge and are ingested by grazing animals.

Nematodirus battus is a gastrointestinal nematode (GIN) that affects both small and large ruminants. It was first described in UK sheep in 1951 (Crofton and Thomas, 1951) and remains a major threat to lamb health throughout the UK and many other temperate regions of the world. *N. battus* is believed to have originated in arctic regions and transferred via animal movements (Hoberg, 2005). This nematode has a direct parasitic lifecycle similar to those of other roundworms but with the major difference that the infective larval stage develops within the egg rather than on pasture with the egg-development phase therefore taking place over a much longer period (e.g. months in *N. battus* versus up to a week in other roundworms; Figure 1. After migration of infective larvae (L_3), onto pasture, they are ingested with herbage, the larvae exsheath within the abomasum and progress to the small intestine (Mapes and Coop, 1972). Progression from an exsheathed L_3 to L_4 to L_5 and finally to sexually mature adult can occur in as little as 14 days post infection (Mapes and Coop, 1972).

Adult worms mate and females produce eggs which are then excreted in the host faeces.

Infection is common in lambs around 6-8 weeks of age, causing acute yellow/green diarrhoea and subsequent dehydration (Figure 2) (Kingsbury, 1953). Given a high larval challenge, immunity to *N. battus* infections typically develops rapidly and dramatically, with the majority of adult worms being expelled 24-34 days post-infection after which lambs recover (Mapes et al., 1973; Martin and Lee, 1976). Severe damage to the small intestine can result in lambs being unable to retain water and thus succumb to dehydration quickly, often before significant number of *N. battus* eggs are present in the faeces. The intensity of the infection varies between lambs; the majority will recover within a month, however deaths from *N. battus* infection can be as high as 10-30% of the lamb crop (Kingsbury, 1953). Other clinical signs include sunken eyes and rough wool, lambs have been observed to have a 'tucked up' appearance, signalling abdominal pain and an unwillingness to move unless forced (Gibson and Everett, 1973; Kingsbury, 1953; Stamp and Dunn, 1955).

Figure 2. Lamb with breech soiling, a clinical sign of N. battus infection.

The timing of *N. battus* egg development and hatching generally depends on climatic conditions. Development occurs within the temperature range of 11.5 - 27˚C and generally takes around 4 weeks (Thomas, 1959a; van Dijk and Morgan, 2008) but hatching does not occur immediately upon completion of development (Boag and Thomas, 1975; Gibson, 1963; Thomas, 1959b). The eggs typically require a period of chilling (below 11°C) prior to hatching, with maximum hatch occurring after a chill

of 12 weeks or more (van Dijk and Morgan, 2008), which would be easily reached in nature throughout the winter months. When spring day/night temperature stabilises within the optimum range for *N. battus*; estimated to be between 11 and 17˚C (van Dijk and Morgan, 2008) for around 10 consecutive days, eggs undergo synchronous mass-hatching, resulting in very high challenge from contaminated pastures.

The exact role of the chill stimulus in *N. battus* hatching remains unclear. Chilling eggs converts energy reserves within the egg into sugars which increases the cold-hardiness of the eggs (Ash and Atkinson, 1983; Jagdale and Grewal, 2003). The requirement for chilling may therefore be a relic of the arctic origins of this species (Hoberg, 2005). Cold-hardiness of eggs would protect the larvae from extremely low winter temperatures and prevent eggs hatching in late summer/autumn when suitable hosts may not be grazing; adaptations which may be less important in UK intensive farming environments.

The required chill stimulus typically restricts *N. battus* hatching to the spring (Boag and Thomas, 1975; Gibson, 1963; Thomas, 1959b) however in recent years, reports of eggs being observed in faecal samples throughout the year, a secondary peak of infection in autumn and clinical cases of *Nematodirosis* in older animals (Sargison et al., 2012) appear to represent a change in the hatching behaviour of some *N. battus* populations.

Control of N. battus

Benzimidazole compounds (white drenches) have been used to control *N. battus* since it was first licensed for use in livestock in 1960 (Brown et. al., 1961) and remains the anthelmintic of choice to date. The white drenches have a high safety index therefore making them ideal for treatment of young stock (Lacey and Gill, 1994) and unlike other *Nematodirus* species (Jackson, 1982; Middelberg and McKenna, 1983; Vlassoff and Kettle, 1985; Beveridge et al., 1990; Obendorf et al., 1991; Oliver et al., 2016), resistance had not been identified in *N. battus* despite heavy usage of this drug. The first case of white drench-resistance in *N. battus* was detected in 2010 on a commercial farm in Northern England (Mitchell et al., 2011). Faecal samples were submitted to the APHA parasite surveillance centre for analysis following treatment failures, the *N. battus* population was then isolated and white drench-resistance was confirmed by a controlled efficacy test (CET) at the Moredun Research Institute (Morrison et al., 2014). Initial genetic analysis identified one of the key mutations associated with white drench-resistance in other nematode species; F200Y (Morrison et al., 2014; Kwa et al., 1994). A further two mutations

have also been associated with white drench-resistance in other roundworm species; F167Y and E198A (Ghisi et al., 2007; Silvestre and Cabaret, 2002).

Detection of anthelmintic resistance

The 'gold standard' test for anthelmintic resistance is the controlled efficacy 'dose and slaughter' test however, it is not an appropriate method for regular anthelmintic efficacy testing on farm. The faecal egg count reduction test (FECRT) is a more usable on-farm test. Animals are treated with the anthelmintic of interest and faecal egg counts are compared on the day of treatment and 3-14 days post-treatment dependent upon the anthelmintic used and the parasite species of interest (Coles et al., 1992). Anthelmintic resistance was characterised by the world association for the advancement of veterinary parasitology (WAAVP) as a reduction in faecal egg count by less than 95% with a lower confidence interval of less than 90% (Coles et al., 1992). FECRT is simple to perform however, in acute *N. battus* infection, worm burden can be reduced rapidly due to hypersensitivity reactions within the host. It can therefore be difficult to determine whether egg reduction was due to effective treatment or the host immune response. FECRT could therefore underestimate white drench-resistance in this species. The time between samples and repeated animal handling also make FECRT labour-intensive. Genetic tests (molecular tools) look for the mutations which confer resistance in the worms and are typically conducted on a single sample without the need for anthelmintic treatment of animals, reducing labour and providing a rapid result. As the genetic markers of white drench-resistance are known, these tools can be used to detect and quantify the mutations to give an estimation of white drench-efficacy within a population. There is currently no 'gold standard' method for the molecular detection and quantification of mutations and many tests have been developed. Within this project we evaluated pyrosequencing and deep amplicon sequencing.

Several pyrosequencing assays have been developed for the detection and quantification of mutations in roundworm species of veterinary importance including, white drench-resistance associated mutations in *T. circumcincta, H. contortus, Trichostrongylus colubriformis and N. battus* (Morrison et al., 2014; Ramunke et al., 2016; Skuce et al., 2010; von Samson-Himmelstjerna et al., 2009). These tests have been widely used to in surveys worldwide aimed at quantifying the resistance-associated mutations (Esteban-Ballesteros et al., 2017; Ramunke et al., 2016).

Deep amplicon sequencing is a powerful, high throughput method, capable of producing reliable genetic sequence data from 384 farms within a single run. Assays and analysis pipelines have been developed for medical diagnostics such as typing

of HPV viral strains (Nilyanimit et al., 2018) and diagnosis of respiratory infections (Thorburn et al., 2015). Assays have also been developed for veterinary use, most notably the development of the nemabiome assay which identifies roundworm species from pooled eggs in faecal samples (Avramenko et al., 2015) and quantifying white drench-resistance mutations from a pool of different roundworm species (Avramenko et al., 2018).

3. Materials and methods

1.1.1 Sample collection

A total of 381 *N. battus* populations from 348 farms were collected between 2011 and 2016 as detailed in figure 3.

