Characterising gastrointestinal nematode populations and anthelmintic resistance in Scottish dairy calves

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Abstract

Gastrointestinal nematode (GIN) infections are ubiquitous in pasture-based grazing systems in the UK and worldwide, and are detrimental to animal health, welfare, and the productivity of livestock systems. These infections comprise co-infections with multiple species within a single host(s). Helminth species vary in epidemiology, pathogenicity, and anthelmintic sensitivity; however, the interactions among these parasites and the influence of management practices remain poorly understood. The most economically and clinically important GIN species infecting cattle in Europe are the abomasal parasite *Ostertagia ostertagi* and the small-intestinal-dwelling *Cooperia oncophora*, with the former being the more pathogenic. To date, the control of GIN infections has primarily been achieved using three anthelmintic classes: benzimidazoles, imidazothiazoles, and macrocyclic lactones (ML), but over-reliance on treatment has inevitably resulted in the development of resistance. Although resistance in GIN of cattle appears to be developing more slowly than in nematodes infecting small ruminants, reports of resistance in the literature are increasing, suggesting an escalating problem.

For this reason, there is a pressing need to conserve anthelmintic efficacy and develop sustainable control measures. However, recommendations for sustainable parasite control in cattle are often extrapolated from sheep-based research, as cattle-specific research is inherently more challenging. The lack of sensitive tests for anthelmintic resistance limits research and surveillance. The purpose of this thesis was to advance understanding of the GIN communities infecting cattle on Scottish dairy farms, with a particular focus on characterising species composition and assessing anthelmintic resistance status. *In vivo*, *in vitro*, and molecular diagnostic approaches were used to characterise the GIN populations, and a genome-wide association study was used to identify genomic regions under ML selection within *Os. ostertagi* field populations. A longitudinal study examining the influence of management practices and anthelmintic treatment demonstrated that many of the established patterns of the most clinically important GIN species of cattle remain consistent when assessed using modern molecular techniques, despite the passage of fifty to sixty years and substantial changes in dairy farming practices since the original empirical studies.

The faecal egg count reduction test and egg hatch test highlighted the complexities involved in interpreting resistance tests, particularly with mixed species communities. These tests underscored the need for clearly defined criteria and thresholds if these methods are to be applied reliably in the field. Finally, the forward genetic approach identified a novel QTL associated with ML resistance on chromosome 5. The detection of this QTL in a major cattle parasite is the first major step toward understanding the genetic basis of ML resistance and will be used to advance our understanding of resistance in the field.

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Authors Declarations

The work presented in this thesis was performed by the author unless otherwise stated. The work described herein is unique and will not be submitted elsewhere for any other degree or qualification at any other university.

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Definitions / Abbreviations

ABZ	Albendazole
AChR	Acetylcholine receptor
ASV	Amplicon sequence variant
AR	Anthelmintic resistance
BLAST	Basic local alignment search tool
BUSCO	Benchmarking universal single-copy orthologs
BZ	Benzimidazole
CI	Confidence interval
COWS	Control of worms sustainably
dpi	Days post-infection
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOR	Doramectin
$EC_{50:}$	Half maximal effective concentration
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EHT	Egg hatch test
EPG	Eggs per gram of faeces
EPM	Eprinomectin
FAO	Food and Agriculture Organization of the United Nations
FBZ	Fenbendazole
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
FGS	First grazing season
F_{ST}	Fixation index
gDNA	Genomic DNA
GIN	Gastrointestinal nematode
GWAS	Genome-wide association study
ITS-2	Internal transcribed spacer 2
IVM	Ivermectin
kb	Kilobase (denotes base pair length)
kbp	Kilobase-pair (denotes genomic location)
L_1	First-stage larvae
L_2	Second-stage larvae
L_3	Third-stage larvae
L_4	Fourth-stage larvae
LDA	Larval development assay
LEV	Levamisole
ML	Macrocyclic lactone
MOX	Moxidectin
MOX-LA	Long-acting moxidectin formulation
MT-PCR	Multiplexed-tandem PCR
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NGS	Next-generation sequencing
NST	Neo-suppressive treatment
NTC	No template control
OFZ	Oxfendazole

PCR	Polymerase chain reaction
PE	Paired-end
PGE	Parasitic gastroenteritis
PGP	P-glycoprotein
PPP	Pre-patent period
rDNA	Ribosomal DNA
RR	Resistance ratio
RT-PCR	Real-time PCR
SCOPS	Sustainable control of parasites in sheep
SD	Standard deviation
SNP	Single-nucleotide polymorphism
SPT	Strategic prophylactic treatment
SRA	Sequence read archive
TBZ	Thiabendazole
TST	Targeted selective treatment
WAAVP	World Association for the Advancement of Veterinary Parasitology

Chapter 1

1. Introduction

In this introduction, I provide the background and justification for this work. In this thesis, I present the results of: (1) seasonal patterns of faecal egg counts and gastrointestinal nematode species composition in Scottish dairy calves; (2) anthelmintic resistance to benzimidazoles and macrocyclic lactones in gastrointestinal nematode populations of cattle farms in Scotland; (3) the development of a mixed amplicon sequencing marker panel for surveillance of anthelmintic resistance in *Ostertagia ostertagi*; (4) the genome-wide analysis of the response to anthelmintic treatment by field populations of *Os. ostertagi*; (5) the inefficacy of ivermectin and moxidectin treatments against *Dictyocaulus viviparus* in dairy calves. Collectively, this work advocates for the development of molecular diagnostic tools for assessing anthelmintic resistance in livestock and serves as an important resource illustrating their practical application in the field.

1.1. Parasitic gastroenteritis – A priority disease for livestock producers

Agriculture - particularly livestock production - is crucial to economic and nutritional security and foundational to socio-cultural development worldwide. In response to growing challenges - including population growth, declining natural resources, and climate change – the Food and Agriculture Organization of the United Nations (FAO) has set a target of increasing global food production by 70% by 2050 ("FAO's Director-General on How to Feed the World in 2050," 2009). To reach this target, livestock producers must balance increasing societal and economic pressures, including an unstable economic climate and decreasing profit margins, while prioritising efficiency, sustainability, and the health and welfare of their animals. Gastrointestinal nematode (GIN) infections are ubiquitous, as is their associated disease, parasitic gastroenteritis (PGE), in both wild and domestic ruminants, posing a significant obstacle to these objectives. The annual cost associated with helminth infections to the ruminant industry in Europe is €1.8 billion (Charlier et al., 2020b) and is estimated to be tens of billions of dollars worldwide. Thus, the livestock

industry could achieve significant economic benefits by improving control of helminth infections, and such efficiencies of production would also reduce environmental costs.

1.2. Gastrointestinal nematodes of the bovine

A single parasite species does not exist in isolation; rather, co-infection with multiple GIN species, forming complex communities, is ubiquitous in pasture-based livestock systems. More than twenty GIN species are known to infect cattle worldwide (Charlier et al., 2020a), although their clinical significance varies by species, host age, and immune status (Ma and Michailides, 2005). The GINs of greatest clinical significance to the livestock industry are of the superfamily *Trichostrongyloidea*: *Cooperia*, *Haemonchus*, *Ostertagia*, *Teladorsagia* and *Trichostrongylus*.

Each species typically occupies a specific site within the gastrointestinal tract, either the abomasum, small intestine, or large intestine, and tends to be host-specific, infecting only cattle. However, *Haemonchus* and *Trichostrongylus* spp. can cross-infect between small ruminants and cattle. The most clinically significant GIN species of cattle, along with their distribution and other characteristics, are summarised in Table 1.1.

Table 1.1 Characteristics of the most clinically significant gastrointestinal nematodes of cattle

Species	Distribution (climate)	Localisation	Pre-patent period (days)	Clinical significance	Study
Cooperia oncophora	Temperate	Small intestine	14-17	++	
Cooperia pectinata	(Sub)tropical	Small intestine	14-17	++	(Isenstein, 1963)
Cooperia punctata	(Sub)tropical	Small intestine	14-17	++	
Haemonchus placei	(Sub)tropical	Abomasum	29-32	+++	(Bremner, 1956)
Nematodirus helvetianus	Temperate	Small intestine	21-26	+	(Herlich, 1954)
Nematodirus spathiger	Temperate	Small intestine	14-16	+	(Kates and Turner, 1955)
Oesophagostomum radiatum	Worldwide	Large intestine	37-41	+	(Andrews and Maldonado, 1941)
Oesophagostomum venulosum	Worldwide	Large intestine	28-30	+	(Wood et al., 1995)
Ostertagia ostertagi	Temperate	Abomasum	26-28	+++	(Rose, 1969)
Trichostrongylus colubriformis	Worldwide	Small intestine	21	++	(Mönnig, 1926)
Trichostrongylus axei	Worldwide	Abomasum	21	+	(Douvres, 1957)

1.1. Epidemiology of gastrointestinal nematodes

1.1.1. Gastrointestinal nematode abundance and distribution

The two most clinically significant GIN species are *Cooperia oncophora* and *Os. ostertagi,* as both are common and relatively pathogenic. *Cooperia oncophora* is particularly common in youngstock during their first grazing season (FGS) and contributes to the majority of the faecal egg count (FEC) until at least late summer in temperate regions. Cattle can mount an effective immune response to this nematode; consequently, both adult burden and faecal egg output typically decline towards the end of the first grazing season and remain low in subsequent years.

The "nemabiome" refers to the community of GIN inhabiting a single host or environmental niche analogous to the microbiome (Avramenko et al., 2015). Interactions between co-infecting GIN species are relatively poorly understood, as most research has focused on individual species in isolation (Evans et al., 2023). However, most natural infections involve complex co-infections of the host's gastrointestinal tract by multiple GIN species. In temperate regions, the composition of GIN communities varies seasonally and geographically, with additional variation observed between age groups and among different management groups on the same farm. These complex co-infection dynamics may influence disease pathogenesis, the response to anthelmintic treatment, and the development of anthelmintic resistance (Evans et al., 2023).

Significant geographical variation exists in the composition of parasite communities. Epidemiological studies from northern temperate regions, such as Europe and specifically the UK (Roeber et al., 2017a), demonstrate that the predominant species are *Os. ostertagi* and *Cooperia oncophora*. However, other species are also present in specific regions. This contrasts with tropical and subtropical regions, where GIN species composition is more diverse, and *Cooperia pectinata*, *Cooperia punctata*, and *Haemonchus* spp. are more abundant (Albrechtová et al., 2020; Avramenko et al., 2017; Roeber et al., 2017a).

1.1.2. Life cycle of gastrointestinal nematodes

All GIN species have a direct life cycle (Figure 1.1). Eggs produced by patent females are passed in the host's faeces and develop to the infective stage (L_3) within

the faecal pat on pasture. Gastrointestinal nematodes develop through a series of progressive moults; with development from $L_{1\ to}$ L_3 occurring over 1-2 weeks under optimal conditions (Khadijah et al., 2013). Translocation takes place during moist conditions when the L_3 migrate from the faecal pat to the surrounding herbage (Dijk and Morgan, 2011). The L_3 possesses a thick cuticle, an outer covering that protects the larva from the external environment, particularly from desiccation, while maintaining a homeostatic interior (Basyoni and Rizk, 2016). This retained cuticle enables survival on pasture for a few months to over a year for some species; however, like all free-living stages, survival is highly dependent on weather conditions and microclimate (Slocombe, 1974).

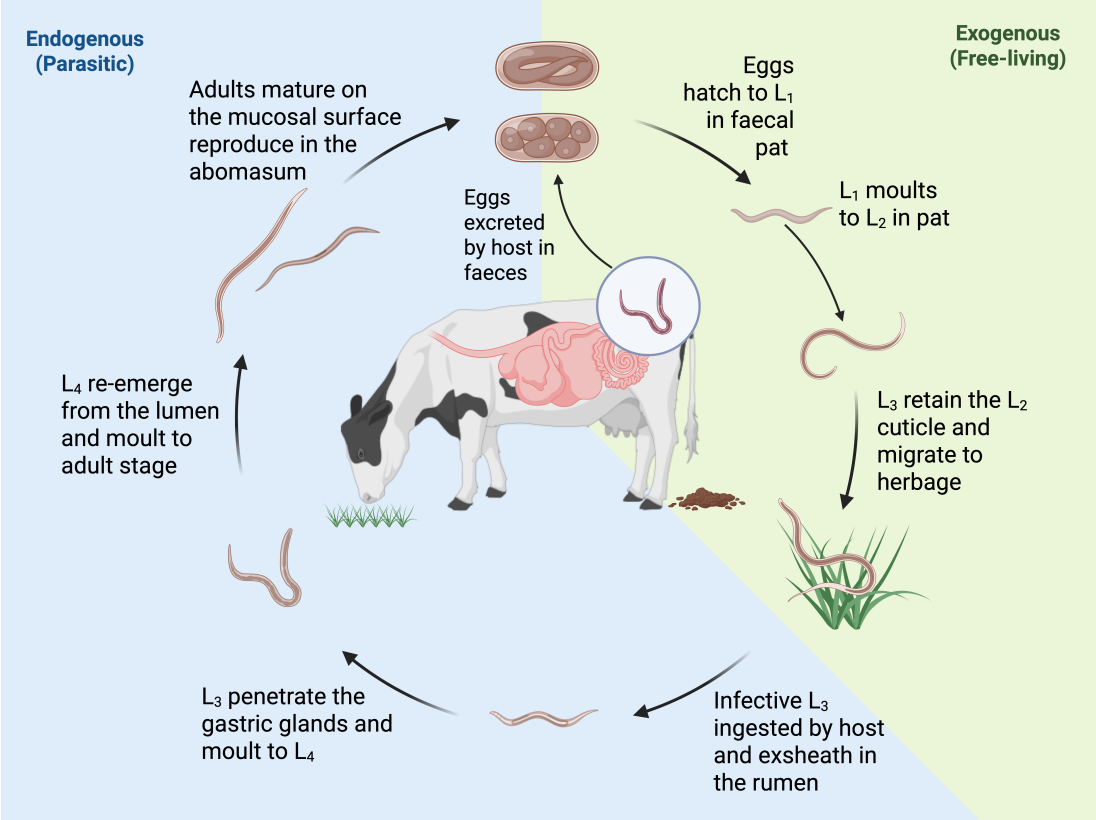

Figure 1.1 | The life cycle of *Ostertagia ostertagi* is an example of a typical Trichostrogylid.

Eggs are passed in faeces and develop to the infective L_3 stage, through successive moults. The L_3 retains its L_2 cuticle, migrates from the faecal pat to the herbage and is subsequently ingested by the host. In the rumen, L_3 exsheathes and subsequently migrates to the abomasum, its predilection site, where it penetrates the gastric glands and develops into the L_4. The nematode then emerges from the gastric glands as an immature adult and reaches sexual maturity on the mucosal surface. *Created with BioRender.com*

Following ingestion of the L_3 by a suitable host, the larva undergoes a major biological transformation from its free-living to the parasitic stage. This transition is initiated by exsheathment of the cuticle in response to stimuli from the host rumen or abomasum (Bekelaar et al., 2019; Sommerville, 1957). In *Os. ostertagi*, the exsheathed L_3 passes into the abomasum, where moulting occurs within the mucosa of the abomasum or, in other species, the intestinal mucosa. Development to L_4 follows invasion of the gastric glands, typically 7 to 10 days post-infection (dpi). The larvae re-emerge into the lumen between 10 and 14 dpi, where they reach sexual maturity as adults (Fox, 1997; Klesius, 1993). Gravid *Os. ostertagi* females can be detected as early as 16 dpi, with a pre-patent period of approximately 21 days (Rose, 1969).

1.1.3. Hypobiosis

Hypobiosis is a key phenomenon in the epidemiology of GIN infections in ruminants, particularly in *Os. ostertagi* and *Haemonchus contortus*, which exhibit a high propensity for developmental arrest. The facultative cessation of development occurs at a defined stage post-infection, most commonly at early L_4 (*Os. ostertagi*, *H. contortus* (Fernández et al., 1999), L_4 (*Trichostrongylus* spp., (Gibbs, 1986)) or early L_5 (*Dictyocaulus viviparus*, (Strube et al., 2007)) stage. Arrested development serves as a survival strategy, allowing GIN to preserve reproductive potential during periods of adverse environmental conditions until circumstances improve for development and transmission of progeny.

The occurrence of hypobiosis is influenced by climate, host management, and immune responses, although no single causative factor has been identified (Fernández et al., 1999). Climatic conditions can produce two general patterns: “winter inhibition”, in which larvae arrest prior to winter in temperate climates, and “dry-season inhibition”, which occurs during arid periods. For example, in Canada, where winters are severe, most *Trichostrongyloid* larvae ingested in late autumn become arrested, whereas in the UK, where winters are milder, only 50 to 60% undergo arrest (Armour et al., 1996). Hypobiosis may also result from host immune responses that regulate parasite establishment, development, fecundity, and survival (Verschave et al., 2014).

1.1.4. Parasitic gastroenteritis

Clinical PGE is characterised by profuse diarrhoea, dull demeanour, poor hair coat, and progressive loss of body condition. However, overt clinical disease is relatively uncommon; more frequently, GIN infections exert subclinical effects, reducing production efficiency without obvious signs of illness. Clinical PGE often reflects co-infections of both the abomasum and the intestines. These co-infections can have synergistic effects, as parasitised small intestines cannot fully compensate for the impaired protein digestion caused by abomasal parasitism (Snider et al., 1985). The severity of the disease depends on the infecting species and their predilection sites.

The pathophysiological effects of GIN infection (Figure 1.2) arise from three main mechanisms:

1. Direct effects - physical damage of tissues (pathological)
2. Indirect effects – host immune responses to infection (physiological)
3. Behavioural effects - reduced feed intake due to infection-associated hormonal changes

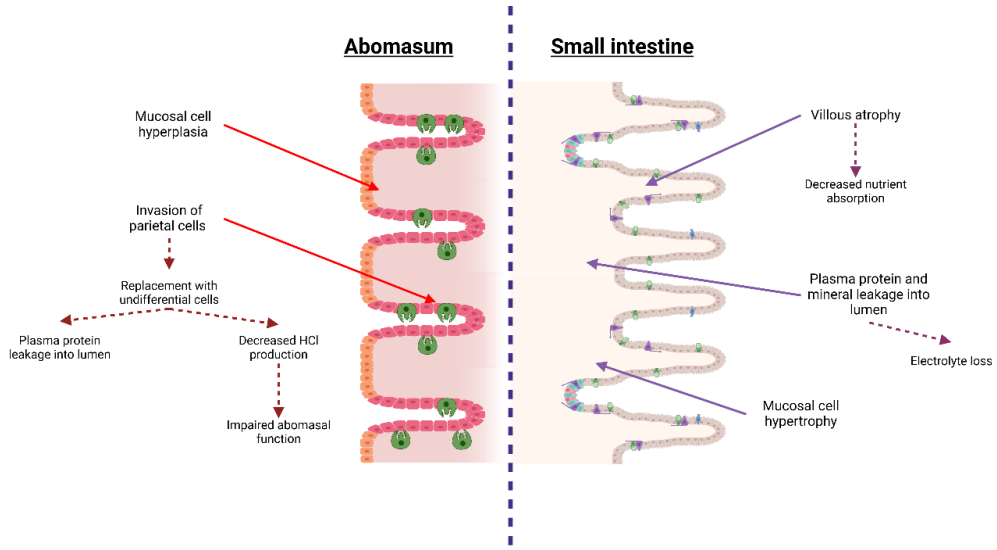

Figure 1.2 | Schematic of the potential causes of pathology during infection of the bovine gastrointestinal tract by gastrointestinal nematodes.

1.1.4.1. Infection with *Ostertagia ostertagi* (Ostertagiosis)

Clinically, ostertagiosis is characterised by diarrhoea, submandibular oedema, weight loss, and hypoalbuminemia, which, in severe cases, can result in mortality. Two distinct forms of the disease are recognised: Type I and Type II ostertagiosis.

Type I ostertagiosis is the classic presentation, typically observed in susceptible youngstock during their first grazing season. Infections occur when infective L_3 are ingested daily from contaminated pasture and invade the gastric glands of the abomasum in a continuous and cumulative process. This syndrome is associated with relatively high morbidity and is most common during late summer and autumn, usually from mid-July onwards. Clinical signs include green, profuse diarrhoea and, in some cases, weight loss and anorexia. Faecal egg counts may not always be markedly elevated.

Type II ostertagiosis occurs primarily in yearlings after a large number of L_3 are ingested late in the grazing season and subsequently undergo hypobiosis. The disease typically manifests in late winter (January–February), when inhibited larvae resume development into adults. The clinical presentation is similar to Type I in severely affected animals, but the severity depends on the scale of larval emergence from the abomasal glands. Gradual emergence can lead to a protracted disease course, whereas massive synchronous development results in acute, severe disease that can be rapidly fatal (Myers and Taylor, 1989). The incidence of Type II disease, as well as other clinical forms of ostertagiosis, has declined with the introduction of modern anthelmintics that are highly effective against inhibited and other early developmental stages.

The primary gross lesions associated with both Type I and Type II ostertagiosis consist of multiple white, raised, umbilicated nodules, typically 2 to 10 mm in diameter, on the mucosal surface of the abomasum. These lesions are frequently accompanied by mucosal reddening and oedema. In severe infections, these nodules may coalesce, producing the characteristic “Morocco leather” appearance of the abomasal mucosa (Figure 1.3). Histologically, larval invasion of the gastric glands by larvae results in epithelial hyperplasia, glandular distension, and loss of differentiation between parietal and chief cells.

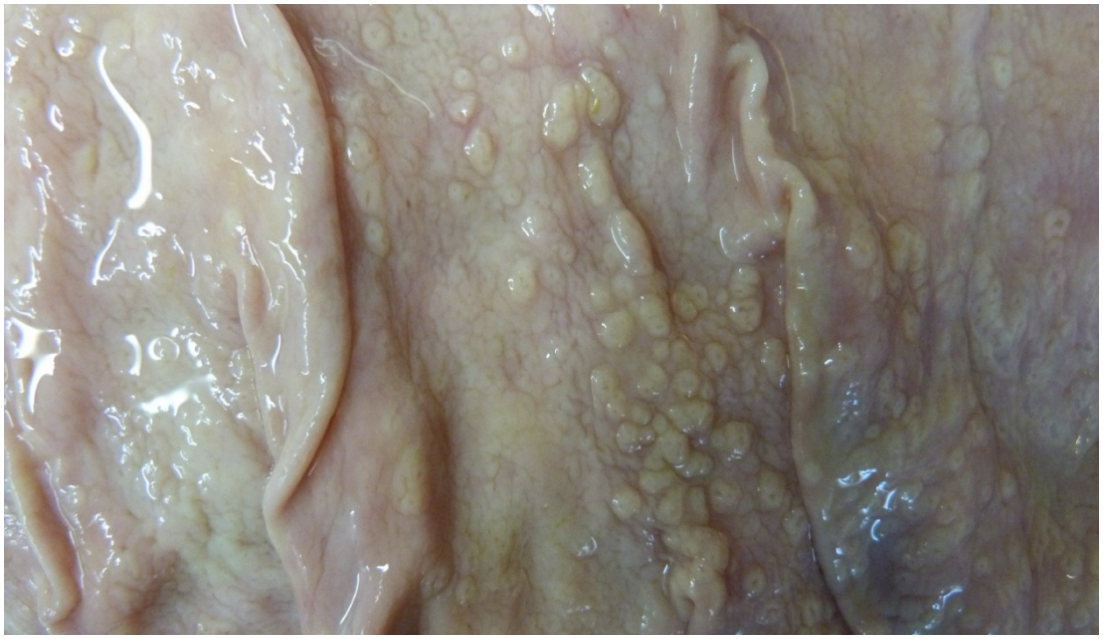

Figure 1.3 | *Ostertagia ostertagi* abomasal legions

Multifocal and coalescing hyperplastic abomasal lesions of parasitised gastric glands associated with *Ostertagia ostertagi* larval invasion. Image provided courtesy of Prof. Andrew Forbes.

1.1.4.2. Infection with *Cooperia* spp. (Cooperiosis)

Cooperia oncophora is generally regarded as only mildly pathogenic; even moderate infections typically cause no more than transient diarrhoea and softening of the faeces (Evans et al., 2023; Kloosterman et al., 1984). Nevertheless, subclinical infections may still influence host behaviour, with evidence suggesting a significant reduction in grazing time (Forbes et al., 2000). Villous atrophy, together with a reduction in brush-border enzyme activity, has been observed only in experimentally infected animals. The relatively low pathogenicity of this species is likely attributable to the superficial nature of the intestinal lesions it induces, combined with the host's rapid acquisition of immunity.

1.2. The bovine lungworm, *Dictyocaulus viviparus*

The bovine lungworm, *Dictyocaulus viviparus*, although not strictly part of the GIN complex due to its primary pathogenesis in the respiratory tract, shares important epidemiological and management considerations with GIN. It is a Clade V parasitic nematode with part of its life cycle occurring in the gastrointestinal tract. Although its disease presentation is distinct, it should be addressed alongside GIN in integrated

parasite control programmes. Anthelmintic treatment options are the same as for GIN, and a similar risk-based framework can be applied, evaluating both the animal's immunity status and environmental risk factors such as pasture contamination.

Parasitic bronchitis caused by *D. viviparus* can present in a range of clinical forms, from acute morbidity and mortality to clinically normal carriers. Clinical disease is characterised by bronchitis, with associated oedema, emphysema, and an inflammatory response (Forbes et al., 2000). In moderately affected cattle, signs include frequent intermittent coughing at rest, tachypnoea (respiratory rate >60 breaths/min), and hyperpnoea. Severely affected animals may exhibit pronounced tachypnoea (respiratory rate >80 breaths/min) and dyspnoea, often adopting the 'air-hunger' position - mouth breathing with head and neck outstretched. Residual lesions may persist after apparent recovery, and lung damage may be irreversible, with the loss of ciliated epithelium potentially predisposing individuals to secondary respiratory infections (Schnieder et al., 1991).

Although a vaccine (Bovilis Huskvac, MSD Animal Health) and long-acting, broad-spectrum anthelmintic drugs are available, clinical disease due to bovine lungworm remains an ongoing concern. Since the 1990s, outbreaks have increased in frequency (David, 1997), and the epidemiology has become increasingly unpredictable, with sporadic outbreaks occurring throughout the year and considerable variation between regions and seasons (McCarthy and van Dijk, 2020).

