November 2019

Student Final Report No. 61110034

Markers of Anthelmintic Resistance in Gastrointestinal Parasites of Ruminants

McIntyre, J[1]

[1]IBAHCM, MVLS, University of Glasgow, Glasgow, G61 1QH

Supervisors: Prof. Eileen Devaney, Dr. Roz Laing, Dr Mary Vickers, Dr Lis King

This is the final report of a PhD project (AHDB Beef and Lamb Ref: 61110034) that ran from October 2015 to November 2019. The work was funded by AHDB Beef and Lamb.

CONTENTS

1. Abstract

Parasitic gastroenteritis (‘scours’) is the primary production limiting disease of sheep in the UK and is a considerable welfare concern. A global problem, it is caused by nematode parasites and mixed species infections can be common. In the UK, the primary pathogen in growing lambs is *Teladorsagia circumcincta*, an abomasal parasite of small ruminants, causing severe pathology and reduced weight gain. *T. circumcincta* is expertly adapted to both the host and the farming year and control is extremely difficult. The majority of UK farmers will use anthelmintics to manage parasitic gastroenteritis. Nevertheless, anthelmintic resistance is increasing, reducing control options. One of the most commonly used anthelmintics to treat *T. circumcincta* is ivermectin.

Unfortunately, over fifty percent of farms in recent UK studies had detectable ivermectin resistance. There is a pressing need to conserve anthelmintics for future use. However, the mechanism of ivermectin resistance is unknown, and the lack of a sensitive test for ivermectin resistance limits research into resistance spread and development. Many excellent studies have investigated ivermectin resistance in nematode parasites, but mutations responsible for ivermectin resistance remain elusive. The purpose of this PhD was to perform a genome wide association study to identify regions of the *T. circumcincta* genome potentially under ivermectin selection within UK *T. circumcincta* field populations. Such regions would contain mutations which could be used as genetic markers to diagnose resistance. *T. circumcincta* eggs collected pre- and post-ivermectin treatment were cultured to the infective larval stage (L3). These L3 were sequenced using next generation sequencing techniques (ddRAD-Seq and Pool-Seq) and population genetics analyses were performed.

Multiple loci were genetically differentiated between pre- and post-ivermectin populations. However, the reference genomes used were highly fragmented and the number of loci under selection could not be established. Genes identified included those with neuronal functions, metabolic functions and regulatory genes. Many genes had associations with pharyngeal structures and chemosensory behaviour. Nevertheless, multiple copies of genes expected to be single copy were detected in both reference genomes and these may have affected read alignment and results. The work performed here provides an important basis for future studies, and has generated high quality next generation sequenced resources from two UK field populations of *T. circumcincta*.

2. Glossary

Abomasal	The abomasum is the 4th stomach of a ruminant, where protein digestion occurs. An abomasal nematode is found within the abomasum.
Allele	An allele is a variation of a particular sequence or nucleotide base within the genome. One allele may make a worm resistant to a drug, another may make it sensitive.
Anthelmintic	A drug used to treat worms, a 'wormer'.
Anthelmintic resistance	When the anthelmintic is no longer able to kill all worms. With continuing anthelmintic use, the number of worms in the population which are resistant gradually increases over time, and the level of anthelmintic resistance can be diagnosed by testing.
bp, kb, Mb	bp = Basepair, denoting a single DNA nucleotide base in the genome. kb = Kilobase (1000 bases), Mb = Megabase (one million bases).
BZ	Benzimidazole, the 1-BZ anthelmintic group (white drench).
ddRAD-Seq	Sequencing of a small fraction of the genome, 'double digest Restriction-site associated DNA sequencing'. DNA is digested using enzymes, which cut at specific places. DNA fragments are selected by size and sequenced.
DNA sequencing	An organism's DNA is the genetic code which provides the information to produce all proteins and molecules required for life. Sequencing determines the order of the bases within the DNA, providing information about that individual. By comparing sequences of DNA between individuals or populations, scientists can identify mutations – differences between individuals, which may produce changes and resistance to drugs.
epg	Eggs per gram of faeces
FEC	Faecal egg count
F_{ST}	A statistical measure, known as a fixation index. It describes how fixed alleles are between two samples compared with the overall population. If sample one has only Allele A, and sample two has only Allele B, then F_{ST} = 1. Each allele is 'fixed' within it's sample. However, if each sample has equal proportions of alleles A and B then F_{ST} = 0. In reality, for worm populations F_{ST} values are often much smaller than 1, as individuals are highly diverse, and a mixture of alleles exists within each sample population.
Gene	A short DNA sequence within the genome which encodes information for an individual protein, or other molecule.
GWAS	Genome wide association study. A study which considers the entire genome, comparing between samples or individuals to identify regions of the genome which may be associated with a particular trait e.g. anthelmintic

	resistance. In this PhD a GWAS was performed to identify regions of a worm genome important in ivermectin resistance.
IVM	Ivermectin, a 3-ML anthelmintic (clear drench).
L1, L2, L3, L4, L5	Larval stages of strongyle worms. L1-L3 are free-living, L3 is the infective stage. L4 and L5 are within the host.
LEV	Levamisole, the 2-LV anthelmintic (yellow drench).
Nematode	A roundworm
MOX	Moxidectin, a 3-ML anthelmintic (clear drench).
PGE	Parasitic gastroenteritis (‘scours’)
Pool-Seq	Sequencing pools of individuals, as opposed to sequencing individuals separately. The DNA sequenced provides information about the alleles present within the sample population as a whole, but alleles belonging to specific individuals cannot be identified.
PPR	Peri-parturient rise in strongyle faecal egg output.
SNP	Single nucleotide polymorphism. A mutation within the DNA of a single nucleotide base. The mutation can be ‘non-synonymous’, which means that it introduces a change in a protein.
Strongyle	Trichostrongylid species of nematodes. A ‘Strongyle FEC’ describes those eggs of a typical ‘strongyle’ appearance and includes all trichostrongylid gastrointestinal nematodes apart from *Nematodirus*. The primary strongyle pathogen in growing lambs in the UK is *Teladorsagia circumcincta*.

3. Introduction

3.1. Parasitic Gastroenteritis

The Agricultural and Horticultural Development Board (AHDB) Beef and Lamb estimated that parasitic gastroenteritis (PGE) reduced the price per lamb by £10 (Lovatt and Stubbings, 2018). This severe production limiting disease is also known as 'scours'. PGE has consistently been the commonest cause of death diagnosed by the Animal and Plant Health Agency (APHA) in both sheep and goats (APHA, 2018). With both clinical and sub-clinical presentations, PGE manifests with various clinical signs, including: poor weight gain, anorexia, diarrhoea, low milk yield, pallor and death (Durham and Elliott, 1975). PGE is caused by a wide range of parasites including *Teladorsagia circumcincta*, the 'small brown stomach worm', responsible for Teladorsagiosis, and the commonest cause of PGE in growing lambs over the summer in the UK (Burgess et al., 2012; Mitchell, 2016). Although the presentation varies, PGE is considered ubiquitous in sheep farming, and is a worldwide concern of farmers (Kaplan and Vidyashankar, 2012).

Collectively, the nematodes (roundworms) which cause PGE are known as 'strongyles'. All, apart from *Nematodirus*, produce eggs of a similar appearance, and would be considered together as part of a 'strongyle' faecal egg count (FEC). Each of these strongyle species have slight differences in their lifecycles affecting the timing, presentation, management and severity of PGE. Farm management practices can impact upon which species survive and thrive on a holding.

3.2. *Teladorsagia circumcincta*, the 'brown stomach worm'

T. circumcincta (previously known as *Ostertagia circumcincta*) is an abomasal trichostrongylid nematode of sheep and goats. *T. circumcincta* is ubiquitous on UK sheep farms, and is a particular problem for UK farmers as it is expertly adapted to both the sheep host and the farming year.

The lifecycle of *T. circumcincta* is shown in Figure 3.1. Development from the egg to the infective L3 larval stage can take between 14 days to 10 weeks dependent on weather conditions (Gibson and Everett, 1972). The parasite is present within all sheep on a holding, but the egg output attributable to *T. circumcincta* is very low in non-pregnant adult sheep. Nevertheless, in late pregnancy, immunity against *T. circumcincta* and other strongyles (excluding *Nematodirus battus*) is reduced. Egg output from all strongyles, but especially *T. circumcincta,* increases dramatically (Gibson, 1973; Jansen, 1987). This phenomenon is known as the peri-parturient rise (PPR) and is key to the maintenance of disease-inducing and production-limiting levels of *T. circumcincta* on farm (Coop et al., 1982). Following lambing, the PPR continues for six weeks, before the ewe's immune system reasserts itself leading to a reduction in egg output (Gibson, 1973).

Lambs initially eat very little grass, but over time begin to ingest greater quantities, increasing their intake of infective L3 from the pasture. Due to the nature of the PPR, and the time taken for eggs to develop to L3 in the UK spring, pasture infectivity is usually high at the time at which lambs begin feeding on grass in earnest (Gibbs, 1986). Lambs take between four to six months to develop immunity to *T. circumcincta* (Smith et al., 1985), and during this time they greatly multiply the number of larvae residing on pasture. This lack of immunity towards parasites causing PGE severely impacts on lamb growth rate if PGE is not appropriately managed within the flock (Coop et al., 1982).

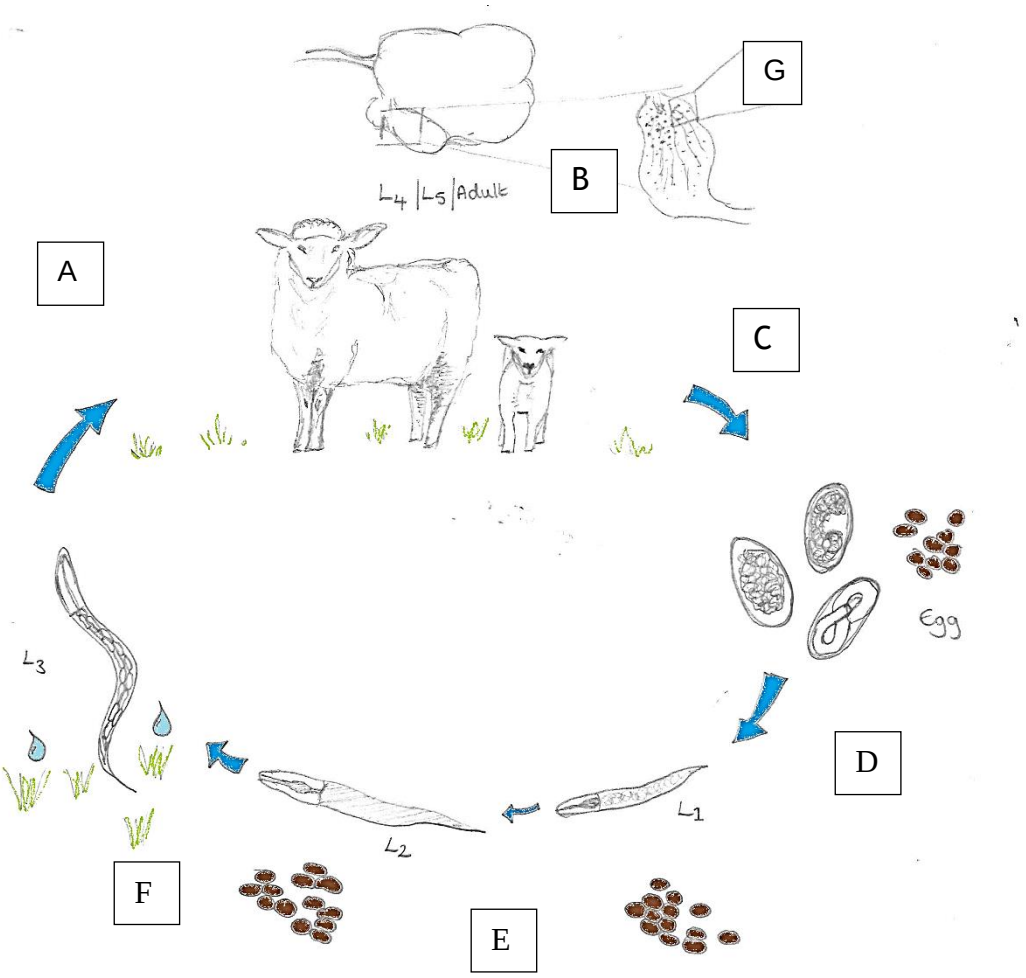

Figure 3.1: *Teladorsagia circumcincta* lifecycle as an example of a typical Trichostrongylid lifecycle. A. An infective L3 on herbage is ingested. B. The L3 exsheaths within the rumen (Bekelaar et al., 2018) and enters the gastric glands of the abomasum (Sommerville, 1953). Here it moults twice and emerges as an L5 before reaching patency as an adult. C. Following patency (typically estimated to take 17-21 days, although 14 days has been observed for *T. circumcincta*), eggs are shed into the faeces (Barrett et al., 1998). D. *T. circumcincta* eggs develop at temperatures above 4 °C (Crofton, 1965). E. The L1 larvae hatch and moult to L2, usually within the space of a few days. F. Following climatic cues, the infective L3 emerges from the faecal pat onto herbage (Gibson and Everett, 1972). Here it may move up the sward, or down into the soil dependent on local environmental conditions (van Dijk and Morgan, 2011). G. Close up of an opened abomasum, illustrating the mucosal folds (lines), with the multi-papillary raised lesions associated with an infection of *T. circumcincta*. These lesions are due to the presence of maturing L4 within the gastric glands, predominantly within the fundic region. Not all L4 will mature – some will be suppressed by the host's immune response, others will become hypobiotic overwinter and re-emerge in spring.

3.3. Anthelmintics and anthelmintic resistance

Management options for PGE vary, and not all practices will be available to all farmers. Almost all farmers rely to a greater or lesser extent on anthelmintics ('wormers') to treat PGE (Burgess et al., 2012). There are five anthelmintic classes which can be used to treat *T. circumcincta* in sheep in the UK (Table 3.1) (Abbott et al., 2012). The earliest modern anthelmintic class introduced in 1961 was the benzimidazole class (1-BZ), however these drugs now have poor efficacy against *T. circumcincta* on most farms due to widespread resistance. The second class of anthelmintic is the imidazothiazoles (2-LV), of which only the anthelmintic levamisole (LEV) is licensed for use in UK sheep.

The third class are the macrocyclic lactones (3-ML), which consist of two sub-classes: avermectins and milbemycin oximes. Avermectins include ivermectin (IVM), doramectin, eprinomectin and abamectin, the latter only sold as a combined product with derquantel. IVM is increasingly used by sheep farmers in the UK specifically to treat PGE in lambs, with moxidectin (MOX), a milbemycin oxime, also commonly used at the time of the PPR (Burgess et al., 2012). MOX is marketed in both 0.1% oral and 1% injectable formulations with five weeks persistency against re-infection with *T. circumcincta*, and a 2% injectable formulation with over 13 weeks persistency (Kerboeuf et al., 1995; Abbott et al., 2012). Macrocyclic lactones are also used to treat the sheep scab ectoparasite, *Psoroptes ovis*, both prophylactically and reactively, simultaneously affecting the strongyle population within the sheep (Doherty et al., 2018). In addition, although they do not treat liver fluke, 3-ML anthelmintics can be purchased as a combination product with a flukicide.

In 2010, monepantel (MPTL) (an amino-acetonitrile derivative, class 4-AD), was released onto the UK market (Hosking et al., 2009; Bartley et al., 2019). In 2011, derquantel (a spiroindole, class 5-SI) was released as a combined product with abamectin (Little et al., 2011). Farmers vary in their use of anthelmintics, both in frequency of dosing and choice of anthelmintic used for sheep by age, and time of year (Burgess et al., 2012).

Advice on management of PGE is provided to farmers by the knowledge transfer group, 'Sustainable Control of Parasites in Sheep' (SCOPS) and the levy board AHDB Beef and Lamb. In addition, other levy boards and organisations provide guidance and veterinary surgeons can assist with flock health planning on a farm-by-farm basis. Research has assessed both the uptake and effect of the SCOPS recommendations. SCOPS guidelines are followed by some farmers, however a significant proportion of farmers still use traditional practices (McMahon et al., 2013b). One study compared the effect of using SCOPS guidelines to treat sheep with anthelmintics, to traditional treatment management practices. No significant difference was noted in worm burden or lamb weight gain over three years between the farms monitored, however SCOPS farmers used significantly less anthelmintic than traditional farmers (Learmount et al., 2016).

Table 3.1: Anthelmintic classes licensed to treat *T. circumcincta* in sheep in the UK.

Anthelmintic class	Anthelmintics licensed in the UK for sheep	Date first introduced	First reports of resistance
1-BZ (benzimidazoles, 'white drenches')	Albendazole, fenbendazole, mebendazole, oxfendazole and ricobendazole	1961 (thiabendazole, no longer used to treat sheep) (Gordon, 1961)	(Drudge et al., 1964; Smeal et al., 1968; Britt, 1982)
2-LEV (imidazothiazoles, 'yellow drench')	Levamisole	c.1970 (Thienpont et al., 1966; Kaplan, 2004)	(Sangster et al., 1979; Hong et al., 1994, 1996)
3-ML (macrocyclic lactones, 'clear drenches', also injectable forms); sub-class avermectins	Ivermectin, doramectin, eprinomectin and abamectin	1981(Chabala et al., 1980; Campbell et al., 1983)	(van Wyk and Malan, 1988; Jackson et al., 1992a)
3-ML (macrocyclic lactones, 'clear drenches', also injectable forms); sub-class milbemycin oximes	Moxidectin	1991 (Kerboeuf et al., 1995)	(Sargison et al., 2005; Sargison et al., 2010)
4-AD (amino-acetonitrile derivative, orange)	Monepantel	2009 (2010 in UK) (Hosking et al., 2009; Kaminsky et al., 2009; Abbott et al., 2012)	(Scott et al., 2013; Van den Brom et al., 2015; Hamer et al., 2018)
5-SI (spiroindole, purple)	Derquantel (only available as a combination product with abamectin)	2011 (Little et al., 2011)	(Lamb et al., 2017)[1]

The anthelmintics available within each class are shown, and an indication of the first introduction of the anthelmintic class into the UK is provided, with early reports of anthelmintic resistance both worldwide and in the UK. [1]This may be due to the presence of *H. contortus* L4 achieving patency, rather than adult resistance and the authors advised further follow-up work was required to fully diagnose resistance.

Anthelmintic resistance is defined as the ability of a worm to survive concentrations of an anthelmintic which are higher than that which might be expected to kill the worm, or which some individuals within a normal population, with natural tolerance towards the anthelmintic might be expected to survive (Le Jambre et al., 1976). On farm, and also in many research studies, anthelmintic efficacy is tested using a faecal egg count reduction test (FECRT) (Coles et al., 2006). This involves the collection of faeces from a group of animals both before and after treatment with an anthelmintic. FECs are performed at each sampling time point and the percentage reduction in egg output is calculated (Coles et al., 2006). Anthelmintic resistance is generally considered when tests show that the anthelmintic is less than 95% effective, with the 95% lower confidence interval calculated at 90% reduction in egg output or less (Coles et al., 1992). If only one of these two conditions is met, resistance is suspected but cannot be confirmed (Coles et al., 1992).

The prevalence of anthelmintic resistance is increasing, with resistance detected to 1-BZ, 2-LV, 3-ML and 4-AD anthelmintic classes within the UK (Table 3.1 and Table 3.2). Multi-drug resistant populations have been increasingly reported, following the first European diagnosis in Scotland in 2001 (Sargison et al., 2001; Sargison et al., 2010; Hybu Cig Cymru, 2015). Worryingly, despite this, few farmers appear to regularly test for anthelmintic efficacy (Morgan et al., 2012). Importantly, the presence of anthelmintic resistance restricts the ability of farmers to treat sheep affected by PGE, and limits their control over pasture contamination with larvae. Although a FECRT can indicate the presence of anthelmintic resistance it is not the most sensitive test, and necessitates the use of the anthelmintic on the holding. For the 1-BZ class of anthelmintics, resistance will only be detected by FECRT once 25% of worms on the holding are resistant (Martin et al., 1989). To better understand how different management practices affect the development of anthelmintic resistance within a flock, more sensitive and specific tests are desirable. These will also enable a better understanding of how the worm population *in refugia* (worms not exposed to drug, e.g. on pasture, or in untreated sheep), may assist in maintaining a susceptible population on the farm. The hope is that *refugia* populations may limit the development of resistance. If they do, then management practices which encourage and exploit *refugia* populations would be highly beneficial to sustainable sheep production in the UK.

Table 3.2: Prevalence of anthelmintic resistance on farms in the British Isles.