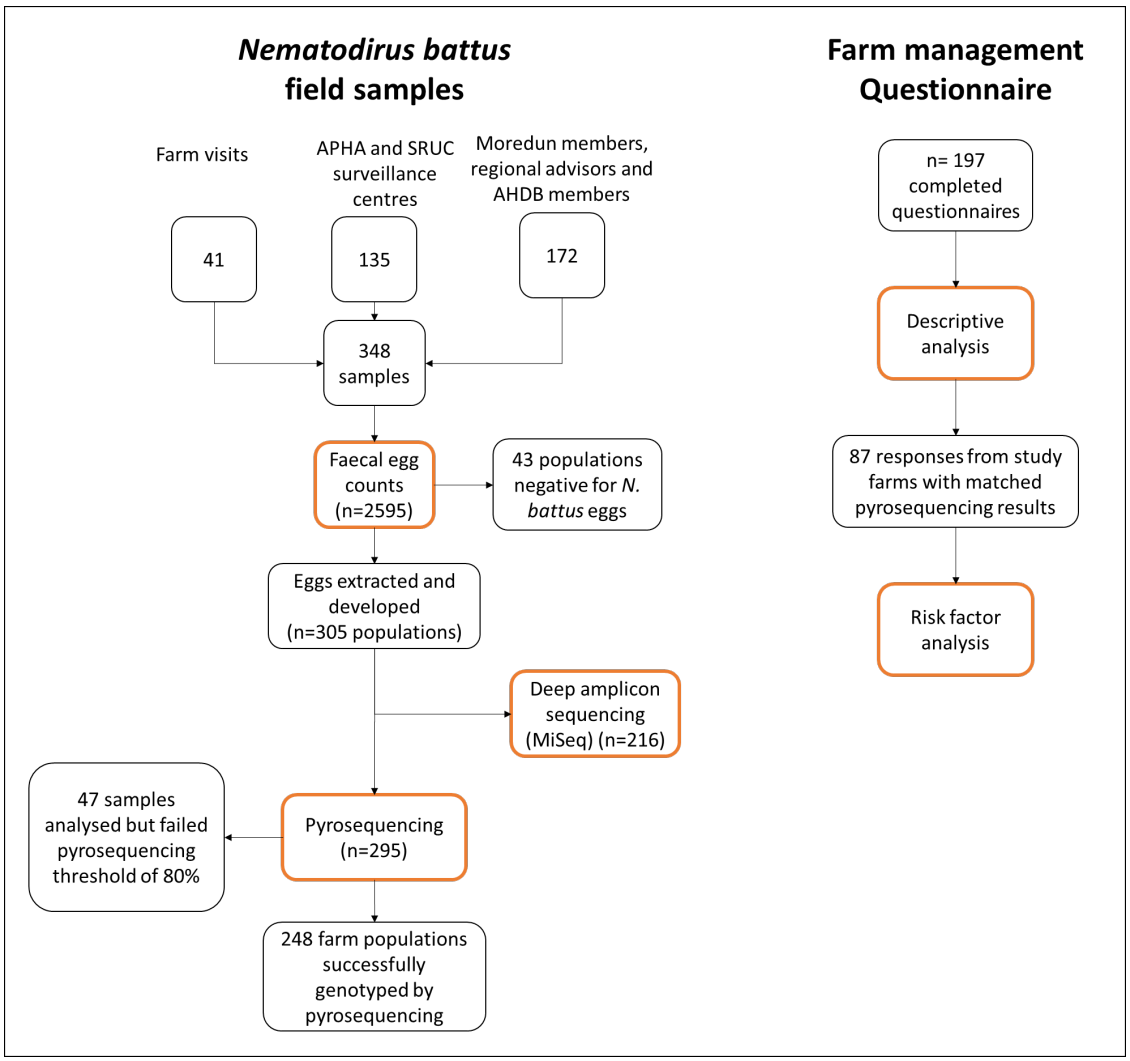

Figure 3. Flow diagram of how many farm samples were collected from surveillance centres, AHDB and Moredun members and in-person farm visits during each sampling year.

1.1.2 Sample preparation and DNA extraction

Faecal egg counts (Jackson and Christie, 1972) were conducted on all samples to confirm the presence of *N. battus* eggs prior to egg extraction, samples were stored at 4°C for up to 5 days before processing.

Eggs were extracted from faeces by differential sieving (212µm, 125µm and 53µm). *N. battus* eggs and fine faecal debris collected on the 53µm sieve were washed into 12ml tubes to clean the filtrate by centrifugation and salt floatation. Eggs were poured over a 53µm sieve and washed with excess tap water to remove remaining salt and placed into non-air-tight jars with tap water, one culture per field or farm population. Egg cultures were stored at ambient room temperature, protected from direct sunlight to allow for larval development. Cultures were monitored microscopically for development and hatching (Figure 5).

Larvated eggs and third stage larvae (L_3) were concentrated and fixed in ethanol (final concentration >70% EtOH) prior to molecular analysis.

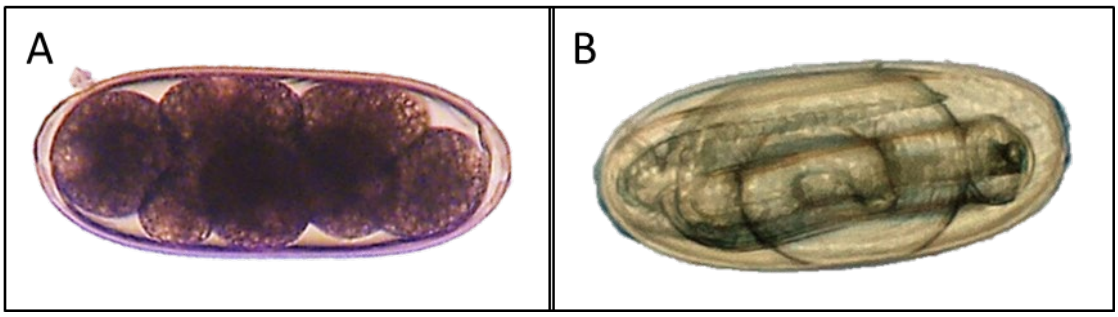

Figure 5. N. battus eggs at different development stages.

N. battus eggs (a) pre-developed and (b) embryonated stage, L_3 visible inside the egg.

3.1.1. Pyrosequencing

Sample preparation

Thirty individual parasites from each farm population were picked at random in 1µl into individual wells of a 96 well plate (Axygen, USA), containing 15µl lysis buffer (50mM KCl, 2.5mM $MgCl_2$, 10mM Tris (pH 8.3) 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% Gelatine) (Kwa et al., 1995). As parasite cultures were pooled by field or farm, eggs/L_3 selected for genotyping were representative of the population rather than any individual animal. Samples containing eggs were then subjected to 8 cycles of 30 second freeze in liquid nitrogen followed by one minute incubation at 100°C to weaken the egg shells. A further 15 µl of worm lysis buffer containing 0.2 mg/ml

proteinase K was added to each well of the plate for both egg and larvae samples. Plates were incubation at 56°C overnight, the temperature was increased to 92°C for 10 minutes to deactivate the proteinase K. Crude lysates were used directly as template in PCR reactions.

DNA amplification and pyrosequencing

The *N. battus*-specific 198/200 SNP assay used was previously detailed by Morison *et al.* (2014). The F167Y assay followed the same PCR and pyrosequencing protocol using the following primers; forward (biotinylated) CGT GAG GAG TAC CCC GAT AGGA, reverse AGT TCG GGA TTT AAC GAA GAGC and sequencing GGC GAC GGA ACG ACA. PCR reactions for 198/200 and 167 were conducted using NovaTaq Hot start master mix (Merck, USA) following the method detailed in Morrison *et al.* (2014). When analysing pyrosequencing results, populations in which fewer than 80% of the individual egg/L_3 DNA lysates were successfully genotyped were removed from downstream analysis.

A subset of populations were selected for 167 analysis based on the results of Illumina MiSeq analysis.

3.1.2. Deep Amplicon Sequencing

A total of 214 *N. battus* populations were included in the deep amplicon sequencing, pools of 500-1000 ethanol-fixed parasites were used per population. Samples were subjected to 8 cycles of freeze/thaw (30 seconds liquid nitrogen/1 minute at 100°C) to crack the outer shell of the egg for enzyme digestion. Proteinase K (Promega, USA) was added to each sample to provide a final concentration of 0.8mg/ml. Samples were incubated on a heatblock at 56°C overnight for digestion to take place, the temperature was then increased to 95°C for 10 minutes to deactivate the proteinase K enzyme. Lysates were cleaned using the quick DNA extraction kit (Zymo, USA) following the manufacturers protocol and extracted DNA was eluted in 1xTE buffer (Sigma, USA) to stabilise DNA for transport to University of Calgary, Canada for sequencing.