1.2.1. *Dictyocaulus viviparus* life cycle

Similar to other GIN species, *D. viviparus* has a direct life cycle (see Figure 1.4). However, unlike GIN, adult females are ovoviviparous, producing eggs that contain fully developed larvae which hatch almost immediately. These L_1 migrate up the trachea, are ingested, and are subsequently excreted in the host's faeces. The development from L_1 to L_3 occurs rapidly on pasture. Under favourable environmental conditions, this transformation is typically completed within one week (Jørgensen, 1980). This rapid development is thought to be supported by the larvae's relatively large lipid reserves, allowing these pre-infective stages to survive without feeding (Croll, 1973).

After ingestion by the host, the infective L_3 penetrate the wall of the small intestine and migrate to the mesenteric lymph nodes (Jarrett et al., 1957), where they

moult into L_4. From there, they travel to the lungs via the lymphatic and/or circulatory systems. If environmental conditions are unfavourable, L_4 may undergo hypobiosis, remaining dormant for up to five months.

Initially, L_4 colonise the alveoli, then move into the bronchioles, where they undergo a final moult to become mature adults. These adults settle in the bronchi and at the base of the trachea, where, in large numbers, they may obstruct the airway lumen. Female lungworms are highly fecund, producing an estimated 1,000 to 25,000 L_1 per day (Ploeger and Eysker, 2000). The host coughs up these eggs/L_1, which are subsequently swallowed, hatch, and are passed as L_1 in the faeces.

This parasitic nematode also has a unique relationship with *Pilobolus* spp. of fungi. When *Pilobolus* spp. are present in the faeces, the L_3 migrate to the tips of the fungal fruiting bodies. Upon spore release, the infective larvae are passively propelled onto the surrounding pasture, reaching distances of up to 1 metre (Jørgensen et al., 1982).

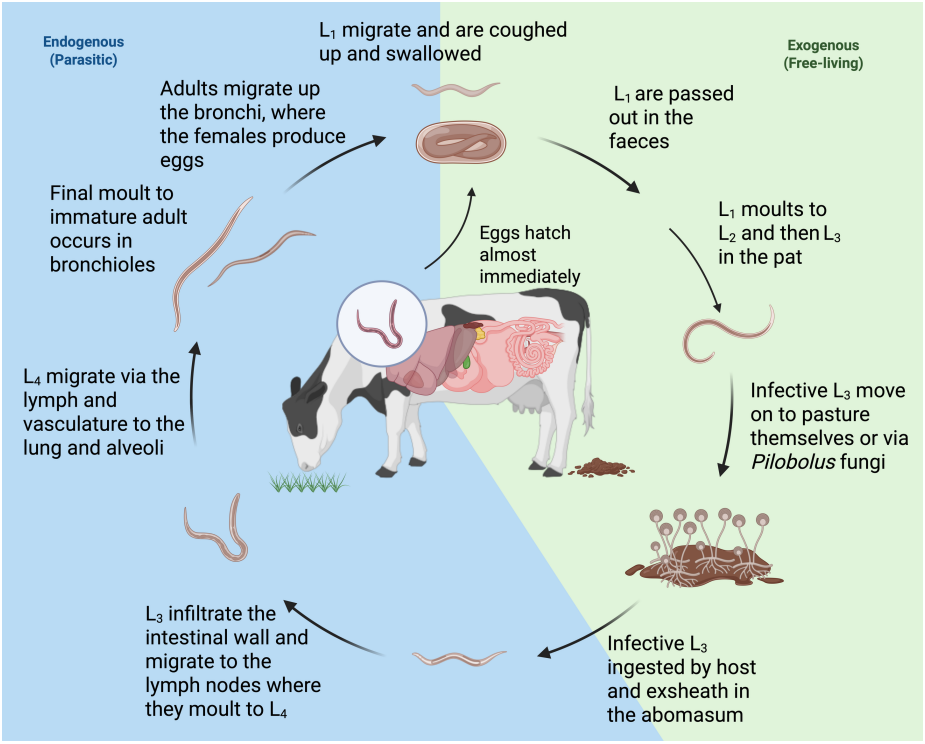

Figure 1.4 | The life cycle of the bovine lungworm *Dictyocaulus viviparus*

Adult worms reside in the lungs, specifically within the bronchi and bronchioles, where ovoviviparous females release eggs that hatch immediately. The L_1 are swallowed and subsequently passed in the faeces. These L_1 rapidly develop through to L_3, typically within seven days. The infective L_3 migrate either unassisted or are dispersed via sporulating *Pilobolus* fungi, and are ingested by the host. Upon ingestion, the L_3 exsheath in the abomasum, penetrate the intestinal wall, migrate to the mesenteric lymph nodes, and moult to L_4. The L_4 then migrate via the lymphatic and/or circulatory systems to the lungs, where they undergo a final moult to become immature adults within the bronchioles. Female lungworms have a pre-patent period of ~25 days. Created with BioRender.com

1.2.2. *Dictyocaulus viviparus* treatment inefficacy

There is growing awareness and concern regarding reports of a suspected lack of efficacy of anthelmintic treatments against lungworm infection in UK cattle (Carty, 2024). However, it remains unclear whether this is due to anthelmintic resistance, administration errors, or apparent treatment failure resulting from persistent respiratory disease and pathology following treatment. To date, only one confirmed case of anthelmintic-resistant *D. viviparus* has been reported worldwide (Molento et al., 2006). Nevertheless, two cases of suspected lack of efficacy have been documented for eprinomectin in adult dairy cows in the UK (APHA, 2020; Jewell et al., 2019).

Currently, the detection of anthelmintic resistance in *D. viviparus* relies solely on the controlled efficacy test, which cannot be conducted under field conditions. As a result, resistance cannot be identified on-farm until treatment failure becomes evident.

1.3. Livestock management and dairy systems

There are two fundamental types of cattle production systems: beef and dairy. However, the distinctions between them have become increasingly blurred, with management practices in both systems directly influencing the epidemiology of GIN infections. In dairy systems, calves are typically removed from their dams shortly after birth and managed separately. In contrast, in beef systems, calves remain with their dams until weaning at six to eight months of age.

1.3.1. The UK dairy industry

Approximately 17.3 million hectares of land are utilised for agriculture in the UK, covering 71% of the country's land area. Of this, 11.2 million hectares consist of either temporary or permanent grassland (DEFRA, 2024). This grassland supports 9.6 million cattle - of which 1.85 million are dairy cows – and 32 million sheep (DEFRA, 2024). The UK is the thirteenth-largest milk producer in the world, with milk accounting for 16.4% (£4.4 billion) of the country's total agricultural output in 2020.

As of January 2024, there are 794 dairy herds in Scotland, most of which are located in the southwest. These herds comprise approximately 180,648 cows with an

average herd size of 227 cows (SDCA, 2024), collectively producing 1.4 billion litres of milk per year (DEFRA, 2024). The Scottish dairy industry encompasses a diverse range of systems, from extensively grazed, lower-input/output systems that yield approximately 6,000 litres per lactation, to intensive, high-yielding cows housed year-round, producing over 10,000 litres per lactation. Most British dairy herds calve year-round (72%), while 8% calve in autumn, 9% in spring, and the remaining 11% follow a dual-block calving system (CHAWG, 2020). The most common breed of dairy cow is the Holstein-Friesian, accounting for 78% of the Great British milking herd (Rusk, 2020). However, in recent years, there has been growing interest in other breeds and crossbreeds - such as Ayrshire, Jersey, and Guernsey - to improve the butterfat and protein content of their milk. In general, milk production in Scotland exhibits seasonal variation, increasing in spring, peaking in May, and then declining in autumn.

1.3.2. Organic vs. non-organic systems

As of 2023, there were 30 certified organic dairy farms in Scotland, operating under one of six organic certification bodies. These organisations work with farms and food processors to ensure compliance with legal organic standards. In practice, organic certification prohibits the use of artificial pesticides and fertilisers, as well as the prophylactic use of chemically synthesised allopathic veterinary medicines - except in cases requiring analgesia, anaesthesia, or vaccination (Soil Association, 2023)

While individual animals or groups may receive reactive veterinary treatments on a case-by-case basis, regulations discourage the use of group treatments as a disease control strategy. Consequently, whole-group anthelmintic treatments for the control of GIN are strongly discouraged. In organic systems, permitted treatments for GIN include benzimidazole or levamisole products (Soil Association, 2023), while the use of macrocyclic lactones is permitted only if resistance to other anthelmintic classes is demonstrated. For a comprehensive discussion of anthelmintic products, see Section 1.5. The use of avermectin products is restricted due to concerns about the excretion of residues in the faeces of treated animals (Errouissi and Lumaret, 2010). Such residues have been correlated with deleterious effects on dung-breeding insects and with a reduced rate of biomass loss from the dung of treated animals (Beynon et al., 2015).

1.3.3. Parasite control and youngstock management

Gastrointestinal nematode infections are a major economic concern for livestock production worldwide, resulting in annual financial losses of nearly £1.5 billion in Europe and £270 million in the UK alone (Charlier et al., 2020b). On-farm, to overcome this impact, the objective is not to eliminate GIN infection but to suppress the parasite burden to the extent that (i) they do not cause clinical disease (PGE) and (ii) ensure that production (daily live-weight gain) is financially sustainable and acceptable. Eliminating GIN infections is likely impossible, given the ubiquity of these parasites, and would be disastrous to the future sustainability of anthelmintic treatments. This reality of requiring a burden of infection that has a limited impact on livestock production while maintaining the sustainability of anthelmintic treatment has numerous trade-offs and would require a subclinical threshold that has yet to be determined. This reality of GIN control is further complicated by the need to suppress other parasitic infections, such as *D. viviparus* (bovine lungworm), *Fasciola hepatica* (liver fluke), and arthropods (*Bovicola bovis* and *Chorioptes bovis*), which necessitates an integrative approach to parasite control (Forbes, 2023).

1.4. Diagnosis and identification of helminth infections

Clinical PGE is typically the result of heavy infections, in which overt clinical signs of disease are evident, and minimal background on the animal's management history is required for diagnosis. In contrast, subclinical infections pose a greater diagnostic challenge due to the absence of visible symptoms, yet they exert a substantial and often underestimated impact on livestock productivity.

Consequently, the development and implementation of diagnostic tests capable of detecting infections at subclinical thresholds are critical for enabling timely intervention and informed treatment decisions. This approach supports the dual goals of maximising production efficiency and minimising unnecessary anthelmintic use, thereby contributing to sustainable parasite control strategies.

Accurate species-level identification is also essential. *Os. ostertagi* and *Cooperia* spp., are among the most clinically relevant GIN species in temperate regions, while *Haemonchus* spp. predominates in subtropical climates. Although less pathogenic species as *Nematodirus* spp., are rarely associated with clinical disease, they nonetheless contribute to the overall parasitic burden.

1.4.1. Traditional parasitological techniques

Historically, human and veterinary parasitology have relied on copromicroscopy for the diagnosis of parasitic infections. The principles underlying the FEC, first described over a century ago (Bass, 1909), remain the cornerstone of veterinary parasitology, wherein parasite ova are recovered from faecal samples via salt flotation. Contemporary industry standards for diagnosing infection and assessing anthelmintic resistance continue to depend on this foundational technique.

Until recently, species identification of GINs relied exclusively on microscopic examination of the morphology of cultured L_3 (MAFF, 1986). This labour-intensive method has now been largely superseded by molecular approaches (Avramenko et al., 2015; Bisset et al., 2014; Roeber et al., 2017a). However, these techniques have not yet been commercialised due to their high cost unless performed at scale, rendering them financially impractical for most producers.

The primary advantage of retaining microscopy as the principal diagnostic tool lies in its universal applicability; it can be used across all helminth species, host species, and anthelmintic drug classes, without the need for specialised equipment or advanced technical training.

1.4.2. The faecal egg count

The FEC is an indirect measure of an animal's GIN burden. However, the correlation between the number of eggs per gram (epg) of faeces and the true worm burden is weak (Eysker and Ploeger, 2000; Murrell et al., 1989), and FECs do not consistently reflect clinical signs or growth rates. Nevertheless, in calves that have not yet developed acquired immunity, pre- first-grazing-season, FECs can offer valuable insight into their potential future larval challenge, arguably the most important predictor of production losses.

Faecal egg counting techniques can be categorised into two principal methods: the counting chamber and the coverslip-based methods. The McMaster technique (Gordon and Whitlock, 1939) is the most widely used chamber-based method and serves as the industry standard from which all other chamber-based techniques are derived. Variations in this method yield diagnostic sensitivities ranging from 1 to 50 epg. The coverslip method, first described in 1928 (Lane, 1928), involves centrifuging eggs suspended in a flotation medium and then counting them on a coverslip.

Adaptations of this method, such as the Wisconsin (Cox and Todd, 1962) and Cornell-Wisconsin (Egwang and Slocombe, 1982) techniques, are widely used in research due to their high sensitivity (as low as one epg), but they are labour-intensive and require centrifugation. In practice, diagnostic techniques such as the classic McMaster (Food, 1986), Mini-FLOTAC (Cringoli et al., 2017), and FECPACG2 (Bosco et al., 2014) are attractive due to their minimal equipment requirements and laboratory expertise.

Interpretation of FEC results presents several challenges. A high FEC may suggest a large worm burden, but does not account for species-specific differences in fecundity or pathogenicity. Conversely, a low FEC does not necessarily indicate a low parasite burden, as only patent adult females produce eggs; males, immature worms, and hypobiotic larvae are not detected. Additional variability arises from sampling factors and the over-dispersed distribution of parasites among hosts. Therefore, a single FEC, whether from an individual or a group, has limited clinical value without supporting contextual information. Furthermore, the development of immunity to GIN reduces female worm fecundity (Viney, 2002), making FECs an unreliable indicator of true parasite burden in mature animals.

1.4.3. PCR-based techniques

Describing the complex, multispecies composition of GIN communities is challenging due to the limited number of distinct morphological features among GIN and the added complexity of intraspecific phenotypic variation. PCR-based techniques, however, are well-established for the major helminth species of veterinary importance offering high sensitivity and specificity for detecting parasite DNA. These methods vary in resolution and complexity, ranging from conventional PCR (Schnieder et al., 1999), which amplifies a single target sequence using one primer pair, to multiplex PCR (Bisset et al., 2014), which amplifies multiple targets simultaneously, and real-time quantitative PCR (Roeber et al., 2017a). Which quantifies target DNA by tracking the accumulation of tagged amplicons during the reaction.

Most widely used assays target the internal transcribed spacer region 2 (ITS-2) of ribosomal DNA (rDNA), along with its flanking 5.8S and 28S rDNA genes, which together form the rDNA cistron (Figure 1.5). The highly conserved 5.8S and 28S genes provide suitable regions for designing pan-nematode primers, while the

ITS-2 region, although less conserved overall, contains subregions of high conservation that enable species-level resolution. These different regions of the rDNA cistron can thus be used to discriminate between taxonomic levels based on their degree of sequence conservation (Charrier et al., 2024). Furthermore, because the rDNA cistron exists as a multicopy tandem array, it is particularly amenable to PCR amplification.

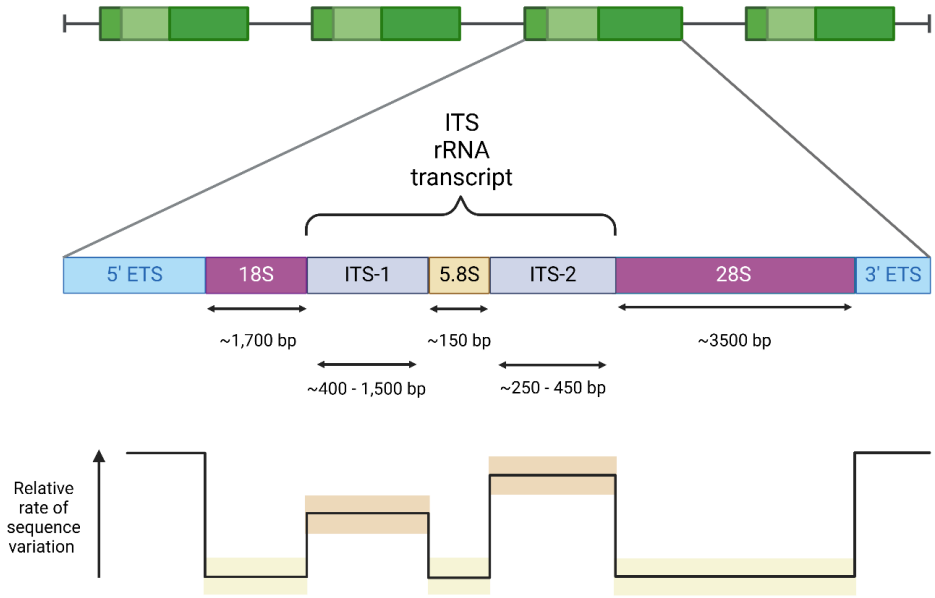

Figure 1.5 | Schematic representation of the nematode rDNA cistron and the relative rate of sequence variation between taxa

The rDNA cistron appears as a multicopy tandem array within the nematode genome, comprising three highly conserved coding regions – 18S, 5.8S, and 28S. These coding regions are separated by the less conserved internal transcribed spacer regions, ITS-1 and ITS-2. These regions of the rDNA can be used to resolve different levels of taxonomy based on their level of conservation across different taxa. Adapted from (Dorris et al., 1999). *Created with BioRender.com.*

1.4.3.1. Deep amplicon sequencing

ITS-2 rDNA metabarcoding, also known as the "Nemabiome" approach (Avramenko et al., 2015), employs short-read Illumina sequencing (MiSeq), and has been widely adopted in parasitological research. It is conceptually analogous to the well-established 16S rDNA sequencing used in bacterial microbiome studies (Johnson et al., 2019). This method has facilitated large-scale studies that offer improved species-level resolution of GIN communities compared to traditional morphological identification or low-throughput (q)PCR assays (Roeber et al., 2017c).

Nemabiome sequencing utilises primers targeting this highly conserved region of the rDNA cistron, which is specific to Clade V nematodes. While conventional and real-time PCR assays are only partially quantitative and limited in their ability to identify species comprehensively, Nemabiome sequencing provides a high-throughput and comprehensive solution for characterising Clade V GIN species.

1.5. Anthelmintic treatments for cattle

Only three classes of broad-spectrum anthelmintics are authorised for the treatment of GIN infection of cattle in the UK: benzimidazoles (BZ), macrocyclic lactones (ML), and levamisole (LEV) (Table 1.2). Monepantel, an amino-acetonitrile derivative, and derquantel, a spiroindole, are licensed for the treatment of GIN infection in sheep but are not commercially available for use in cattle in the UK.

Anthelmintics are classified as prescription-only medicines which may only be prescribed by veterinary surgeons, pharmacists, or a suitably qualified person. Their use in livestock production systems is nearly ubiquitous, mirroring the widespread prevalence of GIN infections themselves.

Table 1.2 Anthelmintic products licensed for use in cattle in the UK.

Class	Compounds	Total number of products	Pour-on formulations (n)	Oral formulations (n)	Injectable formulations (n)
BZ	ABZ	10	-	10	-
	FBZ	4	-	4†	-
	OFZ	3	-	3*	-
LEV	LEV	5	-	4	1
ML	DOR	4	3	-	1
	EPM	9	8	-	1
	IVM	17	8	-	9
	MOX	5	3	-	2

ABZ, Albendazole; DOR, Doramectin; EPR Eprinomectin; FBZ, Fenbendazole; IVM, Ivermectin; LEV, Levamisole; MOX, Moxidectin; Oxfendazole, OFZ

* Two of which are a pulse-release bolus

† One of which is a bolus

On many farms, the control of GINs infecting livestock is primarily based on the prophylactic administration of broad-spectrum anthelmintics. As a result of the availability of relatively inexpensive and easily administered treatments, anthelmintics have been widely adopted to enhance productivity through frequent prophylactic mass administration regimes.

This widespread usage of macrocyclic lactones has led to notable changes in husbandry practices, including increased stocking densities and a reduced reliance on pasture management strategies to mitigate parasitism. Macrocyclic lactones have revolutionised cattle management due to their high efficacy, broad spectrum of activity, and substantial safety margin. Their availability facilitated the development of strategic parasite control programs, which effectively reduced the pasture parasite challenge, thereby improving overall animal productivity and enhancing return on investment. For a comprehensive discussion of macrocyclic lactones and their properties, please refer to Section 1.5.3

1.5.1. Benzimidazoles

The introduction of thiabendazole in 1961 marked a significant breakthrough in the treatment of GIN infections in livestock. It was the first highly efficacious broad-spectrum anthelmintic with a high therapeutic index, substantially transforming helminth treatment in both human and veterinary medicine.

Benzimidazoles are active against both larval and egg stages, particularly targeting adult and immature nematodes. During its development, fenbendazole was considered 100% effective against inhibited L_4. However, inconsistencies in BZ efficacy have been attributed to small experimental group sizes and the use of different

parasite isolates in controlled efficacy studies. The consensus regarding fenbendazole's effectiveness against inhibited *Os. ostertagi* is further complicated by UK product data sheets, which ambiguously state that fenbendazole is "usually effective" without specifying the level of efficacy.

All BZ/pro-BZ compounds share similar but distinct modes of action, as they are extensively metabolised by the host (Prichard et al., 1985). Thiabendazole is metabolised first into the inactive compound netobimin, which is then converted into active moieties such as albendazole. Albendazole is further metabolised into its active form, albendazole oxide. Thiabendazole has since been succeeded by its derivatives – oxfendazole, fenbendazole, and albendazole – which are effective against lungworms, securing their initial pre-eminence as therapeutic agents.

Benzimidazoles exert their anthelmintic effect by binding with high affinity to the colchicine-binding site of helminth β-tubulin, thereby inhibiting the polymerisation of α/β-tubulin dimers and preventing microtubule formation (see Figure 1.6). Microtubules exist in a dynamic equilibrium within the cell, and this inhibition leads to their dissociation as dimers are continually lost. This disruption of cytoskeletal integrity and intracellular transport impairs cellular metabolism, ultimately resulting in nematode death. However, the actions of BZs are relatively slow compared to other anthelmintics that target the nervous system of larvae and adult nematodes.

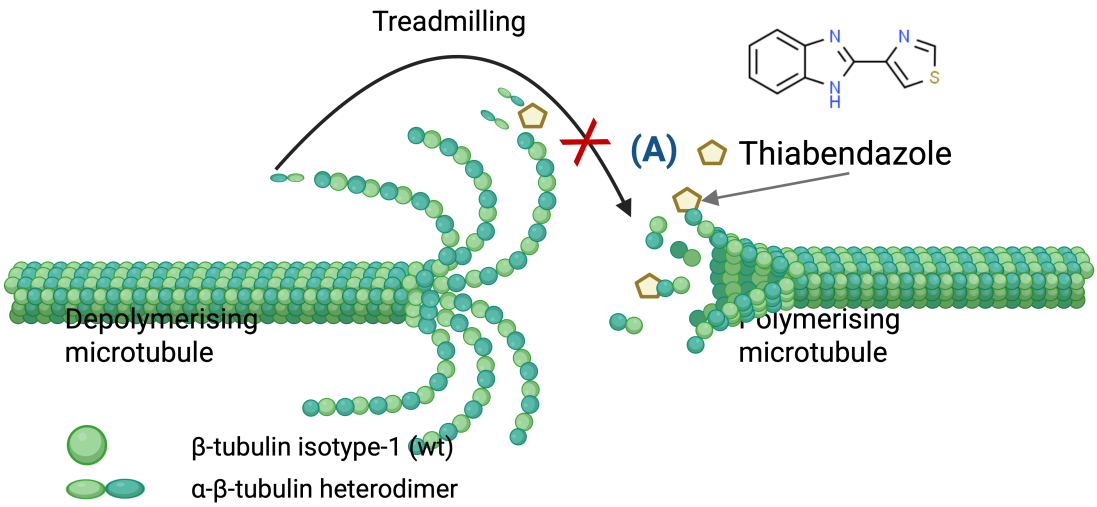

Figure 1.6 | Mode of action of benzimidazole compounds

This schematic diagram illustrates the mode of action of benzimidazole compounds. The microtubule complex existing in a dynamic equilibrium of (de-)polarisation in the absence of benzimidazole compounds. In the absence of the drug, tubulins polymerise to form tubulin dimers, which are added to the associating end of the microtubule. At the dissociating end, the tubulin dimers depolymerise, forming a dynamic equilibrium within the microtubulin complex. (A) illustrates the depolymerisation of the microtubulin complex in the presence of a benzimidazole. The benzimidazole compound binds to the colchicine binding domain of β-tubulin dimers, thereby inhibiting microtubulin polymerisation and leading to the disintegration of the complex. Created with BioRender.com.

1.5.2. Levamisole

Levamisole, introduced in 1968, is a broad-spectrum anthelmintic that belongs to the nicotinic agonist group, which also includes pyrantel. It is effective against nematodes, but not cestodes or trematodes. Levamisole functions as a cholinergic agonist, selectively binding to nematode ligand-gated ion channels, specifically levamisole-sensitive acetylcholine receptors (L-AChRs) located at the neuromuscular junction in body wall muscle cells of the helminth (see Figure 1.7) (Kopp et al., 2009). These drugs mimic the action of naturally produced acetylcholine but with a potency approximately 100 times greater (Qian et al., 2006). The binding of levamisole induces sustained neuromuscular depolarisation, resulting in spastic paralysis of the nematode and subsequent expulsion from the host (Martin et al., 2005). The activity of levamisole is directed exclusively against the adult and larval stages of nematodes, with no ovicidal activity.