Study	Location	Prevalence (percentage) of Anthelmintic Resistance (Total number of farms tested)					Method used
		BZ	LEV	IVM	MOX	MON	
(Cawthorne and Cheong, 1984)	SE England	13.5% (52)	0% (52)	ND	ND	ND	FECRT[1]
(Grimshaw et al., 1994)	S England	100% (5)	40% (5)	0% (5)	ND	ND	FECRT
(Hong et al., 1996)	England and Wales	32.6% (138)	1 farm (total tested unclear)	0% (26)	ND	ND	FECRT/EHT/LDT/ Lab sheep infected[1]
(Bartley et al., 2003)	Scotland	64.4% (90)	ND	ND	ND	ND	EHT
(Bartley et al., 2006)	Scotland	ND	ND	35.3% (17)	ND	ND	FECRT
(Fraser et al., 2006)	SW England	28% (90)[2]	?	?	?	ND	Survey
(Mitchell et al., 2010)	Wales	56.3% (122)	36% (122)	ND	ND	ND	LDT
(Burgess et al., 2012)	UK	17.5% (118)	3.4%(118)	ND	ND	ND	Survey
(Good et al., 2012)	Ireland	88.2% (17)	38.9% (18)	ND[3]	ND	ND	FECRT
(McMahon et al., 2013a)	N Ireland	81% (26)	14% (7)	50% (14)	62% (21)	0% (3)	FECRT[4]
(Keane et al., 2014)	Ireland	70% (155)	48% (82)	24% (132)		ND	FECRT
(Hybu Cig Cymru, 2015)	Wales	94% (47)	68% (47)	51% (47)	19% (47)	ND	FECRT
(Kenyon et al., 2016)	S Scotland	100% (4)	75% (4)	100% (4)	100% (1)	ND	FECRT (Mini-FLOTAC)
(Glover et al., 2017)	SW England	96% (25)	60% (25)	67% (27)	ND	ND	FECRT
(Keegan et al., 2017)	Ireland	68.5% (550)[5]	48.1% (316)[5]	37.5% (405)[5,6]	16% (163)[5]	ND	FECRT

For each study included, data is shown in terms of the percentage of farms with detectable anthelmintic resistance, as defined by the study authors. For each anthelmintic, the percentage of farms with resistance is provided, in addition to the total number of farms tested or surveyed in the study. The method used to determine prevalence is provided. In some studies multiple methods were used, and not all are provided here. [1]Note post-drench check at day 7, potential for under-estimation. [2]Two tests used combination products. [3]Suspected by Larval development test. [4]Excluded combination product results. [5]Number of tests, not farms. [6]Avermectins. ND = Not done. FECRT = Faecal egg count reduction test. EHT = Egg hatch test. LDT = Larval development test.

3.4. Ivermectin

IVM is part of the 3-ML class of anthelmintics. One of the most common anthelmintics in use by farmers, IVM is increasingly used in both the UK and Ireland (Burgess et al., 2012; Keane et al., 2014). There is a considerable prevalence of IVM resistance worldwide (Kaplan and Vidyashankar, 2012), and in the UK over 50% of farms tested had detectable IVM resistance in recent studies (McMahon et al., 2013a; Hybu Cig Cymru, 2015).

IVM induces a flaccid paralysis in the worm (Arena et al., 1995), leading to expulsion from the host. It is thought to act via neurological receptors called 'glutamate gated chloride channels' (GluCl) (Cully et al., 1994). IVM has also been shown to interact with other neurological receptors, including GABA receptors (Holden-Dye and Walker, 1990). Using a model non-parasitic nematode (*Caenorhabditis elegans*), researchers showed that simultaeneous mutations in three GluCl genes, which encode these receptors, conferred high level resistance (4000 fold) to IVM compared to non-mutated, wildtype worms (Dent et al., 2000). IVM is known to inhibit feeding (Gill et al., 1995; Bartley et al., 2009) and these genes were expressed in the pharynx of *C. elegans*, the worm's feeding organ. However, when investigating GluCls in *Haemonchus contortus* (Blackhall et al., 1998; Rezansoff et al., 2016), *T. circumcincta* (Martinez-Valladares et al., 2012) or the cattle parasites *Cooperia oncophora* and *Ostertagia ostertagi* (Njue and Prichard, 2004; El-Abdellati et al., 2011), researchers did not find conclusive evidence that these genes are involved in IVM resistance in ruminant parasites.

IVM also interacts with proteins called P-glycoproteins (P-gps) (Godoy et al., 2015; Janssen et al., 2015; Godoy et al., 2016; Mani et al., 2016). These proteins are important in removing drugs from cells, and changes in these proteins can confer multi-drug resistance in cancer patients (Juliano and Ling, 1976; Lespine et al., 2012). Studies of *T. circumcincta* P-gps have identified three genes in this gene family to potentially contribute to IVM resistance (Dicker et al., 2011; Choi et al., 2017; Turnbull et al., 2018). However, these studies have compared multi-drug resistant populations with drug sensitive populations. As such, differences identified may be related to inherent differences between populations or multi-drug resistant mechanisms rather than IVM specific differences. Other genes which may be important in IVM resistance include those involved in the sensory organs of nematodes – amphidial genes (Dent et al., 2000; Urdaneta-Marquez et al., 2014; Page, 2018). Although the amphids appear important in IVM resistance in *C. elegans*, it is uncertain whether similar mechanisms are associated with IVM resistance in parasitic nematodes as studies investigating these sensory organs and genes in *H. contortus* have produced inconsistent results (Urdaneta-Marquez et al., 2014; Laing et al., 2016).

Therefore, although many excellent studies have been performed, the genetic basis of IVM resistance in parasitic nematodes remains unknown. As such, no genetic test is available which

could sensitively detect IVM resistance in a field poplation. In contrast, genetic tests are available for 1-BZ anthelmintics, based on mutations in the DNA sequence known as 'single nucleotide polymorphisms' (SNPs) which produce changes in the protein sequence of the drug target. The focus of this PhD was to identify genetic markers (SNPs) which could be used to test for IVM resistance in *T. circumcincta* UK field populations.

3.5. Genome wide ssociation studies – identifying novel genes involved in resistance

The studies referred to above have investigated single genes or gene families. A gene is a short stretch of DNA which contains the information required to produce a protein. Mutations in different parts of the gene can lead to changes in the protein structure and its ability to perform its role in the worm's body. In addition, other mutations, outside of a gene, can produce changes in nearby genes. These changes are likely to be missed in single gene studies.

A different type of study, known as a Genome wide association study (GWAS), has the power to investigate the entire genome of a worm. These studies compare between samples or individuals, to identify differences, such as SNPs, anywhere in the genome. As such they are unbiased, and allow novel mutations, genes and gene families related to IVM resistance to be identified. In 2013, a GWAS investigating the cause of oxamniquine resistance in the human fluke parasite *Schistosoma mansoni* successfully identified the gene involved in resistance (Valentim et al., 2013). As part of the study the authors used next generation sequencing techniques to sequence the fluke genomes. Next generation sequencing techniques enable the DNA code to be rapidly determined, and SNPs identified. Valentim et al. identified regions containing several genes potentially under selection by oxamniquine. From the genes identified, with specific knowledge of how resistant flukes differed biochemically from susceptible flukes, they were able to identify the gene conferring oxamniquine resistance.

More recently, UK studies using next generation sequencing have performed GWAS to investigate IVM resistance in *H. contortus* (Doyle et al., 2019) and triclabendazole resistance in the liver fluke, *Fasciola hepatica* (Hodgkinson et al., 2013; Hodgkinson et al., 2018). In *H. contortus*, IVM resistance has been associated with a large region of the worm's genome, on Chromosome V, containing hundreds of genes (Doyle et al., 2019). This work was performed using a genetic cross between susceptible and resistant *H. contortus* lab isolates (Redman et al., 2012). Ongoing work, including the sequencing of outbred *H. contortus* field populations, is reducing the number of genes within this region which may be associated with IVM resistance (Laing, *pers comm*). This PhD performed a GWAS to identify genetic markers of IVM resistance in UK field populations of *T. circumcincta*. These markers could be the cause of IVM resistance, or simply closely associated with IVM resistance conferring mutations.

4. Aims of the PhD

4.1. Locate a suitable *T. circumcincta* UK field population which could be used to identify markers of IVM resistance

Farms were tested for IVM resistant *T. circumcincta*, with samples sourced from across the UK. As part of this work, one farm was closely monitored over a year. During this time current tests of anthelmintic efficacy were compared for suitability, and results are reported here. It was important that the field populations chosen for sequencing were not fully susceptible or fully resistant to IVM. This was because the identification of markers of IVM resistance in the worm's genome relied upon identifying differences in allele frequency between a pre- and a post-IVM population.

4.2. Validation of a reduced representation sequencing method to identify genetic markers of IVM resistance

The genome of *T. circumcincta* is very large. Sequencing the entire genome of a worm is therefore expensive. DNA is sequenced in many short stretches ('reads') and once sequenced, these reads must be aligned to a 'reference genome', to identify where they belong within the genome. A reference genome is one which has been assembled, using computer software, to represent the real genome of a worm. The only reference genome of *T. circumcincta* available at the commencement of this PhD was 700 Mb long, and was in over 80,000 'contigs' (fragments), as opposed to six chromosomes. Such a genome makes analysis challenging because DNA reads will not align well to the genome, and the true position of contigs relative to one another within the genome is unknown.

'double digest Restriction-site Associated DNA sequencing' (ddRAD-Seq), uses enzymes to cut up a worm's genome into lots of small fragments (Peterson et al., 2012). Fragments are then size selected and sequenced. It is expected that the same genome fragments will be selected from each individual allowing a comparative analysis. ddRAD-Seq sequences individual worms to identify SNPs which could be linked with IVM resistance, and used as diagnostic markers. Due to the nature of the technique, less than 1% of the genome is sequenced, which helps to keep costs low. ddRAD-Seq was trialled and validated to identify markers of IVM resistance.

4.3. Validation of a whole genome re-sequencing method to identify genetic markers of IVM resistance.

Since the start of this PhD, an improved draft reference genome, hereafter referred to as the 'Tci2 genome' was assembled by Dr S. Doyle at the Wellcome Sanger Institute. This reference genome was still 685 Mb long, but was in only 8000 contigs. In addition, sequencing costs have been

reducing and recent studies used a technique called ‘Pool-Seq’. Pool-Seq sequences the entire genome, however a large pool of worms are sequenced at the same time, so each individual worm is sequenced very little. Sequencing the same number of individuals separately would be prohibitively expensive. Pool-Seq enables the frequency of each allele within the population as a whole to be estimated, and includes the entire genome during sequencing. A Pool-Seq technique to identify genetic markers of IVM resistance, for use as a diagnostic test of resistance, was validated.

5. Materials and methods

Note that full details for all methods are available in McIntyre, J., 2019 (a PhD thesis held by the University of Glasgow).

5.1. Parasite material

5.1.1. Farm 1

A lowland sheep farm in southeast Scotland was visited between March 2016 and March 2017, every three weeks, with the agreement of the shepherd and knowledge of the presence of anthelmintic resistance. Ethical consent was obtained under the guidance of the University of Glasgow. Farm 1 was used to assess current methods available for monitoring anthelmintic resistance.

5.1.2. Farm 2

A Faecal egg count reduction test (FECRT) was performed on a farm in southwest England in August 2016, coordinated by colleagues from the University of Bristol (Prof E. Morgan, Dr H. Vineer and Miss K. Bull), who provided coprocultured L3 pre- and post-IVM treatment. Farm 2 was used for both ddRAD-Seq and Pool-Seq.

5.1.3. Farm 3

A FECRT was performed on a farm in southeast Scotland in July 2015, coordinated by colleagues at the Moredun Research Institute and coprocultured L3 were provided for use in this study. Farm 3 was used for Pool-Seq.

5.2. Sample Collection and Processing

5.2.1. Collection of faecal samples

The strongyle population on Farm 1 was monitored over time, in both ewes and lambs. Starting in March 2016, faecal samples were collected from ten ewes every three weeks until March 2017. From May 2016 until November 2016, samples were also collected from lambs; the first samples

were from only four lambs, but after this time point at least ten lambs were sampled every three weeks. Samples were collected as soon as possible after voiding by sheep, and care was taken to minimise contamination with free-living nematode species.

5.2.2. Faecal egg counts

A cuvette method, sensitive to 1 egg per gram (epg) was used to perform strongyle faecal egg counts (FECs) (Christie and Jackson, 1982) as described in (McIntyre et al., 2018). A schematic diagram is shown in Figure 5.1.

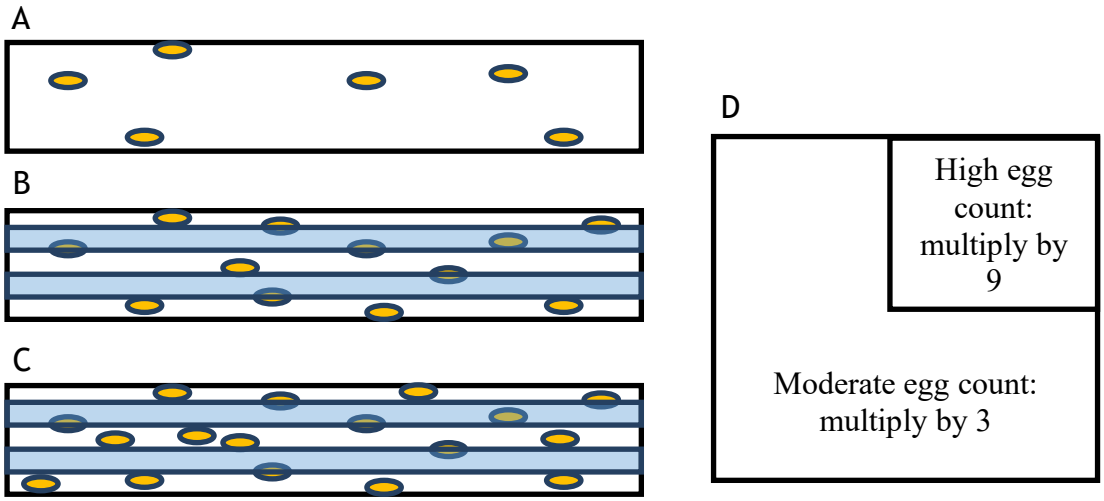

Figure 5.1: Cuvette method for FECs. Each of A, B and C represent a cuvette, containing eggs (yellow ovals). Due to the specific gravity of the NaCl solution all eggs floated to the top of the cuvette for counting (Rinaldi et al., 2011). As the equivalent of one gram of faeces was used, each total count is equivalent to the epg. D represents a Miller eyepiece graticule, which can be placed in the microscope eyepiece to enable counting of moderate to high numbers of eggs. A. Low number of eggs present – counted all eggs present within the surface area. For both moderate (e.g. > 50, B) and high (e.g. >300, C) numbers of eggs two rows (shown in blue) were counted using the graticule. Eggs were counted if they were fully within the upper and lower boundaries of the square. In addition, eggs partially covering one of either the upper or the lower boundary were also counted. For moderate egg counts (B), the larger graticule square was used and the sum of both rows multiplied by three. For high egg counts (C), the smaller graticule square was used, resulting in 'thinner' rows so that the sum of both rows was multiplied by nine.

5.2.3. Coprocultures and Baermannisation of L3

Individual faecal samples were pooled separately by age group (ewes or lambs) for culture. If faeces were too moist, vermiculite was added and faeces formed into small balls for culture at 25 °C for 10 days. Following culture, faeces were immersed in warm water for 6 h before a modified Baermannisation method was used to collect L3. Briefly, the faeces in water were poured over a fine muslin mesh, which was placed in a jar containing warm water and left for 24 h, following which the L3 could be collected from the bottom of the jar. L3 were stored in culture flasks in water at 8 °C. Spare L3 were snap frozen in liquid nitrogen and stored at -80 °C.

5.2.4. Faecal egg count reduction test

A FECRT was performed on Farm 1 in September 2016. Thirty-five, five-and-a-half month old lambs were set aside by the farmer and from these, animals were selected randomly for use in the FECRT, but with the aim of balancing gender. Faeces were collected *per rectum* for FECs pre-treatment, with additional material gathered for further analysis, if voided immediately following treatment. Ten lambs were allocated to the albendazole (BZ) treatment group (5 mg/kg body weight (BW), Albex™ 2.5% w/v SC oral suspension, Chanelle UK), ten lambs to the LEV treatment group (7.5 mg/kg BW, Levacide Drench 3% Oral solution, Norbrook) and twelve lambs to the IVM treatment group (0.2 mg/kg BW, Noromectin® 0.08% w/v Drench Oral Solution, Norbrook). All were weighed individually, using the shepherd's weigh scales (EID weigh crate, Shearwell), and dosed *per os* according to their individual weights using a syringe. Repeat faecal samples were collected on days 7 and 14 post-treatment and faeces transferred anaerobically to the laboratory. The FECR percentage and Bayesian CIs were calculated using R Shiny 'eggCounts' web interface (Wang and Paul, 2017; Wang et al., 2017).

5.3. Bioassays

To assess whether free-living lifecycle stages were resistant to 1-BZ or 3-ML anthelmintics, laboratory tests known as 'bioassays' were used. The 1-BZ test is the 'Egg Hatch Test' (EHT) and has been well described for laboratory populations of strongyles (Coles et al., 1992; von Samson-Himmelstjerna et al., 2009). Eggs are exposed to increasing concentrations of a BZ anthelmintic and if they are resistant, they will hatch to L1 larvae within 48 hours. Otherwise they will remain unhatched. To test IVM resistance, a 'Larval Development Test' (LDT) was used. This measures the ability of eggs to hatch and develop to the infective L3 stage within a week in the presence of IVM. A method adapted from Varady et al. (1996) was used, which is liquid based. Both bioassays are described in detail in (McIntyre et al., 2018).

5.3.1. Egg Hatch Test

EHTs were performed throughout the season on Farm 1. Drug concentrations of thiabendazole were included in triplicate and plates were incubated in a humid environment at 25 °C for 48 h. The number of unhatched eggs and L1 were recorded at 48 h. Eggs collected as part of the September BZ and IVM FECRTs on Farm 1 were tested as follows. Four EHTs were performed using pre-treatment samples for each of the BZ and IVM treated lamb groups. On day 14 post-treatment a reduced EHT was performed for the BZ treated population as follows: all thiabendazole concentrations were incorporated but a reduced quantity of eggs restricted replicate numbers. Two replicates were carried out for each of 0.05 µg/ml, 0.1 µg/ml and 0.2 µg/ml. The rest were single wells. Due to a paucity of eggs post-IVM treatment of lambs, it was not possible to perform a full EHT and only two replicates of the 'definitive dose' wells (0.1 µg/ml) were included.

The ED_{50}, the effective dose at which 50% of larvae fail to hatch, was calculated using a binomial (probit) general linear model in R Studio (version 1.1.383 – © 2009–2017 RStudio, Inc; R version 3.4.3 © 2017). The script used for the GLM is available on GitHub (https://github.com/SheepwormJM). All data were first corrected for the percentage hatch in the DMSO control wells and the thiabendazole concentrations were log_{10} transformed. If the ED_{50} was greater than 0.1 µg/ml thiabendazole, then the sample population was considered resistant to BZ.

5.3.2. Larval development test

The LDT was performed at the time of the September FECRT on Farm 1 using strongyle populations obtained pre- and post-IVM treatment (day 0 and day 14) and pre-BZ treatment. In total, two tests were performed for each FECRT pre-treatment (total four), and one test was performed post-IVM treatment. Insufficient eggs were recovered post-BZ treatment to allow a LDT to be carried out. Pre-treatment each concentration was included in triplicate in each assay, but post-IVM treatment each concentration was included only twice due to fewer eggs being available. The assay was incubated for 7 days at 25 °C in a humid environment. Eggs, L1/L2 and L3 larvae were counted. Those with obvious failure (e.g. no development, fungal or bacterial overgrowth) were discarded. Following correction for development in the control wells, an ED_{50} for each sample was calculated using a general linear model constructed in R Studio as above.

5.4. Molecular Biology work

For all samples gathered during the PhD, from all farms, individual L3 (or eggs/L1) were picked and lysates made to obtain DNA. PCRs to identify the species of each strongyle nematode were performed, adapting previously used techniques to identify individual species (Wimmer et al., 2004; Redman et al., 2008; Burgess et al., 2012) and a multiplex PCR (Bisset et al., 2014).

For Farm 1 samples, using the same lysates, pyrosequencing to identify the 1-BZ resistance associated SNPs of *T. circumcincta* individuals was performed. Samples were compared for changes over the season and pre- and post-treatment during the September FECRT. In addition, microsatellites (short DNA repeat patterns used for genetic fingerprinting) were PCR amplified and analysed to identify changes in the Farm 1 *T. circumcincta* population over time and following different management interventions.

Sequencing libraries for both the reduced representation sequencing method (ddRAD-Seq) and the whole genome re-sequencing method (Pool-Seq) were produced. DNA was sequenced at the Wellcome Sanger Institute.

5.5. Bioinformatics analysis

Detailed bioinformatics analyses were performed for each of the sequencing methods used. These analyses assessed and validated the methods. Using population genetic analyses, genetic markers of IVM resistance were identified. This was done primarily by calculating F_{ST} values for SNPs. It is expected that IVM treatment would select for certain alleles, increasing F_{ST} values. Described in this report is just a small part of these analyses. For full methods and results please see the thesis, McIntyre, J., 2019. Bioinformatics scripts are available on GitHub (https://github.com/SheepwormJM). Raw sequencing data can be accessed via ENA using accession numbers ERS1770619, ERS1770620 and ERS2487257 to ERS2487262.

6. Results

6.1. On Farm 1 the ewe strongyle FEC varied over the season.

Farm 1 was a commercial, 150 acre lowland farm in southeast Scotland with previously diagnosed anthelmintic resistance. The farm was studied from March 2016 to March 2017. The breeding flock consisted of approximately 370 Cheviot Mule ewes which were bred to Texel, Suffolk and Beltex rams. Lambs were finished on pasture before being sold at market in the autumn. Lambing began in late March, with ewes housed on straw from early January, and continued for approximately five to six weeks, with an average annual lambing percentage of 180%.