DNA amplification, library preparation, sequencing and analysis were conducted following the method described by Avarmenko *et al*. (2019).

3.1.3. Farm management questionnaire

Questionnaire design

Interviews were conducted on seven study farms known to carry white drench-resistant *N. battus* (observed during pyrosequencing analysis) to inform question development. The interviews were semi-structured, i.e. free conversation guided by a series of broad questions, conducted in an informal setting on each farm. All participants were briefed and signed a consent form prior to the interview.

A 42-question questionnaire was developed. It was divided into four sections; i) farm demographics, ii) farmer perceptions of *N. battus* and specific control practices, iii) grazing management and iv) general anthelmintic usage and quarantine. The questionnaire was developed, disseminated and responses collected and collated using the *SurveyMonkey* platform.

Questions were designed to gather information on respondent's perceptions of *N. battus* on their farm, the severity of disease, in which season signs of *N. battus* were typically observed and their perception of if/how the disease had changed within the five years prior to the survey. General management practices focused on husbandry decisions which may interfere with or promote the lifecycle of *N. battus* and control strategies aimed at *N. battus*, other roundworm species and liver fluke throughout the grazing season.

The online questionnaire was pilot tested by four volunteer farmers. Pilot testers reported any issues encountered or unclear language choices and recorded the length of time spent completing the questionnaire.

Dissemination

Farmers submitting samples for inclusion in the genotyping survey were contacted by direct email including a link to the online questionnaire. The questionnaire was disseminated to the wider farming community through social media, veterinary newsletters and publication of short articles included in the national sheep association weekly email newsletter and the farming press. The link was shared on twitter by several agricultural groups including the Scottish farmer, members of the AHDB research team and veterinary practices. Moredun foundation regional advisors also circulated it to their local farming community.

3.1.4. Analysis of potential drivers and barriers of white drench-resistance in *N. battus*

Climatic data collection

Climatic data for risk factor analysis was obtained from the Met office DataPoint (Met Office, 2018). The data was collected from a large number of weather stations and sensors across the UK and the information was downloaded as an interpolated data layer. Data layers were downloaded for temperature (minimum and maximum for each month of the year and mean annual values), precipitation (mm, annual average), ground frost (average annual days), sun hours (average number of hours of sunshine) and relative humidity (%). Evapotranspiration (mm, annual mean) data was sourced from the 'Atlas of the biosphere' (Centre for Sustainability and the Global Environment, 2018) and elevation (meters above sea level) at the point of the farmhouse was also included as a rough approximation of elevation of the farm in general, data obtained from DIVA-GIS (Annon., 2018). All environmental data was mapped using open source qGIS software (Las Palmas version 2.18), the farm positions were then mapped on top of the data and values from each data layer were extracted at the farm locations.

Potenital drivers and barriers of white drench-resistance

Farm management data from completed questionnaires, F200Y resistant allele frequency from pyrosequencing and climatic data for each farm were used to identify potential drivers and barriers of white drench-resistance development in *N. battus*.

Univariable analysis was conducted initially to assess which factors (farm management and climatic variables) were statistically associated with resistant allele frequency. Generalised linear mixed effect models (GLMM) were then constructed using factors with p-value <0.2 in univariable analaysis. Factors were added to the model individually and retained if the factor was found to have a statistically significant impact at the 5% level. Multiple models were developed during the analysis based on the order in which variables were added.

Fitted models were compared using a chi-square test and F-test to compare the amount of residual variance in resistant allele frequency not explained by the management and climatic factors included in the model.

3.1.5. Statistical Analysis

Populations were divided into regions for analysis based on the map boundaries detailed in Figure 2.3 where regional analysis was required.

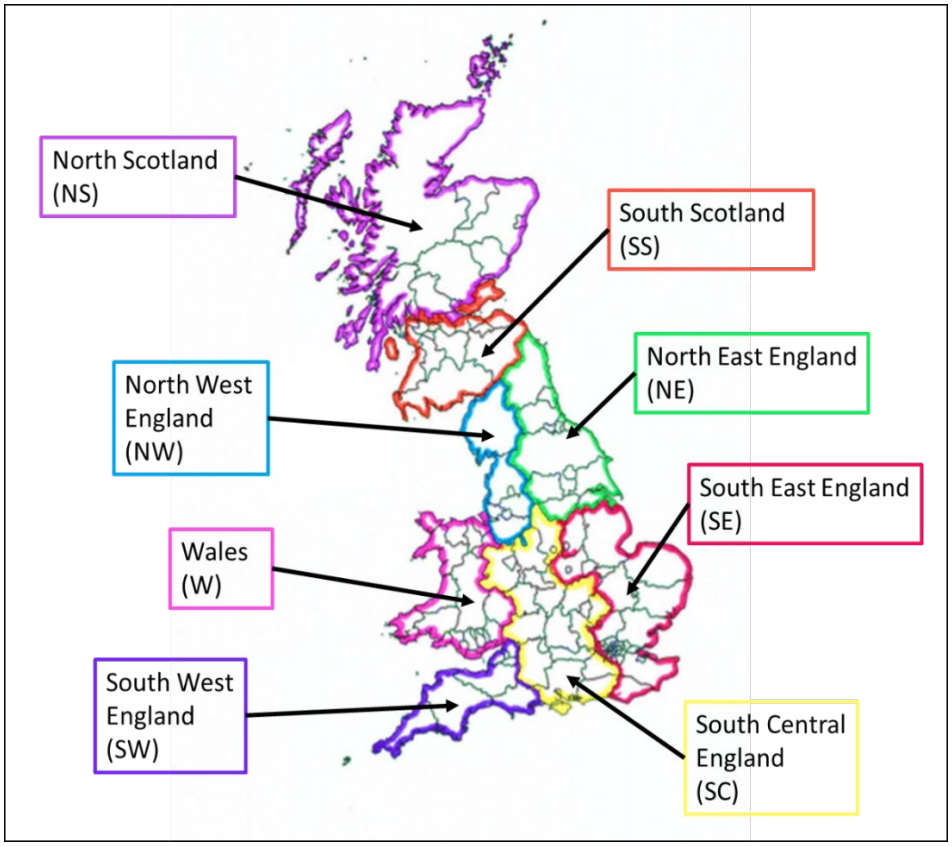

Figure 4. Map of UK regional boundaries used to divide sample populations for data analysis throughout the project.

Genotyping data from pyrosequencing

Binomial logistic regression analysis was carried out to calculate the increase in risk of identifying the F200Y mutation between regions using pyrosequencing results. Analysis was carried out using R version 3.2.5. Hardy-Weinberg analysis was performed to determine whether the loci were under active selection at the point of the study. Observed and expected homo/hetero-zygote frequencies were compared using a chi-squared analysis, performed in Microsoft Excel.

Comparison of deep amplicon sequencing and pyrosequencing results

Allele frequencies of replicate samples analysed by deep amplicon sequencing were compared using a Kruskal Wallis test and the resistant allele frequencies obtained from pyrosequencing and deep amplicon sequencing were compared by linear

regression analysis. A Fisher exact test was also used to compare the presence or absence of F200Y resistant alleles between deep amplicon sequencing and pyrosequencing results. All analyses were carried out using R version 3.2.5.

Farm management questionnaire

Questionnaire responses were downloaded from *SurveyMonkey* into Microsoft Excel for data cleaning, coding and analysis. Descriptive analysis was conducted for each question, initially using the full data set then split by regional location (Figure 4). Chi-squared analysis was conducted in R (version 3.2.5) to explore significant geographical differences in management factor application frequency. Where factors were compared between North and South, responses from North Scotland, South Scotland, North East and North West England comprised “the North” and South East, South West, South central England and Wales comprised “the South”. A statistical significance threshold of 5% (p=0.05) was used in all analyses.

4. Results

4.1. Prevalence of mutations associated with white drench resistance in UK *N. battus* populations

A total of 2595 faecal samples from 348 farms were collected and processed from throughout the UK in 2011 to 2016. Of the farms tested, 94% (n = 329) were positive for *N. battus* with the overall average faecal egg count of 161 eggs per gram (EPG), individual positive counts ranged from 1 to 3330 EPG. Strongyle eggs were also counted; average 141 EPG, range 0 – 2322 EPG.