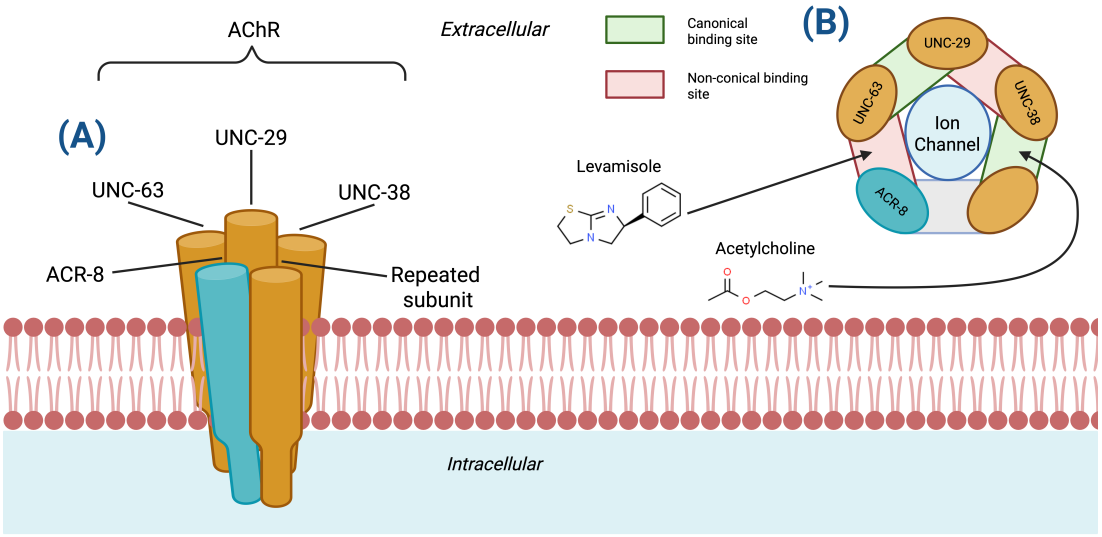

Figure 1.7 | Haemonchus contortus L-AChR subunit composition and ligand binding sites.

The Haemonchus contortus L-AChR is a pentameric Cys-loop ligand-gated ion channel receptor that plays a pivotal role in neurotransmission at neuromuscular synapses, controlling motility and feeding behaviour in nematodes. Levamisole is a selective cholinergic agonist that induces prolonged activation of the receptor and sustained influx of cations, resulting in continuous depolarisation. The canonical agonist binding sites are formed at the interface of two subunits, between the positive surface of the subunit that contributes to the acetylcholine binding surface and the negative surface of the adjacent subunit. (A) L-nAChR side view (B) L-nAChR from above with binding sites. *Created with BioRender.com.*

1.5.3. Macrocyclic lactones

The macrocyclic lactone class comprises two drug families, avermectins and milbemycins, which share similar structural and physicochemical properties, exhibiting broad-spectrum anthelmintic activity against nematodes and arthropods at very low dosages. The avermectin family includes natural and semisynthetic compounds, such as abamectin, ivermectin (IVM), doramectin (DOR), and eprinomectin (EPR), while the milbemycin family includes moxidectin (MOX). Both families are widely used in veterinary medicine, and IVM is an essential drug in human medicine for the treatment and control of onchocerciasis (river blindness) (Cupp et al., 2011) and lymphatic filariasis (Brown et al., 2000). Consequently, IVM is included on the World Health Organisation's Model List of Essential Medicines (World Health Organization, 2023).

In the UK, four ML compounds; DOR, EPR IVM and MOX , are currently licensed for the treatment of GIN infections in cattle and are available in 35 different products.

The avermectins, including IVM, were first derived from the soil bacterium *Streptomyces avermitilis* and commercialised for veterinary use. Their success is attributed to their broad spectrum of activity, safety and ease of administration (Egerton et al., 1979). Ivermectin, introduced in 1981 by Merck & Co. as Ivomec®, revolutionised parasite control and, due to its unparalleled potency against both endoparasites and ectoparasites, led to the coining of the term endectocide, being the first drug of its kind (Laing et al., 2017).

The potency and persistence of activity differ between ML compounds, following the order: IVM < EPR < DOR < MOX. These differences are believed to be related to the lipophilicity and excretion rates of each compound, with MOX being approximately 100 times more lipophilic than IVM. These properties enable higher tissue distribution and therapeutic concentrations in the gastrointestinal tract, while increased binding affinity to glutamate-gated chloride channels also contributes to its potency (Prichard and Geary, 2019). All MLs have a high lipid affinity, with half-lives of the unchanged drugs ranging from 4 to 15 days in cattle for IVM and MOX, respectively (McKellar and Benchaoui, 1996).

Our understanding of the mode of action of MLs against GINs is primarily derived from studies on the non-parasitic Clade V nematode *Caenorhabditis elegans* and the pharmacokinetics of IVM. Macrocyclic lactones, particularly IVM, pseudo-irreversibly and allosterically activate invertebrate-specific Cys-loop receptor family members of membrane-spanning neurotransmitter-gated ion channels. These channels are activated by various neurotransmitters and include α7 nACh receptors (Krause et al., 1998), acetylcholine-gated chloride channels, GABA-gated chloride channels (Bokisch and Walker, 1986, p. 936), histamine-gated chloride channels (Zheng et al., 2002), P2X4 receptors (Khakh et al., 1999), and glycine receptors (Shan et al., 2001). Ivermectin exhibits high affinity for glutamate-gated chloride channels (GluCls) and γ-aminobutyric acid (GABA) receptors, leading to hyperpolarisation-induced inhibition of pharyngeal pumping, suppression of secretory-excretory pore activity, paralysis of body wall musculature, and a reduction in egg laying (Crump, 2017).

Studies have identified GluCls as the primary drug target for IVM in nematodes (Cully et al., 1994; Dent et al., 2000a; McCavera et al., 2009; Wolstenholme and Rogers, 2006). The characterisation of IVM's interaction with GluCl channels in *C. elegans* by Dent et al., 2000a, is seminal in identifying receptor subunits and genes involved in ML activity. Glutamate-gated chloride channels are

believed to be heteropentameric transmembrane structures, assembled similarly to nAChRs, although the stoichiometric arrangement in parasitic nematodes has not been undetermined (see Figure 1.8).

In *C. elegans*, six genes encode GluCl subunits: *Cel-avr-14, Cel-avr-15, Cel-glc-1, Cel-glc-2, Cel-glc-3,* and *Cel-glc-4*. Both *avr-14* and *avr-15* are thought to encode two subunits each through alternative splicing (Dent et al., 2000a). The *H. contortus* genome encodes seven GluCl subunit-encoding genes, with homologues for only *glc-2*, *glc-3*, *glc-4*, and *avr-14* identified. Other *H. contortus* subunits show less similarity to *C. elegans*.

The subunit composition of GluCl channels varies by species and tissue, influencing IVM's site-specific effect. For example, *C. elegans* GluClβ homomeric channels, cloned in *Xenopus* oocytes, are insensitive to IVM, whereas GluClα1 homomeric channels are highly sensitive to IVM (Etter et al., 1996). The pharyngeal muscles of *C. elegans* are particularly sensitive to IVM; likely due to the presence of GluClα2 subunit encoded by *avr-15* (Dent et al., 1997; Pemberton et al., 2001). These differences in subunit composition and expression across nematode species are believed to contribute to the species-specific effects of IVM (Holden-Dye and Walker, 2006).

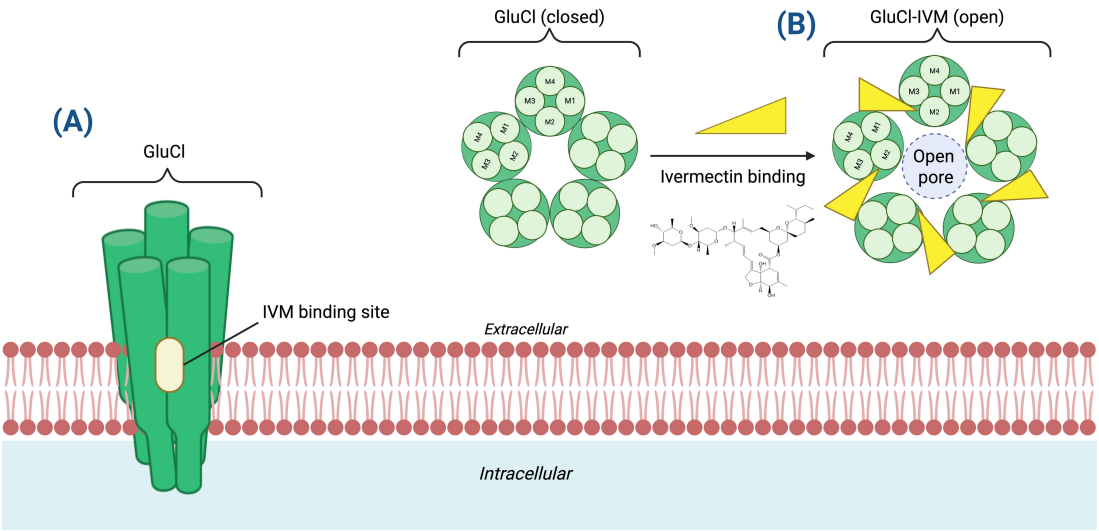

Figure 1.8 | The mode of action of ivermectin on glutamate-gated chloride channels.

Ivermectin binds to the adjacent M1 and M3 membrane-spanning domains, causing the regions to separate and facilitating the opening of the GluCl channel. (A) GluCl side view (B) GluCl from above with binding sites. This figure was adapted from (Hibbs and Gouaux, 2011). *Created with BioRender.com.*

1.6. Anthelmintic resistance and our current understanding of the genetic basis of resistance

Drug resistance poses a significant challenge to the treatment of many infectious diseases, including those caused by parasites. In the absence of highly efficacious vaccines against GINs, treatment and control will continue to rely almost exclusively on anthelmintics for the foreseeable future. The paucity of new anthelmintic compounds for treating GIN infection in cattle, combined with the global rise in resistance to all three major drug classes, represents a significant threat to future livestock production systems.

Anthelmintic resistance is a heritable trait defined as a reduction in a parasite population's sensitivity to a drug's action. Resistance is unlikely to arise from the emergence of new mutations; rather, it is typically driven by the selection of pre-existing mutations within genetically diverse populations of parasites. The rate of selection for resistant parasites in treated hosts depends on several factors, including the frequency, efficacy, and dosage of treatment, the allele frequency of resistance mutations, and the presence of any fitness costs associated with resistance. Over time, continued selection of a resistant subpopulation leads to clinical resistance and ultimately treatment failure.

There are many compelling reasons to investigate the genetic basis of resistance. These include the development of sensitive and specific markers to identify and monitor the emergence and spread of drug-resistant parasites in the field, gaining a deeper understanding of drug modes of action, and accurately assessing treatment regimens designed to minimise the spread of resistance. Furthermore, early detection of drug resistance could offer a valuable opportunity for interventions, such as acting before resistance genotypes predominate.

Given the limited number of anthelmintic drugs available for treating parasitic infections in cattle, preserving the efficacy of existing treatment in the face of rising resistance is critically important.

Our current understanding of the genetic basis of anthelmintic resistance varies depending on the specific drug, but remains limited overall. Knowledge of how resistance arises and spreads within a parasite population is also incomplete (Doyle and Cotton, 2019; Gilleard, 2006; Gilleard and Beech, 2007). The following sections

will review the current understanding of the genetic basis underlying resistance to the three classes of broad-spectrum anthelmintics available for cattle.

1.6.1. Benzimidazole resistance

Our understanding of the genetic basis of BZ resistance in strongylid nematodes has enabled the use of molecular approaches to investigate the prevalence and distribution of resistance to this drug class. Genome-wide studies in *H. contortus* have confirmed the *β-tubulin isotype-1* gene as the single most important resistance locus (Doyle et al., 2022), consistent with earlier findings (Samson-Himmelstjerna et al., 2007). Multiple studies have identified mutations at codons 167, 198, and 200 as conferring resistance in *H. contortus* and other related parasitic nematode species (Coles et al., 2006; M. S. G. Kwa et al., 1994; Ramünke et al., 2016; Redman et al., 2015).

The frequency and distribution of the resistance mutations - F167Y (TTC>T**A**C), E198A (GAA>G**C**A or GAG>G**C**G), E198L (GAA>**TT**A), E198V (GAA>G**T**A), and F200Y (TTC>T**A**C) - vary by nematode species and geographical region. These mutations have also been shown to confer similar levels of BZ resistance in fungi and in transgenically modified *C. elegans* gene where the *ben-1* gene is a co-ortholog of *β-tubulin isotype-1* and *isotype-2* (Dilks et al., 2021, 2020; Pallotto et al., 2022; Saunders et al., 2013; Venkatesan et al., 2023).

A mutation at codon 134 (Q134H (CAA>CA**T**)), in the *β-tubulin isotype-1* gene of *Ancylostoma caninum* has recently been functionally validated as a resistance mechanism. This mutation was not previously associated with BZ resistance in any field population or organism (Venkatesan et al., 2023).

The potency of benzimidazole compounds depends on which β-tubulin isoforms the drug binds to. Nematodes typically possess multiple *β-tubulin* isoforms that are differentially expressed across tissues and life stages (Saunders et al., 2013; Tydén et al., 2016; Wright and Hunter, 2003). The number of isoforms varies by species: *C. elegans* has six β-tubulin genes (*ben-1*, *tbb-1*, *tbb-2*, *tbb-4*, *tbb-6*, and *mec-7*), while *H. contortus* has four, which are differentially expressed across life stages and tissues (Saunders et al., 2013). These additional isoforms may compensate for downregulated ones, although they may also serve distinct functions.

The E198V variant of the *β-tubulin isotype-2* gene is proposed to mediate high-level BZ resistance (Doyle et al., 2022). This mutation is significantly associated with increased resistance to thiabendazole, particularly when the isotype-1 F200Y variant has reached fixation within a population. Although deletion of the *β-tubulin isotype-2* gene was previously linked to BZ resistance (Kwa et al., 1993), recent genome-wide studies have found no supporting evidence for such deletions (Doyle et al., 2022; Wit et al., 2022).

1.6.2. Levamisole resistance

Recently, the genetic basis of levamisole resistance was functionally validated in *H. contortus* (Antonopoulos et al., 2024; Doyle et al., 2022). A non-synonymous SNP in exon four of the *acr-8* gene, which encodes an acetylcholine receptor subunit, was strongly associated with levamisole-resistant *H. contortus*. This SNP encodes a serine-to-threonine substitution (S168T) and was identified in four geographically distinct levamisole-resistant strains (Doyle et al., 2022).

Furthermore, evidence supporting this SNP as a conserved resistance mechanism came from re-analysis of whole-genome sequencing data from levamisole-resistant *Teladorsagia circumcincta*, where the same serine-to-threonine substitution was identified at the analogous position (McIntyre et al., 2025). To validate this resistance marker functionally, wild-type and mutant ACR-8 were reconstituted in *Xenopus* oocyte expression system. It was demonstrated that, in the presence of the S168T variant, levamisole acts only as a partial receptor agonist and is no longer a superagonist. However, a significant number of adult *H. contortus* worms lacking the S168T mutation have been observed to survive levamisole treatment, suggesting the existence of an additional yet unresolved resistance mechanism.

1.6.3. Macrocyclic lactone resistance

Despite decades of research, the mechanisms underlying macrocyclic lactone resistance, including IVM resistance, remains poorly understood in helminths. Much of our understanding is derived from studies in *C. elegans*. This gap in knowledge is likely due to the uncertainty surrounding IVM's mode of action, compounded by the

genetic complexity of expanded and divergent gene families, as well as the highly polymorphic genomes of helminths.

This complexity is reflected in the lack of consistency among studies investigating the genetic basis of IVM resistance, which have largely focused on candidate genes encoding drug targets, metabolic enzymes, and drug transporters. For example, mutations and differential expression of the GluCl gene, *avr-14,* have been reported to confer ML resistance in *C. elegans* (Dent et al., 2000b) and *C. oncophora* (Njue and Prichard, 2004). In *C. oncophora*, three SNPs were described; however, these have not been found to be relevant to avermectin resistance in field populations. In these cases, the SNPs were not found to be relevant to avermectin resistance in field populations of *H. contortus* or *Te. circumcincta* (Baltrušis et al., 2022; Doyle et al., 2019; El-Abdellati et al., 2011; Laing et al., 2022; Rezansoff et al., 2016). While the absence of GluCl subunit mutations conferring resistance across species does not disprove a role for GluCl channels in ML resistance, it only highlights the complexity of the issue.

A recent investigation into IVM resistance, using a genetic cross of susceptible MHco3(ISE) and multi-drug-resistant MHco18(UGA) strains of *H. contortus,* implicated the transcription factor *cky-1* (Doyle et al., 2022; Laing et al., 2022). The mammalian ortholog of *cky-1 Npas4*, encodes an activity-dependent basic helix-loop-helix (bHLH)-PAS family transcription factor (Doyle et al., 2022). *Npas4* expression is rapidly activated by excitatory synaptic activity. It plays a role in the development of inhibitory synapses by regulating activity-dependent genes, which in turn control the number of GABA-releasing synapses formed on excitatory neurons (Lin et al., 2008). This ultimately leads to inhibition of excitatory neurons and excitation of inhibitory neurons (Spiegel et al., 2014). Similarly, other field populations of *H. contortus* have also exhibited a high degree of differentiation at the *cky-1* locus (Baltrušis et al., 2022). If function conservation exists between CKY-1 and NPAS4, it is plausible that cky-1 could contribute to avermectin resistance through the regulation of excitatory-inhibitory neural circuits. In *C. elegans*, knockout of *cky-1* is lethal, and knockdown results in hypersensitivity to IVM. However, our understanding of CKY-1 remains limited. To fully elucidate its role, genomic, transcriptomic, and phenotypic data must be expanded across a large number of individual worms and complemented with functional studies.

1.7. Detection of anthelmintic resistance phenotypes

The controlled efficacy test (CET) is considered the 'gold standard' and the most reliable method for determining anthelmintic efficacy against GIN infections. Efficacy is assessed by comparing the total parasite burdens of randomly assigned groups of treated and untreated individuals at post-mortem, following either natural field infections or artificial infections. The CET allows evaluations of efficacy against all stages of the parasitic life cycle, as parasites are recovered, identified, and counted during postmortem examination. It is the definitive method for detecting anthelmintic resistance within a population. However, due to the high costs and ethical considerations associated with animal use, the CET is not feasible for routine use outside of research settings. Consequently, it is primarily employed in research to characterise new parasite isolates or evaluate novel treatments.

1.7.1. Faecal egg count reduction test

The faecal egg count reduction test (FECRT) remains the only field-based method capable of evaluating anthelmintic efficacy for GIN *in vivo* and is feasible in most commercial farm settings. The FECRT provides a phenotypic measure of anthelmintic resistance, expressed as the percentage reduction in faecal egg count compared to an untreated control group. Resistance is indicated when the reduction is less than 95% with a confidence interval below 90% (Kaplan et al., 2023).

In brief, the test involves collecting faecal samples per rectum from all enrolled individuals on the day of treatment and then resampling at the optimal time points post-treatment. Animals are randomly allocated into treatment groups or assigned via black randomisation based on prior FEC and body weight. Each animal's anthelmintic dose is calculated individually to ensure accurate dosing.

The interval between pre-treatment and post-treatment sampling depends on the drug class. This reflects the time required for the complete egg expulsion following the death of female worms, the resumption of egg production by the surviving females, and the establishment of newly patent infections. This timing is particularly important when assessing MLs, as temporary egg suppression has been reported in both cattle and small ruminants. For example, MOX-induced egg suppression may last up to 14 days, with surviving *Cooperia* spp. females laying significantly fewer eggs at necropsy (Condi et al., 2009; Watson et al., 1996).

As a result, drug- and formulation-specific sampling guidelines have been developed. However, no single time point can fully account for the entire GIN life cycle and the egg suppression effect. A 14-day interval is sufficient for non-persistent drugs such as BZ and LEV, while a 14 to 17-day interval is recommended for avermectin compounds. For MOX, this is extended to 17 to 21 days, and for long-acting formulations, a 21–28 day interval is required. In a commercial setting, where multiple visits may not be feasible, a 14-day interval is considered a practical compromise (Kaplan et al., 2023).

Although the FECRT is an indirect measure of adult GIN survival and can be influenced by factors other than resistance, significant efforts have been made to standardise protocols to maximise accuracy (Coles et al., 1992; Kaplan et al., 2023; Martin et al., 1989). These standardised procedures have established the FECRT as the principal method for detecting anthelmintic resistance in the field.

1.7.2. *In-vitro* bioassays

In-vitro bioassays assess the phenotypic response of specific parasite developmental stages to anthelmintic treatment by exposing them to serial dilutions of the drug. Various methods are available to diagnose resistance, typically relying on comparison of egg-hatching, developmental, and phenotypic phenotypes. These assays are widely used as screening tools in anthelmintic drug development.

One of the simplest and most cost-effective methods for detecting anthelmintic resistance is the egg hatch test, first described by Le Jambre (1976) and later standardised by Samson-Himmelstjerna (2009) using *C. oncophora* and *Os. ostertagi* isolates. This test evaluates the efficacy of BZs by measuring their ovicidal activity, specifically, their ability to inhibit egg hatching, typically using thiabendazole.

As with all bioassays applied to naturally acquired infections, the presence of mixed-species populations introduces interpretational issues. These arise from differences in developmental timing and the inherent tolerance of each species to the anthelmintic or other reagents used in the assay. Further complications occur when one species in the mixed population is susceptible to an anthelmintic, while another is resistant.

1.8. Molecular diagnosis of anthelmintic resistance

Molecular screening of drug-resistant parasites offers several advantages over *in-vivo* and *in-vitro* phenotypic assays. It is well-suited for large-scale studies and can be more easily standardised as a diagnostic technique.

1.8.1. Targeted amplicon sequencing to detect and determine anthelmintic resistance marker frequency

Deep amplicon sequencing utilises Next-Generation Sequencing (NGS) technology to accurately detect sequence variations. This highly scalable approach is commonly used in microbial ecology, enabling the sequencing of many samples in a single run (D'Amore et al., 2016; Weisburg et al., 1991; Woese and Fox, 1977). The deep amplicon sequencing method can be applied similarly to Nemabiome metabarcoding by incorporating amplicons generated from anthelmintic resistance genes that harbour resistance mutations. In this context, sequence data can be used to determine the relative frequency of resistance mutations, or alleles, within a parasite population and may also be used as a taxonomic marker.

Beyond providing insights into the resistance status of GIN populations, this approach has important applications in understanding the evolution and spread of resistance, as well as in supporting the maintenance of refugia (for a comprehensive discussion of the concept of refugia, see Section **Error! Reference source not found. Error! Reference source not found.**). Using this approach, an amplicon sequencing method has been developed to investigate the frequency of BZ resistance mutations at the three key codons (167, 198, and 200) in the *β-tubulin isotype-1* gene for all GINs infesting livestock (Avramenko et al., 2019). This method can reliably detect resistance alleles with a frequency as low as 0.1%, enabling the early detection of resistance.

1.8.2. Parasitic nematode genomic resources

The free-living nematode *C. elegans*, the first multicellular organism to have its complete genome sequenced in 1998 (The *C. elegans* Sequencing Consortium, 1998), is a preeminent model organism fundamental to understanding many aspects of biology. As the most extensively studied species within the phylum Nematoda, *C. elegans* serves as the model organism for this diverse group, many of which are

parasitic. Its genome is 100.3 Mbp in size (Hillier et al., 2005), and despite being distantly related to parasitic nematodes, it shares extensive orthology and synteny with the much larger 283 Mbp genome of *H. contortus* (Laing et al., 2013, 2011; Schwarz et al., 2013). While *C. elegans* is an excellent model for understanding nematode biology, it primarily serves as a comparative model (Gilleard, 2006; Laing et al., 2013; Stevens et al., 2020).

The establishment of *H. contortus* as a model parasitic organism (Gilleard, 2006; Laing et al., 2013) and with the assembly of a high-quality chromosome-scale reference genome (Doyle et al., 2020) has provided a powerful platform for researching anthelmintic resistance. Chromosome-scale genome assemblies have been instrumental in understanding genetic diversity and selection across the genome, supporting previously identified candidate genes and identifying new gene associations with anthelmintic resistance. In *H. contortus,* genes associated with resistance to most broad-spectrum anthelmintics have been identified, including benzimidazoles, IVM, levamisole, and monepantel (Doyle et al., 2022; M. S. Kwa et al., 1994; Rufener et al., 2009). These high-quality genomic resources have also been critical in advancing our understanding of resistance mechanisms in other helminths of human and veterinary importance, such as *Fasciola hepatica* (triclabendazole resistance), *Dirofilaria immitis* (IVM resistance), *Schistosoma mansoni* (praziquantel resistance), and *Onchocerca volvulus* (IVM resistance) (Beesley et al., 2023; Chevalier et al., 2024; Doyle et al., 2017; Gandasegui et al., 2024).

The principal repository for helminth genomic data, WormBase ParaSite (Howe et al., 2017), hosts genomes and annotations from 181 species (version 19, 2024). It was developed in response to the growing availability of helminth genomic resources (e.g., the 50 Helminth Genome Initiative) and the specific needs of parasitologists. WormBase ParaSite extends the *C. elegans* resource, WormBase (Harris et al., 2019), enabling comparative analysis and genomic interrogation, including the mapping of functional data and homologous genomic regions between parasitic nematodes and *C. elegans*.

Until recently, few helminth genomes had been assembled into discrete chromosomes. Most genomic assemblies were represented by contigs and scaffolds, often numbering in the hundreds of thousands in draft genomes. Of the helminth assemblies available from WormBase ParaSite release 19, only 59 out of 274 (21.5%) have been assembled into fewer than 1,000 scaffolds, with considerable variation in

completeness (see Figures 1.9 & 1.10 for a comparison of Clade V genome assemblies and BUSCO completeness). These draft genomes, almost certainly, contain artefacts that do not reflect the true genome, such as multiple haplotypes, collapsed paralogs, and poorly resolved repetitive regions.