Generally, FECs over time on Farm 1 were comparatively low, remaining below 200 epg for much of the year in both ewes and lambs. Ewe FECs rose from a mean of 452.1 epg on the 20th March, to 912.0 epg three weeks later (Table 6.1, Figure 6.1). The increase was not statistically significant, likely reflecting the wide range of individual counts – the median FECs varied less, being 409.5 and 675.0 epg respectively. The majority of ewes were treated with MOX at lambing. Further ewe mean FECs remained low within the pasture group followed, ranging between mean 2.8 epg and 129.8 epg. This latter value was from a group sampled on the 7th June 2016 and was higher compared with other samples collected throughout the summer and early autumn. A rise in egg output from ewes during the autumn breeding period was also observed, rising from a mean 23.0 epg on the 10th October to 106.2 epg on the 23rd November. FECs subsequently reduced, such that in January, February and early March 2017 FECs were low, prior to rising again in late March at lambing time (Table 6.1, Figure 6.1).

6.2. On Farm 1 the lamb strongyle FEC remained low, but rose post-weaning.

Lamb faecal samples were collected from 16th May 2016, from just four lambs in the initial sampling group, when lambs would have been eight weeks old or younger. The initial mean strongyle FEC was only 11.8 epg, but the presence of *N. battus* eggs were noted in the faeces

(Table 6.2, Figure 6.1). Following this, ten lambs were sampled at each time point. Mean FECs remained low, before rising from 40.3 epg on the 8th August to 205.9 epg on the 31st August 2016 (median 30 epg rising to 157.5 epg). Between these two dates the lambs had been weaned, combined into one large group of approximately 300 lambs and moved to silage aftermath. Following this time point, lamb FECs remained higher, averaging between 151.8 and 232.5 epg until the end of November 2016, when very few lambs were left on the farm. Only one sample was low during this time period, with a mean 38.9 epg, collected on the 10th October 2016, 20 days after a LEV treatment was given (

Table 6.2, Figure 6.1).

Table 6.1: Farm 1 ewe strongyle faecal egg counts.

Date	Number of ewes sampled	Arithmetic mean strongyle epg	SD strongyle epg	Range strongyle epg	Median strongyle epg
20/03/2016	10	452.1	318.9	(2-1062)	409.5
07/04/2016	10	912.0	822.7	(60-2484)	675.0
21/04/2016	10	3.4	5.7	(0-15)	0.0
16/05/2016	9	36.4	84.9	(0-261)	3.0
07/06/2016	12	129.8	208.7	(0-711)	30.0
27/06/2016	11	9.8	11.2	(0-36)	6.0
18/07/2016	10	2.8	4.0	(0-11)	1.0
08/08/2016	10	3.7	5.3	(0-16)	1.0
01/09/2016	13	20.3	43.2	(1-162)	5.0
20/09/2016	10	3.0	3.0	(0-8)	3.0
10/10/2016	10	23.0	16.0	(1-51)	19.5
01/11/2016	10	77.0	79.9	(4-207)	32.0
23/11/2016	10	106.2	95.3	(6-270)	84.0
14/12/2016	10	109.5	91.8	(39-297)	67.5
04/01/2017	9	53.0	53.2	(12-180)	30.0
27/01/2017	10	25.3	46.6	(1-153)	8.0
26/02/2017	10	75.9	59.1	(9-195)	58.5
16/03/2017	10	432.9	446.9	(42-1512)	354.0

Samples collected on the 7th and 21st of April were collected *per rectum* as part of a moxidectin faecal egg count reduction test. Epg = eggs per gram. SD = Standard deviation.

Table 6.2: Farm 1 lamb strongyle faecal egg counts.

Date	Number of lambs sampled	Arithmetic mean strongyle epg	SD strongyle epg	Range strongyle epg	Median strongyle epg
16/05/2016	4	11.8	15.1	(0-33)	7.0
07/06/2016	11	31.5	34.0	(0-99)	24.0
27/06/2016	14	25.1	30.4	(0-99)	13.0
18/07/2016	10	5.0	8.6	(0-24)	1.0
08/08/2016	10	40.3	42.6	(0-129)	30.0
31/08/2016	14	205.9	179.4	(9-630)	157.5
12/09/2016	6	232.5	149.2	(114-504)	175.5
20/09/2016	32	172.4	158.2	(27-672)	115.5
10/10/2016	10	38.9	32.3	(3-102)	28.5
01/11/2016	10	151.8	175.9	(2-468)	64.5
23/11/2016	10	203.7	86.9	(27-330)	205.5

Samples collected on the 20th September were collected *per rectum* as part of a faecal egg count reduction test, for which post-treatment results are shown separately. Epg = eggs per gram. SD = Standard deviation.

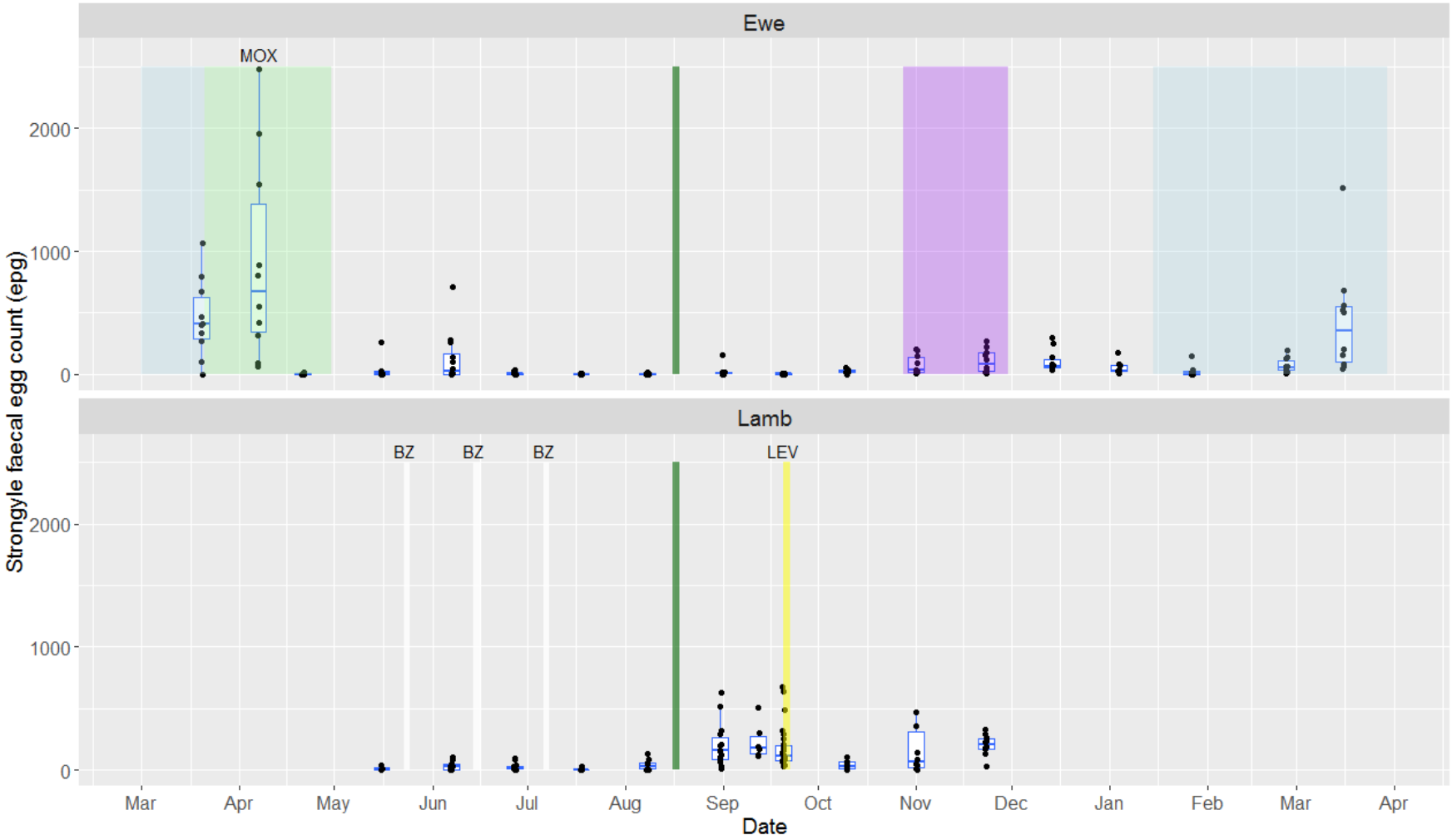

Figure 6.1: Strongyle faecal egg counts over time on Farm 1. A box and whisker plot is shown for each sampling time point. In addition, each individual's epg is plotted at each time point (dots). Key to annotations: light blue = ewes housed, light green = lambing, purple = breeding, dark green = weaning, white = albendazole (BZ) drench, yellow = LEV drench. Note, MOX indicates day zero of the MOX FECRT performed by farm veterinary surgeons. At time of LEV treatment of remaining lambs, 32 lamb FECRTs were performed (post-treatment samples shown elsewhere).

6.3. The species which made up the strongyle FEC changed over time, and were different between ewes and lambs

Species identification of L3 strongyles by PCR revealed considerable variation and diversity within the strongyle population over time and between sheep age groups (Figure 6.2). The following strongyle species were identified: *Cooperia curticei, Chabertia ovina, Oesophagostomum venulosum, N. battus, T. circumcincta, Trichostrongylus vitrinus,* and *Trichostrongylus axei* (Figure 6.2). The proportions of these nematode species varied throughout the year and were generally different between the ewes and the lambs. It is important to note here that species proportions do not necessarily reflect abundance – species proportions when egg counts are high will have a very different impact to when egg counts are low.

Generally, either *T. circumcincta* or *C. curticei* predominated. Species common in the ewe samples were later seen to predominate in the lamb samples. However, usually for a given sampling time point, the majority species were different for ewes and lambs. Indeed, *C. curticei* was not apparent in the lamb samples until mid-September, despite being detected in pre-lambing samples and accounting for over 63% of strongyles speciated in ewes in July (Figure 6.2). Taking species diversity into account, the epg attributable to highly pathogenic species, such as *T. circumcincta*, was often considerably lower than that calculated by the overall strongyle FEC (Figure 6.2).

In the ewes, *T. circumcincta* accounted for only 38 to 53% of strongyles speciated pre-lambing in 2016 (Figure 6.2). Post-MOX treatment, only four strongyle larvae were harvested post-coproculture, of which three were *T. circumcincta* and the other *N. battus*. In early June, coinciding with a small but noticeable rise in FEC, *T. circumcincta* accounted for 58% of strongyle L3 speciated. But in early July, when the FEC dropped to extremely low levels, *T. circumcincta* contributed only 4.6% in the ewe sample (Figure 6.2). Subsequent ewe FECs were low, with negligible proportions of *T. circumcincta*. Despite the rise in FEC in the autumn, the proportion of *T. circumcincta* did not increase, remaining low, and this species was not detected in a ewe sample collected in early January (Figure 6.2).

Trichostrongylus species were detected throughout the year, but were not particularly prevalent in any coproculture. Two large intestinal species, considered to be mild pathogens, were apparent over time in both the ewes and the lambs. *C. ovina* was present in the ewe samples at 29.0% and 31.4% pre-lambing and in the July lamb sample at 37.7%. Only one ewe sample did not include *C. ovina*. Lastly, *O. venulosum* was present in ewe samples pre-lambing, although interestingly did not re-appear in either ewe or lamb samples until late August/early September. However, it was a minor species, so may have been present at low levels, undetectable by the methods used in this study.

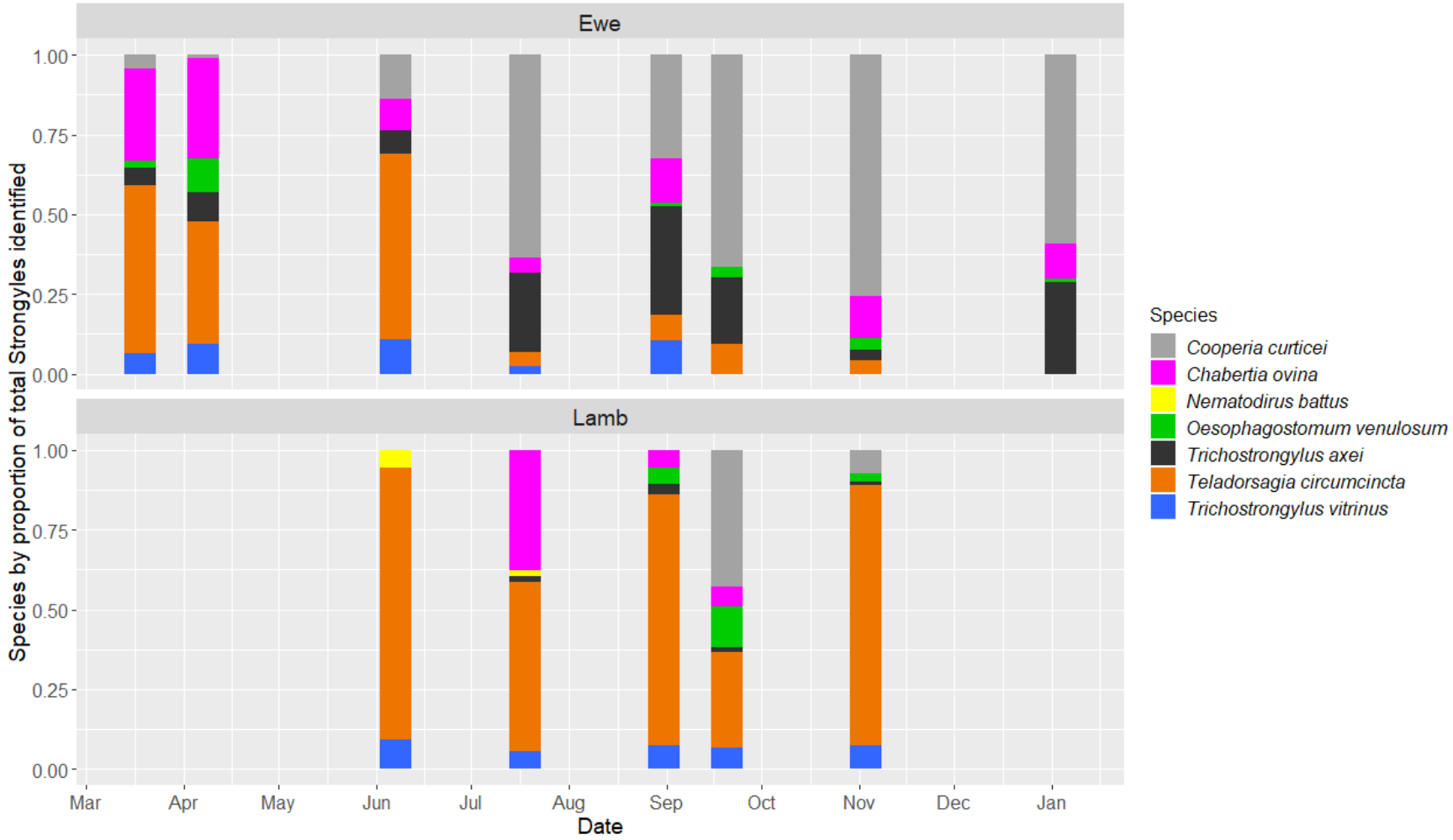

Figure 6.2: Species of cultured strongyle larvae on Farm 1 over time. From left to right the number of total strongyles speciated at each time point was; Ewes: 93, 86, 93, 88, 86, 63, 91 and 91; Lambs: 54, 53, 93, 222, 81. Note that 20th September 2016 is an average of samples cultured and speciated separately from the three lamb pre-treatment samples from the faecal egg count reduction tests. Those speciated post-treatment are shown later (Figure 6.3).

BEEF & LAMB

6.4. How useful were available tests to determine anthelmintic efficacy?

6.4.1. Faecal egg count reduction test

In September 2016, a FECRT was performed (see 5.2.4). Resistance to all three anthelmintic classes (BZ, LEV and IVM) was detected. FECs suggested an overall efficacy of 65% for albendazole (95% High Posterior Density Interval (HPD, a bayesian statistic for a confidence interval): 14.5, 86.2), 80.8% efficacy for LEV (95% HPD Interval: 58.0, 91.1) and 77% efficacy for IVM (95% HPD Interval: 45.5, 91.6).

Pre-treatment, a diverse mix of strongyle species was detected (Figure 6.2 and Figure 6.3). Post-treatment, molecular speciation was performed for albendazole and IVM at days seven and fourteen. Only *T. circumcincta* was detected post-IVM, however *T. circumcincta* and *C. curticei* were detected on day seven post-BZ, with *T. axei* and *O. venulosum* also identified on day 14 (Figure 6.3). Adjustment of the FEC to represent the proportion of larvae identified as *T. circumcincta* indicated no change in the egg output of this species following treatment by either BZ or IVM (Figure 6.4). *C. curticei* epg had fallen to 5 epg post-BZ from an initial 148 epg on day 0 (Figure 6.4). Overall, there was a significant reduction in egg output from strongyle species other than *T. circumcincta* ($p = 0.00$). Post-LEV, from the day seven sample, larvae were selectively picked under low power to obtain *T. circumcincta* individuals for other work, and too few larvae were recovered in total to also permit an un-biased speciation of the strongyle population.

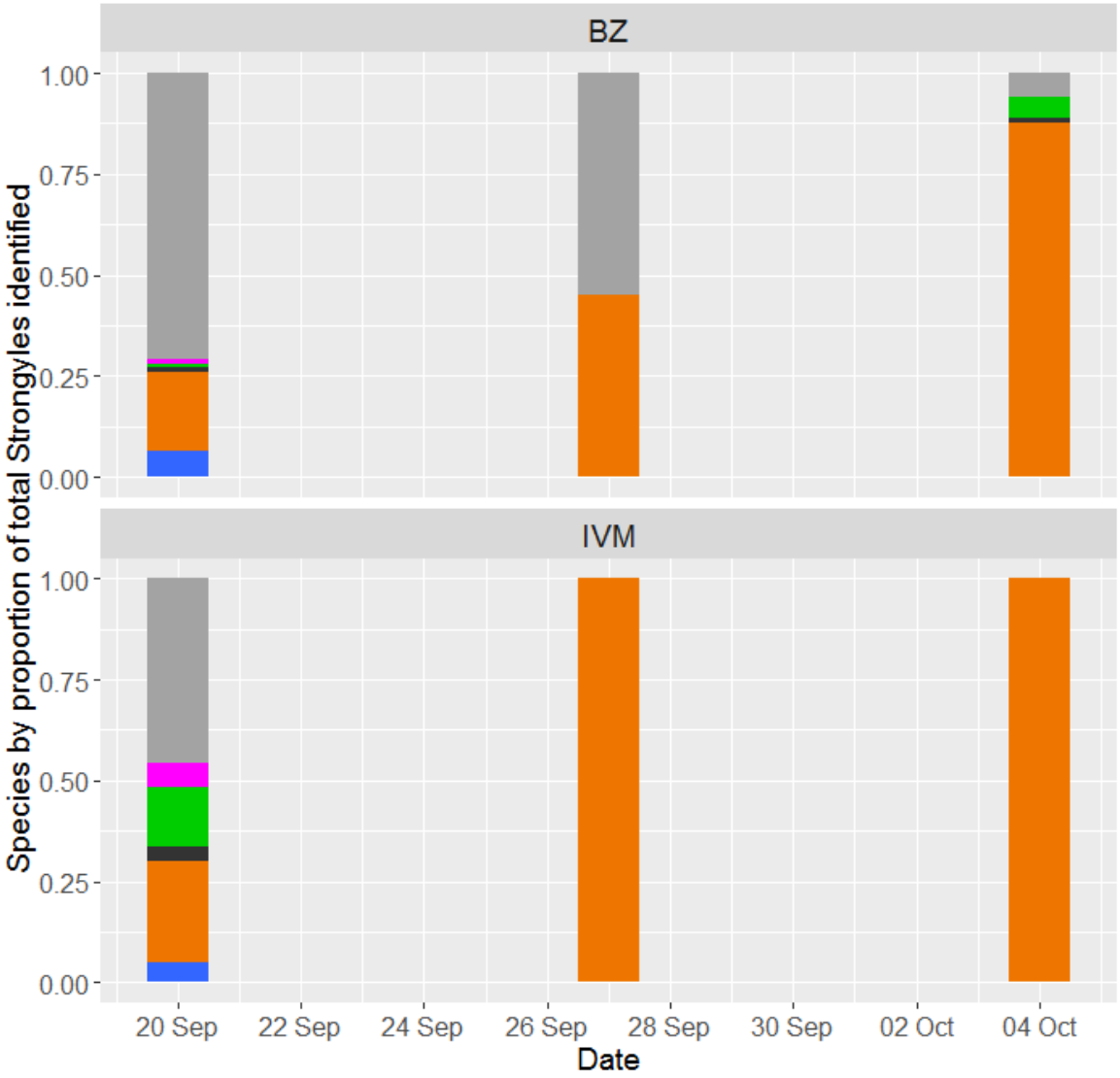

Figure 6.3: Farm 1 BZ and IVM FECRT strongyle species proportions. The proportions of strongyles as speciated by PCR of the ITS2 region are shown for each sampling time point (days 0, 7 and 14 of each FECRT). Key to colours: grey = *Cooperia curticei*, pink = *Chabertia ovina*, green = *Oesophagostomum venulosum*, black = *Trichostrongylus axei*, orange = *Teladorsagia circumcincta*, blue = *Trichostrongylus vitrinus*. From left to right the following number of total strongyles was speciated at each time point; BZ: 96, 62, 82; IVM: 83, 83, 42. L3 were used on days 0 and 7 but on day 14, individuals sampled were eggs and L1 larvae from the wells of an EHT (in equal proportions to the percentage hatch).