The current study identified two mutations in UK *N. battus* populations, previously associated with white drench-resistance in other roundworm species. F200Y was found to be the predominant mutation, as in several other roundworm species. This mutation was identified in one in four of the populations tested (26%). The overall frequency of F200Y was low (mean frequency 2.1%) however, a small number of farms were identified with high F200Y frequency (frequency range 0-93%). The observation of few farms with high frequency F200Y suggests that this mutation does not confer a fitness cost for the parasite. Therefore, given the appropriate selection pressure, the F200Y resistant mutation could increase in frequency, potentially leading to clinical white-drench resistance in the field. The F200Y mutation was widely

distributed throughout the UK with apparent ‘focal regions’ in which several local populations possessed higher than average resistant allele frequency. Analysis of the distribution of resistant mutations identified that F200Y was more likely in North West England compared to other regions of the UK (estimate 2.7; 95% CI. 2.1 - 3.5, $p<0.001$).

The F167Y mutation was identified for the first time in *N. battus* during this project. The frequency of this mutation was low (0-13%) and it was identified in only four of the populations tested, suggesting that the F167Y mutation has newly emerged in this species.

To determine whether the F200Y and F167Y mutation sites were under active selection at the point of sampling, the ratio of semi-resistant (heterozygote) and fully-resistant (homozygote) individuals in each population was compared to the Hardy-Weinberg equilibrium. The analysis indicated that the F167Y mutation site was not under active selection ($\chi^2=0.06$, $p=1$) however, the F200Y mutation site was ($\chi^2=1212$, $p<0.001$). Active selection suggests that the mutation frequency has not reached equilibrium and so could change in response to selection pressures such as anthelmintic treatments.

4.2. Comparison of deep amplicon sequencing and pyrosequencing

There is currently no ‘gold standard’ method for detecting and measuring single nucleotide mutations, such as the F200Y and F167Y mutations associated with white-drench resistance in *N. battus*. However, in the current study we demonstrated that both pyrosequencing and deep amplicon sequencing were equally suitable methods for analysing these muations.

The results obtained using pyrosequencing and deep amplicon sequencing targeting the F200Y mutation were statistically comparable by linear regression ($R^2=0.963$). Variation in the results between the two technologies occurred largely at low mutation frequency (Figure 7). The technologies agreed on the presence or absence of F200Y in 83% of the populations tested however, 17% were suspected to contain the resistant mutation as it was detected by only one test. Resistant mutations were identified in 22 populations by pyrosequencing and not deep amplicon sequencing (F200Y range 1.7 - 6%) and a further six populations by deep amplicon sequencing and not pyrosequencing (F200Y range 0.4 - 1.8%). Low level variation was expected as pyrosequencing and deep amplicon sequencing were not performed on the same

individual parasites but different aliquots from a large population. Additional variation may have been due to PCR/sequencing bias, haplotype variation or handling errors.

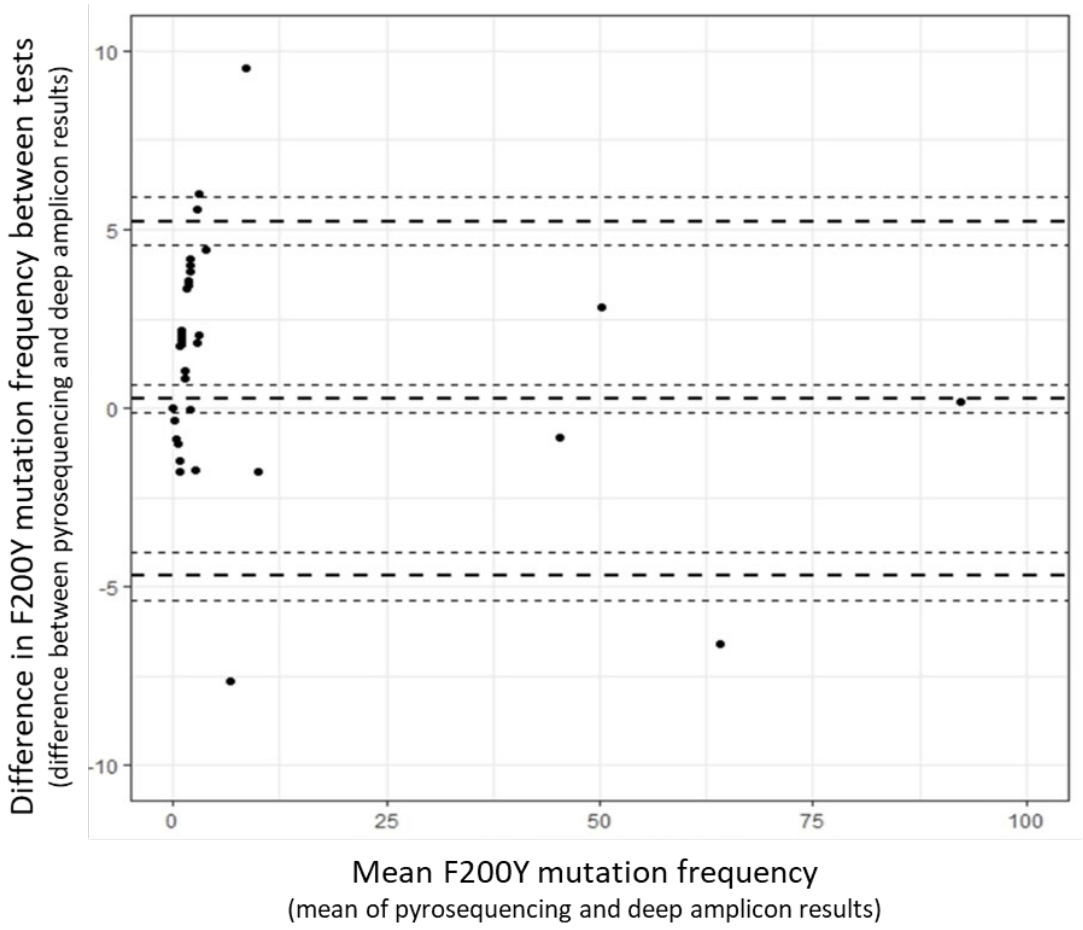

Figure 7. Bland Altman comparison of F200Y allele frequency results obtained from pyrosequencing and deep amplicon sequencing (MiSeq).

The difference in F200Y allele frequency obtained from pyrosequencing and deep amplicon sequencing plotted against the mean F200Y allele frequency from each platform. The dashed lines (---) represent the mean difference in results between the two methods and ±1.96 standard deviation of the difference, with the 95% confidence intervals of each indicated by the dotted lines (…).

4.3. Farm management questionnaire

The aim of the questionnaire study was to gather information on farmers' perceptions of *N. battus* disease and to investigate how control measures and general farm

management practices vary throughout the UK. Differences were identified in the perceived disease severity, timing and sheep management North to South.

Timing of N. battus disease symptoms

Respondents were asked which season(s) they typically observed signs of *N. battus* on farm. Characteristic scouring and lamb losses were observed most commonly in spring however, signs were identified throughout the year in different systems. Spring-only transmission (March–May) was observed by 51% of respondents, 21% reported observing signs exclusively in summer months (June–August) and 3% in autumn only (September–November). Figure 8 shows the overall proportion of respondents who observed signs of *N. battus* in each season. The results suggested a regional trend to *N. battus* disease symptoms however, despite more reports of disease incidence in autumn and winter in the south compared to the north, no statistically significant difference was identified (χ^2= 2.3, d.f. = 1, p= 0.1).