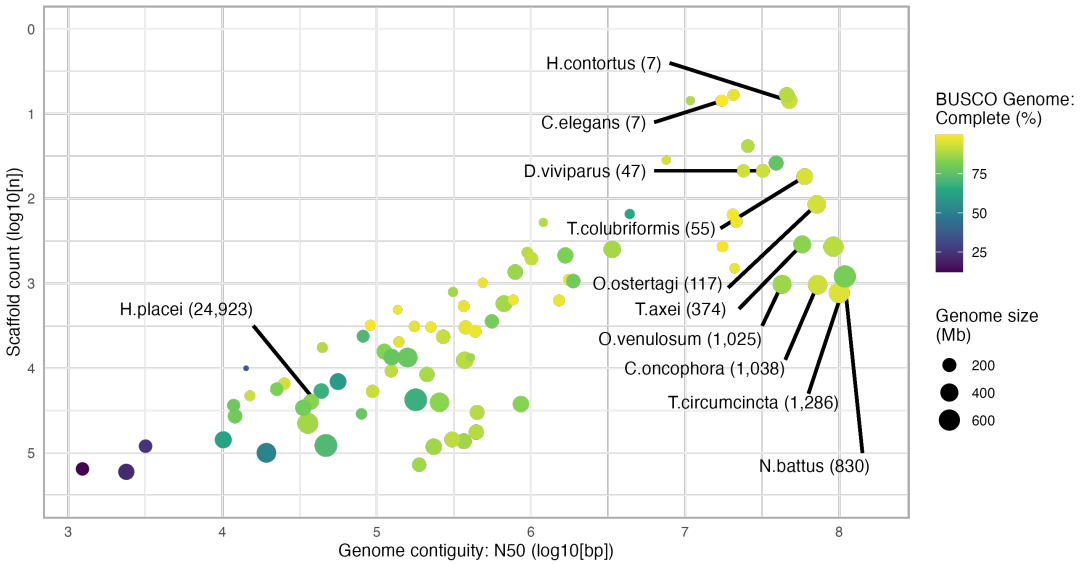

Figure 1.9 | Comparison of Clade V nematode genome assemblies

Comparison of Clade V nematode genome assemblies from WormBase ParaSite release 19 and select assemblies of gastrointestinal nematodes of livestock from the Darwin Tree of Life project. Each point represents a genome assembly (n = 85), comparing the number of scaffolds (y-axis; log10 transformed) against genome contiguity, represented by the scaffold N50 value, which is the shortest scaffold length at which 50% of the total assembly is contained (x-axis; log10 transformed). The point size represents the assembly size, and the colour represents an estimation of the genome completeness measured using BUSCO. The most contiguous genome assemblies of parasitic nematodes of livestock and *C. elegans* are named (n = 11), with the number of scaffolds or contigs in parentheses.

1.8.3. Genome assembly of *Ostertagia ostertagi* and other parasitic nematodes of livestock.

During the course of this PhD project, a highly contiguous chromosome-scale genome assembly for *Os. ostertagi* (nxOstOste4.1) became publicly available. This assembly, along with five other major GIN of livestock, were produced by the Darwin Tree of Life Project, a large-scale initiative aiming to sequence the genomes of all ~70,000 eukaryotic species in Britain and Ireland (The Darwin Tree of Life Project Consortium, 2022). This initiative forms part of the broader Earth BioGenome Project, which seeks to sequence all known eukaryotic life (Lewin et al., 2018).

These projects aim to generate high-quality, reference genome assemblies using long-read sequencing technologies and Hi-C (high-throughput chromosome conformation capture with deep sequencing). The combination of long-read

sequencing platforms (such as PacBio HiFi and Oxford Nanopore) with Hi-C scaffolding has enabled the rapid generation of chromosome-scale assemblies. This advancement significantly enhances the potential for comparative genomic studies involving species beyond *H. contortus* and *C. elegans (*see Table 1.3*)*.

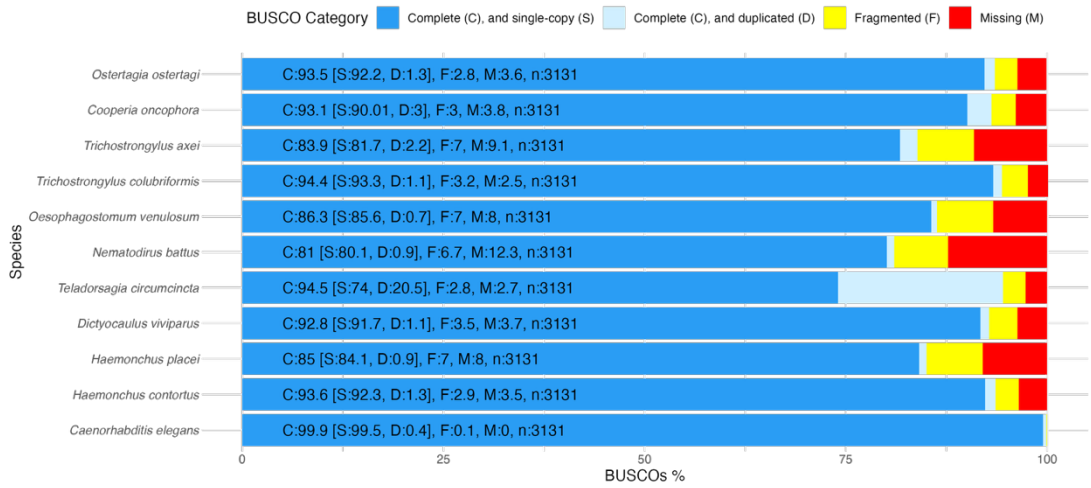

Figure 1.10 | Comparison of the BUSCO genome scores of the available genome assemblies of parasitic nematodes of livestock.

Comparison of BUSCO analysis of genome completeness of the available genome assemblies of parasitic nematodes of domestic livestock and *Caenorhabditis elegans*. Genome completeness was assessed using *BUSCO* v5.8.2 and the nematoda_odb10 lineage dataset.

Table 1.3 Genome assembly statistics for selected nematode species

	Assembly name	Reference	Assembly size (Mb)	Scaffold Count (n) c	Scaffold N50 (Mb) c	Gaps (n) c	Total masked length (%) b	BUSCO Complete Genome (%) a	Gene count (n)
Ostertagia ostertagi	nxOstOste4.1	Unpublished (The Darwin Tree of Life Project Consortium, 2022)	407	117	71.4	767	53.3	93.5	-
Cooperia oncophora	nxCooOnco1(Draft)	Unpublished (The Darwin Tree of Life Project Consortium, 2022)	467	1,038	72.3	705	54.4	93.1	-
Haemonchus contortus	H._contortus_MHCO3ISE_4.0	(Doyle et al., 2020)	283	6	47.4	185	36.5	93.6	19,623
Teladosargia circumcincta	T._circumcincta_MTci2	(McIntyre et al., 2024)	573	1,286	84.47	10,977	54.8	94.5	22,948
Trichostrongylus colubriformis	nxTriColu2.1	Unpublished (The Darwin Tree of Life Project Consortium, 2022)	310	55	59.6	349	49.3	94.4	-
Caenorhabditis elegans	WBcel235	(Davis et al., 2022)	100	6	17.5	0	16.4	99.9	19,983
Dictyocaulus viviparus	ngDicVivi2.1	Unpublished (The Darwin Tree of Life Project Consortium, 2022)	181	47	31.9	445	31.3	92.8	-
Trichostrongylus axei	nxTriAxei3 (Draft)	Unpublished (The Darwin Tree of Life Project Consortium, 2022)	374	374	57.5	-	-	-	-
Nematodirus battus	nxNemBatt (Draft)	Unpublished (The Darwin Tree of Life Project Consortium, 2022)	656	830	108.7	-	-	-	-
Oesophagostomum venulosum	nxOesVenu3 (Draft)	Unpublished (The Darwin Tree of Life Project Consortium, 2022)	432	1,025	42.5	-	-	-	-
Haemonchus placei	H_placei_MHpl1_0011_upd	(Coghlan et al., 2019)	259	24,923	0.38	40,367	29.3	85	21,928

[a] Genome completeness was assessed using *BUSCO* v5.8.2. (parameter: - genome) using the nematoda_odb10 lineage reference datasets

[b] Repetitive sequences were detected using *RepeatModeler* v2.0.1 with default parameters followed by *RepeatMasker* 4.1.0 (parameters: -s -html -gff -small -poly) to annotate the genome and summarise the repeat classes.

[c] Scaffold count, N50 and gaps were assessed using *assembly-stats* v1.0.1

1.9. Current prevalence of anthelmintic resistance

Anthelmintic resistance in livestock GIN is widespread and has been steadily increasing globally for many years, with reports of resistance across all host species to most available broad-spectrum anthelmintic classes (Baiak et al., 2018; Kaplan and Vidyashankar, 2012; Sutherland and Leathwick, 2011). In regions with intensive livestock production systems, multi-drug resistance is increasingly observed in GIN species infecting small ruminants. Unsurprisingly, there are no reports of simultaneous resistance to all three major classes in cattle (Sauermann et al., 2024).

Until recently, cattle producers in the UK had little incentive to assess the anthelmintic resistance status of their herd. Anthelmintic efficacy had been perceived as a lesser concern in cattle than in the sheep industry. Instead, the sector has focused on improving antimicrobial stewardship. However, there is now growing attention on the responsible use of anti-parasitic veterinary products in food-producing animals. In 2022, the British Cattle Veterinary Association published a policy statement on the use of parasiticides, outlining how the successes of the antimicrobial stewardship framework could be applied to anthelmintics. In 2023, the year in which the FECRTs were conducted in this PhD project, the Scottish government introduced an incentive for producers to conduct anthelmintic efficacy testing for both GIN and liver fluke through a grant scheme. Producers were encouraged to apply for an Animal Health and Welfare Interventions grant of up to £500 after conducting an FECRT under the guidance of their veterinarian or an expert advisor. In September 2024, all anti-parasitics were reclassified as prescription-only medicines in Ireland, requiring producers to obtain a veterinary prescription to purchase anthelmintics.

1.9.1. Prevalence of clinical parasitism and anthelmintic resistance in the UK

The prevalence of parasitic diseases in UK cattle is assessed through a passive surveillance system, known as VIDA (Veterinary Investigation Diagnosis Analysis), coordinated by the Animal and Plant Health Agency. This system collects data from post-mortem examinations and laboratory diagnostic submissions. However, as a passive surveillance system, it is inherently limited by underreporting and selection bias. These limitations are particularly evident in the reporting of clinical parasitism, as such cases are often observed by farmers or local veterinarians, but are not

systematically reported. The extent of underreporting is illustrated in Figure 1.11, which shows that fewer than 150 reported cases of PGE or parasitic bronchitis were reported in a single year.

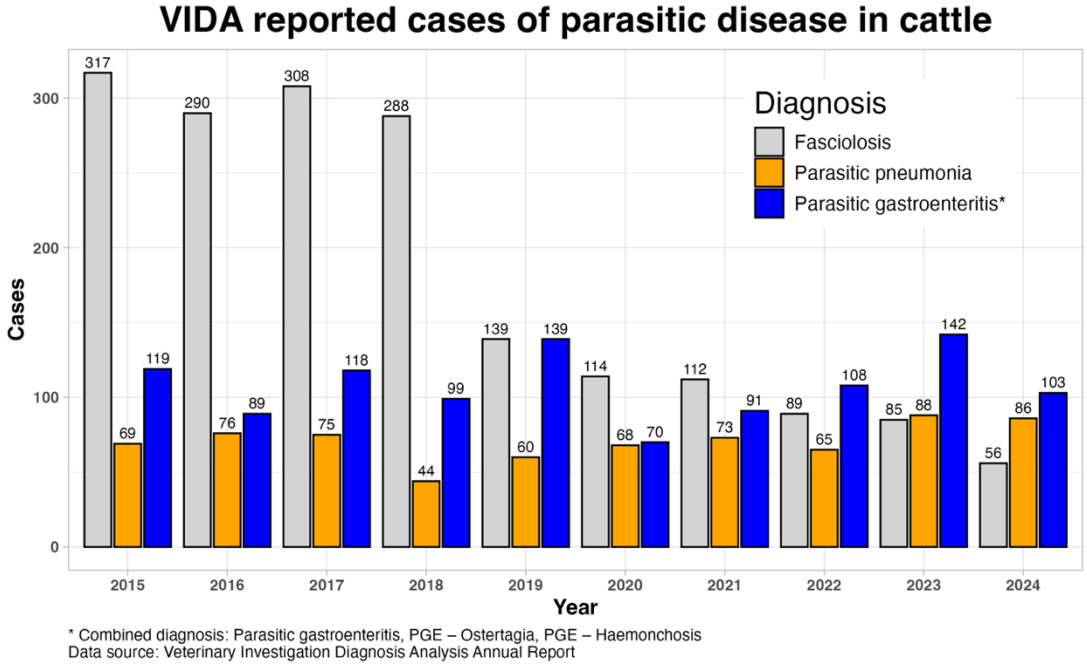

Figure 1.11 | VIDA reported cases of helminth infections in cattle across the UK from 2015 to 2024.

Given the underreporting of PGE, it is not surprising that our confidence in determining the prevalence and anthelmintic status of cattle in the UK remains low (Hannah Rose Vineer et al., 2020). However, due to the widespread use of MLs, some degree of resistance is almost certainly present, although it appears to be less prevalent than in sheep. There is currently no systematic monitoring of anthelmintic resistance in the UK. Nevertheless, resistance has been documented in multiple helminth species across all anthelmintic classes, as evidenced by scattered reports in all domestic livestock species. To date, no large-scale studies on anthelmintic resistance in UK cattle have been conducted. Small-scale and active surveillance studies have primarily focused on ML resistance, with no published surveillance studies on BZ or LEV (see Table 1.4). The first case of anthelmintic resistance in GIN of cattle in the UK was reported in 1998, when IVM resistance was confirmed in a field population of *C. oncophora* (Coles et al., 1998). The extent of underreporting is highlighted by the fact that a benzimidazole-resistant cattle GIN population was not reported in the UK until 2021 (Bartley et al., 2021).

Table 1.4 Studies of anthelmintic resistance of GIN of cattle in the UK.

Anthelmintic	Study	Location	Year	Farms test (n)	Resistant GIN species	Proportion of farms with resistance (%)
FBZ	(Bartley et al., 2021)	South-west England	2018	1	*Os. ostertagi*	100
IVM	(Geurden et al., 2015a)	Northumberland West Sussex	2015	10	*C. oncophora* *Os. ostertagi*	30
	(Bartley et al., 2012)	Dumfriesshire Ayrshire	2012	2	*C. oncophora*	100
	(McArthur et al., 2011)	Scotland	2011	4	*C. oncophora*	75
	(Stafford and Coles, 1999a)	South-west England	1999	8	*C. oncophora*	12.5
	(Coles et al., 1998)	Somerset	1998	1	*C. oncophora*	100
MOX	(Geurden et al., 2015a)	Northumberland West Sussex	2015	10	*C. oncophora* *Os. ostertagi*	10
	(Bartley et al., 2012)	Dumfriesshire Ayrshire	2012	2	*C. oncophora*	100

FBZ, fenbendazole; IVM, ivermectin; MOX, moxidectin

1.10. Sustainable control measures

In the veterinary field, the long-term control of nematode infections with periodic mass anthelmintic administration is not viable due to the inevitable trend towards anthelmintic resistance. Few new anthelmintic compounds are in development, and novel compounds are unlikely to be approved at a rate which matches the pace of emerging anthelmintic resistance. This has sparked renewed interest in alternative and sustainable control measures, including vaccination, biological control, and targeted selective treatment.

1.10.1. Vaccination

Immunological control through vaccination is considered a promising control strategy in terms of sustainability and cost-effectiveness. The principle of vaccination is to induce immunological protection in the host against future infections. The immune system can be stimulated by a weakened (attenuated) or killed pathogen (inactivated), as well as the protein or antigens from it (subunit). Unfortunately, only two nematode vaccines have reached commercialisation. These are vaccines against *D. viviparus* (Bovilis Huskvac, MSD Animal Health) and *H. contortus* (Barbervax, WormVax), which utilise irradiated larvae and crude antigen mixtures, respectively.

Numerous attempts have been made to immunise cattle against *Os. ostertagi* with live attenuated larvae (Burger and Pfeiffer, 1969), somatic antigens (Herlich and Douvres, 1979), or unfractionated excretory-secretory products (Hilderson et al., 1995). However, none of these approaches have resulted in significant levels of protection. The most promising experimental GIN vaccines have been based on activation-associated secreted proteins, which have also succeeded in immunisation against *C. oncophora* (Vlaminck et al., 2015) and *Te. circumcincta* (Nisbet et al., 2013).

The commercial bovine lungworm vaccine, developed in the 1950s, is a live-attenuated vaccine composed of irradiated L_3 *D. viviparus*, which offers significant protection and reduces adult worm burdens by 95 to 98% (Benitez-Usher et al., 1976; Jarrett et al., 1959). Two oral doses of approximately 2,000 L_3 are required, administered four weeks apart and completed at least two weeks before turnout to pasture, in order to allow for a subsequent "trickle" infection. Although the vaccine produces significant protection, it has several disadvantages, including ethical concerns (larvae production requires the use of donor animals), a short shelf life, the requirement for refrigeration, oral administration, and difficulties for producers in implementing the vaccination schedule. Several experimental recombinant subunit vaccines have been developed to overcome these issues, but with limited success (Holzhausen et al., 2018; Matthews et al., 2001; Strube et al., 2015).

1.10.2. Biological control

Biological control is generally defined as "any activity of one species that reduces the adverse effect of another". Regarding GIN, biological control utilises natural pathogens or predators of GINs to reduce the presence of the free-living stages on pasture. Today, isolates of nematophagous fungi have received considerable interest as a sustainable means of control and have been successfully demonstrated in controlling GIN that infects small ruminants. A commercial formulation of the nematophagous fungus *Duddingtonia flagrans* (Bioverm®, GhenVet Animal Health, Paulínia, São Paulo, Brazil) represents a promising option, as it has demonstrated efficacy in sheep, goats, horses, and buffalo (Braga et al., 2020; Fausto et al., 2021; Mendes et al., 2023). However, such commercial products are not yet available in the UK.

1.10.3. Targeted selective treatment

Targeted selective treatment (TST) primarily involves treating only those individuals in a herd or flock that exhibit a high parasite burden or are at the greatest risk of disease (summarised in Kenyon et al., 2009). This approach contrasts with blanket or whole-herd treatment, where all animals receive an anthelmintic regardless of their individual need, a strategy widely practised by the industry. The concept of TST is based on two key principles: refugia and the over-dispersed distribution of GINs, where only a small subset (20 - 30%) of animals harbour the majority of parasites (80%) (Sréter et al., 1994). In the context of GIN, a refugium refers to the untreated hosts that maintain the drug-sensitive parasites. By minimising anthelmintic exposure, such refugia-based strategies aim to conserve the sensitive alleles within the population. The resultant mixing of sensitive and resistant genotypes on pasture allows for the potential of cross-breeding within the host, thereby “diluting” the frequency of resistant genotypes.

1.11. Aims and objectives

Given the escalating challenges posed by parasitic infections in the UK, particularly in the face of climate change and the ongoing development of anthelmintic resistance, alternative approaches are needed for both parasite control and the investigation of resistance. The number and composition of a GIN population are key determinants of pathogenicity, and understanding the dynamics of such infections in response to anthelmintic treatment is a prerequisite for effective and sustainable control of parasites. Novel molecular diagnostic tools are needed to advance GIN and anthelmintic resistance surveillance and proof-of-concept studies, promoting their adoption beyond research settings. Candidate gene-based approaches have failed to identify reliable markers of macrocyclic lactone resistance; however, genome-wide association studies offer a comprehensive and unbiased approach, confirming both known and novel loci that confer drug resistance in helminths.

The overarching aims of this PhD project were to further our understanding of GIN species prevalence in Scottish dairy farms, the impact of parasite control practices on the development of anthelmintic resistance, and to explore the practical application of NGS sequencing technologies to study anthelmintic resistance in the field. The PhD project was based on two large field studies undertaken in year 1 and year 2: the first was a longitudinal surveillance of GIN infections in first-grazing-season dairy calves across an entire grazing season, and the second was a programme of FECRTs and EHTs on a subset of farms. The objectives of the project were:

- To describe gastrointestinal nematode infections in first-grazing-season calves of Scottish dairy herds across various management practices.

- To measure resistance to benzimidazoles (fenbendazole) and macrocyclic lactones (ivermectin and moxidectin) using the faecal egg count reduction test and egg hatch test in naturally acquired mixed-species infections.

- To apply mixed amplicon metabarcoding and sequencing approaches to determine species composition, anthelmintic resistance SNP frequency, at anthelmintic resistance loci in *Os. ostertagi*.

- To apply whole-genome sequencing to investigate the genomic signatures of anthelmintic selection in *Os. ostertagi* field populations.

Chapter 2

2. Seasonal patterns of faecal egg counts and gastrointestinal nematode species composition in Scottish dairy calves

Author Declaration & Contribution

Full paper citation, incl. authors and their affiliation (or link/ref to preprint server for papers not published)	Campbell, P., McIntyre, J., O'Neill, K., Forbes, A., Laing, R., Ellis, K., 2025. Seasonal patterns of faecal egg counts and gastrointestinal nematode species composition in Scottish dairy calves. Vet. Parasitol. 110574. https://doi.org/10.1016/j.vetpar.2025.110574

Please state your author contribution below for each category in accordance with the principles of the Contributor Roles Taxonomy system (CRediT). Please refer to the descriptors of each category before completion (https://credit.niso.org/)

Conceptualisation	PC, JM, AF, RL, KE
Data Curation	PC
Formal Analysis	PC
Investigation	PC, KO
Methodology	PC, JM, AF, RL, KE
Project Administration	PC, RL
Visualisation	PC
Writing – original draft	PC
Writing – review & editing	PC, JM, AF, RL, KE

We declare that the author contribution statements, in accordance with the CRediT system, are an accurate representation for this chapter/paper:

Author signature	
Primary supervisor signature (or corresponding author if different)	

Abstract

Gastrointestinal nematode (GIN) infections impact livestock production globally. In pasture-based systems, GIN infections are ubiquitous, typically comprising co-infections with several different species within a single host. Nematode species vary in their epidemiology, pathogenicity, and anthelmintic sensitivity, which in turn can be influenced by weather, host factors, and management practices. The epidemiology of parasitic gastroenteritis in young cattle in temperate regions has been thoroughly researched. However, many studies were conducted more than fifty years ago, before the advent of modern molecular techniques and the widespread use of macrocyclic lactone (ML) anthelmintics.

This study's objective was to survey dairy farms with different management profiles, using faecal egg counts (FEC) and GIN L_3 identification, to determine if any changes had occurred since these original studies. The longitudinal study of 23 Scottish dairy farms included 131 monthly sampling points, from which 1,967 individual FECs were conducted, and a minimum of 94 L_3 from pooled coprocultures identified by PCR (n = 13,297) per visit. Species composition and FEC followed expected patterns yet varied considerably in relation to management and anthelmintic use; *Cooperia oncophora* was more abundant earlier in the grazing season, while *Ostertagia ostertagi* became more abundant as the season progressed. Other GIN observed included *Trichostrongylus* spp., *Oesophagostomum* spp., and *Haemonchus contortus*. The majority of farms relied entirely on ML products and had done so for many years. Farmer concerns regarding anthelmintic resistance were minimal, and few farms routinely employed FECs to aid management decisions. Regardless of treatment strategy, the groups exhibited no evidence of clinical disease, and FECs remained relatively low throughout, even on farms not using any anthelmintic treatment (0-480 eggs per gram).

Keywords:

- Gastrointestinal nematodes
- Dairy cattle
- Species composition
- Faecal egg counts
- Anthelmintic treatment

Highlights:

- Longitudinal sampling of gastrointestinal nematode species and faecal egg counts on 23 dairy cattle farms.
- Ten gastrointestinal nematode species from eight genera were identified.
- Faecal egg counts remained relatively low throughout (0 – 480 EPG).

2.1. Introduction

Infection with gastrointestinal nematode (GIN) species is ubiquitous in pasture-based livestock systems. In first-grazing-season (FGS) calves, the intensity of infection and species composition broadly determine the risk of clinical disease and the severity of subclinical production losses. First-grazing-season calves are naïve and particularly susceptible to such infections when grazing for the first time, typically occurring after weaning and within the first year of life. Parasitic gastroenteritis (PGE) in cattle is unlikely to cause mortality in UK systems, but it is a significant production-limiting infection, primarily due to the reduced growth rate in youngstock (Vercruysse and Claerebout, 2001). In the UK and other temperate regions, the most commonly observed GIN species infecting young cattle are *Ostertagia ostertagi* and *Cooperia oncophora*, located in the abomasum and small intestine, respectively. *Ostertagia ostertagi* is the more pathogenic species, but its impact can be increased when animals are co-infected with *C. oncophora* (Kloosterman et al., 1984; Parkins et al., 1990). The number and composition of a GIN nematode population are key determinants of their pathogenicity, the onset and development of immunity within the host (Vercruysse and Claerebout, 1997), and their propensity to tolerate or develop resistance to an anthelmintic (Coles, 2002a). Many non-biological factors, such as climatic and environmental conditions, grazing and pasture management, and the age and diversity of hosts, will also influence the epidemiology of such infections (Armour, 1989; Forbes, 2017; Githigia et al., 2001; Stromberg, 1997). Scotland experiences a northern temperate climate, with an annual average temperature of 8.5 °C in 2022, characterised by relatively constant humidity and an average annual rainfall of 1,574 mm(Kendon et al., 2023). The pasture grazing of dairy farms is primarily composed of improved grassland with high proportions of ryegrass-based swards; these pastures are often regularly fertilised with inorganic fertilisers, reseeded, and high-yielding.

Understanding the dynamics of GIN infections throughout the grazing season in different production systems and regions is a prerequisite for effective and sustainable parasite control. There have been many changes in dairy farming since the 1960s and 70s, when many of the epidemiological studies were conducted, including cow genetics, milk yield, calving patterns, feeding regimes, pasture management, and, in terms of PGE, the introduction of the macrocyclic lactone parasiticides. In addition,

factors such as climate change and the increasing threat of anthelmintic resistance could all influence the epidemiology and future control of GIN infections (Morgan et al., 2019; Skuce et al., 2013). Already, changing epidemiological patterns in the UK have been observed in *Nematodirus battus* in sheep and *Dictyocaulus viviparus* in cattle (McCarthy and van Dijk, 2020; Melville et al., 2021).