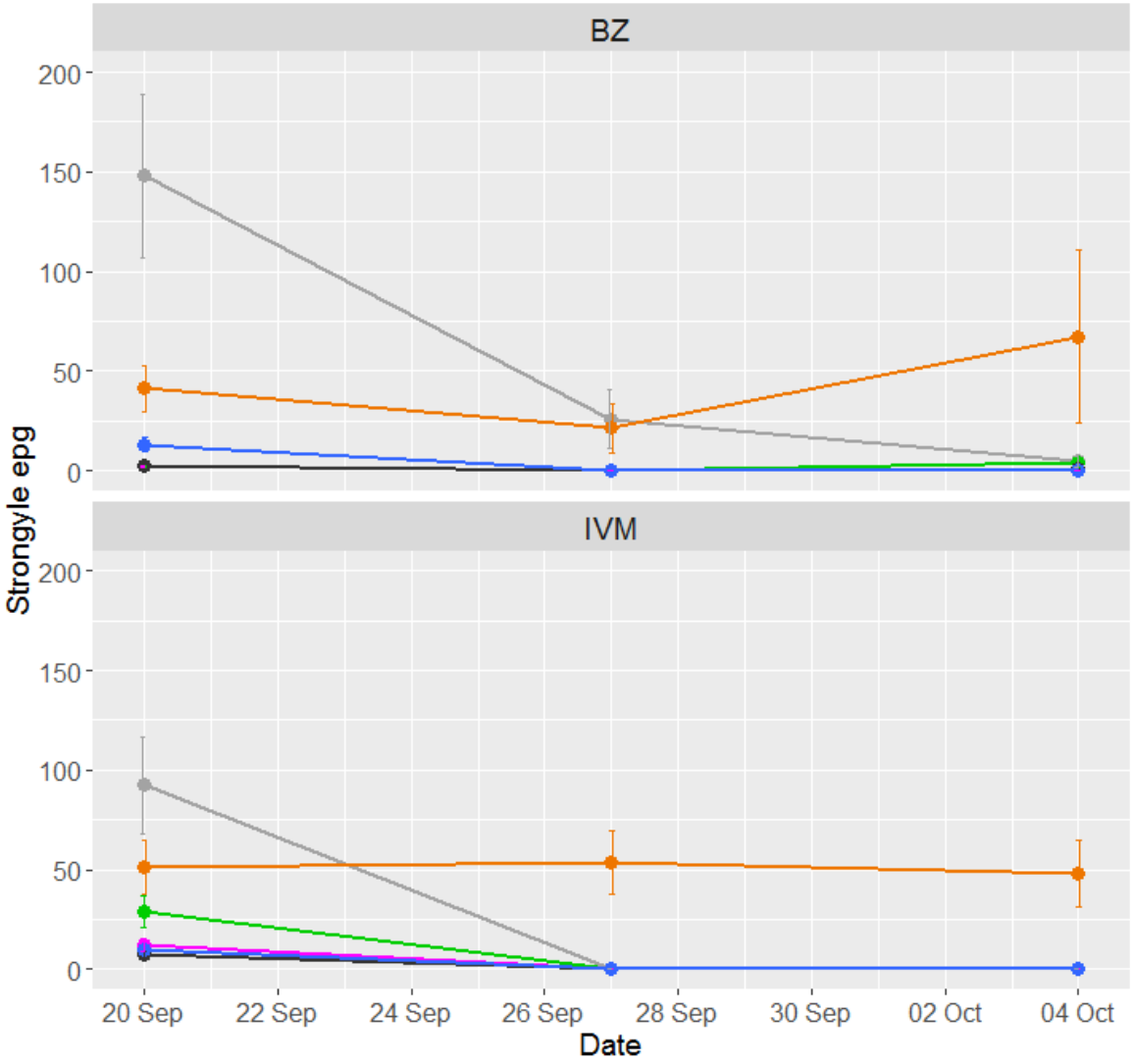

Figure 6.4: Farm 1 BZ and IVM FECRT mean strongyle epg by species proportion. Individual lamb FECs were extrapolated by L3 strongyle species proportions identified. The arithmetic mean for each species is shown, with the SEM plotted. Key to colours: grey = *Cooperia curticei*, pink = *Chabertia ovina*, green = *Oesophagostomum venulosum*, black = *Trichostrongylus axei*, orange = *Teladorsagia circumcincta*, blue = *Trichostrongylus vitrinus*. From left to right the following number of total strongyles was speciated at each time point; BZ: 96, 62, 82; IVM: 83, 83, 42. L3 were used on days 0 and 7 but on day 14, individuals sampled were eggs and L1 larvae from the wells of an EHT (in equal proportions to the percentage hatch).

6.4.2. Bioassays – determining anthelmintic efficacy using free-living stages

Anthelmintic resistance can also be detected using *in vitro* bioassays, testing sensitivity of free-living lifecycle stages to the anthelmintic (Coles et al., 2006). Samples were tested using an EHT for BZ resistance and a LDT for IVM resistance. These bioassays were used both pre- and post- the BZ and IVM FECRTs performed in September 2016 on the farm to correlate the *in vivo* phenotype observed (the reduction in egg output following treatment) with the *in vitro* phenotype of the offspring.

The ‘definitive dose’ is the drug concentration at which 99% of susceptible larvae fail to hatch in the EHT. From studies this has been identified as 0.1 µg/ml (Coles et al., 2006). Larvae hatching at this concentration could be considered resistant to BZ. After adjustment for the response in the control wells, it was determined that 39% of eggs from the pre-BZ strongyle sample hatched in the EHT at the definitive dose of 0.1 µg/ml. Post-BZ, 93% hatched (Table 6.3), giving a resistance ratio (RR) of 2.4 (% hatch post-treatment/% hatch pre-treatment). Calculating the ED_{50}, at which 50% of eggs hatched, also revealed a difference pre- and post-BZ treatment. Post-BZ, 50% hatched at a higher concentration of thiabendazole/DMSO (0.615 µg/ml) compared with pre-BZ (0.048 µg/ml) (Table 6.3, Figure 6.5), a RR of 12.8 (ED_{50} post-treatment/ED_{50} pre-treatment). The pre-IVM strongyle sample was similar to the pre-BZ sample, with an ED_{50} of 0.043 µg/ml (Table 6.3), and a percentage hatch in the 0.1 µg/ml definitive dose well of 32.6% (Table 6.3). Post-IVM, not enough material was available to perform both a full EHT and a full LDT, and so only the definitive dose well was assessed post-IVM, with no control well used. Although imperfect, this still revealed an uncorrected percentage hatch of 82.4% post-IVM (Table 6.3), indicating that offspring of IVM survivors were also BZ resistant.

Table 6.3: Egg hatch test results.

Test	EHT ED_{50} (µg/ml)[1] (95% CI)	Percentage hatch in 0.1 µg/ml[2] (SD)
Pre-BZ FECRT (four plates combined analysis)	0.048 µg/ml (0.045, 0.052)	39.1% (8.58)
Post-BZ FECRT	0.615 µg/ml (0.538, 0.730)	93.1% (0.23)
Pre-IVM FECRT (four plates combined analysis)	0.043 µg/ml (0.040, 0.046)	32.6% (6.06)
Post-IVM FECRT	NA	82.4%[3] (3.43)

Effective doses (ED_{50}) of thiabendazole required to prevent hatching of 50% of eggs are reported for each full test performed. The average percentage hatch in definitive dose wells (0.1 µg/ml) is given for each test. [1]Raw data was adjusted for response in 0.5% DMSO control wells. [2]Adjusted for response in 0.5% DMSO control wells. [3]No DMSO well was included post-IVM and only two replicate wells for 0.1 µg/ml were included. This therefore is the uncorrected percentage hatch. The uncorrected percentage hatch in the 0.1 µg/ml wells pre-IVM was 27.52% (5.0 SD).

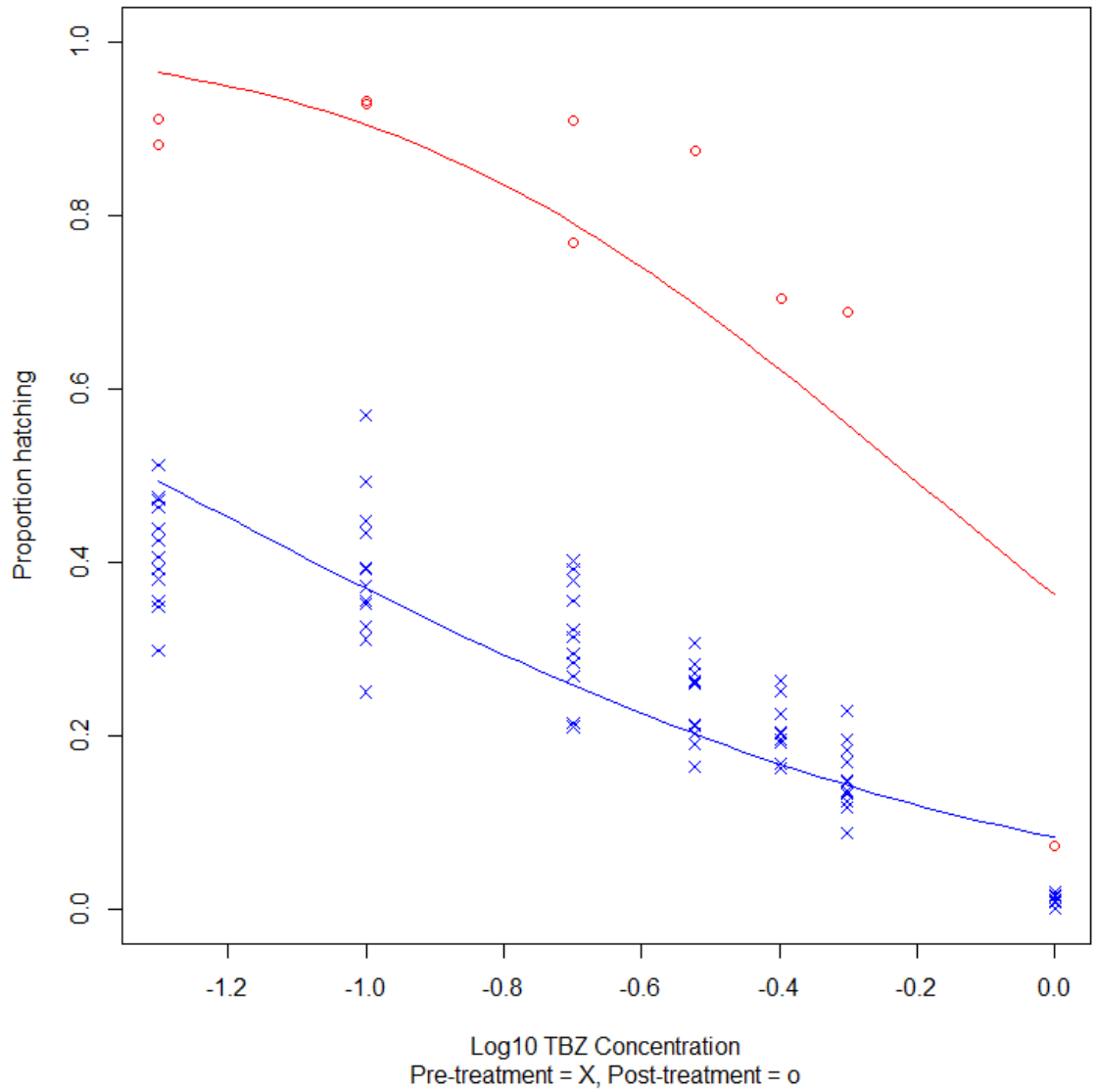

Figure 6.5: Dose response curve of the egg hatch test performed using samples from the benzimidazole faecal egg count reduction test. The corrected EHT data is shown, with log_{10}(thiabendazole concentration) plotted against the proportion hatching. The data was modelled in R using a binomial GLM (probit), and the regression line is plotted here. Pre-BZ (X, blue), four tests were performed, each with three replicate wells for each concentration included. Each well is plotted. On day 14 post-BZ, lambs were sampled and a further EHT performed. Fewer eggs were available and for some concentrations only one or two replicate wells were included. The proportion hatching in each well is shown (O, red).

No definitive dose has been established for the IVM LDT used in this study, but the ED_{50} can be determined. Pre-IVM this was 2.43 nM (95% CI: 2.14, 2.81) and post-IVM this rose to 4.07 nM (95% CI: 2.87, 6.36) (Table 6.4, Figure 6.6), giving a RR of 1.7. Pre-BZ treatment an ED_{50} of 2.27 nM was calculated, comparable to that of the pre-IVM group (Table 6.4).

Table 6.4: Larval development test results.

Test	IVM LDT ED_{50} (nM)[1] (95% CI)
Pre-BZ FECRT (two plates combined analysis)	2.27 nM (2.05, 2.56)
Pre-IVM FECRT (two plates combined analysis)	2.43 nM (2.14, 2.81)
Post-IVM FECRT	4.07 nM (2.87, 6.36)

Effective doses (ED_{50}) of ivermectin required to inhibit 50% development of eggs to L3 are reported for each test performed. [1]Raw data was adjusted for response in 2% DMSO control wells.

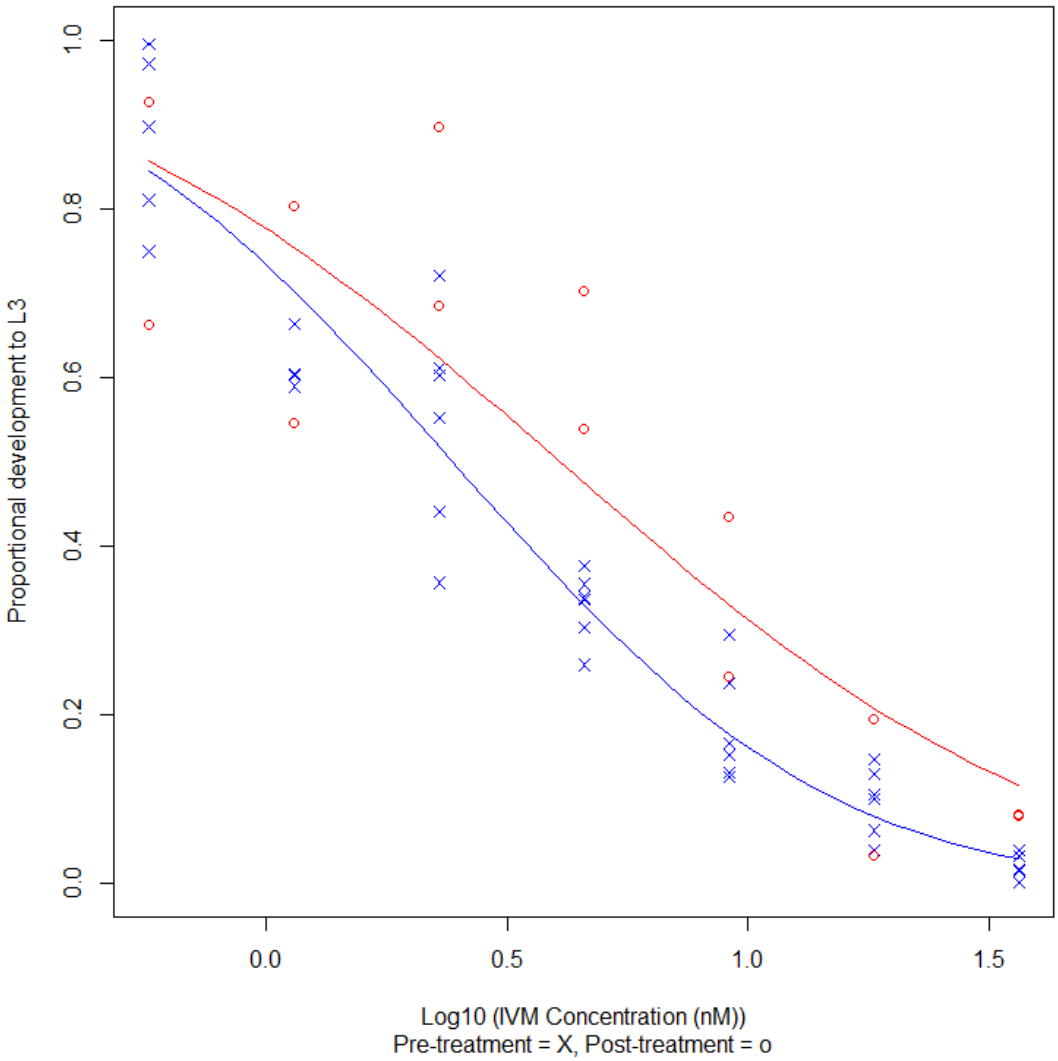

Figure 6.6: Dose response curve of the larval development test performed using samples obtained during an ivermectin faecal egg count reduction test. The corrected LDT data is shown, with log_{10}(IVM concentration) plotted against the proportion developing to L3. The data was modelled in R using a binomial general linear model (probit), and the regression line is plotted here. Pre-treatment (X, blue), two tests were performed, each with three replicate wells per concentration included. Each well is plotted. On day 14 post-IVM (O, red), fewer eggs were harvested and concentrations were only performed in duplicate.

Over the season the EHT was used to assess the BZ sensitivity of the lamb strongyle population. For each sample tested, the hatch percentage in the control wells, and the corrected hatch percentage of the definitive dose wells (0.1 µg/ml) are shown in Figure 6.7. From May to July, percentage hatch in both the control wells and the definitive dose wells (0.1 µg/ml) averaged greater that 80%, and variation between replicate wells within a test was minimal. However, in August and September within test variation increased considerably, especially at the definitive dose. Lower percentage hatch was recorded in August and September at the definitive dose, compared with earlier in the year (Figure 6.7). In contrast, in October, 14 days following BZ treatment, both the egg hatch in the control well and the definitive dose wells were very similar, and hatching percentages were similar to those in early summer (Figure 6.7).

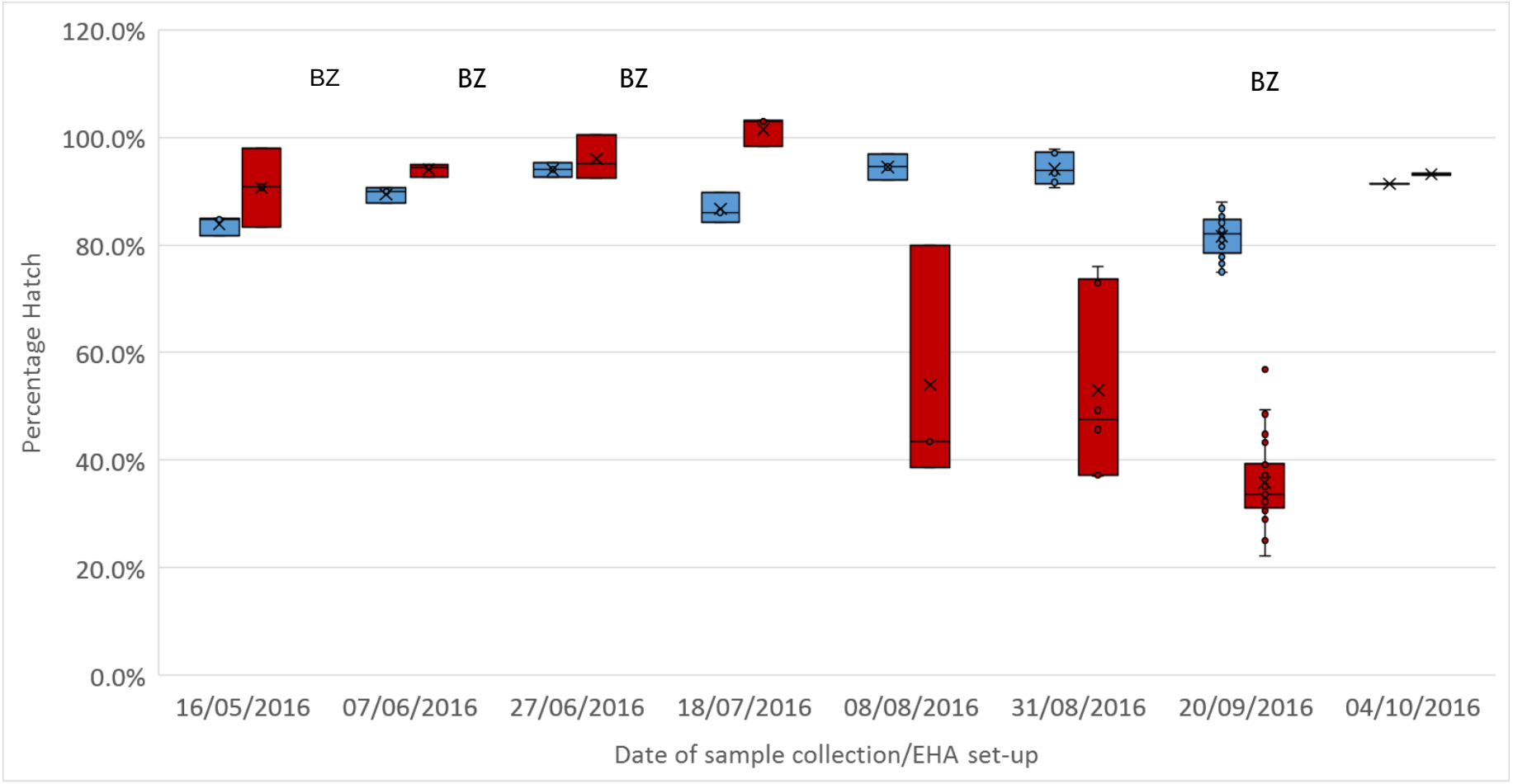

Figure 6.7: Lamb egg hatch test results over the season. The plot shows the percentage hatch in the DMSO control wells (blue, uncorrected) and the definitive dose wells (0.1 µg/ml, corrected by average percentage hatch in control wells, red) of EHTs performed at each sampling time point. Number of tests performed (each with wells included in triplicate) at each sampling time point from 16th May 2016 onwards: 1, 1, 1, 1, 1, 2, 8, 1 (the latter had just one DMSO well and two definitive dose wells). Individual points are shown (each point represents one well), and the X indicates the mean value. BZ = Lambs treated with BZ.