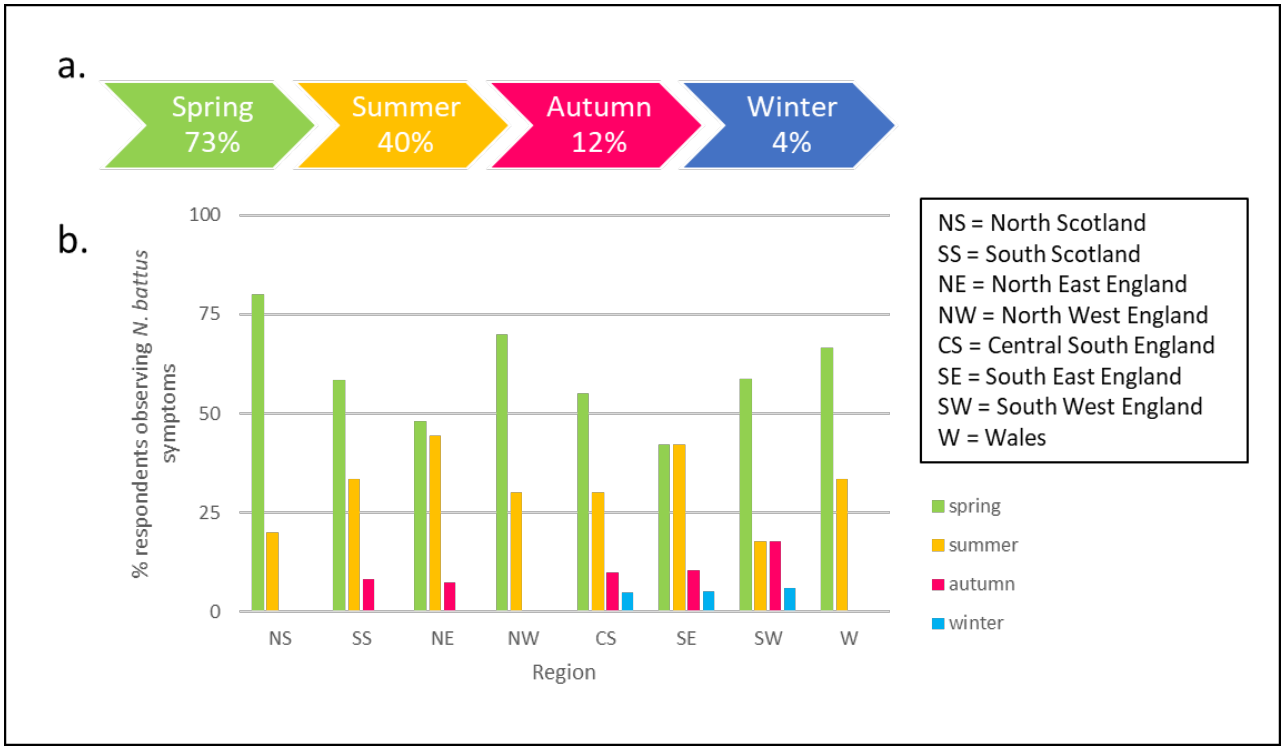

Figure 8. Summary of the season in which respondents typically observed symptoms of N. battus.

The proportion of respondents observing typical signs of N. battus during each season (a) overall and (b) by region. Green bar (spring); Yellow bar (summer), Red bar (autumn); Blue bar (winter).

Perception of disease symptom severity

Regional variation in respondent's perceptions of *N. battus* disease severity was found to be statistically significant (χ^2= 16.38, d.f. = 5, p= 0.006) (Figure 9). Respondents from North England and Scotland observed changes in the severity of disease and overall higher prevalence of scouring and lamb losses attributed to *N. battus* infection in recent years. Subclinical disease and changes in the timing of *N. battus* were more commonly perceived in the south.

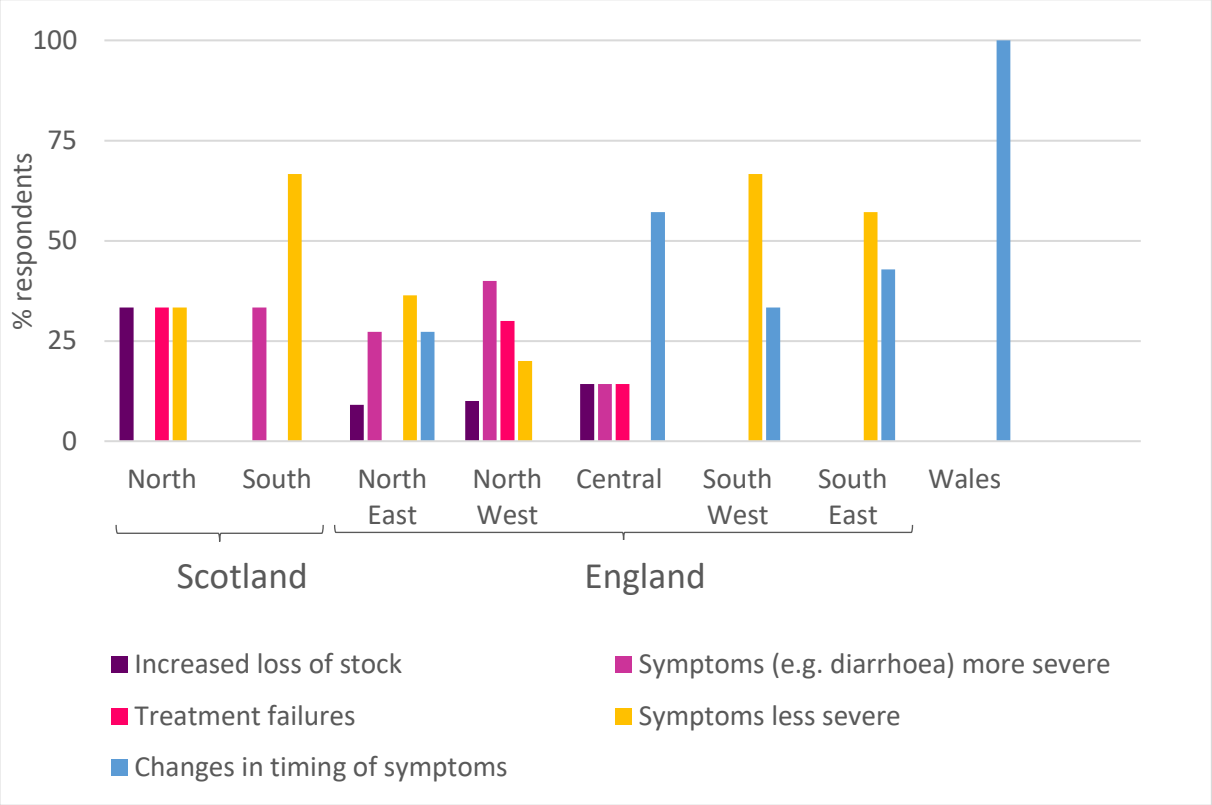

Figure 9. Summary of the changes in N. battus infection reported by respondents.

Proportion of respondents in each geographic region observing different changes in N. battus disease on farm (timing/severity of symptoms and outcome of anthelmintic treatment) within the five years prior to the study.

Monitoring methods to determine anthelmintic treatment timings

Figure 10 summarises the use of FEC and yearly prophylactic (pre-planed) treatments between regions. Variation in the use of FEC monitoring was statistically significant between regions (χ^2= 19.7, d.f.= 7, p= 0.006) most commonly employed in South West and South Central England (80% and 81% of respondents respectively).

The use of prophylactic, yearly treatments also appeared to vary North to South with 40% and 39% of respondents in South Scotland and North East England employing yearly treatments respectively, compared to 22% and 20% of respondents in Wales and South West England respectively however, regional variation was not found to be statistically significant (χ^2= 7.4, d.f.= 7, p= 0.4).

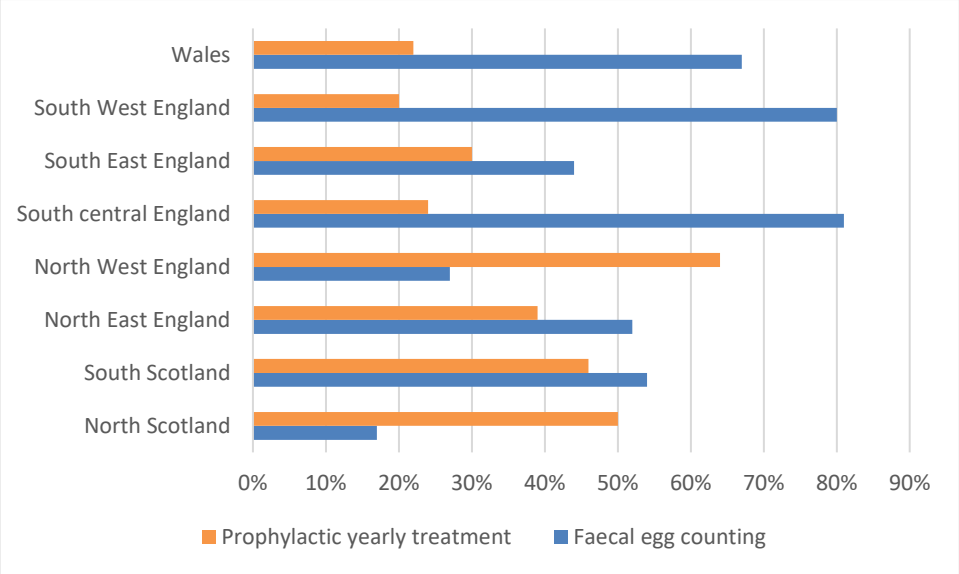

Figure 10. Regional use of faecal egg counting and yearly prophylactic treatments in the control of GIN.