In the UK, limited recent research has examined the seasonal patterns of cattle GIN infection, leaving apparent gaps in our understanding. While previous studies have investigated the seasonal dynamics of GIN infections (Armour et al., 1979; Eysker and Van Miltenburg, 1988; Lancaster and Hong, 1987; Michel, 1969a, 1969b, 1969c; Rose, 1970), these historical studies relied on the morphological identification of infective larvae and were conducted before the advent of macrocyclic lactone products, which are now widely used in all livestock sectors. In contrast, extensive research has been conducted on sheep GIN, both historically and more recently, emphasising the prevalence of anthelmintic resistance (Hannah Rose Vineer et al., 2020). Notably, Rose Vineer et al, (2020) found that, in most European countries, the detected prevalence of anthelmintic resistance was positively correlated with research labour. The interactions between co-infecting parasitic helminth species are still poorly understood (Evans et al., 2023), and assessment of species prevalence is a critical element of the host-parasite interaction. Mathematical modelling is increasingly being applied to parasitic helminth infections in livestock (Filipe et al., 2023; Rose et al., 2015; H. Rose Vineer et al., 2020), and therefore, updated and comprehensive investigations are required, using modern molecular techniques applied to various management systems.

This study aimed to characterise the GIN infection patterns of a range of dairy calf management practices using modern molecular techniques and to generate data useful in guiding future research and parasite control initiatives. We compare the GIN communities and FEC patterns of calves throughout their first grazing season in 23 commercial Scottish dairy herds.

2.2. Materials and methods

The University of Glasgow MVLS College Ethics Committee (Project No: 200210097) approved all research procedures involving animal use.

2.2.1. Study design, farm recruitment and selection criteria

Using a non-interventionist approach, this longitudinal study was designed to monitor faecal egg counts (FEC) and estimate GIN species prevalence in first-grazing-season (FGS) dairy calves at monthly intervals. Simple inclusion criteria enabled the recruitment of a broad range of farms, requiring minimal input from participants to minimise burdens and promote participation. To participate, farms had to have turned out a minimum of 30 first-grazing season dairy calves before July 2022. They were also required to complete an animal husbandry questionnaire and allow for faecal sample collection on a monthly basis. No advice regarding anthelmintic treatments was provided, and farms were encouraged to manage animals as they normally would; FEC data were provided promptly, and participants could act on this information with guidance from their veterinarian. Twenty-three dairy farms completed an entire grazing season of sampling, of which four were organic and 19 were non-organic, located in Southwest, Central Scotland or Aberdeenshire (**Error! Reference source not found.**.). Farms were recruited through outreach through relevant stakeholder groups, milk suppliers, and private veterinary practices.

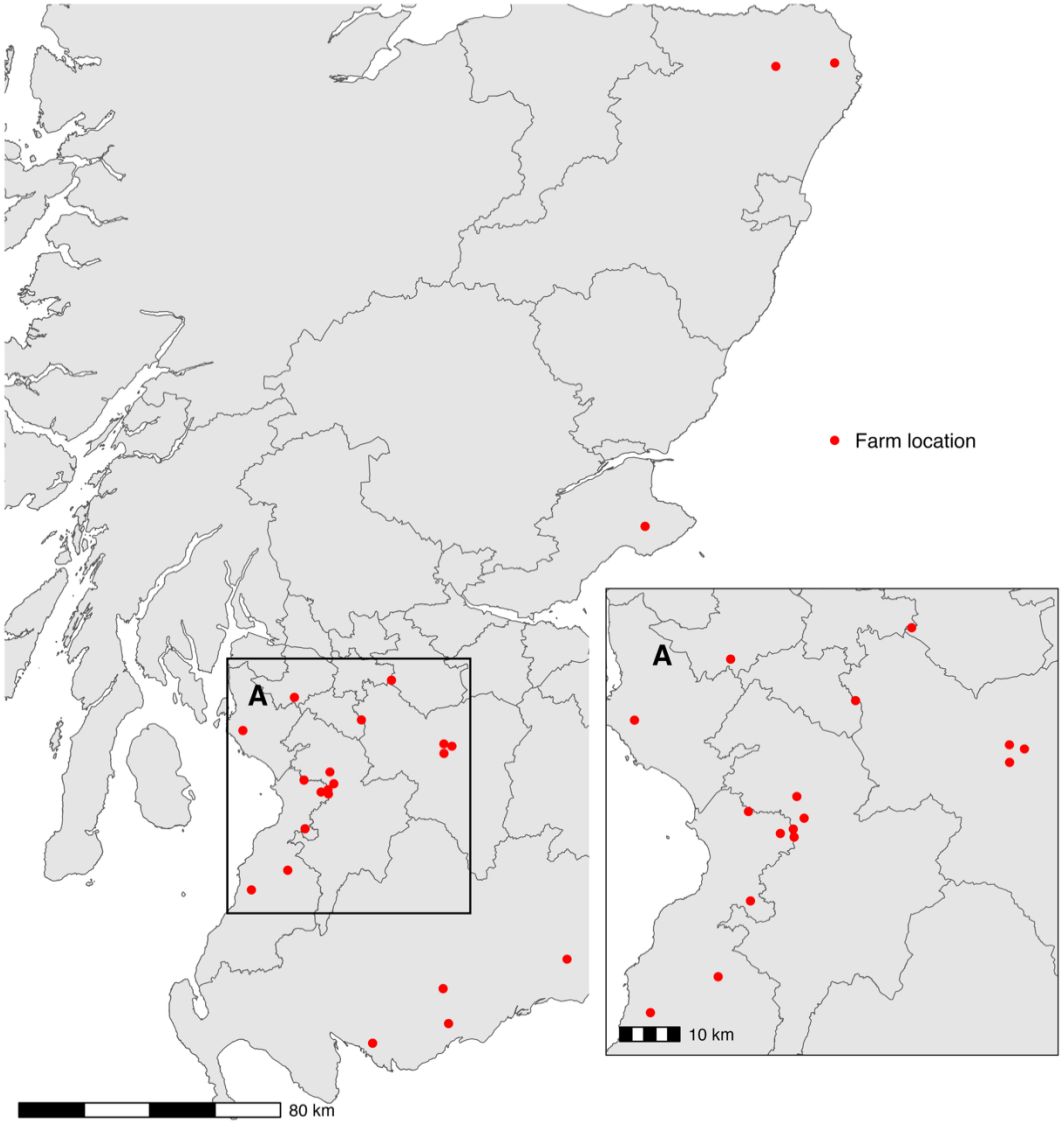

Figure 2.1 | Map of the study area and locations in mainland Scotland.

The map shows the distribution and approximate locations of dairy farms participating in the study across mainland Scotland. (A) represents the zoomed inset of Southwest Scotland, where many of the farms were located. Freshly voided faecal samples were collected from May to November 2022 at the sites indicated. Shapefiles for Scotland were obtained from Boundaries Scotland (OS_BL_ScottishLocalAuthority).

2.2.2. Sample collection and faecal egg counts

Sample collections began six weeks post-turnout of the calves to pasture on each farm and were repeated approximately every four weeks until the end of the grazing season. Each farm was visited 4 to 7 times between May and October 2022, resulting in a total of 131 sampling time points. At each farm visit, 15 individual fresh faecal samples were randomly collected directly from the pasture by surveying the field grazed by FGS dairy youngstock. Only samples identifiable as fresh (i.e., glossy and warm to the touch) were collected, and fresh faeces with similar appearance and consistency that were less than 3 m apart were ignored to minimise the possibility of repeat-sampling the same individual. Faeces were collected from the centre of the pat and transferred to 120 ml sterile specimen containers, sealed, and transported to the University of Glasgow on the same day. In total, 1,967 faecal samples were collected over the 2022 grazing season. Aliquots for the FEC were prepared on arrival and stored at 4 °C, while samples for coprocultures were stored at room temperature and processed within 24 h of collection.

A FEC was conducted on every individual sample collected using a modified, highly sensitive three-chamber McMaster technique (Paras et al., 2018) with a detection limit and multiplication factor of 8 eggs per gram (EPG). Briefly, 4 g of faeces were homogenised with water in a 1:10 faeces-to-water suspension. The suspension was then passed through a 250 µm sieve and further diluted to a 1:15 suspension by rinsing the sieve. A 15 ml aliquot was centrifuged at 1,061 xg for 5 min, and the supernatant was removed. The pellet was resuspended in a saturated salt solution (sodium chloride) and inverted several times. Immediately after inversion, an aliquot was removed to fill a three-chamber McMaster slide. McMaster slides were read after waiting a minimum of five minutes, only counting eggs at least partially within the inner and outer gridlines. GIN eggs were identified morphologically and counted as strongyle-type or *Nematodirus* spp.

2.2.3. Coproculture and individual crude lysates

Pooled larval coprocultures from each visit were set up by hand-mixing approximately equal volumes of faeces with vermiculite to form a well-aerated, uniform paste-like consistency and incubated at 27 °C for 14 days. Cultures were sprayed with water during incubation to ensure adequate moisture for L_3 development.

After the incubation period, the larvae were harvested using a modified Baermann technique as described in Roberts and O'Sullivan, 1950; pooled aliquots of L_3 in double-distilled H_2O were stored at –80 ˚C.

Crude individual worm lysates (n = 13,292) were produced from single strongyle larvae in 96-well plates using a modified proteinase K lysis reaction. Briefly, 100x volume solution of lysis buffer was made as follows: 1,000 µl DirectPCR Lysis Reagent (Cell) (Viagen Biotech), 50 µl 1M DTT (Invitrogen), and 10 µl Proteinase K (100 mg/ml) (Invitrogen) and 10 µl was aliquoted per well of a 96-well PCR plate. Using a stereo microscope, individual larvae were transferred into each well in a volume of <1 µl. Lysates were then incubated at 60 ˚C for 2 h, followed by 85 ˚C for 45 min to denature the Proteinase K. Crude lysates were aliquoted in 1:20 dilutions using nuclease-free water.

2.2.4. Strongyle species identification

For each sampling time point, a minimum of 94 recovered larvae were identified to species or genus level by PCR using the ITS2 region. A multiplex PCR method (Bisset et al., 2014) was employed, using primers designed to amplify the strongyle ITS2 region and regions specific to GIN species of interest (see Appendix A). Two reaction sets were employed; set one included primers targeting *Haemonchus spp.*, *Os. ostertagi*, *C. oncophora*, *Oesophagostomum venulosum*, and *Trichostrongylus. axei*. Reaction set two targeted *Ostertagia leptospicularis*, *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis* and was used for strongyles not identified by reaction set one.

The multiplex PCRs were performed in 96-well plates with 94 individual worm lysates, one PCR-negative control (no genomic DNA template), and one lysis-negative control (lysate without larva). Eurofins Genomics sequenced the amplicons of the conserved ITS2 region of any strongyle not identified to the species level by either reaction set. Then, a BLASTn search was performed using a curated ITS2 ribosomal DNA database (Workentine et al., 2020) for identification. To confirm the accuracy of the primers employed, five positively identified GIN for each primer pair had their entire ITS-2 region amplified and sent for capillary sequencing. All samples were positively identified as the correct species, and primer HACOFD3 identified only *H. contortus*. *Haemonchus placei* has never been reported in the UK to date, and UK-

wide Nemabiome datasets have exclusively identified *H. contortus* (Hogg et al., 2010; Jewell et al., 2024; McGregor, 2024)*;* therefore, we have assigned all HACOFD3-positive GIN as *H. contortus*.

2.2.5. Management questionnaire design and analysis

A questionnaire (n = 36 questions) was developed to collect farm management data in three sections (details provided below) (Appendix B**Error! Reference source not found.**).

(i) Farm demographics and key performance indicators. (Q1 – Q8)
(ii) General husbandry and pasture management practices. (Q9 – Q18)
(iii) Anthelmintic usage and decision-making. (Q19 – Q36)

The researcher completed the questionnaire through a semi-structured interview, allowing for free conversation guided by the questionnaire. The questionnaire was piloted with four volunteer farmers to sense-check the questions and ensure clarity.

2.3. Faecal egg count and multispecies abundance data analysis

All statistical analysis and visualisation were performed using R studio version 2024.12.1+563, and the code used to analyse raw data and generate figures is available from GitHub (https://github.com/pau1campbe11/Seasonal-patterns-of-FEC-and-GIN-composition).

2.4. Results

2.4.1. Farm profiles

The basic farm demographics and anthelmintic use information are summarised in Table 2.1. Twenty-six farms were initially recruited for this study, of which 23 completed the entire sample collection season. Of the 23 farms, 11 were dairy-only systems, nine were dairy and beef, one was dairy and sheep, and two were dairy, beef and sheep systems. Four organically certified farms participated in the study, two of which had a "cow-with-calf" policy where calves were kept at foot with the dam until weaning at 5 to 6 months of age as opposed to most dairy systems where calves are removed from the dams within the first 24 h and weaned at ~8 weeks of age or older. An extensive range of FGS calf group sizes were included, ranging from 29 to 105 individuals, with ages ranging from 3 to 11 months, of which 63 to 100% were female.

Macrocyclic lactones were the most commonly used anthelmintic class during the study, with limited use of benzimidazoles and no reported use of levamisole (Table 2.1). Across all farms, there were a total of 25 anthelmintic treatments given during the study (including treatments given at turnout but excluding treatments at housing). Of these treatments, eight were IVM, six MOX (three were MOX long-acting injectable products), seven DOR, and four FBZ. The most anthelmintic treatments administered in a single grazing season on an individual farm were three doses of IVM given by Farm 1. In contrast, four farms (three organic and one non-organic) did not treat any animals during the grazing season. There were three broad categories of treatment regimens employed by the farms in the study, which we define as follows:

- Neo-suppressive: treatment to limit the establishment of a parasitic infection and minimise pasture larval contamination
- Prophylactic: treatment of an at-risk group in anticipation of clinical or production-limiting parasitism based on previous management experience, but without the use of diagnostic indicators
- Tactical: treatment of a group based on an indicator of sub-clinical infection that may be production-limiting.

Table 2.1 Farm demographics summary.

Farm	Organic status	Dairy System	Calving pattern	No. of calves in study group	Age of calves in study group	Anthelmintic product used	No. of treatments	Anthelmintic total theoretical period of protection from *Os. ostertagi* infection
1	Non-organic	Dairy only	Spring-block	59	Spring-born	IVM	3	42
2	Non-organic	Dairy, beef, & sheep	AYR	78	Spring-born	MOX	1	35
3	Non-organic	Dairy only	Autumn block	79	Spring-born	FBZ	1	0
5	Non-organic	Dairy & beef	Spring block	105	Spring-born	IVM	1	21
7	Non-organic	Dairy & beef	Spring block	80	Spring-born	DOR	2	70
8	Non-organic	Dairy only	AYR	49	Spring-born	DOR	1	35
9	Non-organic	Dairy only	Dual-block	72	Autumn-born	None	0	0
10	Non-organic	Dairy only	AYR	43	Spring-born	IVM	1	21
11	Organic	Dairy & sheep †	Dual block	36	Spring-born	None	0	0
12	Non-organic	Dairy only	Dual block	53	Spring-born	IVM	1	21
13	Non-organic	Dairy & beef	AYR	48	Spring-born	MOX LA	1	120
14	Non-organic	Dairy & beef	AYR	70	Spring-born	MOX LA	1	119
15	Non-organic	Dairy & beef	AYR	63	Spring-born	DOR	1	35
16	Non-organic	Dairy & beef	AYR	91	Spring-born	MOX	1	35
17	Non-organic	Dairy & beef	AYR	39	Spring-born	MOX LA	1	120
18	Organic	Dairy, beef, & sheep	Dual block	39	Autumn-born	None	0	0
19	Non-organic	Dairy & beef	AYR	42	Autumn-born	DOR	2	70
20	Non-organic	Dairy only	AYR	54	Autumn-born	IVM	2	21
21	Non-organic	Dairy only	Spring block	89	Spring-born	DOR	1	35
22	Non-organic	Dairy & beef	AYR	70	Autumn-born	MOX	1	35
23	Organic	Dairy only	AYR	76	Spring-born	FBZ	2	0
25	Non-organic	Dairy only	Dual-block	114	Spring-born	FBZ	1	0
26	Organic	Dairy only †	Dual block	29	Spring-born	None	0	0

†Cow-and-calf policy where calves were reared with the dam until 5-6 months of age
AYR: All-year-round, DOR: Doramectin, FBZ: Fenbendazole, IVM: Ivermectin, MOX: Moxidectin, MOX LA: Moxidectin long-acting formulation

All treatments using the long-acting MOX formulation were classed as neo-suppressive (n = 3, Farms 13, 14, 17). Four farms utilised a tactical treatment regimen where animals were treated after veterinary advice based on an FEC, or after an observed reduction in growth rates (Farms 3, 12, 23 and 25). The remaining 16 farms utilised a prophylactic regimen where animals were treated at a specific time point in anticipation of a reduction in growth rates or increased risk of parasitic bronchitis (*Dictyocaulus viviparus* infection). All anthelmintic treatments were administered at the group level, and no farm employed a targeted selective treatment strategy. All farms utilised pour-on formulations of ML, except for those using long-acting MOX products, which are administered by subcutaneous injection. All benzimidazole treatments were administered orally, and no farms utilised an intraruminal bolus.

Of the seven farms that indicated that they had changed anthelmintics during the previous five years, only one was concerned about ineffective treatment/resistance in GIN. Three farms indicated they had changed anthelmintic drugs based on veterinary advice as part of a planned rotation/herd health plan, and four were influenced by their milk buyers to reduce ML use (specifically long-acting MOX formulations). Effective quarantine was not routinely practised on any farm in this study: only two farms stated that they gave quarantine treatments to bought-in cattle, and both stated that they do not routinely treat every animal.

2.4.2. Pasture Management

The majority of farms (18/23 farms) turn out calves to graze on the same pasture in spring every year, potentially creating grazing areas of “higher risk” for GIN infection. Of these, 78% (14/18 farms) would re-graze the same individuals on the same pasture in the autumn. These pastures of potentially “higher risk” were also permanent pastures, not cropped or re-seeded in the previous five years on most farms (13/14). Over half (15/23) of farms employed a form of rotational grazing, of which 33% (5/15) rotated after a set number of days, and the remaining farms regularly rotated based on grazing availability. Seventeen per cent (4/23) of farms set-stocked their calves for the majority of the grazing season, all of which changed pasture within the last 34 days of their respective grazing season. The remaining farms (4/23) changed pasture on an ad hoc basis, averaging two changes per season.

2.4.3. Farmer Attitudes

Only one farm expressed concern about anthelmintic resistance on their own farm, and no farm had ever previously tested for anthelmintic efficacy using the faecal egg count reduction test (FECRT). Four farms routinely employed FECs as a decision-making tool. Eight farms were actively aiming to reduce the frequency of anthelmintic treatment or to minimise treatment frequency. Only 22% of farms regarded GIN infections as of particular concern, but more (65%) regarded lungworm infection as of particular concern.

2.4.4. Species composition of GIN communities in Scottish dairy calves

Faecal egg counts were performed using individual animal samples, and GIN species identification was performed using a multiplex PCR of L_3 cultured from pooled faeces from each calf group. Overall, FECs remained low throughout the grazing season on all farms (0 to 480 EPG), with the FEC distribution summarised in Table 2.2. The majority of GIN eggs were Strongyle-type but with *Nematodirus spp.* eggs observed frequently on 22/23 farms and in 23% of samples. The *Nematodirus spp.* FEC ranged from 0 to 32 EPG but only accounted for 5.2% of the total GIN FEC in this study.

Table 2.2 | Frequency of occurrence of Strongyle and *Nematodirus* spp. type eggs in faecal egg counts

Strongyle-type			***Nematodirus* spp. -type**		
FEC	**Frequency**	**Proportion of all samples (%)**	**FEC**	**Frequency**	**Proportion of all sample (%)**
0	463	23.5	**0**	1,492	75.9
8-56	939	47.7	**8**	332	16.9
64-104	309	15.7	**16**	117	5.9
104-152	124	6.3	**24**	24	1.2
152-200	83	4.2	**32**	2	0.1
200-304	41	2.1			
304+	8	0.4			

Despite differences in both the magnitude and timing of increasing FEC between each farm and variations among farms using similar treatment regimens and anthelmintics, clear trends and patterns were evident upon visual inspection (Figures 2.2-2.8). The population dynamics of GIN infection throughout the grazing season are shown as individual farms grouped by anthelmintic product used. The efficacy of treatments could be clearly observed as decreases in FEC, but it was not calculated as the sampling intervals were not equivalent to those of an FECRT. These data illustrate that all prophylactic and strategic treatments were associated with dramatic decreases in FECs. On the farms that did not treat calves during the grazing season, a gradual increase in FECs was observed, except for Farm 11 (Figure 2.), where FECs were consistently very low and did not increase as the season progressed.

The occurrence of *Nematodirus* spp. in the FECs was transient and generally only present during the first three months of the grazing season. *Nematodirus* spp. were not observed during the period of protection provided by MOX-LA but did appear transiently in September on these farms. Except in samples taken during the periods of residual activity, the individual FECs often appeared over-dispersed throughout the dataset, the extent of which is highlighted by Farm 14 on the sixth consecutive timepoint (day 194), where the FEC had a mean of 34 and ranged from 0-168 epg (**Error! Reference source not found.**).

The composition of gastrointestinal nematode species present in pooled faecal samples was determined for all 131 sampling visits, with 13,297 individual larvae identified to species level; the observed species frequencies are summarised in Table 2.3. Ten gastrointestinal nematode species from seven genera were detected: *Os. ostertagi, C. oncophora, Tr. axei, Nematodirus helvetianus, Tr. colubriformis, Os. leptospicularis, Te.* circumcincta, *H. contortus*, *Oesophagostomum radiatum*, and *Oe. venulosum*. The two most prevalent species were *Os. ostertagi* and *C. oncophora* ($p < 0.04$), which were present on all farms and detected in 90.1% and 99.2% of the 131 pooled samples, respectively (Table 2.3). The next most prevalent species was *Tr. axei*, detected on 20/23 farms and in 30.5% of pooled samples, showed no significant association with any herd demographic characteristic (>0.05). *Haemonchus contortus* was present on 47.8% of farms and showed a marked regional distribution, primarily in South and East Ayrshire. *Os. leptospicularis*, *Te. circumcincta*, and *Oesophagostomum* spp. were present in up to seven farms and 10.7% of samples and showed no significant association with any demographic characteristic (>0.05). The

GIN community for each sample is shown in Figures 2.2 to 2.7, grouped by the anthelmintic regimes.

Table 2.3 | Frequency of occurrence and proportion of gastrointestinal nematode species in pooled cultures (n = 131) and at individual farm level (n = 23)

	Pooled samples (n = 131)			Farm (n = 23)	
Species	**Frequency of occurrence No. samples = n (%)**		**Inter-sample range (%)**	**Frequency of occurrence No. farms = n (%)**	
Ostertagia ostertagi	118	(90.1)	0-93.6	23	(100)
Cooperia oncophora	130	(99.2)	0-100	23	(100)
Trichostrongylus axei	40	(30.5)	0-11.5	20	(87)
Nematodirus helvetianus	33	(25.2)	0-18.8	19	(82.6)
Trichostrongylus colubriformis	47	(35.9)	0-8.7	21	(91.3)
Ostertagia leptospicularis	14	(10.7)	0-4.7	7	(30.4)
Teladorsagia circumcincta	11	(8.4)	0-9.3	4	(17.4)
Haemonchus contortus	26	(19.8)	0-5.3	11	(47.8)
Oesophagostomum radiatum	7	(5.4)	0-9.5	5	(21.4)
Oesophagostomum venulosum	11	(8.4)	0-6.8	7	(30.4)

The abundance of the two predominant GIN species, *Os. ostertagi* and *C. oncophora*, varied in proportion over time; in general, on each farm, C. oncophora was the most abundant species observed at the beginning of the grazing season, and as the season progressed, *Os. ostertagi* became more dominant. This pattern is most evident on the farms that did not treat during the grazing season (**Error! Reference source not found.**) and those that administered a fenbendazole product (**Error! Reference source not found.**). This dynamic, however, is disturbed when an anthelmintic treatment is given; in the case of the ML treatments, a dramatic increase in the proportion of *C. oncophora* L_3 observed after treatment coinciding with a decrease in *Os. ostertagi* (Figures 2.3-2.5 and 2.7). The opposite effect was observed after fenbendazole treatment (**Error! Reference source not found.**), where an increase in *Os. ostertagi* proportion and reductions of *C. oncophora* are observed. A resumption of this pattern of *Os. ostertagi* becoming progressively more abundant is clearly observed after the end of the period of residual anthelmintic activity on most farms

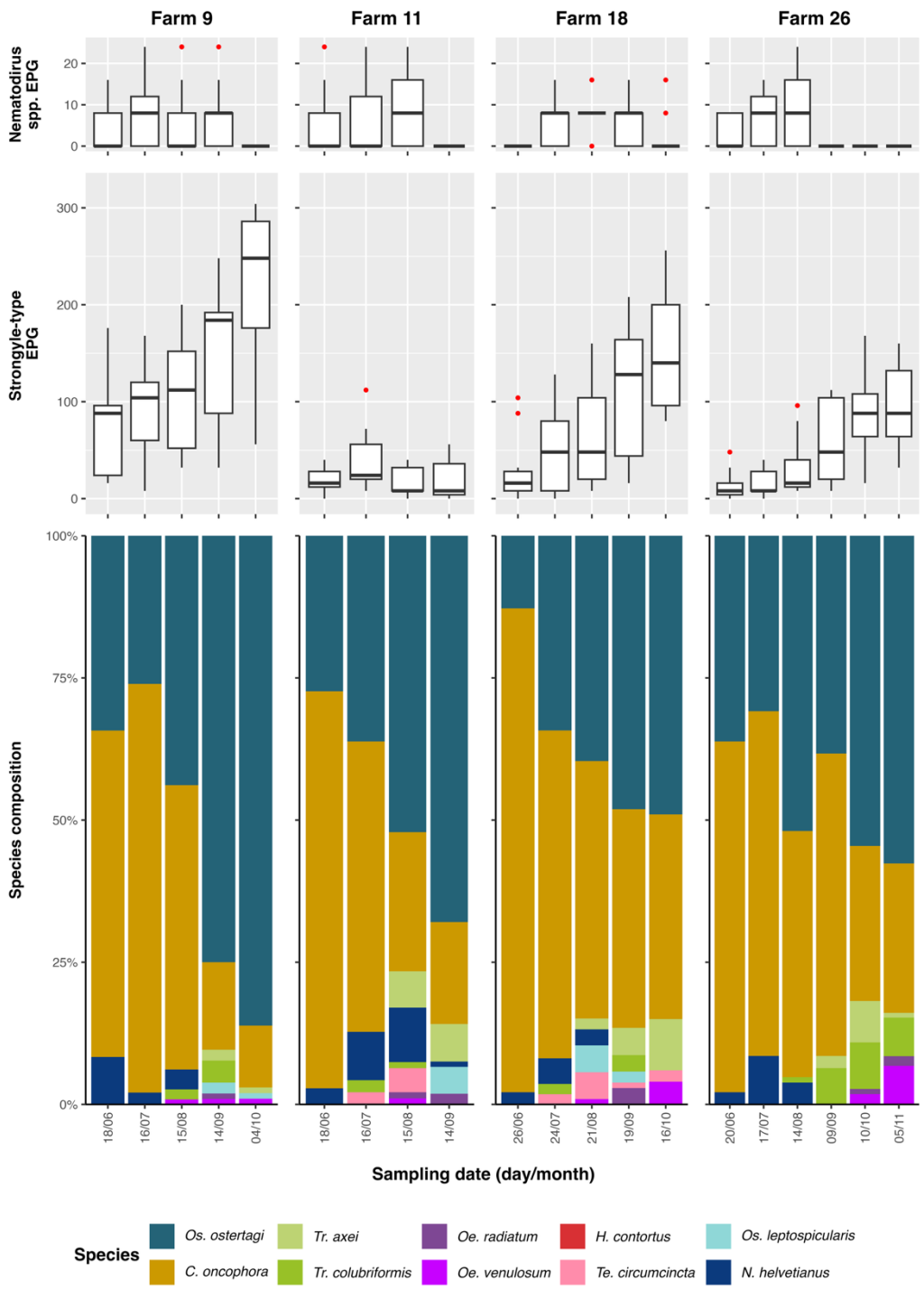

Figure 2.2 | Relative species abundance and faecal egg counts of gastrointestinal nematode communities of FGS calves not treated with an anthelmintic during their grazing season.