BEEF & LAMB

6.4.3. Pyrosequencing – using a genetic marker as a sensitive diagnostic test

A genetic marker can be a mutation directly causing resistance or a mutation closely linked to the mutation causing resistance. This mutation can be tested for in any parasite lifecycle stage, as it uses the DNA. It should provide a rapid and accurate indication of resistance status. BZ resistance is associated with non-synonymous SNPs within the *β*-tubulin isotype-1 gene in many strongyle species (Kwa et al., 1995; Silvestre and Cabaret, 2002; Ghisi et al., 2007). Various genetic tests are available to detect these mutations. For this study, a pyrosequencing method, developed by Skuce et al. (2010) and subsequently used by Redman et al. (2015) in field populations, was used to measure BZ resistance on this farm. Over the year, 386 individual *T. circumcincta* L3 were sequenced at these SNPs, which cause changes in the protein sequence at three amino acids (codons 167, 198 and 200), and prevent BZ drugs from binding correctly.

Each worm has two alleles of the *β*-tubulin isotype-1 gene. Of the 386 individuals sequenced, 64% carried two resistance associated SNP alleles at codon 200, 28.5% were heterozygous at codon 200 (carrying one resistance associated allele and one susceptible allele). Only 7.5% were homozygous susceptible at this position. In total 2.1% of all 386 individuals were homozygous for the resistance associated SNP alleles at codon 198, and 5.2% of all individuals were heterozygous at codon 198. All individuals with resistance-associated SNPs at codon 198 encoded the amino acid leucine. In addition, one individual encoded a stop codon at position 198 (GAA > TAA). Only 6 individuals (1.6%) carried a resistance associated mutation at codon 167 and all were heterozygous.

Included in these 386 individuals sequenced were 30 *T. circumcincta* individuals selected at each sampling time point of the BZ and IVM FECRTs in September 2016, totalling 180 individuals (Figure 6.8 and Figure 6.9). Individuals homozygous or heterozygous resistant were detected in all samples. Those with homozygous resistance mutations at codon 198 were detected on day 0 of the IVM FECRT and on days 7 and 14 of the BZ FECRT. ‘Susceptible’ individuals were identified on days 0, 7 and 14 of the IVM FECRT and on day 14 of the BZ FECRT. For both the BZ and IVM treatment groups no significant differences in genotype proportions were noted between samples before and after treatment (Figure 6.8 and Figure 6.9).

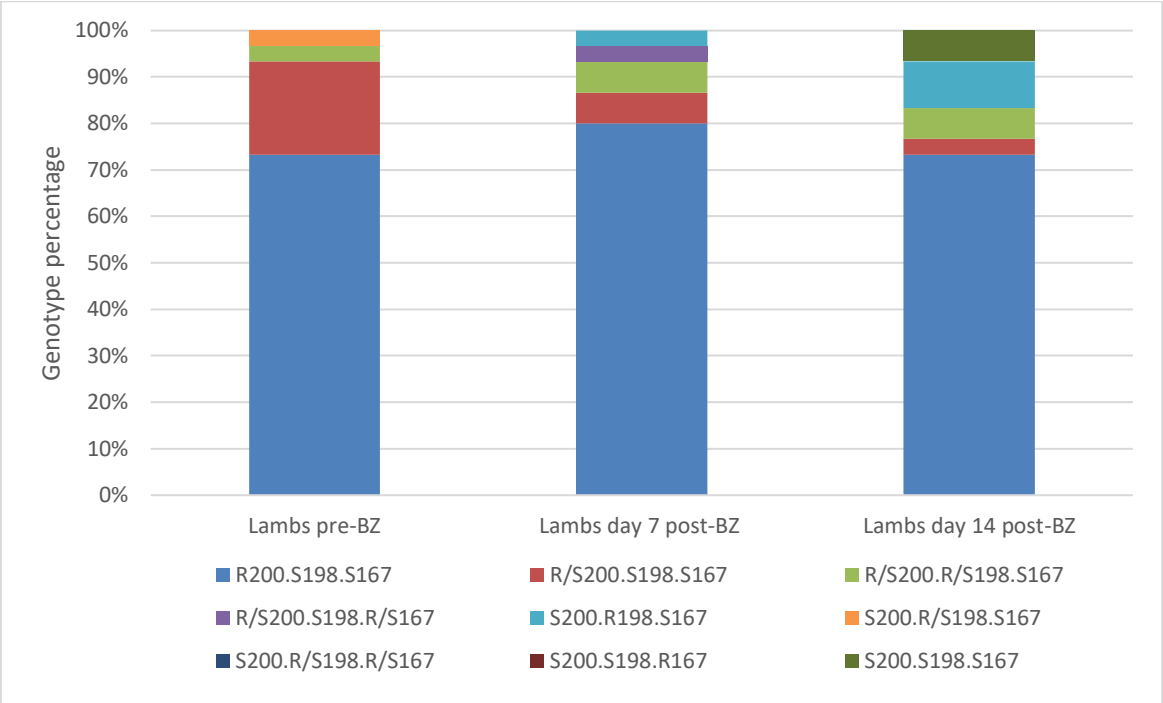

Figure 6.8: *Teladorsagia circumcincta* offspring genotypes determined by pyrosequencing before and after benzimidazole treatment of lambs. At each sampling time point (day 0, 7 and 14 of a BZ FECRT), 30 *T. circumcincta* individuals were genotyped at the 200, 198 and 167 codons of the *β*-tubulin isotype-1 gene. R = resistant allele, S = sensitive allele. R/S = heterozygous.

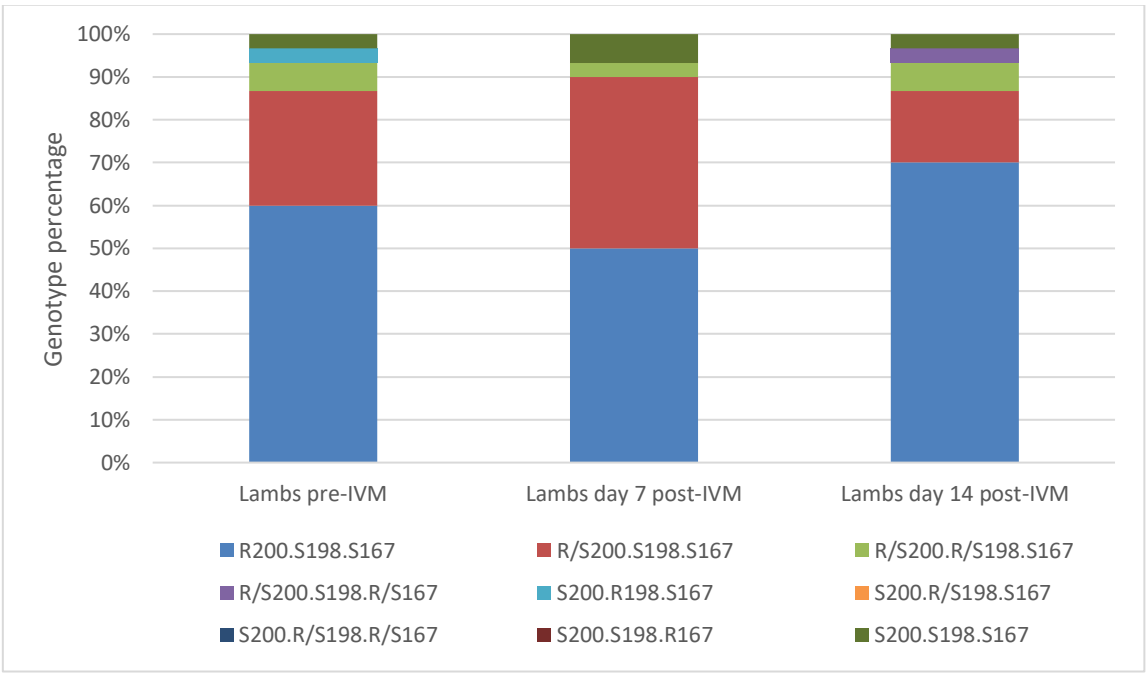

Figure 6.9: *Teladorsagia circumcincta* offspring genotypes determined by pyrosequencing before and after ivermectin treatment of lambs. At each sampling time point (day 0, 7 and 14 of an IVM FECRT), 30 *T. circumcincta* individuals were genotyped at the 200, 198 and 167 codons of the *β*-tubulin isotype-1 gene. R = resistant allele, S = sensitive allele. R/S = heterozygous.

6.5. Assessing population change over time

Ten previously published microsatellites (Grillo et al., 2006; Redman et al., 2008) were used to investigate whether the *T. circumcincta* population on Farm 1 underwent selection over the year, or following management interventions. If sub-populations of *T. circumcincta* existed, this could impact the detection of resistance using genetic markers.

At each sampling time point 30 *T. circumcincta* L3 were genotyped and population genetics analysis was conducted using Arlequin v3.5.2.2 (Excoffier et al., 2005). Two ewe samples (collected at lambing pre-MOX treatment, and in early June) and several lamb samples (days 0 and 14 of the BZ FECRT and days 0, 7 and 14 of the IVM FECRT) were tested. There was high within-sample diversity, with the majority of variation detected was between individual worms rather than between sample groups - only 0.66% of all variation was found to be between samples. No microsatellite appeared to be under selection over the course of the year, suggesting a homogenous population. No obvious selection occurred due to either anthelmintic treatment, indicating that the microsatellites were not linked to either BZ or IVM resistance associated markers. They could therefore not be used as diagnostic tests of resistance.

6.6. Identifying genetic markers of IVM selection using a reduced representation method: ddRAD-Seq

6.6.1. Farm 2 – a farm identified as appropriate to use for ddRAD-Seq

To identify genetic markers of IVM resistance which could be used as a diagnostic test of resistance, a genome wide association study (GWAS) was performed. This allowed assessment of the entire genome to identify SNPs associated with IVM resistance. The GWAS compared offspring pre- and post-IVM treatment of their parents, IVM treatment enriching for resistance associated SNP alleles within the population. These SNPs could then be detected using statistics like F_{ST}. If there is no difference (genetic differentiation) between two populations, or each population contains many alleles, then F_{ST} is 0. If populations are completely different at a SNP then F_{ST} is 1.

To perform a GWAS to detect selection by IVM upon a population, a field population with an appropriate reduction in FEC was needed. Farm 1 had proved too resistant to IVM for use in a GWAS. Instead, a second UK farm was chosen for the analysis. Farm 2 was located in southwest England, and had previously diagnosed multi-drug resistance, detected by FECRT in 2013 (Glover et al., 2017). In August 2016 a further FECRT was performed by the farm's veterinary surgeons using four groups of 20 lambs for each anthelmintic tested. BZ, LEV, IVM and MOX efficacies were

calculated to be 87.7%, 95.4%, 87.4% and 98.6% respectively (Table 6.5). Little is known about how the farm is managed, however it is understood that two separate flocks were merged between 2013 and 2016. In 2016, pre-treatment samples from all groups of lambs were combined for coproculture and post-treatment samples for each anthelmintic treatment group were coprocultured separately. Colleagues at the University of Bristol then sent L3 to Glasgow. From the 2016 samples, L3 strongyles were speciated by PCR. Of these, 69.8% were identified as *T. circumcincta* pre-treatment, and only *T. circumcincta* was identified post-IVM treatment. Extrapolation of the strongyle species percentages to the faecal egg count (FEC) data suggested that IVM was 81.9% effective against the *T. circumcincta* population on Farm 2 in August 2016 (Table 6.5, Figure 6.10).

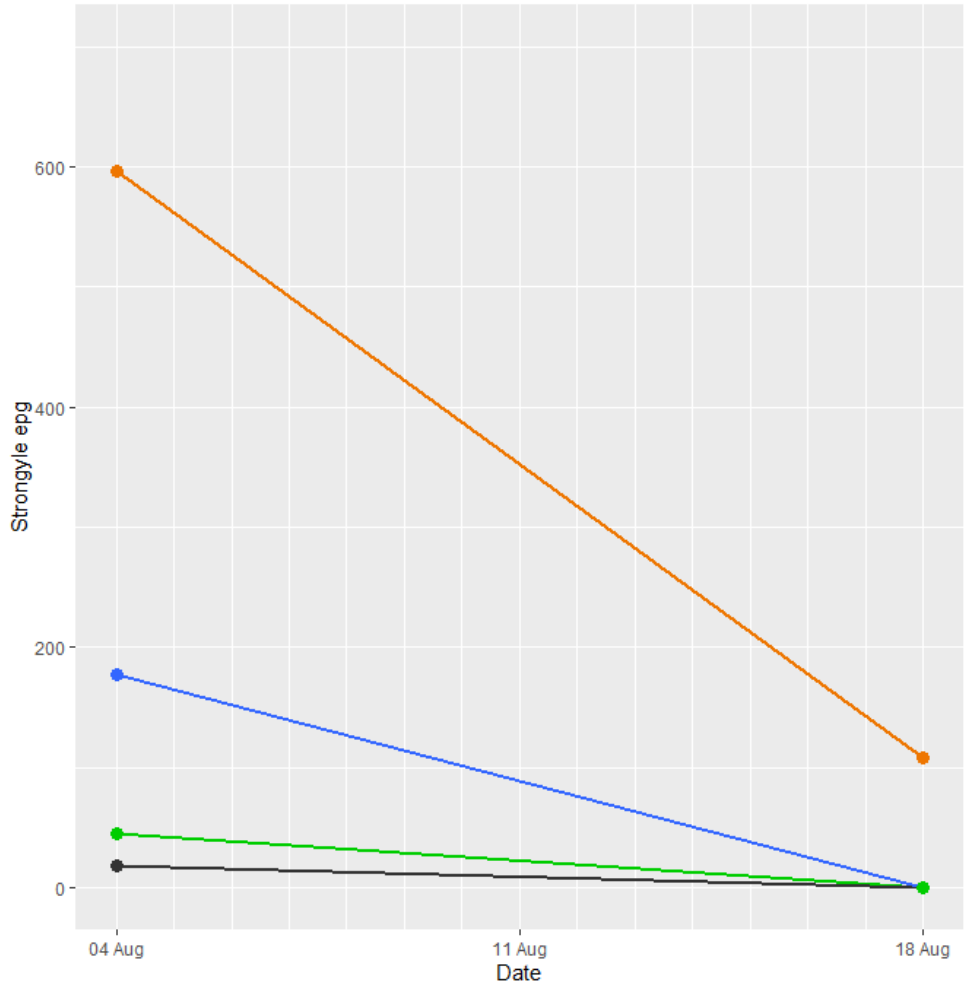

Figure 6.10: Ivermectin faecal egg count reduction test Farm 2, 2016, with eggs per gram attributed by species. Following coproculture, 96 pre- and 89 post-treatment L3 strongyles were speciated by PCR using the ITS2 region. Species percentages were used to extrapolate eggs per gram of faeces by each species present. Key to colours: green = *Oesophagostomum venulosum*, black = *Trichostrongylus axei*, orange = *Teladorsagia circumcincta*, blue = *Trichostrongylus vitrinus*.

Table 6.5: Faecal egg count reduction test results on Farm 2.

Anthelmintic tested	FECRT 2013 (%)	FECRT 2016 (%)	FECRT 2016 against *T. circumcincta* (%)
BZ	83.7%	87.7%	ND
LEV	92.5%	95.4%	ND
IVM	93.0%	87.4%	81.9%
MOX	ND	98.6%	ND

Overall percentage reduction in strongyle egg output is shown, with the proportional reduction in *Teladorsagia circumcincta* faecal egg count indicated for those samples where L3 larvae were speciated by PCR of the ITS2 region. ND = Not done.

30 pre- and 32 post-treatment *T. circumcincta* L3 from Farm 2 were sequenced using ddRAD-Seq next generation sequencing techniques. The sequenced DNA was aligned to the new Tci2 draft reference genome, which was in 8000 contigs (fragments), and was 685 Mb long. Analysis of the data to compare between the pre- and post-treatment samples was then performed.

6.6.2. Identification of suitable RADloci to use for population analysis

Over 3.3 million RADloci, the short DNA sequences adjacent to an enzyme cut site, were sequenced and identified. These 3.3 million RADloci were identified using all 62 individuals. However, just 75,517 RADloci were shared by enough individuals (80% of each of the pre- and post-treatment samples) to be included in an analysis to identify SNPs under IVM selection. Most of these RADloci were non-polymorphic (78.9%), meaning that they did not contain a mutation (a SNP) between the pre- and post-treatment samples, and were thus excluded from further analysis. The 15,912 polymorphic RADloci were spread across the 685 Mb genome and sampled less than 0.01% of it.

6.6.3. Linkage decay was rapid

Worms have two copies of each chromosome. During meiosis, and the formation of an egg or sperm, the genome undergoes ‘recombination’. DNA is exchanged between each pair of chromosomes, increasing genetic variation in the offspring. Sites that are close together on a chromosome are likely to be ‘linked’ and inherited together. Sites that are far apart on a chromosome are more likely to be rearranged during meiosis and alleles mixed between chromosomes. Any two sites in the genome may be inherited separately or together. If they are inherited separately, then they are considered ‘unlinked’ and are in ‘linkage equilibrium’.If they are inherited together they are ‘linked’, and are in ‘linkage disequilibrium’. If IVM selection had occurred recently, then a large region of linkage disequilibrium surrounding the mutation causing IVM

resistance might be expected to be detected, and ddRAD-Seq would be a useful technique to identify this region.

To calculate the linkage disequilibrium, the squared correlation coefficient value (r^2) between each pair of SNPs on a contig was chosen because this will only be high when alleles are commonly linked. A diagnostic test of resistance, to be used in multiple populations, would require alleles to be common as well as linked to a causal SNP. This measure varies from 0 (no linkage) to 1 (always inherited together). The mean r^2 value was 0.090 (range 0.000 - 1.000), while the median value was even lower at 0.016. The distance to the mean intercontig linkage disequilibrium (0.0206) was calculated (Cutter et al., 2006), and was found to be 2805 bp. To put this in context, the average *T. circumcincta* gene is 6145 bp in length. Although some genotypes appeared to be linked over a considerable distance, the majority had very low r^2 values, even over short distances, and the linkage decay was extremely rapid (Figure 6.11). This data will necessarily be affected by the lengths of contigs within the Tci2 genome. The average contig length is only 85.5 kb, but many contigs are only a few thousand basepairs long. If linkage disequilibrium was high on short contigs, but lower on longer contigs, this could overestimate the speed of linkage decay and underestimate the half decay distance.

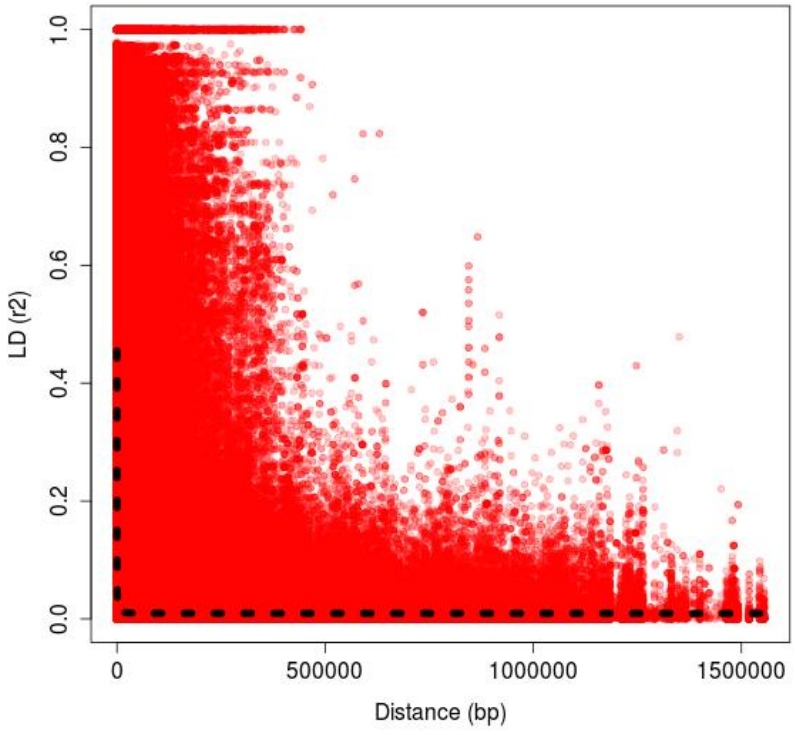

Figure 6.11: Linkage decay plot. Each point represents the linkage disequilibrium (r^2) between one pair of SNPs, within the same contig. Note that points are overlaid and most points lie on the far left of the plot. The distance in basepairs between genotype positions is plotted along the x-axis. The linkage decay is plotted as a black dotted line and falls sharply.

6.6.4. Low and variable SNP density across the genome

The SNP density per kilobase across the genome and contigs 1 to 50 in the Tci2 genome was plotted (Figure 6.12). These were generally the longest contigs, with the first contig measuring 2.08 Mb, however, not all contigs in the Tci2 genome are in size order. From these plots it was clear that the SNP density per kilobase varied widely across the genome, including within a contig, and there were large regions where no SNPs were present (Figure 6.12).