Percentage of respondents from each region employing faecal egg counting to determine the optimal timing of anthelmintic treatment compared to the percentage using yearly, prophylactic anthelmintic treatments to control GIN infection in grazing sheep.

The regional variation in respondent's perceptions of disease timing, severity and changes could be driven by a combination of differences in environmental conditions, disease monitoring practices or anthelmintic treatment strategy which also vary geographically in the UK.

4.4. Potential drivers and barriers of white drench-resistance in UK *N. battus*

The current study identified a range of factors positively and negatively associated with the presence of the resistant mutation F200Y in UK *N. battus* populations. SCOPS recommended practices such as effective quarantine and the use of faecal egg counting to monitor the need for treatment were identified as protective factors against the development of white drench-resistance in *N. battus,* indicating that better

awareness and monitoring of the problem on farm in conjunction with the timing, or number of treatment administrations may be important. Co-grazing of sheep with cattle and supplementary feeding were also associated with fewer resistance mutations being identified whilst the observation of *N. battus* symptoms predominantly in spring was associated with increased likelihood of resistant mutations.

The hypothesis that the development of white drench-resistance in *N. battus* was similar to that of other roundworm species i.e. influenced by the frequency of anthelmintic treatment and quarantine practices was partially correct. From the results of the present study, quarantine appears to play a significant role in determining the presence of the resistance mutation F200Y within the population, suggesting that resistant mutations are more likely to be introduced with incoming or returning stock than they are to originate on farm. Key risk factors highlighted as being selective for resistance development in other roundworms infecting sheep and cattle include; the frequency and timing of anthelmintic treatment and movement of animals onto low contamination fields immediately post-treatment (dose and move) (Falzon et al., 2014; Hughes et al., 2007; Lawrence et al., 2006; Suarez and Cristel, 2014; Suter et al., 2004; Vadlejch et al., 2014), which were not selected by the current analysis.

The observation of *N. battus* symptoms predominantly in spring was highlighted as a potential risk factor in the development of BZ-resistance in this species. The potential association of resistance and spring infection may be due to the frequent use of white drenches to control this speices in spring, the high challenge typically observed at this time or the role of parasite refugia in the development of white drench-resistance in *N. battus*. Refugia describes the parasite population as a whole; i.e. parasites within animals and on pasture. Maximising the population of parasites not exposed to anthelmintic treatment (i.e. roundworms on pasture, in wildlife or in untreated stock) has been identified as an important factor in delaying the development of anthelmintic resistance in other roundworm species (van Wyk, 2001). However, the impact on *N. battus* is difficult to assess given the differences in parasite lifecycle and disease timings between this and other roundworm species.

Co-grazing sheep with cattle was highlighted as significantly reducing the odds of identifying the resistant mutation on farm. The influence of alternate or co-grazing of sheep and cattle on the parasite population is difficult to quantify and will differ dependent upon the roundworm species of interest and its co-infectivity between sheep and cattle (Morley and Donald, 1980). The results of previous studies on anthelmintic resistance in roundworms were varied (Eddi et al., 1996; Lawrence et

al., 2006) suggesting that co-grazing has the potential to enhance or reduce resistance development in different farming systems. The association of both mixed grazing and supplementary feeding of lambs with reduced likelihood of resistance mutations could be explained by reduced exposure to *N. battus*, via decreased pasture contamination or reduced grazing, or the increased nutritional plane of the animals. Additionally, sheep and cattle are unlikely to be treated at the same time, thereby maintaining susceptible worms in the population. Reduced exposure of lambs to parasites may trigger fewer treatments given less severe clinical symptoms, reducing the selection pressure on the population. Less severe symptoms could also safeguard against sub-optimal dose retention of any anthelmintic treatments administered.

5. Discussion

Since the initial characterisation of *Nematodirus battus* in the 1950s (Crofton and Thomas, 1951) this parasite species has become more difficult to control due to less predictable disease timings and the recent emergence of white drench resistance. The aims of this project were to explore the novel resistance-associated changes observed in some *N. battus* populations; quantify white drench-resistance throughout the UK and to explore current control measures to assess whether farm management practices may be associated with the emergence of resistance. *N. battus* remains an important threat to lamb health in the UK. Interest from the agricultural community was evident in the large number of samples volunteered for analysis and queries at agricultural shows and animal health talks throughout the span of the project. The enthusiasm from the livestock sector highlighted the importance of the work and the need for updated information on this topic. It is important to understand the novel aspects of parasite lifecycle to design effective control strategies, minimise production loss and protect animal welfare.

To our knowledge, this project was the largest survey of UK *N. battus* populations conducted to date, the large number of populations included adds power to the findings, reducing sampling bias. A range of applied parasitology techniques were used to collect, isolate and culture *N. battus* populations and a range of state-of-the-art genetic sequencing methods were designed and evaluated for the detection and quantification of mutations associated with white drench-resistance. The laboratory-based deep amplicon sequencing and pyrosequencing methods were both found to be suitable for use as diagnostics or research tools. F200Y was identified as the main

mutation associated with white drench-resistance in *N. battus.* At the farm level, in terms of presence-absence, the mutation was found to be highly prevalent; identified in ¼ of the populations tested. However, currently, the F200Y frequency remains low on most farms. A focal region, in which several local populations possessed a higher than average F200Y frequency, was identified in North West England, the region where the initial case of white drench-resistant *N. battus* was identified. The origin(s) and likely spread of resistant alleles throughout the country cannot be reliably inferred from prevalence data however, further analysis of the genetic sequence data produced during the current project could provide an opportunity to explore the origin and spread of anthelmintic resistance. Exploration of white drench-resistance development in other roundworms has proven difficult due to the high prevalence of resistance in multiple countries (Chaudhry et al., 2015; Redman et al., 2015; Silvestre et al., 2009; Skuce et al., 2010) however, as white drench-resistance appears to be at an early stage in *N. battus* currently, this may be the ideal model system for study. *N. battus* remains a significant threat to lamb health in the UK and with limited anthelmintic options available for safe treatment of young stock, it is pertinent to monitor white drench resistance in this species in an attempt to slow the spread of resistance and safeguard white drench efficacy. Due to the significant differences in the parasite lifecycle and disease timings between *N. battus* and other roundworms, knowledge gained from the study of *N. battus* is not directly transferrable to other species. However, recent changes in the timing of *N. battus*, towards more year-round activity on some farms, may result in this species becoming more similar to other roundworms, including other *Nematodirus* species, at which point information may be more transferrable. With continued variation in *N. battus* disease timings then white drench-resistance in this species may advance in line with *N. spathiger* in 5-10 years time.

Providing information on the prevalence of anthelmintic resistance is interesting and allows for progression to be measured over time but this information is of limited use without understanding the drivers involved. This project explored current farm management practices and the association of different factors with the presence of the F200Y mutation. Exploring the current control measures in use throughout the UK, provided quantitative information on farm management, anthelmintic usage and parasite control as well as qualitative information on farmer's perceptions of disease on their farm. The unique insight into perceptions of disease severity, timings and changes over time together with parasite control plans highlighted knowledge gaps throughout the country which could be addressed in the future using targeted knowledge exchange programs.

The analysis conducted in this project provides an indication of factors which may have contributed to the changes observed in this species. The potential risk factors highlighted could form the basis of future study to verify the associations estimated here and measure the impact of each factor with a view to developing industry recommendations.