Species identity was assigned by ITS-2 rDNA multiplex PCR from group-level pools of L_3 larvae harvested from coprocultures from each visit. A minimum of 94 L_3 larvae were identified per pooled coproculture. For each farm and sampling timepoint, the upper boxplot indicates the *Nematodirus* spp. faecal egg count (FEC). The larger boxplot below represents the respective Strongyle FEC. Each red dot represents an outlier FEC. EPG = eggs per gram of faeces. The main bar chart in each panel shows the species composition of the larval cultures. For interpretation of the time points and references to colour in this figure legend, the reader is referred to the Web version of this article.

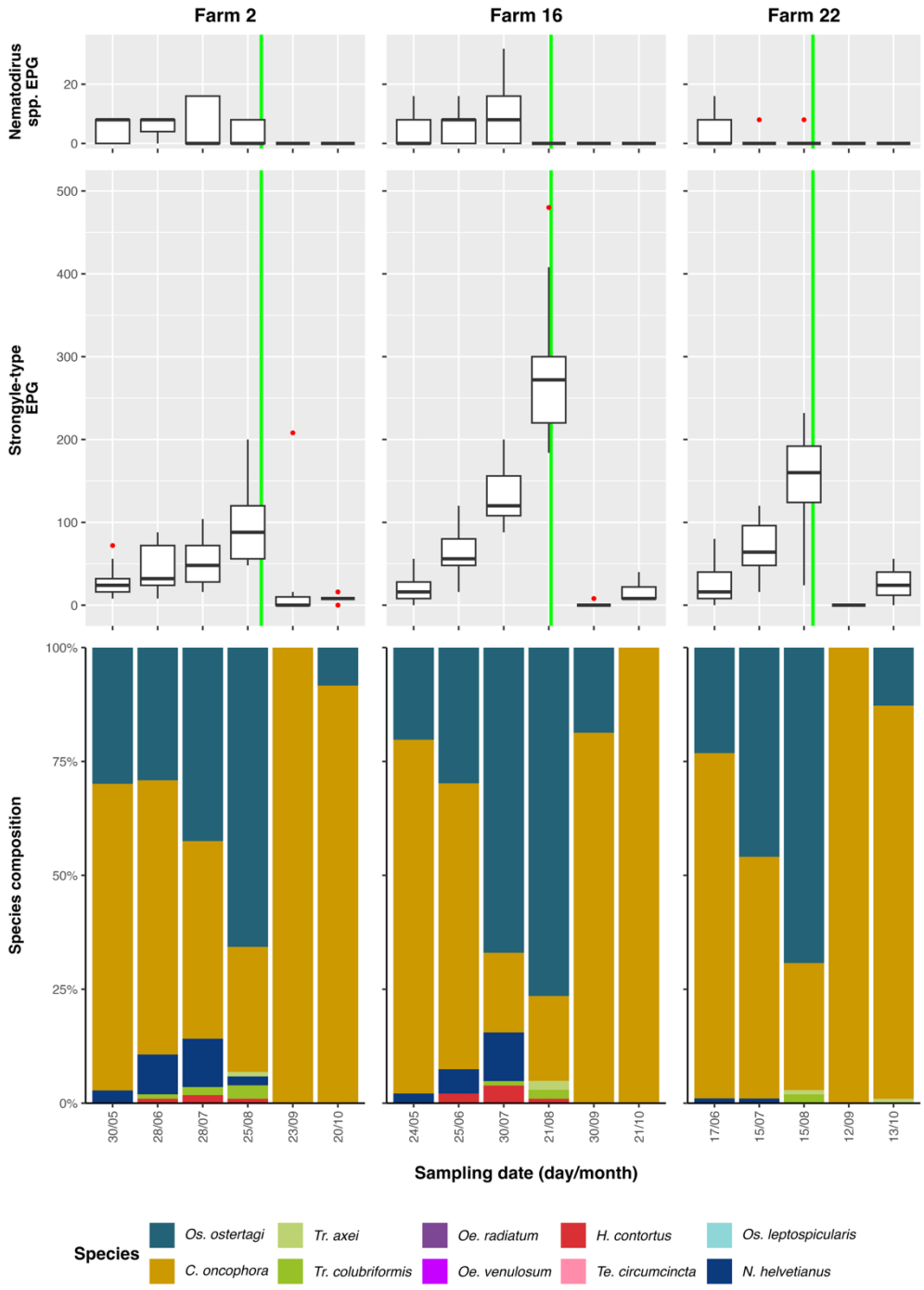

Figure 2.3 | Relative species abundance and faecal egg counts of gastrointestinal nematode communities of FGS calves treated with a moxidectin product during their grazing season.

Species identity was assigned by ITS-2 rDNA multiplex PCR from group-level pools of L_3 larvae harvested from coprocultures from each visit. A minimum of 94 L_3 larvae were identified per pooled coproculture. For each farm and sampling timepoint, the upper boxplot indicates the Nematodirus spp. faecal egg count (FEC). The larger boxplot below represents the respective Strongyle FEC. Each red dot represents an outlier FEC. The vertical green line represents when the moxidectin treatment was administered. EPG = eggs per gram of faeces. The main bar chart in each panel shows the species composition of the larval cultures. For interpretation of the time points and references to colour in this figure legend, the reader is referred to the Web version of this article.

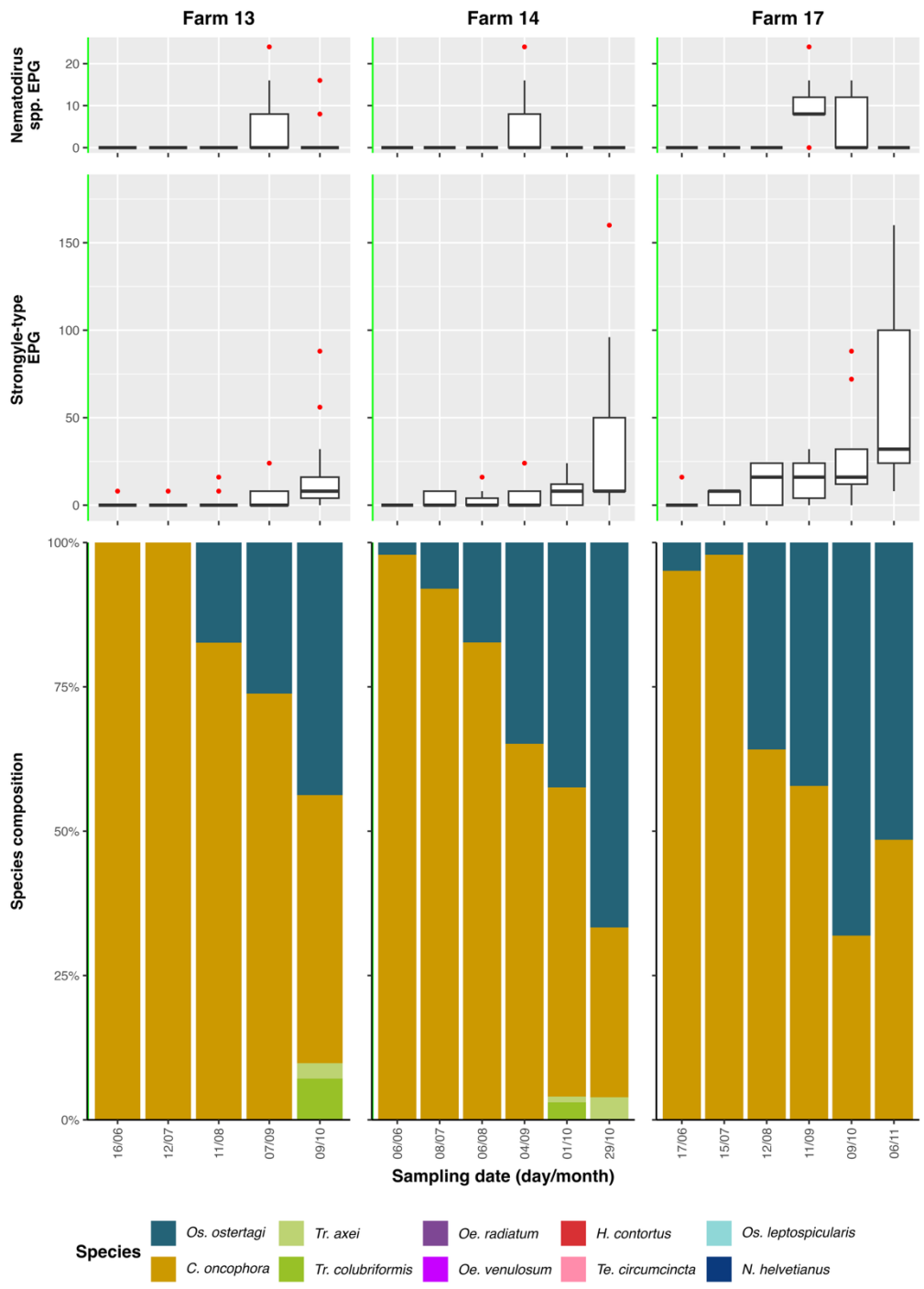

Figure 2.4 | Relative species abundance and faecal egg counts of gastrointestinal nematode communities of FGS calves treated with a moxidectin long-acting product at turnout.

Species identity was assigned by ITS-2 rDNA multiplex PCR from group-level pools of L_3 larvae harvested from coprocultures from each visit. A minimum of 94 L_3 larvae were identified per pooled coproculture. For each farm and sampling timepoint, the upper boxplot indicates the Nematodirus spp. faecal egg count (FEC). The larger boxplot below represents the respective Strongyle FEC. Each red dot represents an outlier FEC. The vertical green line represents when the moxidectin long-acting treatment was administered. EPG = eggs per gram of faeces. The main bar chart in each panel shows the species composition of the larval cultures. For interpretation of the time points and references to colour in this figure legend, the reader is referred to the Web version of this article.

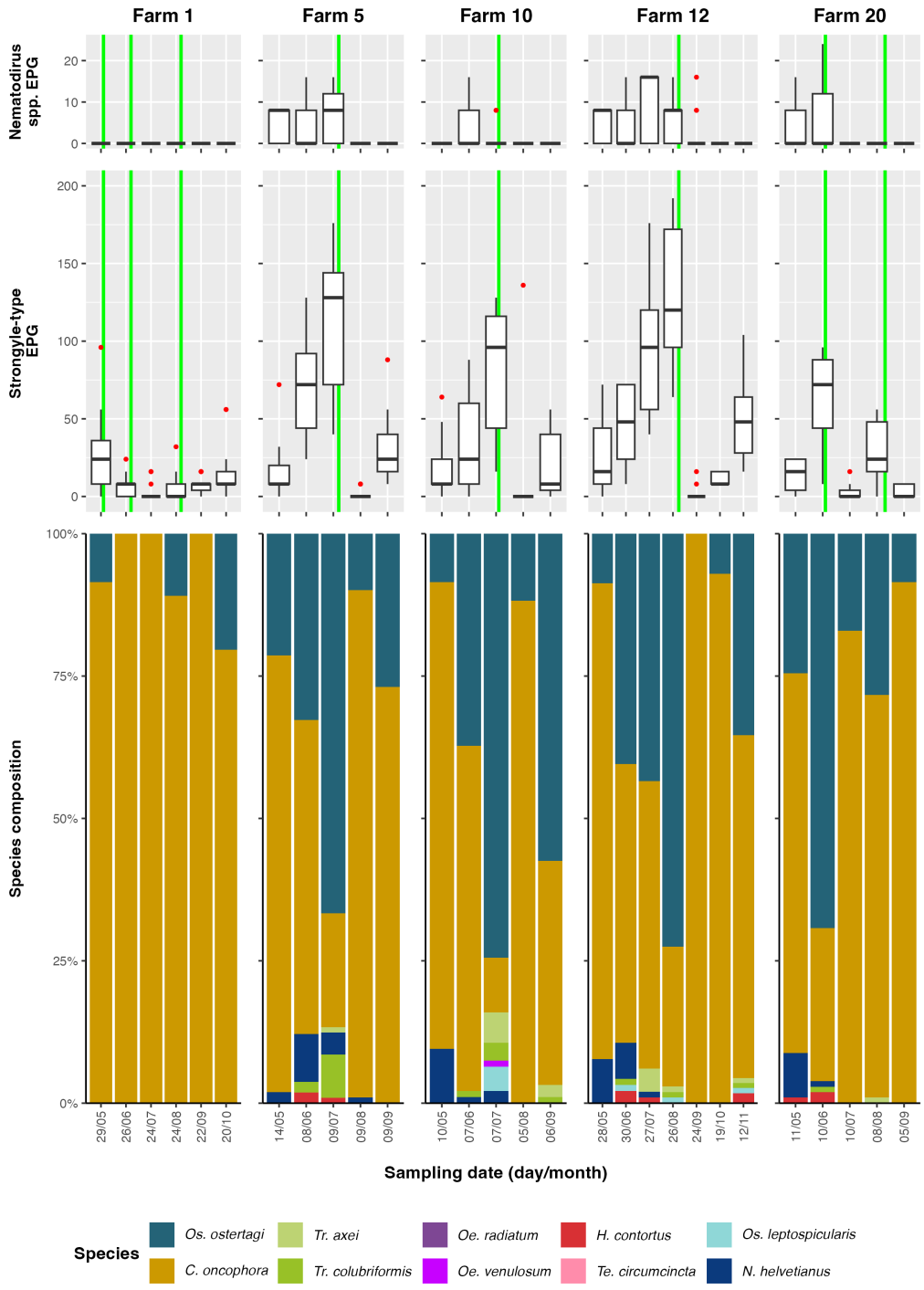

Figure 2.5 | Relative species abundance and faecal egg counts of gastrointestinal nematode communities of FGS calves treated with an ivermectin product during their grazing season.

Species identity was assigned by ITS-2 rDNA multiplex PCR from group-level pools of L_3 larvae harvested from coprocultures from each visit. A minimum of 94 L_3 larvae were identified per pooled coproculture. For each farm and sampling timepoint, the upper boxplot indicates the Nematodirus spp. faecal egg count (FEC). The larger boxplot below represents the respective Strongyle FEC. Each red dot represents an outlier FEC. The vertical green line represents when the ivermectin treatment was administered. EPG = eggs per gram of faeces. The main bar chart in each panel shows the species composition of the larval cultures. For interpretation of the time points and references to colour in this figure legend, the reader is referred to the Web version of this article.

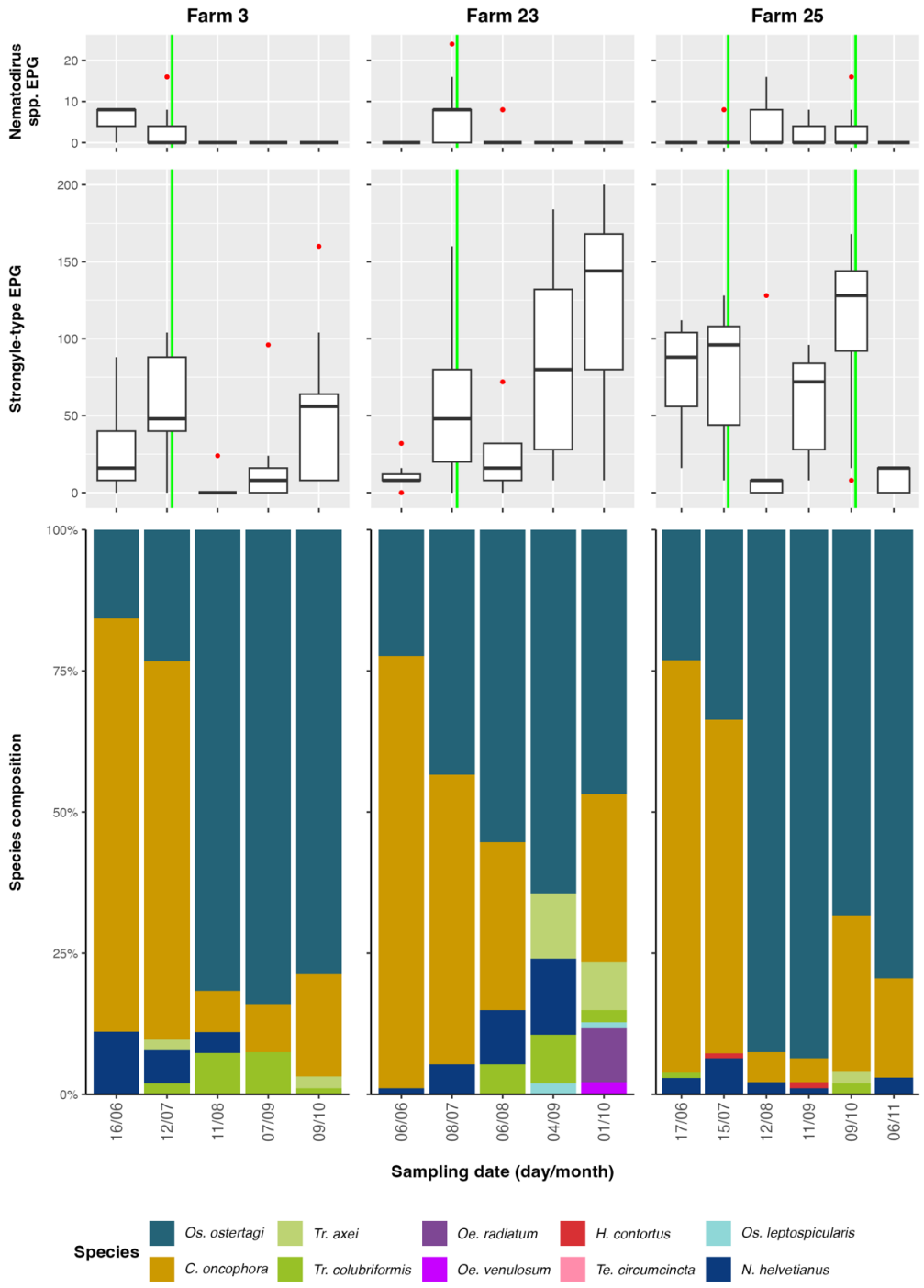

Figure 2.6 | Relative species abundance and faecal egg counts of gastrointestinal nematode communities of FGS calves treated with a fenbendazole product during their grazing season.

Species identity was assigned by ITS-2 rDNA multiplex PCR from group-level pools of L_3 larvae harvested from coprocultures from each visit. A minimum of 94 L_3 larvae were identified per pooled coproculture. For each farm and sampling timepoint, the upper boxplot indicates the Nematodirus spp. faecal egg count (FEC). The larger box plot below represents the respective Strongyle FEC. Each red dot represents an outlier FEC. The vertical green line represents when the fenbendazole treatment was administered. EPG = eggs per gram of faeces. The main bar chart in each panel shows the species composition of the larval cultures. For interpretation of the time points and references to colour in this figure legend, the reader is referred to the Web version of this article.

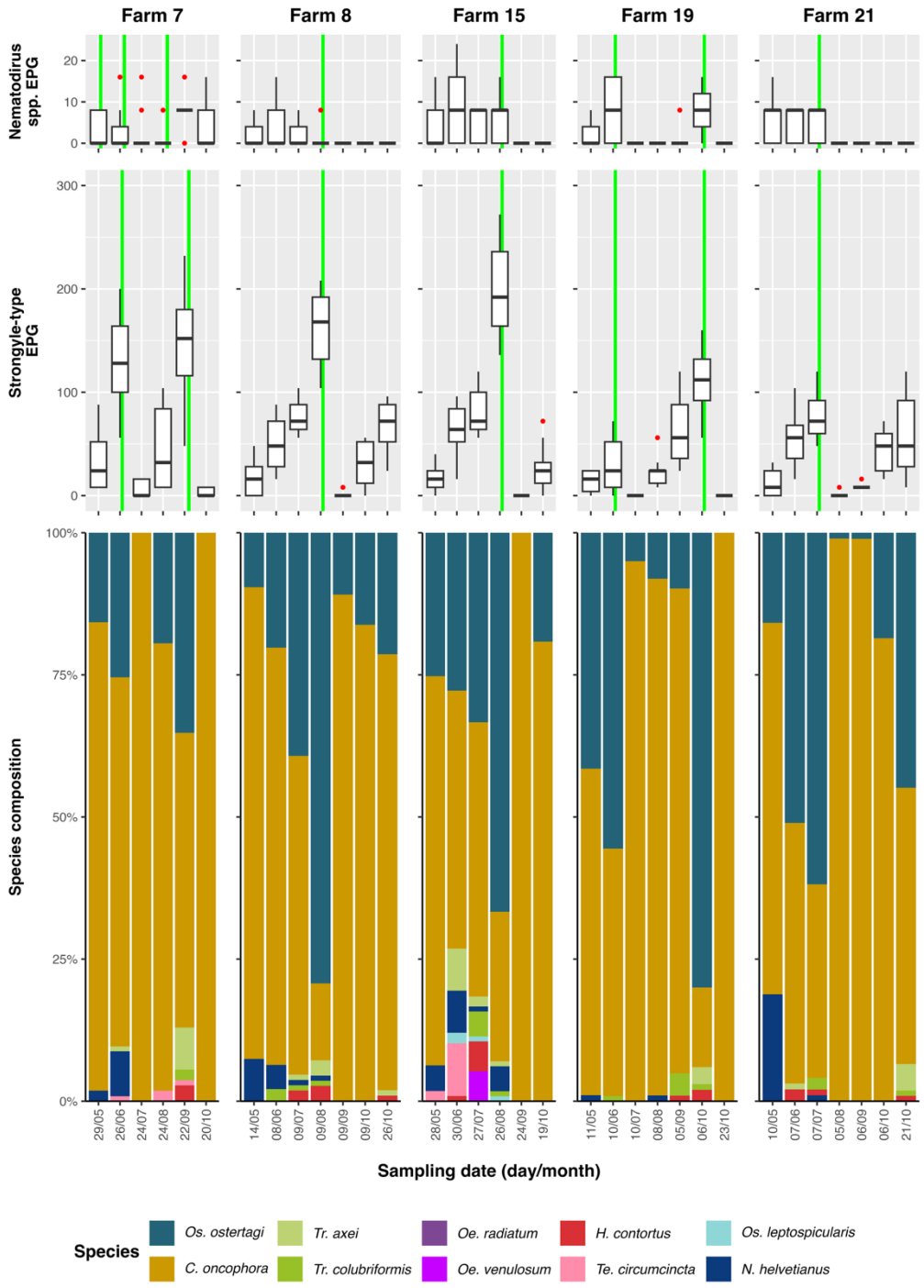

Figure 2.7 | Relative species abundance and faecal egg counts of gastrointestinal nematode communities of FGS calves treated with a doramectin product during their grazing season.

Species identity was assigned by ITS-2 rDNA multiplex PCR from group-level pools of L_3 larvae harvested from coprocultures from each visit. A minimum of 94 L_3 larvae were identified per pooled coproculture. For each farm and sampling timepoint, the upper boxplot indicates the Nematodirus spp. faecal egg count (FEC). The larger box plot below represents the respective Strongyle FEC. Each red dot represents an outlier FEC. The vertical green line represents when the doramectin treatment was administered. EPG = eggs per gram of faeces. The main bar chart in each panel shows the species composition of the larval cultures. For interpretation of the time points and references to colour in this figure legend, the reader is referred to the Web version of this article.

2.5. Discussion

In this study, we investigated the seasonal patterns of FECs and nematode species composition in first-grazing season dairy calves on 23 farms in the northern temperate climate zone of Scotland. Understanding the infection patterns of GINs through the grazing season and the impact of anthelmintic treatments is crucial in supporting the development of evidence-based, sustainable parasite control strategies. Still, contemporary data on GIN species prevalence and infection intensities in UK cattle systems is lacking. Only one published study used a PCR platform to identify GIN (Roeber et al., 2017b), but this study did not collect any herd or farm characteristics. Most information from the UK is based on small-scale studies of one or two groups of cattle using morphological identification and the relatively insensitive McMaster technique with a sensitivity of 50 EPG. This study is in good agreement with these earlier studies, highlighting many similarities between morphological studies regarding GIN population dynamics over a grazing season and the effect of anthelmintic treatment (Armour, 1989; Kloosterman, 1971; Nansen et al., 1988; Steffan and Nansen, 1990). The identification of a wide range of less common and clinically significant genera such as *Trichostrongylus*, *Nematodirus* and *Oesophagostomum* spp. more frequently than previously reported, likely, however, reflects the greater accuracy and higher throughput of assigning species identity by PCR.