6.6.5. Population analysis identified two SNPs on a single contig to be of interest. However these were unlikely to be under IVM selection.

A population genetic analysis was performed. This analysis provided many potential sites which were genetically differentiated between the pre- and post-treatment samples, and potentially under selection by IVM treatment. Of these, one RADlocus in particular was of interest. It contained two SNPs which appeared highly differentiated, and these were identified to be within a gene. However, after aligning the RADloci haplotypes (the DNA sequences) of each individual it became clear that there was no evidence of IVM selection over the region. These SNPs were differentiated, but the post-treatment sample was more diverse than the pre-treatment sample. Therefore, it was unlikely that IVM had applied selection pressure over the region.

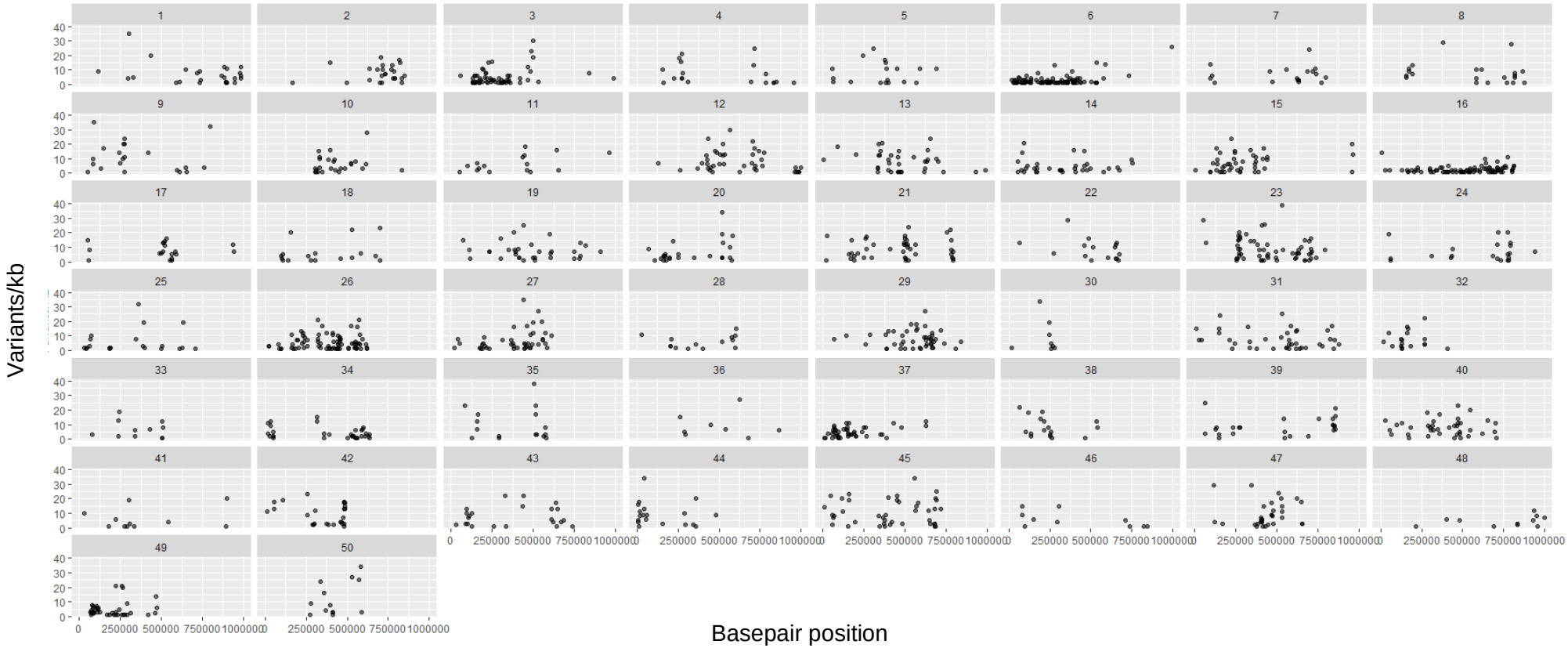

Figure 6.12: ddRAD-Seq SNP density across the genome. The first 50 contigs are plotted, each on a separate plot. Each dot represents a 1 kilobase window, which contains at least one variant site. For each contig the first million basepairs are included, however some contigs shown will be shorter in length than this.

6.7. Identifying genetic markers of IVM selection: Validation of a Pool-Seq technique

ddRAD-Seq had generated good quality data. However, the RADloci which were shared by enough individuals were sparsely distributed across the genome. As the linkage disequilibrium was low, and the the linkage between sites rapidly decayed, large regions of the genome were effectively not sampled. In addition, for various reasons it was determined that the sample size was too small, and a larger number of individuals needed to be sequenced due to the rapid linkage decay and high within-sample diversity. Pool-Seq, which has recently been employed as a next generation sequencing technique in GWAS allowed an increased number of individuals to be sequenced within the budget. It also provided the important benefit of sequencing the entire genome. For Pool-Seq, both Farm 2 and a new farm, Farm 3, were chosen.

Farm 3 was in southeast Scotland and in July 2015 a FECRT was performed by colleagues at the Moredun Research Institute. Anthelmintic efficacies were -34.4%, 96.0% and 22.4% for BZ, LEV and IVM respectively (Table 6.6). Pre-treatment egg counts were 135.8 epg and 181.0 epg for BZ and IVM treatment groups respectively. All L3 strongyles provided by the Moredun Research Institute and speciated by PCR pre- and post-IVM were *T. circumcincta*, and all but one post-BZ L3 were also *T. circumcincta*. A further two groups of lambs were used to test the combined efficacy of co-administering BZ and IVM, and LEV and IVM. These demonstrated improved anthelmintic efficacies compared with BZ or IVM given as a single active alone, reducing egg output by 54.5% and 89.1% respectively but highlighted the presence of dual resistant worms (Morrison, *pers comm*, Table 6.6).

Table 6.6: Faecal egg count reduction test results on Farm 3, 2015.

Anthelmintic	FECRT 2015 (%)	FECRT 2015 efficacy against *T. circumcincta* (%)
BZ	-34.4%	-32.5%
LEV	96.0%	ND
IVM	22.4%	22.4%
BZ and IVM	54.5%	ND
LEV and IVM	89.1%	ND

Overall percentage reduction in strongyle egg output is shown, with the proportional reduction percentage of *Teladorsagia circumcincta* indicated for those samples where L3 larvae were speciated by PCR of the ITS2 region. ND = Not done.

6.7.1. Population genetics analysis of Pool-Seq data identified many genomic regions potentially under selection by IVM on each farm.

Pool-Seq worked well for the *T. circumcincta* field populations. A large number of differentiated regions were identified, which were potentially under IVM selection. The nucleotide diversity was calculated as 0.022, similar to other estimates for *H. contortus* (Doyle et al., 2019), and confirming the high individual diversity expected within these sample populations. In essence this indicates that each site in the genome has a 2.2% chance of containing a mutation. Regions of genetic differentiation were identified using the F-statistic, F_{ST}, which is a measure of how fixed an allele is within a sample population (Figure 6.13). If selection by IVM had occurred at a SNP, we could expect a large proportion of individuals in the post-IVM samples to have the same allele at the SNP site. In contrast, the pre-treatment individuals would be more mixed. In addition, a measure known as Tajima's D was used to look for evidence of recent selection over the genome. This showed that rare alleles were more abundant in the pre-treatment sample groups than in the post-IVM samples. In other words, there were fewer rare alleles post-IVM than pre-treatment. This suggests some selection has occurred, reducing the number of alleles shared between individuals resistant to IVM.

Neither the F_{ST} nor the Tajima's D analysis revealed a single, highly differentiated, large region of selection. Using RNA-Seq data provided by the BUG Consortium, the Tci2 draft reference genome was annotated as part of this PhD. The gene information was then used to investigate all regions of 10 kb or wider which were considered to be genetically differentiated above a chosen threshold on either farm (Figure 6.13). Gene ontology information, which predicts the potential role of each gene, and its location within the cell and the organism (i.e. neuronal, intestinal etc), was obtained.

In addition, genes identified were compared with other GWAS of IVM resistance in *H. contortus* (Sallé et al. (2019) and BUG Consortium, unpublished work), *Onchocerca volvulus*, a human parasite (Doyle et al., 2017), and *T. circumcincta* (Choi et al., 2017). For the latter study, the published reference genome had been used. This genome was much more fragmented than the Tci2 reference genome (81,000 contigs vs 8000 contigs), but of a similar length and had protein information. In addition to comparing between the two studies using genes identified as potentially under selection in this PhD, the sequenced DNA was also aligned to the genome used by Choi et al. (2017). The studies were then directly compared.

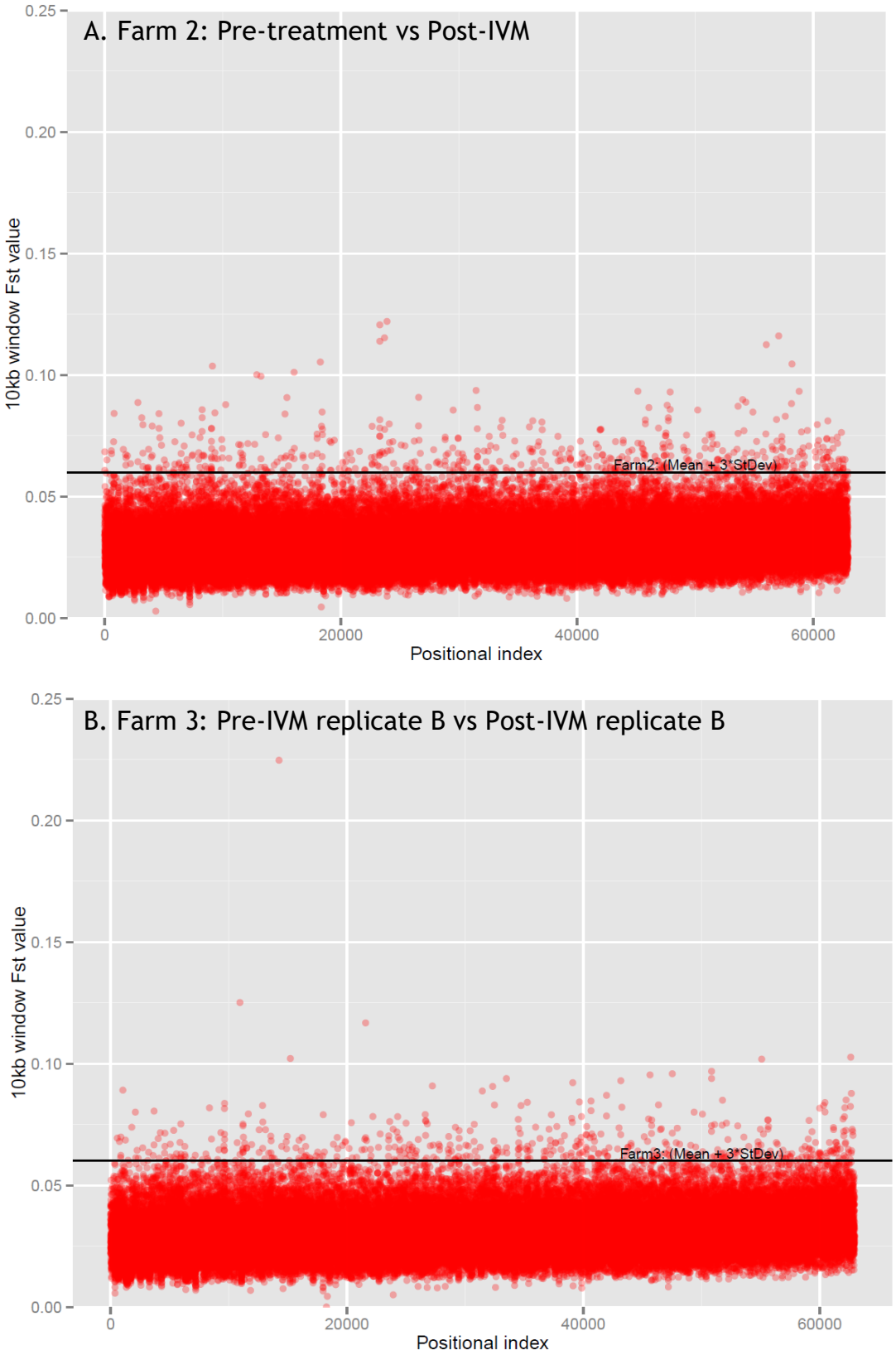

Figure 6.13: Manhattan plots of Farm 2 and Farm 3 Pool-Seq data. Each point is a 10 kb region. The higher the F_{ST} value, the more the region is genetically differentiated between the sample populations. Points above the threshold (black line) were investigated further.

A few genes were identified as potentially under IVM selection in more than one study, or on both farms in this study. However, no gene was common to all studies. Of note was a GluCl gene in the Farm 2 data, *glc-3*, and a P-glycoprotein gene, *pgp-3*, which was common to both studies. However multiple copies of the *pgp-3* gene were identified within the Tci2 draft genome, and different copies appeared under selection on each of Farm 2 and 3. The gene *pgp-3* has been previously implicated to potentially have a minor role in anthelmintic resistance in *T. circumcincta* (Dicker et al., 2011). Multiple neuronal genes were identified, including a number involved in the acetylcholine receptor signalling pathways.

A number of genes identified in this study were involved in chemosensory pathways, or were known to affect the normal architecture or movement of the *C. elegans* pharynx. Other genes were identified as metabolic genes. One metabolic gene, *usp-50*, a ubiquitination enzyme, was identified on both farms, although, as for *pgp-3*, separate gene copies appeared under selection on each farm. Lastly, a number of regulatory genes were identified. Regulatory genes identified may enhance or reduce expression of genes in certain pathways and can be important in normal parasite function. Changes may affect other genes which might directly induce reistance towards IVM.

Over the course of the analysis it became clear that many genes were potentially falsely duplicated within the Tci2 draft reference genome. Lists are available of genes expected to be single copy within a genome. To determine how many of these genes were present as single copies within the Tci2 draft genome, and how many were duplicated, a BUSCO analysis was performed (Simao et al., 2015; Waterhouse et al., 2017). This identified that of the genes expected to be single copy, 57% were single copy, 9% were missing, 11% were fragmented and 23% were duplicated. A second available *T. circumcincta* reference genome also has artefactually duplicated genes. However, DNA was aligned to this second genome for comparison. Very few genes appeared to be potentially under selection by IVM in both alignments. Of note were two neuronal genes; ligand-gated ion channels. One of these, *lgc-42*, was also differentially expressed between IVM treated and IVM untreated *H. contortus* adults (Laing, *pers comm*).

7. Discussion

PGE is a major production limiting disease and a considerable welfare problem (Sargison, 2014; APHA, 2018). Anthelmintic resistance is increasing. The aims of this PhD were to first identify a suitably IVM resistant field population of *T.* circumcincta, and then perform a GWAS to identify genetic markers of IVM resistance. Such markers could be used as a diagnostic test. Whilst a

suitable population was being identified, PGE and anthelmintic resistance were monitored over the course of a year on a commercial farm.

FECs identified a similar pattern in the egg output over the season as has been previously described (Salisbury and Arundel, 1970). The change in species over time and between hosts is in agreement with historical UK data (Morgan et al., 1951; Boag and Thomas, 1977; Wilson et al., 2008). However such species diversity is more commonly seen in unmanaged flocks, or those with little anthelmintic use (Craig et al., 2009; Sinclair et al., 2016). The presence of multiple species on this farm, despite a comparatively frequent use of anthelmintics, may be due to multiple BZ resistant species. In addition, multiple pasture types were present which may encourage niche species (Silvestre et al., 2000). Lastly, although ewes were housed pre-lambing, pastures continued to be grazed by adult sheep throughout the year, which can maintain more vulnerable species, such as *O. venulosum* and *C. ovina*, which have much longer pre-patent periods (Silvestre et al., 2000) (Roeber et al., 2013). Whether diversity is beneficial for the host is uncertain. One study found a reduction in egg output and improved productivity in sheep co-grazed with cattle, alongside increased strongyle diversity (Giudici et al., 1999). Another study investigating co-infection with *T. circumcincta* and *Trichostrongylus vitrinus* (a small intestinal parasite), noted that *T. circumcincta* infection reduced establishment of *T. vitrinus* (Jackson et al., 1992b). A further study reported reduced establishment of *Trichostrongylus colubriformis* when lambs were co-infected with this parasite and *T. vitrinus,* compared to monospecific infections (Roy et al., 2004). Further work to better understand the potential benefits of multiple species in PGE may be useful, especially as anthelmintic resistance increases.

For both the FECRT and the bioassays, employed to monitor anthelmintic resistance on Farm 1, species diversity on the farm was identified as a confounding factor. For FECRTs and even for simple FECs, species identification would add valuable information to aid in flock health planning. This information is not novel, and has been discussed previously (McKenna, 1997), however it is not frequently performed in the UK and speciating strongyles naturally carries additional costs for the farmer. Nevertheless, without it, false conclusions can be drawn and anthelmintics can appear more effective than they truly are against highly pathogenic species. Importantly, as was observed on Farm 1, the strongyle community varied across the year and was different between hosts. It is therefore necessary to identify the species composition, and anthelmintic efficacy, at the time of year and using the sheep age group which will be treated. An alternative to monitoring PGE using FECs with strongyle speciation is to use a production measurement, such as the difference between expected and actual weight gain of sheep (Greer et al., 2009). This is currently used on some farms as a ‘Targeted Selective Treatment’ measure, reducing anthelmintic use whilst

maintaining production (Kenyon et al., 2013; McBean et al., 2016). Future work to investigate the use of such a production measure as part of a FECRT may be beneficial. Potentially, this could circumvent the requirement to identify strongyle species, as it would focus on the ability of an anthelmintic to improve production parameters.

Interestingly, this study identified a small but significant rise in egg output of the ewes in June, after the period when MOX persistence would have ceased. This rise coincided with the last recorded sample containing a large percentage of *T. circumcincta* individuals. Following this time point the ewes FEC was low, and the *T. circumcincta* FEC negligible. This may indicate a delayed PPR following the use of MOX if the resumption of immunity is delayed (Brunsdon, 1966). Importantly, the realisation that the ewes shed proportionately very few *T. circumcincta* eggs following June may suggest that ewes could be used to 'clean' pastures whether co-grazing with their lambs, or grazing separately, after this time point. It would be interesting to determine whether this is true for all adult sheep – i.e. from what age could adults helpfully co-graze with young lambs as a means to reduce pasture contamination with pathogenic species? It would be important to determine whether there is a difference between ewes treated with MOX and those treated with a short acting anthelmintic or given no treatment at lambing time.

The *T. circumcincta* population on Farm 1 was found to be highly diverse, similar to previous UK studies using microsatellites (Grillo et al., 2007; Redman et al., 2015). It did not appear to vary across the year, suggesting one large population is present on the farm. This indicates that management practices may have little impact upon the population and that it is adaptable. Any tests of anthelmintic resistance based on genetic markers are therefore likely to be effective throughout the year and across farms. Individuals within the population were identified to be BZ and IVM dual-resistant. Offspring of IVM treated survivors were identified to be BZ resistant using the EHT and pyrosequencing of BZ resistance associated SNPs. These individuals, which appear to comprise a significant majority, based on the lack of change in FEC, will not be efficiently treated by either 1-BZ or IVM anthelmintics.

IVM resistance is expected to be inherited as a dominant trait in *T. circumcincta* (Sutherland et al., 2002). The next generation sequencing results supported this. A recessively inherited mutation would be expected to have a much stronger signal – a much higher F_{ST} value than was observed in this study. For example, the recessively inherited oxamniquine resistance in *S. mansoni*, had a single strong selection peak (Valentim et al., 2013). In a recent *H. contortus* study (Laing, *pers comm*), a similar tall peak was noted around the BZ resistance locus, inherited as a partially recessive trait (Le Jambre et al., 1976). If IVM resistance is dominant then this has implications for

management on farm. IVM resistant individuals could be rapidly selected for as heterozygotes would survive treatment. However, there is also a greater potential to use *refugia* (untreated and unexposed worms) to maintain individuals sensitive to IVM treatment.

Whether one or many loci contribute to IVM resistance is not possible to determine from the results presented here due to the fragmented state of the available reference genomes. Previous studies have suggested that IVM resistance may be polygenic in both *C. elegans* (Dent et al., 2000) and *O. volvulus* (Doyle et al., 2017). Recent studies of *H. contortus* suggest a single dominant locus conferring IVM resistance (Doyle et al., 2019; Sallé et al., 2019; Laing, *pers comm*). Although this may indicate that IVM resistance is not polygenic in *H. contortus*, this genomic region contains many genes, and it is possible that multiple genes are under selection by IVM within this locus. In the model nematode *C. elegans*, high level IVM resistance is conferred by mutations in three genes (Dent et al., 2000). But using gradual selection, with incremental doses of IVM, further genes were identified as under selection (James and Davey, 2009). This may be important in field populations, where sub-optimal dosing may occur. However, the extent and relevance of this is unknown and further studies are required before conclusions can be drawn.

In this PhD, although a set of highly interesting and promising genes were identified, no single gene was identified on both farms or between studies, which had been previously suggested to be strongly involved in IVM resistance in *T. circumcincta.* One GluCl gene (*glc-3*) and one P-glycoprotein (*pgp*-3) which had been weakly associated with IVM resistance in *H. contortus* (Williamson et al., 2011) and *T. circumcincta* respectively (Dicker et al., 2011), were identified. However, *glc-3* was only identified in the Farm 2 analysis, while different gene copies of *pgp-3* in the reference genome were important on each of Farm 2 and Farm 3. The role of these genes in field resistance is therefore hard to quantify from the data presented here. However the genetic basis of IVM resistance may differ between farm populations, as has been suggested in *O. volvulus* populations (Doyle et al., 2017). Although some genes were identified on both farms, most genes were different.