The main risk factor for the presence of the F200Y mutation associated with white drench-resistance in *N. battus* was the lack of effective quarantine of new and returning stock. The importance of quarantine has been recognised for a long time (Coles and Roush, 1992; Falzon et al., 2014; Leathwick, 2004) and clear guidelines have been created to promote 'best practice' by farmers. Despite the tools being freely available, uptake remains low; a recent survey of UK and Irish sheep farmers found that although quarantine of incoming stock was being implemented on almost half of the farms tested, only 3% used a suitable strategy capable of preventing the introduction of resistant roundworms (Morgan et al., 2012). Effective quarantine is a simple message to relay to the agricultural community, protecting not only against the transmission of white drench-resistant *N. battus* but all infectious diseases of livestock. Recent research into the uptake of recommendations by farmers found that one of the key factors was the confirmation of anthelmintic resistance on their farm, suggesting that action is more likely to be taken after an event such as significant production loss due to drug failure than before (Jack et al., 2017). Perhaps a focus on changing farmer perceptions of industry recommendations and developing novel methods of promoting research outputs, such as integrating animal health advice into on-farm technology or greater use of demonstration farms, would be beneficial.

Recently, terms such as "smart farming" have frequently been discussed, suggesting that the future of the livestock industry relies upon the development of sensors and decision support systems, i.e. computer software which provides management advice. Technology capable of identifying production limiting factors earlier than current strategies, triggering treatment and minimising production losses. Decision support tools for managing parasite risk would undoubtedly be beneficial however, the findings discussed in this project also highlight fundamental issues which could be addressed without the development of new devices. A greater understanding of parasite biology and knowledge exchange on the basic principles and benefits of existing control strategies such as quarantine practices may provide a significant benefit in the control of *N. battus* in the future.

The results presented in this project show the white drench-resistant mutations are present on a large number of farms but are predominant in only a limited number of cases. If *N. battus* is thought of as having a low refugia i.e. the population is largely

active at the same time and all exposed during whole-flock treatments then it may have been expected that once present, white drench-resistant mutations would quickly expand within the population. However, this was not observed in the majority of the populations sampled, suggesting that additional factors may be influencing the development of resistance in this species. Given the high farm-level prevalence of F200Y in *N. battus* populations throughout the UK and the continued favour of white drenches for the control of *N. battus*, it could be hypothesised that the mutation would be likely to increase in both prevalence and frequency in the near future. However, it is difficult to predict a timescale for the development and spread of resistance in this species due to the lack of understanding on the presence, size and importance of refugia in *N. battus* and the correlation between mutation frequency and anthelmintic efficacy.

The findings discussed in this project showcase the complexity and variation observed in *N. battus* populations in the UK. This parasite species cannot be effectively controlled using a 'one-fits all' approach any longer, with the launch of online risk maps and recent changes in industry recommendations beginning to reflect this. The interest and support from the livestock industry has been highly motivating throughout the project and will hopefully continue as research progresses on this topic. The risk factors highlighted by the current analysis should now provide the basis for in-depth continued study of *N.* battus. The fascinating plasticity of this species is of great academic interest but it is also economically important to the livestock industry as a clear understanding of the drivers of anthelmintic resistance are key to the design of effective control strategies to minimise production losses and protect animal welfare.

6. Industry messages

Finding	What it means	Potential action points
Prevalence results		
Mutations associated with white drench-resistance are present in UK *N. battus* populations at a low frequency	Anthelmintic resistance is currently at an early stage in this species	- The early stage of resistance in this species offers the opportunity to investigate the origin(s) of anthelmintic resistance
F200Y present in ¼ of farm populations tested	Despite the low frequency overall, the widespread distribution of the F200Y mutation could develop into clinical drug failure in the future given the appropriate selection pressure	- Use the results of risk factor analysis to reduce the likelihood of further development and dissemination of resistance
Potential barriers to the development of white drench resistance		
Quarantine	Mutations associated with white drench-resistance in *N. battus* are more likely to be introduced from another source than originate on farm	- Knowledge exchange programs highlighting the importance of effective quarantine - Practical, easy to follow guides promoting best practice quarantine

7. References

Annon. 2018. DIVA GIS [online] available at http://www.diva-gis.org/Data [acccessed 17th November 2018].

Ash, C.P.J., Atkinson, H.J., 1983. Evidence for a Temperature-Dependent Conversion of Lipid Reserves to Carbohydrate in Quiescent Eggs of the Nematode, Nematodirus-Battus. Comparative Biochemisrty and Physiology. 76, 603-610.

Avramenko, R.W., Redman, E.M., Lewis, R., Yazwinski, T.A., Wasmuth, J.D., Gilleard, J.S., 2015. Exploring the Gastrointestinal "Nemabiome": Deep Amplicon Sequencing to Quantify the Species Composition of Parasitic Nematode Communities. PLoS One 10, e0143559.

Avramenko, R.W., Redman, E.M., Melville, L., Bartley, Y., Wit, J., Queiroz, C., Bartley, D.J., Gilleard, J.S., 2018. Deep amplicon sequencing as a powerful new tool to screen for sequence polymorphisms associated with anthelmintic resistance in parasitic nematode populations. International Journal of Parasitology.

Beveridge, I., Ellis, N.J., Riley, M.J., Brown, T.H., 1990. Prevalence of resistance in sheep nematode populations to benzimidazole and levamisole anthelmintics in the high rainfall areas of South Australia. Australian Veterinary Journal. 67, 413-415.

Boag, B., Thomas, R.J., 1975. Epidemiological studies on Nematodirus species in sheep. Research in Veterinary Science. 19, 263-268.

Brown, H.D., Matzuk, A.R., Ilves, I.R., Peterson, L.H., Harris, S.A., Sarett, L.H., Egerton, J.R., Yakstis, J.J., Campbell, W.C., Cuckler, A.C., 1961. Antiparasitic drugs. IV. 2-(4'-thiazolyl)-benzimidazole, a new anthelmintic. Journal of the American Chemical Society 83, 1764-1765.

Centre for Sustainability and the Global Environment, 2018. Atlas of Biosphere [online] available at https://nelson.wisc.edu/sage/data-and-models/atlas/ [accessed 7th Deember 2018].

Chaudhry, U., Redman, E.M., Raman, M., Gilleard, J.S., 2015. Genetic evidence for the spread of a benzimidazole resistance mutation across southern India from a single origin in the parasitic nematode Haemonchus contortus. International Journal of Parasitology 45, 721-728.

Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology 44, 35-44.

Coles, G.C., Roush, R.T., 1992. Slowing the spread of anthelmintic resistant nematodes of sheep and goats in the United Kingdom. Veterinary Record 130, 505-510.

Crofton, H.D., Thomas, R.J., 1951. A New Species of Nematodirus in Sheep. Nature 168, 559-559.

Eddi, C., Caracostantogolo, J., Pena, M., Schapiro, J., Marangunich, L., Waller, P.J., Hansen, J.W., 1996. The prevalence of anthelmintic resistance in nematode parasites of sheep in southern Latin America: Argentina. Veterinary Parasitology 62, 189-197.

Esteban-Ballesteros, M., Rojo-Vazquez, F.A., Skuce, P.J., Melville, L., Gonzalez-Lanza, C., Martinez-Valladares, M., 2017. Quantification of resistant alleles in the beta-tubulin gene of field strains of gastrointestinal nematodes and their relation with the faecal egg count reduction test. BMC Veterinary Research 13, 71.

Falzon, L.C., O'Neill, T.J., Menzies, P.I., Peregrine, A.S., Jones-Bitton, A., vanLeeuwen, J., Mederos, A., 2014. A systematic review and meta-analysis of factors associated with anthelmintic resistance in sheep. Preventive Veterinary Medicine 117, 388-402.

Ghisi, M., Kaminsky, R., Maser, P., 2007. Phenotyping and genotyping of Haemonchus contortus isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Veterinary Parasitology 144, 313-320.

Gibson, T.E., 1963. Experiments on the epidemiology of nematodiriasis. Research in Veterinary Science 4, 11.

Gibson, T.E., Everett, G., 1973. Observations on the control of nematodiriasis and trichostrongylosis in sheep. Journal of Comparative Pathology 83, 125-132.

Hoberg, E.P., 2005. Coevolution and biogeography among Nematodirinae (Nematoda : Trichostrongylina) Lagomorpha and Artiodactyla (Mammalia): Exploring determinants of history and structure for the northern fauna across the Holarctic. Journal of Parasitology 91, 358-369.

Hughes, P.L., Dowling, A.F., Callinan, A.P., 2007. Resistance to macrocyclic lactone anthelmintics and associated risk factors on sheep farms in the lower North Island of New Zealand. New Zealand Veterinary Journal 55, 177-183.