One of the major challenges of undertaking studies assessing GIN infections in cattle is the low FEC of individual cattle and, consequently, low recovery of larvae from faecal samples, particularly post-treatment and within the window of residual activity. To overcome this, a large pooling strategy was used to archive larvae collected during these periods of residual activity; this enabled the collection of significant numbers of larvae (>1,000) from each sampling visit, even when the FEC were below the 8 EPG limit of detection. No clinical disease associated with GIN infection was reported on any farms during the study. However, Farm 10 experienced an outbreak of parasitic bronchitis, characterised by mild clinical signs caused by *D. viviparus* infection, which was diagnosed by Baermann analysis.

It should be noted that the estimates of the relative abundance of different GIN species based on cultured L_3 can yield quite different results from postmortem identification of adult GIN populations in the GI tract. Peterson (1957) found that

faecal examination often overestimates the abundance of *C. oncophora* compared to postmortem results (Anderson et al., 1965; Ciordia et al., 1964; Rose, 1968), with these differences attributed to differences in fecundity and reproductive biology. Consequently, the use of FEC is more likely to better describe future pasture contamination and parasite challenges. Potential limitations arise from the methodology of pooling samples for culture regardless of individual FEC and the variations in the time between sampling points. The approach employed in this study reflects the real-world conditions regarding investigating GIN infections on commercial dairy farms, as well as the numerous confounding variables, including the broad range of management and treatment regimens that complicate comparisons between farms. Given these challenges, this study's results and discussion have been primarily focused on analysing the observed general trends in FEC and L_3 species composition.

Although FEC does not accurately reflect the true adult worm burden of the host, they are useful determinant of pasture contamination and, hence, the future risk of clinical and sub-clinical disease. Mean group EPGs > 200 around eight weeks after turnout are shown to be predictive of clinical PGE later if calves remain on the same pasture (Shaw et al., 1998). Strongyle infections were observed in 76.5% of samples with a mean FEC of 63 EPG, while only 2.5% of FECs were greater or equal to 200 EPG, none occurred during the first eight weeks after turnout.

There were no apparent treatment failures during the study. Although anthelmintic efficacy cannot be assessed from the data, all treatments coincided with a dramatic decrease in FEC. Clear trends can be observed for each anthelmintic treatment regimen. For example, very low FECs were observed during the periods of residual activity of macrocyclic lactone products, and gradual increases in FEC were observed towards the end of these periods in the case of long-acting MOX. On farms that did not treat with an anthelmintic during the study, gradual increases in FEC were observed over the grazing season on all but one farm.

Ten GIN species were detected, and at the species level, species abundance, distribution, and temporal changes agree well with previous studies in temperate zones (Michel, 1969d; Pafčo et al., 2024; Roeber et al., 2017c). *C. oncophora* was the most abundant species during the beginning of the grazing season, but as the season progressed, the prevalence of *Os. ostertagi* increased and became the dominant species in many cases. This is unsurprising as these species are well known to be adapted to

this climate. *C. oncophora* was also the predominant species during the first few months after turnout, correlating well with data from Kloosterman, A., 1971. Several species primarily found in sheep were also observed: *Te. circumcincta* and *H. contortus*, but at low levels, as well as *Os. leptospicularis,* a GIN primarily of wild deer (Lyons et al., 2024).The generalist GIN species *Tr. axei* and *Tr. colubriformis* were observed on the majority of farms (87% and 91.3%, respectively) but only present at a minority of time points (<36%) either transiently or towards the end of the grazing season. The presence of *H. contortus,* primarily in Ayrshire, is noteworthy as a species that can infect a range of ruminant hosts. However, it is relatively uncommon in its preferred sheep hosts in this region (Sargison et al., 2007). No apparent correlation was observed between the GIN species found and co-grazing sheep, albeit with a relatively small sample size. Similar results, however, have been reported where a low prevalence of *H. contortus* was observed in Scottish cattle (McGregor, 2024). The clinical significance of many relatively uncommon species is uncertain and rarely detected by veterinary surveillance in the UK, and is rarely associated with parasitic gastroenteritis (Jewell et al., 2023). Although *H. contortus* and *Oesophagostomum* species are more prevalent and economically important in subtropical and tropical areas, climate change in the UK, with its warmer and more humid conditions, may lead to these species assuming greater clinical significance. It is also challenging to hypothesise specific reasons for the differing species proportions as numerous factors may influence the predominance of one species over another, including climate, farm management and the presence of anthelmintic resistance in one species over others.

Comparing the impact of MLs to BZs reveals the effect of the anthelmintic class on post-treatment population composition. *Cooperia* spp. are known to be dose-limiting for IVM and MOX (Benz and Ernst, 1979; Ranjan et al., 1992) and have been commonly observed predominating after IVM treatment in Europe (Demeler et al., 2009; El-Abdellati et al., 2010; Familton et al., 2001); the data presented is consistent with this for all ML products. An opposite trend can be observed after fenbendazole treatment, where *Os. ostertagi* is the dominant species.

The farms in this study employed a range of different management practices, which likely significantly contributed to inter-herd variation. The use of macrocyclic lactone products is widespread in the UK, as reflected in the high proportion of farms in the study that use such products and the absence of any farm that has used levamisole. The high proportion of pour-on formulations likely reflects their ease of

application, and interestingly, the only non-pour-on products used do not currently have an equivalent pour-on formulation. The two farms with a cow-with-calf policy are likely outliers and do not represent typical dairy systems. This is a very uncommon practice in the UK, and as calves are kept with the dam until weaning, it is likely more akin to a beef-suckler system.

Regarding the groups of FGS calves in this study, several conclusions can be drawn: clinical disease is unlikely to have occurred on these farms, and the impact of subclinical disease on production could not be estimated but was almost certainly occurring to a greater or lesser extent (Shaw et al., 1998). Again, this is complicated by the differences in pathogenicity and fecundity of GIN species. This is why species identification is an important factor and why GIN epidemiology should not be interpreted solely in terms of FEC. The impact of GIN infections could be interpreted as well-managed on all these farms, as FEC were relatively low throughout the study, and there were no outbreaks of clinical PGE. However, it is difficult to determine if the applications of anthelmintic products on these farms were always applied appropriately or if anthelmintic use could be reduced. Bovine lungworm infections are a concern in many systems and are treated with the same anthelmintic products. Outbreaks of clinical disease, however, were noted, but monitoring of sub-clinical infections was outside the scope of this study.

2.6. Conclusion

In summary, this longitudinal study has revealed the intra- and inter-herd differences in GIN species abundance and FEC intensities and the effect of anthelmintic treatments in FGS dairy calves. Species composition and FEC patterns varied in accordance with the expected patterns, yet varied considerably in relation to management and anthelmintic use. Ten species from eight genera were identified, and less common and clinically significant species were observed at greater frequency than expected. These new data represent the most comprehensive overview of the major GIN species across Scottish dairy farms to date. Substantial variance in the relative abundance of the two most predominant and clinically significant GIN species, *Os. ostertagi* and *C. oncophora,* between and within farms, were revealed, as well as the diversity and complexity of farm management systems. This study has provided

updated data on the population dynamics of GIN infection using modern molecular techniques, serving as a baseline for future epidemiological studies.

Ethical Approval

The University of Glasgow MVLS College Ethics Committee (Project No: 200210097) approved all research procedures involving animal use.

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CRediT authorship contribution statement

Paul Campbell: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Jennifer McIntyre:** Conceptualization, Formal analysis, Writing – review & editing. **Kerry O'Neill:** Methodology, Investigation. **Andrew Forbes:** Conceptualization, Formal analysis, Writing – review & editing. **Roz Laing:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Kathryn Ellis:** Conceptualization, Formal analysis, Supervision, Writing – review & editing,

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Chapter 3

3. Characterising anthelmintic resistance against benzimidazoles and macrocyclic lactones in gastrointestinal nematode populations of dairy cattle

Author Declaration & Contribution

Full paper citation, incl. authors and their affiliation (or link/ref to preprint server for papers not published)	Characterising anthelmintic resistance against benzimidazoles and macrocyclic lactones in gastrointestinal nematode populations of dairy cattle. Paul Campbell, Jennifer McIntyre, Kerry O'Neill, Andrew Forbes, Kathryn Ellis, Roz Laing. bioRxiv 2025.09.14.676042; doi: https://doi.org/10.1101/2025.09.14.676042

Please state your author contribution below for each category in accordance with the principles of the Contributor Roles Taxonomy system (CRediT). Please refer to the descriptors of each category before completion (https://credit.niso.org/)

Conceptualisation	PC, JM, AF, KE, RL
Data Curation	PC
Formal Analysis	PC
Investigation	PC, KO
Methodology	PC, JM, AF, KE, RL
Project Administration	PC, RL
Visualisation	PC
Writing – original draft	PC
Writing – review & editing	PC, JM, AF, KE, RL

We declare that the author contribution statements, in accordance with the CRediT system, are an accurate representation for this chapter/paper:

Author signature	
Primary supervisor signature (or corresponding author if different)	

Abstract

Anthelmintic resistance in gastrointestinal nematode populations is endemic across all grazing livestock production systems. In this study, resistance to macrocyclic lactones and benzimidazoles (BZ) in gastrointestinal nematodes from 14 Scottish dairy farms was assessed using multiple approaches. The faecal egg count reduction test (FECRT) remains the primary tool for evaluating anthelmintic resistance in the field. However, differing methodologies and recent guideline updates complicate the interpretation of results across studies; different statistical approaches yield varying confidence intervals, which, in turn, influence conclusions. However, resistance to benzimidazoles and macrocyclic lactones was consistently detected with all methods in 1/3 and 4/4 farms, respectively. The egg hatch test was combined with nematode speciation by PCR and used to test for benzimidazole resistance on 14 farms. The high effective concentrations (EC) at both the population and species levels, particularly for *Ostertagia ostertagi*, were consistent with benzimidazole resistance on 13/14 farms, including the resistant population identified by FECRT. Finally, mixed amplicon sequencing was applied to 10 populations from 7 farms, including pre- and post-treatment populations from the FECRT. Resistance polymorphisms in *beta-tubulin isotype-1* were detected on 5/7 farms and were found at >25% abundance in *O. ostertagi*, *Cooperia oncophora* and *Trichostongylus spp*.

The detection of resistance against both benzimidazoles and macrocyclic lactones on certain farms highlights the urgency of implementing sustainable control strategies. Although the data presented here are from Scotland, given the high rate of animal movement and the similar patterns of anthelmintic use across the UK, these findings are likely to be relevant across much of the UK.

Keywords

- Gastrointestinal nematodes
- Egg hatch test
- Anthelmintic resistance
- Benzimidazole
- Surveillance

Highlights

- Resistance to MLs and BZs was detected in GIN populations of cattle
- Multidrug resistance found in Scottish GIN populations of cattle
- Egg hatch test results suggest, and sequencing confirms high BZ-resistance allele frequencies

3.1. Introduction

Gastrointestinal nematode (GIN) infections are a major cause of reduced productivity and adverse effects on animal health and welfare. Broad-spectrum anthelmintic drugs have been the backbone of parasite management in livestock for 60 years; however, their continuous use has driven the selection of drug-resistant populations worldwide. The high prevalence and rapid emergence of anthelmintic resistance, including multidrug resistance, in the sheep industry have been the focus of extensive research.

In contrast, the ostensibly lower rate of anthelmintic resistance detection in cattle in temperate regions may partly be due to diagnostic challenges, as faecal egg counts are generally low. Egg counts are considerably less reflective of the true worm burden compared with sheep, making detection of resistance using the faecal egg count reduction test (FECRT) inherently more uncertain (Morgan et al., 2022; Sabatini et al., 2023). Simultaneous resistance to all three drug classes, benzimidazoles (BZ), macrocyclic lactones (ML), and imidazothiazoles, has now been identified in the two main GIN species infecting cattle: *Cooperia oncophora* and *Ostertagia ostertagi* in New Zealand (Sauermann et al., 2024).

Reports of anthelmintic resistance in UK cattle remain limited and do not currently suggest a severe problem. Nevertheless, UK studies (Bartley et al., 2012; Geurden et al., 2015b; McArthur et al., 2011; Stafford and Coles, 1999b) have indicated low to moderate levels of ML resistance. By contrast, resistance to BZs appears rare, with only one resistant population identified to date (Bartley et al., 2021). However, the number of studies conducted and farms surveyed is small, and current data are insufficient to provide a representative national picture (Hannah Rose Vineer et al., 2020).

Grazing cattle are particularly susceptible to GIN infection during their first two grazing seasons, after which they typically develop immunity and maintain low FECs. Youngstock, however, shed relatively more eggs and are therefore the most suitable age group for evaluating anthelmintic efficacy using the FECRT. In organic systems, the use of MLs is restricted, and regulations strongly discourage whole-group anthelmintic treatments, instead promoting evidence-based treatment approaches, such as targeted-selective-treatment strategies. Organic producers primarily use BZ or

levamisole (LEV) products to treat GIN infections; therefore, nematode populations on these farms should have little or no exposure to MLs.

To address the knowledge gap regarding anthelmintic resistance in Scottish cattle, we conducted a study investigating the prevalence of resistance in first-grazing-season dairy calves. The FECRT was used to evaluate the efficacy of the most commonly used anthelmintics: IVM, MOX , and FBZ, with the egg hatch test also used as an alternative measure of BZ resistance. In addition, mixed amplicon sequencing was employed to characterise GIN species composition and to determine the frequency of genetic markers associated with BZ and LEV resistance.

3.2. Methods

3.2.1. Ethics statement

The University of Glasgow MVLS College Ethics Committee (Project No: 200210097) approved all research procedures involving animal use.

3.2.2. Selection of farms

All 14 farms were selected on the following criteria: located in Scotland; herd size ≥30 first-grazing-season (FGS) calves, no anthelmintic treatment administered during the current grazing season; and a minimum of two months of grazing before sample collection. Additionally, all farms were required to complete a livestock management survey. The four farms participating in the FECRT were also required to have a cattle crush and handling system available.

All farms were offered free FEC analyses and evaluation of anthelmintic efficacy by FECRT. All farms participating in the FECRT were conventionally managed, whereas those participating only in the EHT were all organically managed

3.2.3. Farm survey

A questionnaire was completed during a semi-structured interview, collecting demographic data, information on pasture management and anthelmintic usage, and experiences with faecal egg counts and other helminth infections. For more details, see Chapter 2, Section 2.5 and Appendix B.

3.2.4. Faecal egg count reduction test protocol

All animals were turned out to pasture in May 2023. From eight weeks post-turnout, faecal egg counts (FECs), were monitored fortnightly until the group mean FEC reached ~100 eggs per gram (EPG). In addition, bovine lungworm (*Dictyocaulus viviparus*) larvae in faeces and body condition were measured regularly. On the day of treatment (Day 0) on each farm, 15 calves were randomly allocated to a treatment group and received either fenbendazole (Panacur® 10% Oral Suspension; MSD Animal Health) *per os*, subcutaneous IVM (IVOMEC® Classic Injection for Cattle and Sheep; Boehringer Ingelheim), or MOX (Cydectin® 10% LA Solution for

Injection; Zoetis) by subcutaneous ear injection, at the manufacturers' recommended dose rates of 7.5, 0.2, and 1.0 mg/kg of body weight respectively. Calves were either individually weighed or their weight estimated using a dairy calf weight band (AHDB), with weights ranging from 187 to 239 kg. Dose calculations were performed by the researchers, and anthelmintics were administered accordingly, with each dosage rounded to the nearest practical measure based on the formulation: 1 ml for fenbendazole, 0.1 ml for IVM, and 0.05 ml for MOX.

Faecal samples were collected *per rectum* from all animals on day 0 (pre-treatment) and day 14 (post-treatment). Samples were sealed immediately after collection and transported to the University of Glasgow on the same day. Aliquots of faecal material were stored at 4 ˚C for FEC analysis, while samples for egg isolation and coproculture were stored at room temperature and processed within 24 hrs of collection. On all farms, calves from the different treatment groups were grazed together on the same pastures until day 14.

3.2.5. Faecal egg count

A faecal egg count was performed on every individual animal sample using a modified salt flotation technique as described in Jackson, 1974, with a detection limit and multiplication factor of 1 egg per gram (epg). Briefly, faecal samples (3g) were homogenised in 10 ml of water per gram of faeces suspension and passed through a 1 mm sieve, followed by rinsing with 5 ml of water to remove debris. The filtrate was then transferred to polyallomer centrifuge tubes and centrifuged at 200 g for 5 minutes. The supernatant was discarded, and the pellet resuspended in saturated salt solution (SSS) (specific gravity of 1.2), vortexed, and centrifuged at 200 g for 10 minutes. The meniscus was then isolated using haemostat clips, and the suspension transferred to a cuvette, which was then completely filled with SSS. Cuvettes were then read after waiting a minimum of five minutes, and GIN eggs were identified morphologically and counted as either strongyle-type or *Nematodirus* spp.

For each individual animal, the number of strongyle eggs present pre- and post-treatment was used to calculate the percentage reduction in FEC, thereby estimating anthelmintic efficacy. In accordance with the revised FECRT guidelines (Kaplan et al., 2023), a minimum mean of 40 strongyle-type eggs per animal pre-treatment was required for reliable assessment. This threshold was achieved by performing one FEC

per individual; however, to facilitate species-specific faecal egg count reduction calculations, it was estimated that a total of three FECs were needed per sample.

3.2.6. Egg hatch test

Egg hatch tests on farms participating in the FECRT were conducted using eggs pooled from all pre-treatment animals. For the organic farms, eggs were pooled from faeces collected from pasture using the methodology described in Chapter 2, section 2.2.

Eggs were isolated from pooled fresh faeces by sieving, centrifugation and flotation in SSS. Briefly, 300g of faeces were mixed with tap water, passed through 500 µm and 210 µm sieves, and centrifuged at 2,500 rpm for 5 minutes. The supernatant was discarded, and kaolin was added to the pellet, and the mixture was vortexed before being resuspended in the SSS. After centrifugation at 1,000 rpm for 10 minutes, the polyallomer tubes were clamped to isolate the eggs, which were then collected in a 38 µm sieve, rinsed thoroughly with deionised water, and examined microscopically to confirm that embryonation had not yet begun.

Each sample was tested in triplicate using six concentrations of thiabendazole (TBZ) (0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 µg TBZ / ml) and a negative control (no drug, 0.5% DMSO), also in triplicate. The EHT was performed following the protocol described by von Samson-Himmelstjerna et al., 2009. After 48 hours, the test was terminated with Lugol's iodine stain, the contents of each well were transferred to a Petri dish, and all eggs and larvae were counted. The contents from each well were then pooled by TBZ concentration for species identification. Up to 94 eggs/larvae were identified to species level for each TBZ concentration as described in Chapter 4, Section 2.5.

3.2.7. Coproculture and species identification by PCR

Pooled larval coprocultures from each pre- and post-treatment group were prepared by hand-mixing approximately 300g of faeces with vermiculite to form a well-aerated, uniform paste-like consistency. The coprocultures were incubated at 27 $^{\circ}$C for 14 days and sprayed with water as needed to maintain adequate moisture for L_3 development. After incubation, larvae were harvested using a modified Baermann

technique as described in Roberts and O'Sullivan, 1950; pooled aliquots of L_3 in ddH_2O were stored at –80 ˚C.

Crude lysates were prepared from single strongyle eggs or larvae in 96-well plates using a modified proteinase K lysis reaction for individual strongyles identified by PCR. Briefly, a 100x stock solution of lysis buffer was made as follows: 1,000 µl DirectPCR Lysis Reagent (Cell) (Viagen Biotech), 50 µl 1M DTT (Invitrogen), and 10 µl Proteinase K (100 mg/ml) (Invitrogen). A 10 µl aliquot of this buffer was dispensed per well of a 96-well PCR plate. Using a stereomicroscope, individual strongyle eggs or larvae were transferred into each well in a volume of ≤1 µl. Lysates were incubated at 60 ˚C for 2 h, followed by 85 ˚C for 45 minutes to denature the Proteinase K. Crude lysates were diluted 1:20 with nuclease-free water.

For each pooled pre-/post- sample, a minimum of 94 recovered larvae/eggs were identified to species level by PCR targeting the ITS2 region. A multiplex PCR method (Bisset et al., 2014) was employed, using primers designed to amplify the strongyle ITS2 region and species-specific regions for GIN species of interest (see Appendix C). The reaction set included primers targeting *Haemonchus contortus*, *Os. ostertagi*, *C. oncophora*, *Oesophagostomum venulosum*, and *Trichostrongylus. axei*. Multiplex PCRs were performed in 96-well plates with 94 individual worm lysates, one DNA-negative control (no genomic DNA template), and one lysis-negative control (lysate without larva).

3.2.8. Genomic DNA isolation and amplicon sequencing library preparation

Genomic DNA from pools of 3,000 L_3 was isolated using the Monarch® Spin gDNA Extraction Kit (T3010S) following the manufacturer's instructions, with a final elution in 30 µl of buffer (10 mm Tris-HCl, pH = 9.0, 0.1 mm EDTA). The isolated gDNA was normalised to a concentration of 25 ng/µl, and all samples were stored at 4 °C.

Anthelmintic resistance mixed amplicon sequencing libraries were generated by individual PCR amplification of the loci as described in Chapter 3, Section 2.4. The primers for the ITS2 and *beta-tubulin isotype-1* loci were modified to include pan-nematode primer pairs developed by Avramenko et al., 2019, 2015. For the complete list of primers and thermocycling parameters used, see Appendix D. The PCR reactions (50 µl total volume) contained: 10 µl 5x Phusion GC buffer (New England

Biolabs), 1 µl of 10 mM dNTPs, 2.5 µl of 10 µM of each forward and reverse primer (or primer mix), 0.5 µl of Phusion DNA Polymerase, 1 µl of gDNA, and 32.5 µl of nuclease-free water. All PCR steps were performed following best practices to minimise aerosol formation, including the use of filter pipette tips, working in a PCR cabinet, and sealing PCR plates with adhesive seals.

The unindexed amplicon libraries were submitted for Illumina MiSeq amplicon sequencing. Stage-2 indexing PCRs, library quantification, normalisation, pooling, and denaturing were performed according to Illumina's 16S Metagenomic Sequencing Library Preparation (Illumina Inc., USA). Illumina 250 bp paired-end (250 PE) sequencing was undertaken on an Illumina MiSeq platform using MiSeq Reagent Kits v2.0 (500 cycles). The MiSeq was set to generate only FASTQ files with no post-run analysis. Samples were automatically demultiplexed by the MiSeq, based on the supplied index combinations.

3.2.9. Bioinformatic analysis

All loci were analysed separately using the same method as described in Chapter 3, Section 2.4. Briefly, amplicon sequence variants (ASVs) were identified for each locus using *dada2 v1.30.0* in RStudio. Filtering was employed to remove reads containing unresolved nucleotides as well as reads exceeding the expected error number and size range, *filterAndTrim* (run parameters: *maxN* = 0, *maxEE* = c(2,2), *truncQ* = 2, *minLen* = 50, *rm.phix* = TRUE, *matchIDs* = TRUE). This filtered dataset was then used for error training, *learnErrors(),* and error correction (denoising) of the dataset. The paired reads were merged, and chimeric sequences were identified before removal *removeBimeraDenovo().* A final table was produced for all the ASVs identified, along with their frequencies within the dataset.

3.2.10. Statistical analysis

All data were analysed and visualised with R Studio and publicly available packages: *tidyverse*, *drc*, *irr*, *tidymodels*, *eggCounts*, *bayescount*, and *ggplot2*. The EC_{50} (effective concentration for 50% inhibition) and EC_{95} values for each EHT were calculated using the *drm()* function of the *drc* package using the LL.4 model (Ritz and Streibig, 2005). The *EDcomp()* function compares effective doses derived from dose-response curves and reports p-values reflecting the statistical significance of the

differences between groups ($p \leq 0.05$ considered significant). The resistance ratio was calculated as the EC_x value of the sample divided by the EC_x of the susceptible isolate (FECRT_3).

For all FEC datasets, statistical analysis was performed to calculate the faecal egg count reduction (FECR) using *eggCounts* v2.4 to estimate the FECR with 90% and 95% confidence intervals (CI), and *bayescount* v0.9.99-9 to calculate the 90% CI. The output of the *eggCounts* and *bayescount* packages were interpreted based on the original FECRT guidelines described by Coles et al., 1992 and the revised guidelines described by Kaplan et al., 2023. The two guidelines are summarised in Table 3.1. To quantitatively compare the agreement between statistical models and the original and revised FECRT guidelines, weighted Cohen's κ coefficients were calculated using *kappa2(weights = "quadratic")* (Normal < Inconclusive < Low resistant < Resistant) and interpreted as described by McHugh, 2012.

Table 3.1 Comparison of the classification criteria for the faecal egg count reduction test.