IVM causes paralysis and can inhibit feeding. Several neuronal genes were within a gene family associated with acetylcholine signalling pathways. Within this family are different classes, or groups, of genes (Holden-Dye et al., 2013). One class has been associated with LEV resistance (Fauvin et al., 2010; Boulin et al., 2011; Blanchard et al., 2018), while another is associated with monepantel (Zolvix) resistance (Kaminsky et al., 2008; Rufener et al., 2009; Niciura et al., 2019) (Turnbull et al., 2019). The genes identified in this study have associations with each of these classes. Interestingly, a recent study identified that abamectin, a drug within the same class as

IVM, was capable of antagonising an acetycholine receptor (Abongwa et al., 2016), which may indicate a role for IVM in these signalling pathways. Alternatively these genes may be present within results due to the selection of dual-resistant parasites.

Other genes identified may be involved in the metabolism of IVM. Many of the metabolic genes were predicted to be involved in lipid storage or metabolism. IVM is a lipophilic drug and affects fat storage in nematodes (Smus et al., 2017). Potentially, differences in how worms handle lipids (fat) may affect their tolerance of IVM. One metabolic gene, an enzyme, *usp-50*, is involved in ubiquitination pathways (Huynh et al., 2016). IVM has been shown to induce ubiquitination of and enzyme, P21-activated kinase 1 (PAK1), resulting in death of breast cancer cells (Dou et al., 2016). Alterations in ubiquitinilation of proteins have been associated with artemisinin resistance in the malaria parasite *Plasmodium falciparum* (Dogovski et al., 2015). Genes with the ability to regulate other genes were also identified. Recent studies continue to identify novel IVM targets. One study identified a regulatory gene in *C. elegans* to be important in tolerance of IVM (Ménez et al., 2019). These non-neuronal genes may be interesting for further study.

Although genes identified in this study are most likely to be associated with IVM resistance, it is possible that individuals sequenced are dual-resistant and that genes identified are due to the previous use of other anthelmintics on these farms. Importantly, the fragmented state of the genomes makes it impossible to determine the relative position of genes within the genome, and many genes identified are likely to be present due to genetic hitch-hiking, rather than being directly selected by IVM themselves. The high genetic diversity and rapid linkage decay indicates that the region(s) under selection by IVM are likely to be comparatively small in these outbred populations. This should enable a more rapid identification of genes directly conferring IVM resistance as the genome assembly improves (Doyle et al., 2019).

The main limitation of this study is the lack of a completed reference genome. At the moment it is unknown as to how much the available reference genomes have affected the analysis. Essentially when DNA reads are aligned to a genome, it is expected they will align to a single region of the genome. If multiple gene copies, which are present as technical artefacts, then DNA reads will align over multiple locations. This will affect comparison between the pre- and post-treatment populations, as allelic copies of genes will be analysed separately. Both reference genomes available are fragmented, and have multiple copies of genes which should be single copy. A key reason for this is the high levels of diversity within the *T. circumcincta* populations used to assemble the current reference genomes. Unfortunately, to generate enough DNA to sequence and assemble a reference genome, multiple worms (250,000 L3 for the Tci2 genome) were

required. Newer techniques may help to reduce the amount of DNA required, and the BUG Consortium is currently working to improve the Tci2 genome.

Overall this PhD has enabled good quality next generation sequencing data to be produced. Importantly this is from UK field populations of *T. circumcincta*, rather than lab isolates, which means that the genetic basis of IVM resistance as it has naturally arisen in the UK can be determined. Unfortunately, the reference genomes have potentially confounded the analysis, and it is wise to treat with caution genes identified. Future work to improve the genome will facilitate data analysis, and the analysis performed here will be repeated to better resolve the genetic basis of IVM resistance in *T. circumcincta*. Once genetic markers are identified, genetic tests of anthelmintic resistance can be developed. These will allow different management practices and the role of *refugia* in delaying or reversing anthelmintic resistance to be investigated. As IVM is considered to be a dominant trait, the potential to delay development, or improve anthelmintic efficacy is greater than for a recessive trait, such as BZ resistance. Finally, work perfomed during this PhD has enabled the detailed study of PGE on a modern commercial farm over an entire year. The findings are surprisingly similar to historical data, despite frequent anthelmintic use. Although the diversity of species is not novel, it is perhaps still uncommon in the UK to speciate when a FEC or a FECRT are performed. Nevertheless, the FECRT work on Farm 1 highlights the importance of speciating strongyles during a FECRT, and the differences encountered between hosts and across the year. Despite high levels of anthelmintic resistance on this farm, FECs remained low. Although liveweight gain was not recorded, this suggests that PGE can be effectively managed, even in the face of anthelmintic resistance.

8. Industry messages

8.1. Monitoring parasitic gastroenteritis

- PGE can be monitored over time using FECs, however these may be comprised of many different species. The species composition of a FEC could vary between sheep ages and over the season. Recent anthelmintic treatments should be taken into account.
- Ideally, strongyles should be speciated to determine the species composition before choosing to treat sheep based on a high FEC alone.
- Given the diversity of species present within the hosts, and their varying pathogenicity, it is important to remember to monitor weight gain +/- BCS over time. This can detect weight loss due to large numbers of pre-patent larvae within the sheep (which are missed by FEC), or help to determine whether a high FEC requires treatment.

8.2. Monitoring anthelmintic efficacy

- A FECRT can be beneficial to determine the ability of an anthelmintic to reduce pasture contamination with eggs.
- A FECRT, without speciation of the strongyles present pre- and post-treatment, will be limited in it's ability to identify anthelmintic resistance on a farm. Highly pathogenic species which are highly resistant can be hidden amongst a more sensitive strongyle community.
- The EHT was confounded by the presence of multiple sensitive species within the strongyle population tested. However, it did reflect the proportion of individuals that were BZ resistant. Speciation by PCR of eggs and L1, obtained from the EHT, would be beneficial.
- The LDT used in this study was not helpful to determine IVM resistance in *T. circumcincta*, or in the mixed species population.
- Genetic tests are only available for BZ resistance, but are currently not commercially available. However, this may change going forward. No genetic tests are available for IVM, but would be useful.

8.3. Identifying markers of IVM resistance for use as a diagnostic test

- Two good quality sequencing resources have been generated, and protocols validated, using two independent UK field populations of *T. circumcincta*.
- The genomic footprint of IVM resistance (the length of the worm's genome selected by IVM) is likely to be quite small in the UK field populations.
- Previous studies have suggested IVM resistance is inherited as a dominant trait in *T. circumcincta*. The sequencing results from this PhD would agree with this. A dominant trait means that individuals can be more rapidly selected, however the potential to maintain susceptible individuals within the population is higher than for a recessive trait.
- IVM causes a flaccid paralysis, and induces starvation in free-living stages. Many interesting genes were identified which might be under selection by IVM. Genes involved in neuronal processes were identified, including those related to feeding and chemosensation. IVM is a lipophilic drug capable of affecting fat stores in worms. Several genes involved in metabolism generally and lipid metabolism and storage specifically were also identified. Other genes which may have regulatory roles were noted within the results.
- Genetic markers of IVM resistance will be identified using an improved genome assembly. The *T. circumcincta* reference genomes available during this PhD were both highly fragmented (they are in 8000-81,000 contigs, instead of six chromosomes). Both contain technical artefacts, including significant duplication of genes which should be single copy.

These genomes may therefore have affected the analysis. When an updated reference genome becomes available the analysis will be repeated. It is hoped that with newer sequencing methodologies, and using protocols to attempt to reduce diversity within *T. circumcincta* populations, it may be possible to improve the genome in the near future.

- When diagnostic markers become available they will enable researchers to understand how IVM resistance changes over time on a farm. An understanding of how different management practices affect IVM resistance and the benefits of *refugia* can then be studied.

9. References

Abbott, K.A., Taylor, M.A., Stubbings, L.A., 2012. Sustainable Worm Control Strategies for Sheep: A Technical Manual for Veterinary Surgeons and Advisers. SCOPS 4th edition.

Abongwa, M., Buxton, S.K., Robertson, A.P., Martin, R.J., 2016. Curiouser and Curiouser: The Macrocyclic Lactone, Abamectin, Is also a Potent Inhibitor of Pyrantel/Tribendimidine Nicotinic Acetylcholine Receptors of Gastro-Intestinal Worms. PLoS One 11, e0146854.

APHA 2018. Veterinary Investigation Diagnosis Analysis (VIDA) Annual report 2017.

Arena, J.P., Liu, K.K., Paress, P.S., Frazier, E.G., Cully, D.F., Mrozik, H., Schaeffer, J.M., 1995. The mechanism of action of avermectins in *Caenorhabditis elegans:* correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. J Parasitol 81, 286-294.

Barrett, M., Jackson, F., Huntley, J.F., 1998. Pathogenicity and immunogenicity of different isolates of *Teladorsagia circumcincta*. Vet Parasitol 76, 95-104.

Bartley, D.J., Jackson, E., Johnston, K., Coop, R.L., Mitchell, G.B., Sales, J., Jackson, F., 2003. A survey of anthelmintic resistant nematode parasites in Scottish sheep flocks. Vet Parasitol 117, 61-71.

Bartley, D.J., Donnan, A.A., Jackson, E., Sargison, N., Mitchell, G.B.B., Jackson, F., 2006. A small scale survey of ivermectin resistance in sheep nematodes using the faecal egg count reduction test on samples collected from Scottish sheep. Vet Parasitol 137.

Bartley, D.J., McAllister, H., Bartley, Y., Dupuy, J., Menez, C., Alvinerie, M., Jackson, F., Lespine, A., 2009. P-glycoprotein interfering agents potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*. Parasitol 136, 1081-1088.

Bartley, D.J., Hamer, K., Andrews, L., Sargison, N.D., Morrison, A.A., 2019. Multigeneric resistance to monepantel on a UK sheep farm. Vet Parasitol: X, 100003.

Bekelaar, K., Waghorn, T., Tavendale, M., McKenzie, C., Leathwick, D., 2018. Carbon dioxide is an absolute requirement for exsheathment of some, but not all, abomasal nematode species. Parasitol Res 117, 3675-3678.

Bisset, S.A., Knight, J.S., Bouchet, C.L., 2014. A multiplex PCR-based method to identify strongylid parasite larvae recovered from ovine faecal cultures and/or pasture samples. Vet Parasitol 200, 117-127.

Blackhall, W.J., Pouliot, J.F., Prichard, R.K., Beech, R.N., 1998. *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. Exp Parasitol 90, 42-48.

Blanchard, A., Guegnard, F., Charvet, C.L., Crisford, A., Courtot, E., Sauve, C., Harmache, A., Duguet, T., O'Connor, V., Castagnone-Sereno, P., Reaves, B., Wolstenholme, A.J., Beech, R.N., Holden-Dye, L., Neveu, C., 2018. Deciphering the molecular determinants of cholinergic anthelmintic sensitivity in nematodes: When novel functional validation

approaches highlight major differences between the model *Caenorhabditis elegans* and parasitic species. PLoS Pathog 14, e1006996.
Boag, B., Thomas, R.J., 1977. Epidemiological studies on gastro-intestinal nematode parasites of sheep: the seasonal number of generations and succession of species. Res Vet Sci 22, 62-67.
Boulin, T., Fauvin, A., Charvet, C.L., Cortet, J., Cabaret, J., Bessereau, J.L., Neveu, C., 2011. Functional reconstitution of *Haemonchus contortus* acetylcholine receptors in *Xenopus oocytes* provides mechanistic insights into levamisole resistance. Br J Pharmacol 164, 1421-1432.
Britt, D.P., 1982. Benzimidazole-resistant nematodes in Britain. Vet Rec 110, 343-344.
Brunsdon, R.V., 1966. A comparison of the spring-rise phenomenon in the faecal nematode-egg counts of housed sheep with that of sheep grazing infective pasture. N Z Vet J 14, 145-151.
Burgess, C.G., Bartley, Y., Redman, E., Skuce, P.J., Nath, M., Whitelaw, F., Tait, A., Gilleard, J.S., Jackson, F., 2012. A survey of the trichostrongylid nematode species present on UK sheep farms and associated anthelmintic control practices. Vet Parasitol 189, 299-307.
Campbell, W.C., Fisher, M.H., Stapley, E.O., Albers-Schonberg, G., Jacob, T.A., 1983. Ivermectin: a potent new antiparasitic agent. Science 221, 823-828.
Cawthorne, R.J., Cheong, F.H., 1984. Prevalence of anthelmintic resistant nematodes in sheep in south-east England. Vet Rec 114, 562-564.
Chabala, J.C., Mrozik, H., Tolman, R.L., Eskola, P., Lusi, A., Peterson, L.H., Woods, M.F., Fisher, M.H., Campbell, W.C., Egerton, J.R., Ostlind, D.A., 1980. Ivermectin, a new broad-spectrum antiparasitic agent. J Med Chem 23, 1134-1136.
Choi, Y.-J., Bisset, S.A., Doyle, S.R., Hallsworth-Pepin, K., Martin, J., Grant, W.N., Mitreva, M., 2017. Genomic introgression mapping of field-derived multiple-anthelmintic resistance in *Teladorsagia circumcincta*. PLoS Genet 13, e1006857.
Christie, M., Jackson, F., 1982. Specific identification of strongyle eggs in small samples of sheep faeces. Res Vet Sci 32, 113-117.
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. Vet Parasitol 44.
Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse, J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol 136, 167-185.
Coop, R.L., Sykes, A.R., Angus, K.W., 1982. The effect of three levels of intake of *Ostertagia circumcincta* Larvae on growth rate, food intake and body composition of growing lambs. J Agr Sci 98, 247-255.
Craig, B.H., Jones, O.R., Pilkington, J.G., Pemberton, J.M., 2009. Re-establishment of nematode infra-community and host survivorship in wild Soay sheep following anthelmintic treatment. Vet Parasitol 161, 47-52.
Crofton, H.D., 1965. Ecology and biological plasticity of sheep nematodes. I. The effect of temperature on the hatching of eggs of some nematode parasites of sheep. Cornell Vet 55, 242-250.
Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H., Schaeffer, J.M., Arena, J.P., 1994. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nat 371, 707-711.
Cutter, A.D., Baird, S.E., Charlesworth, D., 2006. High nucleotide polymorphism and rapid decay of linkage disequilibrium in wild populations of *Caenorhabditis remanei*. Genet 174, 901-913.
Danecek, P., Auton, A., Abecasis, G., Albers, C.A., Banks, E., DePristo, M.A., Handsaker, R.E., Lunter, G., Marth, G.T., Sherry, S.T., McVean, G., Durbin, R., Genomes Project Analysis, G., 2011. The variant call format and VCFtools. Bioinformatics 27, 2156-2158.
Dent, J.A., Smith, M.H.M., Vassilatis, D.K., Avery, L., 2000. The genetics of ivermectin resistance in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 97, 2674-2679.

Dicker, A.J., Nisbet, A.J., Skuce, P.J., 2011. Gene expression changes in a P-glycoprotein (*Tci-pgp-9*) putatively associated with ivermectin resistance in *Teladorsagia circumcincta*. Int J Parasitol 41, 935-942.
Dogovski, C., Xie, S.C., Burgio, G., Bridgford, J., Mok, S., McCaw, J.M., Chotivanich, K., Kenny, S., Gnadig, N., Straimer, J., Bozdech, Z., Fidock, D.A., Simpson, J.A., Dondorp, A.M., Foote, S., Klonis, N., Tilley, L., 2015. Targeting the cell stress response of *Plasmodium falciparum* to overcome artemisinin resistance. PLoS Biol 13, e1002132.
Doherty, E., Burgess, S., Mitchell, S., Wall, R., 2018. First evidence of resistance to macrocyclic lactones in *Psoroptes ovis* sheep scab mites in the UK. Vet Rec 182, 106-106.
Dou, Q., Chen, H.N., Wang, K., Yuan, K., Lei, Y., Li, K., Lan, J., Chen, Y., Huang, Z., Xie, N., Zhang, L., Xiang, R., Nice, E.C., Wei, Y., Huang, C., 2016. Ivermectin Induces Cytostatic Autophagy by Blocking the PAK1/Akt Axis in Breast Cancer. Cancer Res 76, 4457-4469.
Doyle, S.R., Bourguinat, C., Nana-Djeunga, H.C., Kengne-Ouafo, J.A., Pion, S.D.S., Bopda, J., Kamgno, J., Wanji, S., Che, H., Kuesel, A.C., Walker, M., Basanez, M.G., Boakye, D.A., Osei-Atweneboana, M.Y., Boussinesq, M., Prichard, R.K., Grant, W.N., 2017. Genome-wide analysis of ivermectin response by *Onchocerca volvulus* reveals that genetic drift and soft selective sweeps contribute to loss of drug sensitivity. PLoS Negl Trop Dis 11, e0005816.
Doyle, S.R., Illingworth, C.J.R., Laing, R., Bartley, D.J., Redman, E., Martinelli, A., Holroyd, N., Morrison, A.A., Rezansoff, A., Tracey, A., Devaney, E., Berriman, M., Sargison, N., Cotton, J.A., Gilleard, J.S., 2019. Population genomic and evolutionary modelling analyses reveal a single major QTL for ivermectin drug resistance in the pathogenic nematode, *Haemonchus contortus*. BMC Genomics 20, 218.
Drudge, J.H., Szanto, J., Wyant, Z.N., Elam, G., 1964. Field Studies on Parasite Control in Sheep: Comparison of Thiabensazole, Ruelene, and Phenothiazine. Am J Vet Res 25, 1512-1518.
Durham, P.J., Elliott, D.C., 1975. Experimental *Ostertagia* infection of sheep: pathology following a single high dose of larvae. N Z Vet J 23, 193-196.
El-Abdellati, A., De Graef, J., Van Zeveren, A., Donnan, A., Skuce, P., Walsh, T., Wolstenholme, A., Tait, A., Vercruysse, J., Claerebout, E., Geldhof, P., 2011. Altered *avr-14B* gene transcription patterns in ivermectin-resistant isolates of the cattle parasites, *Cooperia oncophora* and *Ostertagia ostertagi*. Int J Parasitol 41, 951-957.
Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 1, 47-50.
Fauvin, A., Charvet, C., Issouf, M., Cortet, J., Cabaret, J., Neveu, C., 2010. cDNA-AFLP analysis in levamisole-resistant *Haemonchus contortus* reveals alternative splicing in a nicotinic acetylcholine receptor subunit. Mol Biochem Parasitol 170, 105-107.
Fraser, D.E., Hunt, P.J., Skinner, R.J., Coles, G.C., 2006. Survey of parasite control on sheep farms in south-west England. Vet Rec 158, 55-57.
Ghisi, M., Kaminsky, R., Mäser, P., 2007. Phenotyping and genotyping of *Haemonchus contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Vet Parasitol 144, 313-320.
Gibbs, H.C., 1986. Hypobiosis and the periparturient rise in sheep. Vet Clin North Am Food Anim Pract 2.
Gibson, T.E., Everett, G., 1972. The ecology of the free-living stages of *Ostertagia circumcincta*. Parasitol 64, 451-460.
Gibson, T.E., 1973. Recent Advances in Epidemiology and Control of Parasitic Gastroenteritis in Sheep. Vet Rec 92, 469-473.
Gill, J.H., Redwin, J.M., Van Wyk, J.A., Lacey, E., 1995. Avermectin inhibition of larval development in *Haemonchus contortus* — Effects of ivermectin resistance. Int J Parasitol 25, 463-470.
Giudici, C., Aumont, G., Mahieu, M., Saulai, M., Cabaret, J., 1999. Changes in gastro-intestinal helminth species diversity in lambs under mixed grazing on irrigated pastures in the tropics (French West Indies). Vet Res 30, 573-581.