Jack, C., Hotchkiss, E., Sargison, N.D., Toma, L., Milne, C., Bartley, D.J., 2017. A quantitative analysis of attitudes and behaviours concerning sustainable parasite control practices from Scottish sheep farmers. Preventive Veterinary Medicine 139, 134-145.

Jackson, F., Christie, M., 1972. Quantitative recovery of floatable helminth eggs from 1 g. of ruminant faeces for counting followed by hatching for identification. Transactions of The Royal Society of Tropical Medicine and Hygine 66, 546.

Jackson, R.A., 1982. Possible *Nematodirus* resistance. Surveillance 9, 24-26.

Jagdale, G.B., Grewal, P.S., 2003. Acclimation of entomopathogenic nematodes to novel temperatures: trehalose accumulation and the acquisition of thermotolerance. International Journal of Parasitology 33, 145-152.

Kingsbury, P.A., 1953. Nematodirus infestation - a probable cause of losses amongst lambs. Veterinary Record 65, 167-169.

Kwa, M.S., Veenstra, J.G., Roos, M.H., 1994. Benzimidazole resistance in Haemonchus contortus is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Molecular and Biochemical Parasitology 63, 299-303.

Kwa, M.S., Veenstra, J.G., van Dijk, M., Roos, M.H., 1995. Beta-tubulin genes from the parasitic nematode Haemonchus contortus modulate drug resistance in Caenorhabditis elegans. Journal of Molecular Biology 246, 500-510.

Lacey, E., Gill, J.H., 1994. Biochemistry of benzimidazole resistance. Acta Tropical 56, 245-262.

Lawrence, K.E., Rhodes, A.P., Jackson, R., Leathwick, D.M., Heuer, C., Pomroy, W.E., West, D.M., Waghorn, T.S., Moffat, J.R., 2006. Farm management practices associated with macrocyclic lactone resistance on sheep farms in New Zealand. New Zealand Veterinary Journal 54, 283-288.

Leathwick, D.M. 2004. Managing drench resistance in sheep parasites. Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association, Annual Seminar Jan 2004. 73-81.

Mapes, C.J., Coop, R.L., 1972. The development of single infections of Nematodirus battus in lambs. Parasitology 64, 197-216.

Mapes, C.J., Coop, R.L., Angus, K.W., 1973. The fate of large infective doses of Nematodirus battus in young lambs. International Journal of Parasitology. 3, 339-347.

Martin, J., Lee, D.L., 1976. Observations on Crystals Found in Intestine of Nematodirus-battus during Development of Immunity to This Nematode in Lambs. Parasitology 72, 75-&.

Met Office, 2018. Data point [online] available at https://www.metoffice.gov.uk/datapoint [accessed 19th November 2018].

Middelberg, A., McKenna, P.B., 1983. Oxfendazole resistance in *Nematodirus spathiger*. New Zealand Veterinary Journal 31, 65-66.

Mitchell, S., Mearns, R., Richards, I., Donnan, A.A., Bartley, D.J., 2011. Benzimidazole resistance in Nematodirus battus. Veterinary Record. 168, 623-624.

Morgan, E.R., Hosking, B.C., Burston, S., Carder, K.M., Hyslop, A.C., Pritchard, L.J., Whitmarsh, A.K., Coles, G.C., 2012. A survey of helminth control practices on sheep farms in Great Britain and Ireland. Veterinary Journal 192, 390-397.

Morley, F.H.W., Donald, A.D., 1980. Farm-Management and Systems of Helminth Control. Veterinary Parasitology 6, 105-134.

Morrison, A.A., Mitchell, S., Mearns, R., Richards, I., Matthews, J.B., Bartley, D.J., 2014. Phenotypic and genotypic analysis of benzimidazole resistance in the ovine parasite Nematodirus battus. Veterinary Research 45, 116.

Nilyanimit, P., Chansaenroj, J., Poomipak, W., Praianantathavorn, K., Payungporn, S., Poovorawan, Y., 2018. Comparison of Four Human Papillomavirus Genotyping Methods: Next-generation Sequencing, INNO-LiPA, Electrochemical DNA Chip, and Nested-PCR. Annals of Laboratory Medicine 38, 139-146.

Obendorf, D.L., Nicholls, J., Koen, T., Lacy, E., 1991. Benzimidazole resistant Nematodirus species in Tasmania. Australian Veterinary Journal 68, 72-73.

Oliver, A., Pomroy, W.E., Leathwick, D.M., 2016. Benzimidazole resistance in Nematodirus spathiger and N. filicollis in New Zealand. New Zealand Veterinary Journal 64, 201-206.

Ramunke, S., Melville, L., Rinaldi, L., Hertzberg, H., de Waal, T., von Samson-Himmelstjerna, G., Cringoli, G., Mavrot, F., Skuce, P., Krucken, J., Demeler, J., 2016. Benzimidazole resistance survey for Haemonchus, Teladorsagia and Trichostrongylus in three European countries using pyrosequencing including the development of new assays for Trichostrongylus. International Journal of Parasitology: Drugs and Drug Resistance 6, 230-240.

Redman, E., Whitelaw, F., Tait, A., Burgess, C., Bartley, Y., Skuce, P.J., Jackson, F., Gilleard, J.S., 2015. The emergence of resistance to the benzimidazole anthlemintics in parasitic nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Neglected Tropical Diseases 9, e0003494.

Sargison, N.D., Wilson, D.J., Scott, P.R., 2012. Observations on the epidemiology of autumn nematodirosis in weaned lambs in a Scottish sheep flock. Veterinary Record 170, 391-347.

Silvestre, A., Cabaret, J., 2002. Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? Molecular and Biochemical Parasitology 120, 297-300.

Silvestre, A., Sauve, C., Cortet, J., Cabaret, J., 2009. Contrasting genetic structures of two parasitic nematodes, determined on the basis of neutral microsatellite markers and selected anthelmintic resistance markers. Molecular Ecology 18, 5086-5100.

Skuce, P., Stenhouse, L., Jackson, F., Hypsa, V., Gilleard, J., 2010. Benzimidazole resistance allele haplotype diversity in United Kingdom isolates of Teladorsagia circumcincta supports a hypothesis of multiple origins of resistance by recurrent mutation. International Journal for Parasitology 40, 1247-1255.

Stamp, J.T., Dunn, A.M., 1955. Lamb Losses from Worms. *Nematodirus* Infestation. Scottish Agriculture 34, 213-215.

Suarez, V.H., Cristel, S.L., 2014. Risk factors for anthelmintic resistance development in cattle gastrointestinal nematodes in Argentina. Revista Brasileira Parasitologia Veterinaria 23, 129-135.

Suter, R.J., Besier, R.B., Perkins, N.R., Robertson, I.D., Chapman, H.M., 2004. Sheep-farm risk factors for ivermectin resistance in Ostertagia circumcincta in Western Australia. Preventive Veterinary Medicine 63, 257-269.

Thomas, R.J., 1959a. A comparative study of the life histories of Nematodirus battus and N. filicollis, nematode parasites of sheep. Parasitology 49, 374-386.

Thomas, R.J., 1959b. Field studies on the seasonal incidence of Nematodirus battus and N. filicollis in sheep. Parasitology 49, 387-410.

Thorburn, F., Bennett, S., Modha, S., Murdoch, D., Gunson, R., Murcia, P.R., 2015. The use of next generation sequencing in the diagnosis and typing of respiratory infections. Journal of Clinical Virology 69, 96-100.

Vadlejch, J., Kopecky, O., Kudrnacova, M., Cadkova, Z., Jankovska, I., Langrova, I., 2014. The effect of risk factors of sheep flock management practices on the development of anthelmintic resistance in the Czech Republic. Small Ruminant Research 117, 183-190.

van Dijk, J., Morgan, E.R., 2008. The influence of temperature on the development, hatching and survival of Nematodirus battus larvae. Parasitology 135, 269-283.

van Wyk, J.A., 2001. Refugia - overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort Journal of Veterinary Research 68, 55-67.

Vlassoff, A., Kettle, P.R., 1985. Register of anthelmintic resistant nematodes in New Zealand. New Zealand Veterinary Journal 33, 71-71.

von Samson-Himmelstjerna, G., Walsh, T.K., Donnan, A.A., Carriere, S., Jackson, F., Skuce, P.J., Rohn, K., Wolstenholme, A.J., 2009. Molecular detection of benzimidazole resistance in Haemonchus contortus using real-time PCR and pyrosequencing. Parasitology, 1-10.