Glover, M., Clarke, C., Nabb, L., Schmidt, J., 2017. Anthelmintic efficacy on sheep farms in south-west England. Vet Rec 180, 378-378.
Godoy, P., Che, H., Beech, R.N., Prichard, R.K., 2015. Characterization of *Haemonchus contortus* P-glycoprotein-16 and its interaction with the macrocyclic lactone anthelmintics. Mol Biochem Parasitol 204, 11-15.
Godoy, P., Che, H., Beech, R.N., Prichard, R.K., 2016. Characterisation of P-glycoprotein-9.1 in *Haemonchus contortus*. Parasit Vectors 9, 52.
Good, B., Hanrahan, J.P., de Waal, D.T., Patten, T., Kinsella, A., Lynch, C.O., 2012. Anthelmintic-resistant nematodes in Irish commercial sheep flocks- the state of play. Ir Vet J 65, 21.
Gordon, H.M., 1961. Thiabendazole: a Highly Effective Anthelmintic for Sheep. Nat 191, 1409-1410.
Greer, A.W., Kenyon, F., Bartley, D.J., Jackson, E.B., Gordon, Y., Donnan, A.A., McBean, D.W., Jackson, F., 2009. Development and field evaluation of a decision support model for anthelmintic treatments as part of a targeted selective treatment (TST) regime in lambs. Vet Parasitol 164.
Grillo, V., Jackson, F., Gilleard, J.S., 2006. Characterisation of *Teladorsagia circumcincta* microsatellites and their development as population genetic markers. Mol Biochem Parasitol 148, 181-189.
Grillo, V., Jackson, F., Cabaret, J., Gilleard, J.S., 2007. Population genetic analysis of the ovine parasitic nematode *Teladorsagia circumcincta* and evidence for a cryptic species. Int J Parasitol 37, 435-447.
Grimshaw, W.T., Hunt, K.R., Hong, C., Coles, G.C., 1994. Detection of anthelmintic resistant nematodes in sheep in southern England by a faecal egg count reduction test. Vet Rec 135, 372-374.
Hamer, K., Bartley, D., Jennings, A., Morrison, A., Sargison, N., 2018. Lack of efficacy of monepantel against trichostrongyle nematodes in a UK sheep flock. Vet Parasitol 257, 48-53.
Hodgkinson, J., Cwiklinski, K., Beesley, N.J., Paterson, S., Williams, D.J., 2013. Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant *Fasciola hepatica*. Parasitol 140, 1523-1533.
Hodgkinson, J.E., Cwiklinski, K., Beesley, N., Hartley, C., Allen, K., Williams, D.J.L., 2018. Clonal amplification of *Fasciola hepatica* in *Galba truncatula*: within and between isolate variation of triclabendazole-susceptible and -resistant clones. Parasit Vectors 11, 363.
Holden-Dye, L., Walker, R.J., 1990. Avermectin and avermectin derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic muscle cells of *Ascaris*; is this the site of anthelmintic action? Parasitol 101 Pt 2, 265-271.
Holden-Dye, L., Joyner, M., O'Connor, V., Walker, R.J., 2013. Nicotinic acetylcholine receptors: a comparison of the nAChRs of *Caenorhabditis elegans* and parasitic nematodes. Parasitol Int 62, 606-615.
Hong, C., Hunt, K.R., Coles, G.C., 1994. Resistance to levamisole. Vet Rec 135, 535-536.
Hong, C., Hunt, K.R., Coles, G.C., 1996. Occurrence of anthelmintic resistant nematodes on sheep farms in England and goat farms in England and Wales. Vet Rec 139, 83-86.
Hosking, B.C., Kaminsky, R., Sager, H., Rolfe, P.F., Seewald, W., 2009. A pooled analysis of the efficacy of monepantel, an amino-acetonitrile derivative against gastrointestinal nematodes of sheep. Parasitol Res 106, 529.
Huynh, J.M., Dang, H., Munoz-Tucker, I.A., O'Ketch, M., Liu, I.T., Perno, S., Bhuyan, N., Crain, A., Borbon, I., Fares, H., 2016. ESCRT-Dependent Cell Death in a *Caenorhabditis elegans* Model of the Lysosomal Storage Disorder Mucolipidosis Type IV. Genet 202, 619-638.
Hybu Cig Cymru, H., 2015. Wales Against Anthelmintic Resistance Development, Final Project Report.
Jackson, F., Jackson, E., Coop, R.L., 1992a. Evidence of multiple anthelmintic resistance in a strain of *Teladorsagia circumcincta* (*Ostertagia circumcincta*) isolated from goats in Scotland. Res Vet Sci 53, 371-374.

Jackson, F., Jackson, E., Coop, R.L., Huntley, J., 1992b. Interactions between *Teladorsagia circumcincta* and *Trichostrongylus vitrinus* infections in young lambs. Res Vet Sci 53, 363-370.
James, C.E., Davey, M.W., 2009. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. Int J Parasitol 39, 213-220.
Jansen, J., 1987. The peri-parturient rise in sheep. Faecal worm egg counts in normal and late lambing ewes. Vet Q 9, 97-102.
Janssen, I.J., Krucken, J., Demeler, J., von Samson-Himmelstjerna, G., 2015. Transgenically expressed *Parascaris* P-glycoprotein-11 can modulate ivermectin susceptibility in *Caenorhabditis elegans*. Int J Parasitol Drugs Drug Resist 5, 44-47.
Juliano, R.L., Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 455, 152-162.
Kaminsky, R., Ducray, P., Jung, M., Clover, R., Rufener, L., Bouvier, J., Weber, S.S., Wenger, A., Wieland-Berghausen, S., Goebel, T., 2008. A new class of anthelmintics effective against drug-resistant nematodes. Nat 452.
Kaminsky, R., Mosimann, D., Sager, H., Stein, P., Hosking, B., 2009. Determination of the effective dose rate for monepantel (AAD 1566) against adult gastro-intestinal nematodes in sheep. Int J Parasitol 39, 443-446.
Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol 20.
Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: global worming and anthelmintic resistance. Vet Parasitol 186, 70-78.
Keane, O.M., Keegan, J.D., Good, B., de Waal, T., Fanning, J., Gottstein, M., Casey, M., Hurley, C., Sheehan, M., 2014. High level of treatment failure with commonly used anthelmintics on Irish sheep farms. Ir Vet J 67, 16.
Keegan, J.D., Keane, O.M., Good, B., De Waal, T., Denny, M., Hanrahan, J.P., Fitzgerald, W., Sheehan, M., 2017. A nationwide survey of anthelmintic treatment failure on sheep farms in Ireland. Ir Vet J 70, 7.
Kenyon, F., McBean, D., Greer, A.W., Burgess, C.G., Morrison, A.A., Bartley, D.J., Bartley, Y., Devin, L., Nath, M., Jackson, F., 2013. A comparative study of the effects of four treatment regimes on ivermectin efficacy, body weight and pasture contamination in lambs naturally infected with gastrointestinal nematodes in Scotland. Int J Parasitol Drugs Drug Resist 3, 77-84.
Kenyon, F., Rinaldi, L., McBean, D., Pepe, P., Bosco, A., Melville, L., Devin, L., Mitchell, G., Ianniello, D., Charlier, J., Vercruysse, J., Cringoli, G., Levecke, B., 2016. Pooling sheep faecal samples for the assessment of anthelmintic drug efficacy using McMaster and Mini-FLOTAC in gastrointestinal strongyle and *Nematodirus* infection. Vet Parasitol.
Kerboeuf, D., Hubert, J., Cardinaud, B., Blond-Riou, F., 1995. The persistence of the efficacy of injectable or oral moxidectin against *Teladorsagia*, *Haemonchus* and *Trichostrongylus* species in experimentally infected sheep. Vet Rec 137, 399.
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*. J Mol Biol 246, 500-510.
Laing, R., Maitland, K., Lecova, L., Skuce, P.J., Tait, A., Devaney, E., 2016. Analysis of putative resistance gene loci in UK field populations of *Haemonchus contortus* after 6 years of macrocyclic lactone use. Int J Parasitol 46, 621-630.
Lamb, J., Elliott, T., Chambers, M., Chick, B., 2017. Broad spectrum anthelmintic resistance of *Haemonchus contortus* in Northern NSW of Australia. Vet Parasitol 241, 48-51.
Le Jambre, L.F., Southcott, W.H., Dash, K.M., 1976. Resistance of selected lines of *Haemonchus contortus* to thiabendazole, morantel tartrate and levamisole. Int J Parasitol 6, 217-222.

Learmount, J., Stephens, N., Boughtflower, V., Barrecheguren, A., Rickell, K., Massei, G., Taylor, M., 2016. Three-year evaluation of best practice guidelines for nematode control on commercial sheep farms in the UK. Vet Parasitol 226, 116-123.
Lespine, A., Menez, C., Bourguinat, C., Prichard, R.K., 2012. P-glycoproteins and other multidrug resistance transporters in the pharmacology of anthelmintics: Prospects for reversing transport-dependent anthelmintic resistance. Int J Parasitol Drugs Drug Resist 2, 58-75.
Little, P.R., Hodge, A., Maeder, S.J., Wirtherle, N.C., Nicholas, D.R., Cox, G.G., Conder, G.A., 2011. Efficacy of a combined oral formulation of derquantel-abamectin against the adult and larval stages of nematodes in sheep, including anthelmintic-resistant strains. Vet Parasitol 181, 180-193.
Lovatt, F., Stubbings, L. 2018. Worm control in sheep for Better Returns (Agriculture and Horticulture Development Board (AHDB) Beef and Lamb).
Mani, T., Bourguinat, C., Keller, K., Ashraf, S., Blagburn, B., Prichard, R.K., 2016. Interaction of macrocyclic lactones with a *Dirofilaria immitis* P-glycoprotein. Int J Parasitol 46, 631-640.
Martin, P.J., Anderson, N., Jarrett, R.G., 1989. Detecting benzimidazole resistance with faecal egg count reduction tests and *in vitro* assays. Aust Vet J 66, 236-240.
Martinez-Valladares, M., Geldhof, P., Jonsson, N., Rojo-Vazquez, F.A., Skuce, P., 2012. *Teladorsagia circumcincta*: Molecular characterisation of the *avr-14B* subunit and its relatively minor role in ivermectin resistance. Int J Parasitol Drugs Drug Resist 2, 154-161.
McBean, D., Nath, M., Lambe, N., Morgan-Davies, C., Kenyon, F., 2016. Viability of the Happy Factor targeted selective treatment approach on several sheep farms in Scotland. Vet Parasitol 218, 22-30.
McIntyre, J., Hamer, K., Morrison, A.A., Bartley, D.J., Sargison, N., Devaney, E., Laing, R., 2018. Hidden in plain sight - Multiple resistant species within a strongyle community. Vet Parasitol 258, 79-87.
McKenna, P.B., 1997. Further potential limitations of the undifferentiated faecal egg count reduction test for the detection of anthelmintic resistance in sheep. N Z Vet J 45, 244-246.
McMahon, C., Bartley, D.J., Edgar, H.W., Ellison, S.E., Barley, J.P., Malone, F.E., Hanna, R.E., Brennan, G.P., Fairweather, I., 2013a. Anthelmintic resistance in Northern Ireland (I): prevalence of resistance in ovine gastrointestinal nematodes, as determined through faecal egg count reduction testing. Vet Parasitol 195, 122-130.
McMahon, C., McCoy, M., Ellison, S.E., Barley, J.P., Edgar, H.W., Hanna, R.E., Malone, F.E., Brennan, G.P., Fairweather, I., 2013b. Anthelmintic resistance in Northern Ireland (III): uptake of 'SCOPS' (Sustainable Control of Parasites in Sheep) recommendations by sheep farmers. Vet Parasitol 193, 179-184.
Ménez, C., Alberich, M., Courtot, E., Guegnard, F., Blanchard, A., Aguilaniu, H., Lespine, A., 2019. The transcription factor NHR-8: A new target to increase ivermectin efficacy in nematodes. PLoS Pathog 15, e1007598.
Mitchell, E.S., Hunt, K.R., Wood, R., McLean, B., 2010. Anthelmintic resistance on sheep farms in Wales. Vet Rec 166, 650-652.
Mitchell, S., 2016. Parasitic gastroenteritis in sheep. Vet Rec 179, 223-224.
Morgan, D.O., Parnell, I.W., Rayski, C., 1951. The Seasonal Variations in the Worm Burden of Scottish Hill Sheep. J Helminthol 25, 177-212.
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. Vet J 192, 390-397.
Niciura, S.C.M., Tizioto, P.C., Moraes, C.V., Cruvinel, G.G., de Albuquerque, A.C.A., Santana, R.C.M., Chagas, A.C.d.S., Esteves, S.N., Benavides, M.V., do Amarante, A.F.T., 2019. Extreme-QTL mapping of monepantel resistance in *Haemonchus contortus*. Parasit Vectors 12, 403.
Njue, A.I., Prichard, R.K., 2004. Genetic variability of glutamate-gated chloride channel genes in ivermectin-susceptible and -resistant strains of *Cooperia oncophora*. Parasitol 129, 741-751.

Page, A.P., 2018. The sensory amphidial structures of *Caenorhabditis elegans* are involved in macrocyclic lactone uptake and anthelmintic resistance. Int J Parasitol 48, 1035-1042.
Peterson, B.K., Weber, J.N., Kay, E.H., Fisher, H.S., Hoekstra, H.E., 2012. Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. PLoS One 7, e37135.
Redman, E., Packard, E., Grillo, V., Smith, J., Jackson, F., Gilleard, J.S., 2008. Microsatellite analysis reveals marked genetic differentiation between *Haemonchus contortus* laboratory isolates and provides a rapid system of genetic fingerprinting. Int J Parasitol 38, 111-122.
Redman, E., Sargison, N., Whitelaw, F., Jackson, F., Morrison, A., Bartley, D.J., Gilleard, J.S., 2012. Introgression of ivermectin resistance genes into a susceptible *Haemonchus contortus* strain by multiple backcrossing. PLoS Pathog 8, e1002534.
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 Negl Trop Dis 9, e0003494.
Rezansoff, A.M., Laing, R., Gilleard, J.S., 2016. Evidence from two independent backcross experiments supports genetic linkage of microsatellite Hcms8a20, but not other candidate loci, to a major ivermectin resistance locus in *Haemonchus contortus*. Int J Parasitol 46, 653-661.
Rinaldi, L., Coles, G.C., Maurelli, M.P., Musella, V., Cringoli, G., 2011. Calibration and diagnostic accuracy of simple flotation, McMaster and FLOTAC for parasite egg counts in sheep. Vet Parasitol 177, 345-352.
Roeber, F., Jex, A.R., Gasser, R.B., 2013. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance - an Australian perspective. Parasit Vectors 6, 153.
Roy, E.A., Hoste, H., Beveridge, I., 2004. The effects of concurrent experimental infections of sheep with *Trichostrongylus colubriformis* and *T. vitrinus* on nematode distributions, numbers and on pathological changes. Parasite 11, 293-300.
Rufener, L., Mäser, P., Roditi, I., Kaminsky, R., 2009. *Haemonchus contortus* Acetylcholine Receptors of the DEG-3 Subfamily and Their Role in Sensitivity to Monepantel. PLoS Pathog 5, e1000380.
Salisbury, J.R., Arundel, J.H., 1970. Peri-parturient deposition of nematode eggs by ewes and residual pasture contamination as sources of infection for lambs. Aust Vet J 46.
Sallé, G., Doyle, S.R., Cortet, J., Cabaret, J., Berriman, M., Holroyd, N., Cotton, J.A., 2019. The global diversity of *Haemonchus contortus* is shaped by human intervention and climate. Nat Commun 10, 4811.
Sangster, N.C., Whitlock, H.V., Russ, I.G., Gunawan, M., Griffin, D.L., Kelly, J.D., 1979. *Trichostrongylus colubriformis* and *Ostertagia circumcincta* resistant to levamisole, morantel tartrate and thiabendazole: occurrence of field strains. Res Vet Sci 27, 106-110.
Sargison, N., Scott, P., Jackson, F., 2001. Multiple anthelmintic resistance in sheep. Vet Rec 149, 778-779.
Sargison, N.D., Jackson, F., Bartley, D.J., Moir, A.C., 2005. Failure of moxidectin to control benzimidazole-, levamisole- and ivermectin-resistant *Teladorsagia circumcincta* in a sheep flock. Vet Rec 156, 105-109.
Sargison, N.D., Jackson, F., Wilson, D.J., Bartley, D.J., Penny, C.D., Gilleard, J.S., 2010. Characterisation of milbemycin-, avermectin-, imidazothiazole- and benzimidazole-resistant *Teladorsagia circumcincta* from a sheep flock. Vet Rec 166, 681-686.
Sargison, N.D., 2014. Sustainable helminth control practices in the United Kingdom. Small Ruminant Res 118, 35-40.
Scott, I., Pomroy, W.E., Kenyon, P.R., Smith, G., Adlington, B., Moss, A., 2013. Lack of efficacy of monepantel against *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Vet Parasitol 198, 166-171.

Silvestre, A., Chartier, C., Sauve, C., Cabaret, J., 2000. Relationship between helminth species diversity, intensity of infection and breeding management in dairy goats. Vet Parasitol 94, 91-105.
Silvestre, A., Cabaret, J., 2002. Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? Mol Biochem Parasitol 120, 297-300.
Simao, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., Zdobnov, E.M., 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31, 3210-3212.
Sinclair, R., Melville, L., Sargison, F., Kenyon, F., Nussey, D., Watt, K., Sargison, N., 2016. Gastrointestinal nematode species diversity in Soay sheep kept in a natural environment without active parasite control. Vet Parasitol 227, 1-7.
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. Int J Parasitol 40, 1247-1255.
Smeal, M.G., Gough, P.A., Jackson, A.R., Hotson, I.K., 1968. The occurrence of strains of *Haemonchus contortus* resistant to thiabendazole. Aust Vet J 44, 108-109.
Smith, W.D., Jackson, F., Jackson, E., Williams, J., 1985. Age immunity to *Ostertagia circumcincta*: comparison of the local immune responses of 4 1/2- and 10-month-old lambs. J Comp Pathol 95, 235-245.
Smus, J.P., Ludlow, E., Dalliere, N., Luedtke, S., Monfort, T., Lilley, C., Urwin, P., Walker, R.J., O'Connor, V., Holden-Dye, L., Mahajan, S., 2017. Coherent anti-Stokes Raman scattering (CARS) spectroscopy in *Caenorhabditis elegans* and *Globodera pallida*: evidence for an ivermectin-activated decrease in lipid stores. Pest Manag Sci 73, 2550-2558.
Sommerville, R.I., 1953. Development of *Ostertagia circumcincta* in the Abomasal Mucosa of the Sheep. Nat 171, 482-483.
Sutherland, I.A., Leathwick, D.M., Moen, I.C., Bisset, S.A., 2002. Resistance to therapeutic treatment with macrocyclic lactone anthelmintics in *Ostertagia circumcincta*. Vet Parasitol 109, 91-99.
Thienpont, D., Vanparijs, O.F.J., Raeymaekers, A.H.M., Vandenberk, J., Demoen, P.J.A., Allewijn, F.T.N., Marsboom, R.P.H., Niemegeers, C.J.E., Schellekens, K.H.L., Janssen, P.A.J., 1966. Tetramisole (R 8299), A New, Potent Broad Spectrum Anthelmintic. Nat 209, 1084-1086.
Turnbull, F., Jonsson, N.N., Kenyon, F., Skuce, P.J., Bisset, S.A., 2018. P-glycoprotein-9 and macrocyclic lactone resistance status in selected strains of the ovine gastrointestinal nematode, *Teladorsagia circumcincta*. Int J Parasitol Drugs Drug Resist 8, 70-80.
Turnbull, F., Devaney, E., Morrison, A.A., Laing, R., Bartley, D.J., 2019. Genotypic characterisation of monepantel resistance in historical and newly derived field strains of *Teladorsagia circumcincta*. Int J Parasitol Drugs Drug Resist 11, 59-69.
Urdaneta-Marquez, L., Bae, S.H., Janukavicius, P., Beech, R., Dent, J., Prichard, R., 2014. A *dyf-7* haplotype causes sensory neuron defects and is associated with macrocyclic lactone resistance worldwide in the nematode parasite *Haemonchus contortus*. Int J Parasitol 44, 1063-1071.
Valentim, C.L.L., Cioli, D., Chevalier, F.D., Cao, X., Taylor, A.B., Holloway, S.P., Pica-Mattoccia, L., Guidi, A., Basso, A., Tsai, I.J., Berriman, M., Carvalho-Queiroz, C., Almeida, M., Aguilar, H., Frantz, D.E., Hart, P.J., LoVerde, P.T., Anderson, T.J.C., 2013. Genetic and molecular basis of drug resistance and species-specific drug action in schistosome parasites. Science 342, 1385-1389.
Van den Brom, R., Moll, L., Kappert, C., Vellema, P., 2015. *Haemonchus contortus* resistance to monepantel in sheep. Vet Parasitol 209, 278-280.
van Dijk, J., Morgan, E.R., 2011. The influence of water on the migration of infective trichostrongyloid larvae onto grass. Parasitol 138, 780-788.

van Wyk, J.A., Malan, F.S., 1988. Resistance of field strains of *Haemonchus contortus* to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. Vet Rec 123, 226-228.
Varady, M., Biorn, H., Nansen, P., 1996. *In vitro* characterization of anthelmintic susceptibility of field isolates of the pig nodular worm *Oesophagostomum spp.*, susceptible or resistant to various anthelmintics. Int J Parasitol 26, 733-740.
von Samson-Himmelstjerna, G., Coles, G., Jackson, F., Bauer, C., Borgsteede, F., Cirak, V., Demeler, J., Donnan, A., Dorny, P., Epe, C., 2009. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. Parasitol Res 105.
Wang, C., Paul, M. 2017. eggCounts: Hierarchical Modelling of Faecal Egg Counts.
Wang, C., Torgerson, P.R., Hoglund, J., Furrer, R., 2017. Zero-inflated hierarchical models for faecal egg counts to assess anthelmintic efficacy. Vet Parasitol 235, 20-28.
Waterhouse, R.M., Seppey, M., Simão, F.A., Manni, M., Ioannidis, P., Klioutchnikov, G., Kriventseva, E.V., Zdobnov, E.M., 2017. BUSCO Applications from Quality Assessments to Gene Prediction and Phylogenomics. Mol Biol Evol 35, 543-548.
Williamson, S.M., Storey, B., Howell, S., Harper, K.M., Kaplan, R.M., Wolstenholme, A.J., 2011. Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of *Haemonchus contortus*. Mol Biochem Parasitol 180, 99-105.
Wilson, D.J., Sargison, N.D., Scott, P.R., Penny, C.D., 2008. Epidemiology of gastrointestinal nematode parasitism in a commercial sheep flock and its implications for control programmes. Vet Rec 162, 546-550.
Wimmer, B., Craig, B.H., Pilkington, J.G., Pemberton, J.M., 2004. Non-invasive assessment of parasitic nematode species diversity in wild Soay sheep using molecular markers. Int J Parasitol 34, 625-631.