Interpretation	**Lower 95 % FECR CI**	**FECR estimate**	**Upper 95 % FECR CI**
Original guidelines described by Coles et al., 1992			
Normal	> 90	> 95	NR
Suspected susceptible	< 90	> 95	NR
Suspected resistant	> 90	< 95	NR
Resistant	< 90	< 95	NR
Interpretation	**Lower 90 % FECR CrI**	**FECR estimate**	**Upper 90 % FECR CrI**
Revised guidelines described by Kaplan et al., 2023			
Susceptible	> 95	NR	> 99
Inconclusive	< 95	NR	> 99
Low resistant	> 95	NR	< 99
Resistant	< 95	NR	< 99
FECR; faecal egg count reduction, CL; confidence limit, NR; not relevant			

3.3. Results

3.3.1. Farms included in the study and their parasite management

The basic farm demographics and anthelmintic use information are summarised in Table 3.2. Fourteen farms participated in this study: four farms participated in the FECRT, and ten organic farms participated in the egg hatch test. All farms were operated as commercial dairy farms, with a majority also engaged in dairy-beef production (13/14), and half (7/14) also had sheep. The groups of FGS calves ranged from 29 to 62 individuals, all of which were spring-born and aged between 4 and 7 months at the time of sampling, with 71% to 100% being female. All non-organic farms were reported to have exclusively used macrocyclic lactone anthelmintics during the previous seven years, while organic farms exclusively used BZ products, and no farms had reported using levamisole. All participating farms reported only administering anthelmintics at the group level. All non-organic farms reported an average of two anthelmintic treatments per year, broadly described as one mid-season treatment while at pasture and one at housing. Of the organic farms, 6/10 averaged one group treatment per year, while four reported fewer than one group treatment per year. There were three broad categories of treatment regimens employed by the farms in the study, which we define as follows:

- Neo-suppressive: treatment to limit the establishment of a parasitic infection and minimise pasture larval contamination
- Prophylactic: treatment of an at-risk group in anticipation of clinical or production-limiting parasitism based on previous management experience, but without the use of diagnostic indicators
- Test-and-treat: treatment based on an FEC that may be production-limiting

All non-organic farms employed a prophylactic treatment regimen; while those that had also administered a MOX long-acting injectable also employed a neo-suppressive regimen. All organic farms used a test-and-treat approach using FECs, while 4/10 organic farms also employed a prophylactic treatment regimen, treating animals in anticipation of a significant parasite challenge and/or burden. Effective

quarantine was not routinely practised on any farm in this study: five farms stated that they gave quarantine treatments to bought-in cattle, and all stated that they do not routinely treat every animal

Table 3.2 Farm characteristics

Farm	Organic status	Dairy system	Calving pattern	No. of calves in study group	Anthelmintic compounds used in previous 7 years	Average number of anthelmintic treatments (group) per year	Treatment strategies previously employed
FECRT1	Non-organic	Dairy & beef	Dual-block	46	IVM, DOR	2	PT
FECRT2	Non-organic	Dairy & beef	Dual-block	48	IVM	2	PT,
FECRT3	Non-organic	Dairy	AYR	33	MOX LA, IVM	2	PT, NS
FECRT4	Non-organic	Dairy, beef, & sheep	Spring-block	45	MOX LA, MOX, IVM	2	PT, NS
Organic01	Organic	Dairy & beef	AYR	37	FBZ	1	TT
Organic02	Organic	Dairy, beef, & sheep	Dual-block	44	FBZ	1	TT, PT
Organic03	Organic	Dairy & beef	AYR	29	FBZ, ABZ	<1	TT
Organic04	Organic	Dairy, beef, & sheep	AYR	33	FBZ	1	TT, PT
Organic05	Organic	Dairy, beef, & sheep	Dual-block	38	FBZ	1	TT,
Organic06	Organic	Dairy & beef	Dual-block	59	FBZ	<1	TT
Organic07	Organic	Dairy, beef, & sheep	AYR	39	FBZ, ABZ	1	TT
Organic08	Organic	Dairy & beef	AYR	40	FBZ	<1	TT, PT
Organic09	Organic	Dairy, beef, & sheep	Dual-block	39	FBZ	1	TT, PT
Organic10	Organic	Dairy, beef, & sheep	Dual-block	46	FBZ	<1	TT

FECRT; Faecal egg count reduction test, IVM; Ivermectin, DOR; Doramectin, ST; Strategic treatment, AYR; All-year-round, MOX LA; Moxidectin long-acting, PT; Prophylactic treatment, MOX; Moxidectin, FBZ; Fenbendazole, TT; Test and Treat Dual block: spring and autumn calving

3.3.2. Faecal egg count reduction test

To meet the required minimum mean eggs counted required by the revised guidelines, one FEC per sample was needed to calculate and assign resistance status to the entire strongyle and *Os. ostertagi* communities. In comparison, three FEC per sample were required to obtain enough eggs to assign the resistance status to the *C. oncophora* populations of all farms. When the mean FEC of a sample was between 0 and 1 EPG, this was always rounded up to 1 EPG.

3.3.3. Non-modelled data

After treatment with any anthelmintic drug, FECs were significantly reduced for all populations (Figures 3.1 – 3.4), on all farms, regardless of the anthelmintic product used; the expected FECR is 99% (Kaplan et al., 2023). The paired pre- and post-treatment faecal egg counts are depicted as violin plots in the first column of Figures 3.1 - 3.4. For all treatments, the pre-treatment data are over-dispersed, with significant variance in FEC. The paired FECR for each individual is shown in the second column, and a substantial difference in FECR was observed between *Os. ostertagi* and *C. oncophora* in each treatment. Species composition of each strongyle population was determined by multiplex PCR, revealing six species from five genera in the study. In every pretreatment population, *Os. ostertagi* was the most prevalent species, with *C. oncophora* always the second most prevalent species. In all post-treatment populations, only two species were observed: *Os. ostertagi* and *C. oncophora*. After FBZ treatment, the prevalence of *Os. ostertagi* consistently increased, whereas after MOX treatment, the prevalence of *C. oncophora* was consistently increased. In six of the eight IVM treatment populations, *C. oncophora* prevalence increased, while in the Farm FECRT_1 and FECRT_4 populations, *Os. ostertagi* was observed to increase post-treatment.

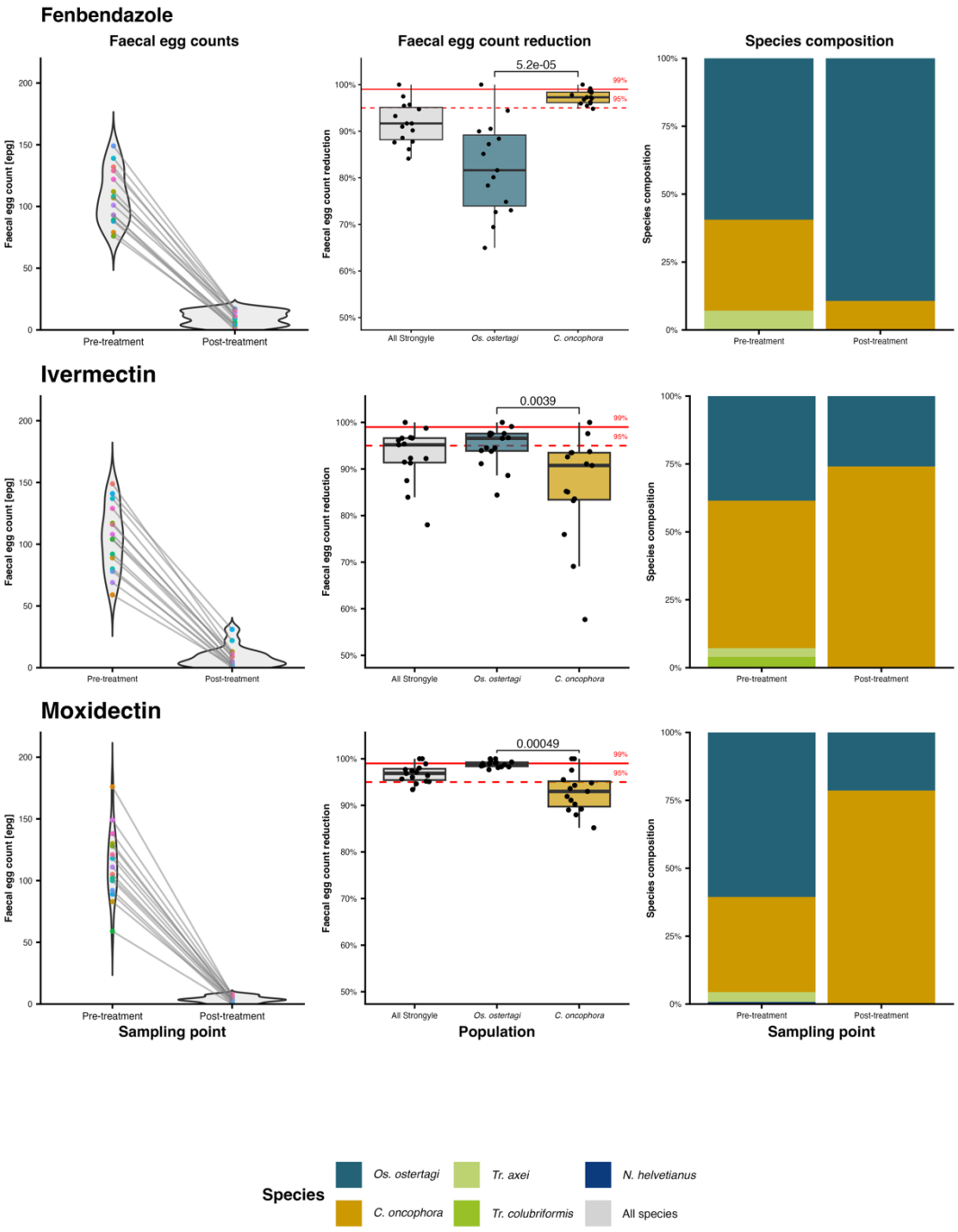

Figure 3.1 | FECRT_1: paired faecal egg count reduction, anthelmintic efficacy and relative species abundance of gastrointestinal nematode communities, pre- and post-treatment.

Species identity was assigned by ITS-2 rDNA multiplex PCR of a pool of L_3 larvae harvested from coprocultures of each cohort and time point. A minimum of 94 L_3 were identified per pooled coproculture. The violin plots with paired points represent the probability and distribution of strongyle-type faecal egg counts (FECs) pre- and post-treatment. Faecal egg counts were conducted using a modified salt flotation technique with a sensitivity of epg 1. The boxplots represent the faecal egg count reduction estimates for each individual, based on the FEC and interpolated species compositions.

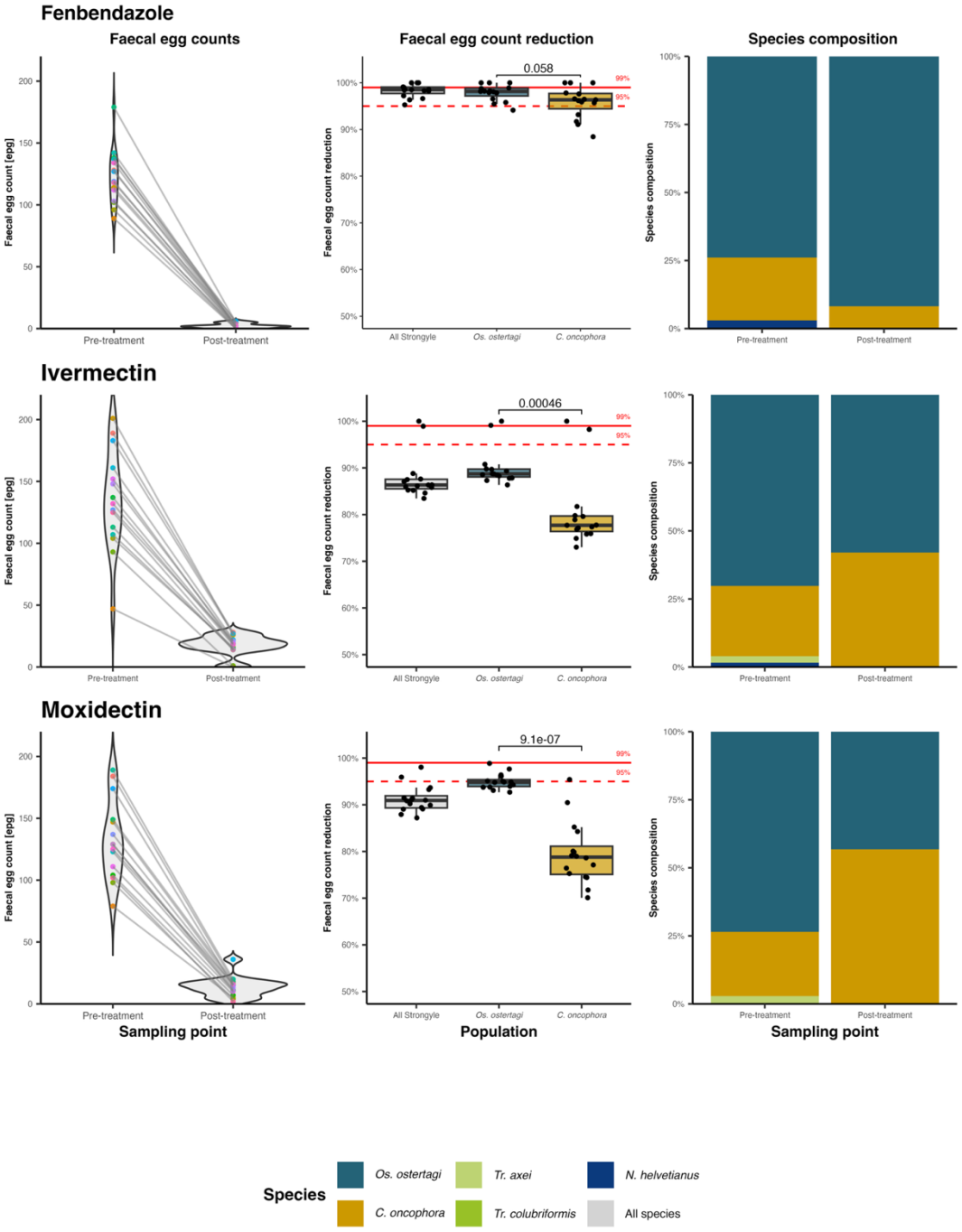

Figure 3.2 | FECRT_2: paired faecal egg count reduction, anthelmintic efficacy and relative species abundance of gastrointestinal nematode communities, pre- and post-treatment.

Species identity was assigned by ITS-2 rDNA multiplex PCR of a pool of L_3 larvae harvested from coprocultures of each cohort and time point. A minimum of 94 L_3 were identified per pooled coproculture. The violin plots with paired points represent the probability and distribution of strongyle-type faecal egg counts (FECs) pre- and post-treatment. Faecal egg counts were conducted using a modified salt flotation technique with a sensitivity of epg 1. The boxplots represent the faecal egg count reduction estimates for each individual, based on the FEC and interpolated species compositions.

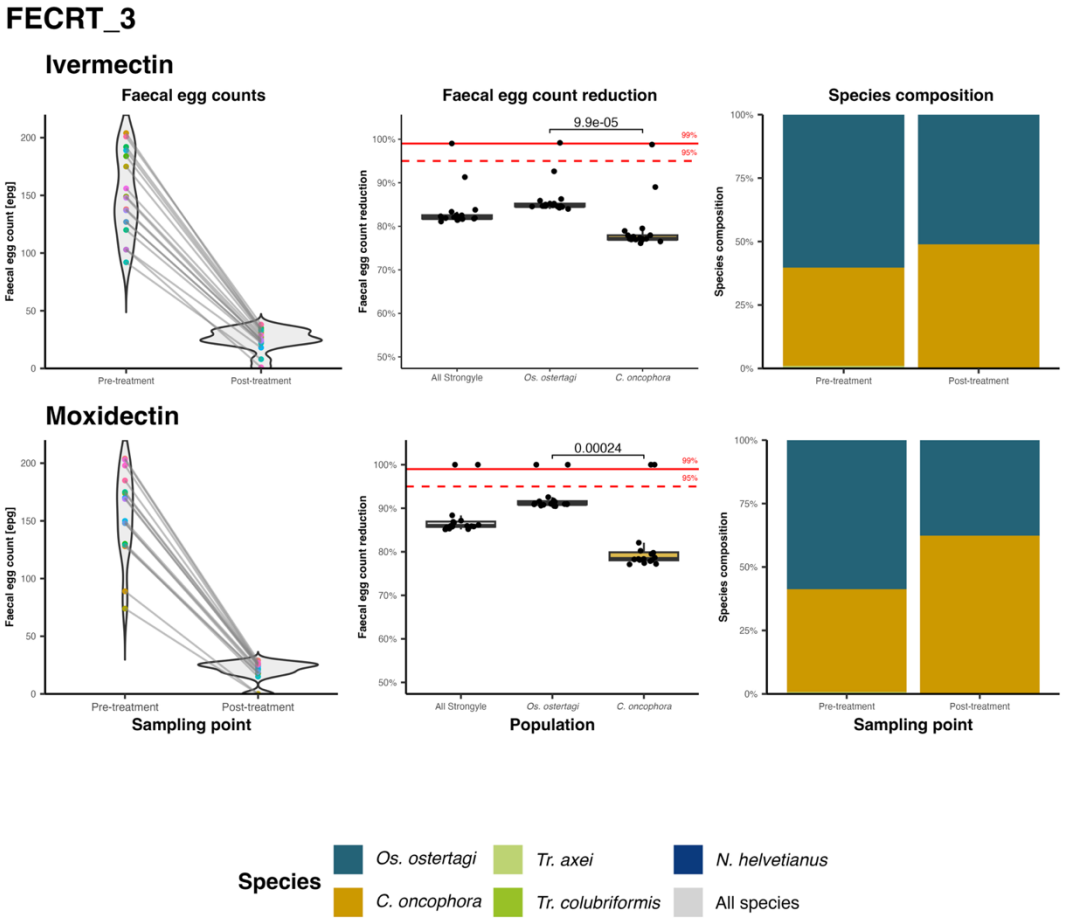

Figure 3.3 | FECRT_3: paired faecal egg count reduction, anthelmintic efficacy and relative species abundance of gastrointestinal nematode communities, pre- and post-treatment.

Species identity was assigned by ITS-2 rDNA multiplex PCR of a pool of L_3 larvae harvested from coprocultures of each cohort and time point. A minimum of 94 L_3 were identified per pooled coproculture. The violin plots with paired points represent the probability and distribution of strongyle-type faecal egg counts (FECs) pre- and post-treatment. Faecal egg counts were conducted using a modified salt flotation technique with a sensitivity of epg 1. The boxplots represent the faecal egg count reduction estimates for each individual, based on the FEC and interpolated species compositions.

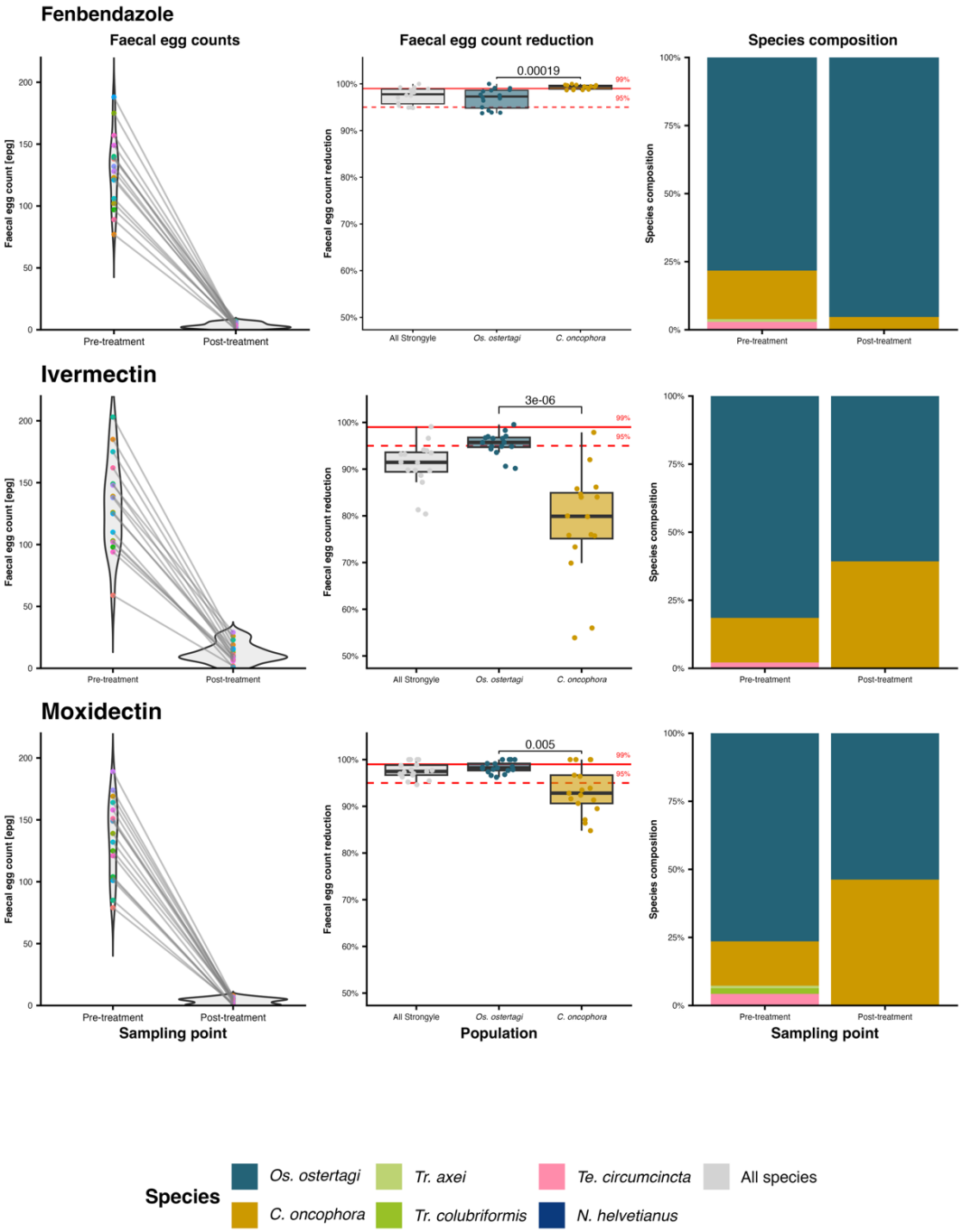

Figure 3.4 | FECRT_4: paired faecal egg count reduction, anthelmintic efficacy and relative species abundance of gastrointestinal nematode communities, pre- and post-treatment.

Species identity was assigned by ITS-2 rDNA multiplex PCR of a pool of L_3 larvae harvested from coprocultures of each cohort and time point. A minimum of 94 L_3 were identified per pooled coproculture. The violin plots with paired points represent the probability and distribution of the strongyle-type faecal egg counts (FECs) pre- and post-treatment. Faecal egg counts were conducted using a modified salt flotation technique with a sensitivity of epg 1. The boxplots represent the faecal egg count reduction estimates for each individual, based on the FEC and interpolated species compositions.

3.3.4. Comparison and interpretation of the faecal egg count reduction test between statistical methods and guidelines

The interpretation of the FECRT using the previous WAAVP guidelines for detecting anthelmintic resistance (Coles et al., 1992) was compared with the recently published revised guidelines (Kaplan et al., 2023). For this purpose, the categories for determining anthelmintic efficacy from the original guidelines - i.e., reduced, suspected resistant, suspected susceptible, and normal - were considered equivalent to those of resistant, low resistant, inconclusive, and susceptible, respectively, from the current revised guidelines. For all comparisons of guidelines and FECRT modelling packages, see Appendix F.

When assigning a resistance status to the entire strongyle population against FBZ using the revised guidelines, only one scenario resulted in a susceptible assignment (FECRT2/BZ/EC/RV), as shown in Figure 3.5. Consistency between all FBZ scenarios was observed only in the status classified to FECRT_1, which classified the populations as resistant. In all IVM treatment scenarios in all farms, the strongyle populations were determined to be resistant. The MOX-treated populations of farms FECRT_2 AND FECRT_3, were consistently determined to be resistant in all scenarios, whereas the MOX-treated populations of FECRT_1 and FECRT_4 were only classified as resistant in scenarios using the revised guidelines.

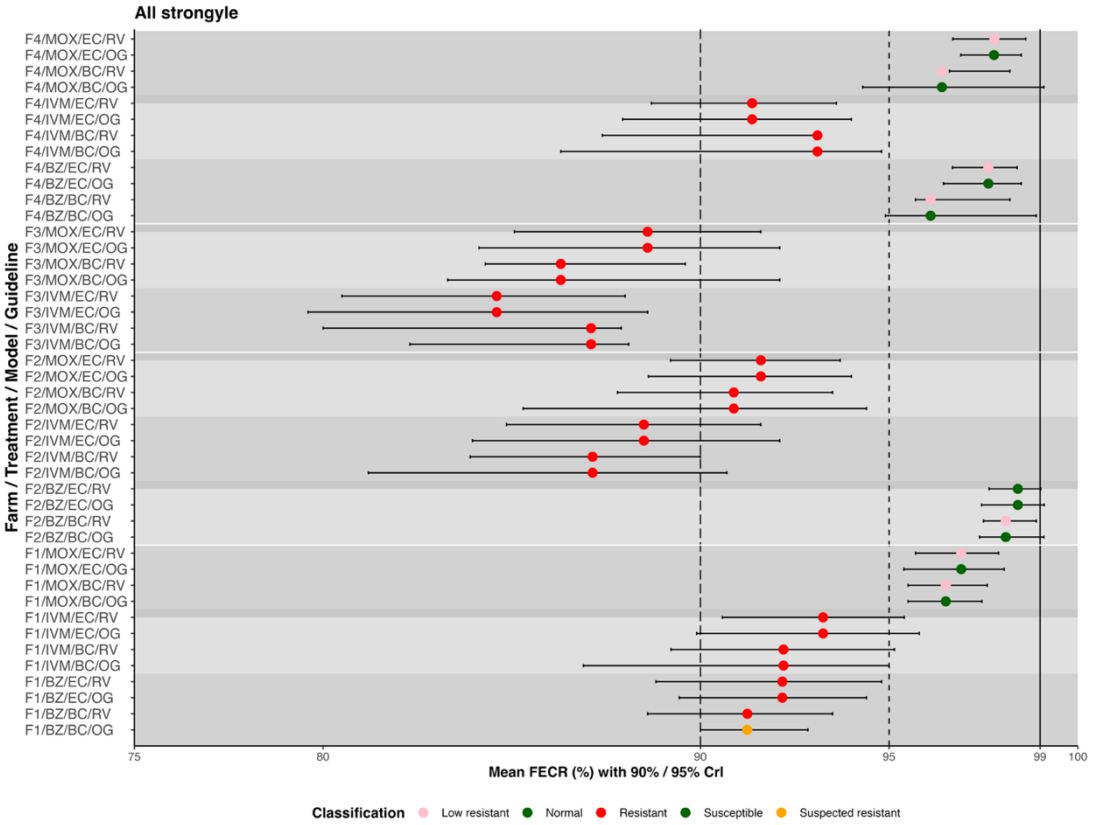

Figure 3.5 | Comparison of the eggCounts and bayescount faecal egg count reduction estimates for the entire strongyle population.

Faecal egg count reductions (FECR) with the credible intervals (CrIs) for anthelmintic treatment against the entire strongyle population. The CrIs were calculated using either *eggCounts* (EC) or *bayescount* (BC) models and interpreted based on either the revised guidelines (RV) for the faecal egg count reduction test (Kaplan et al., 2023) with corresponding 90% CrIs, or on the original guidelines (OG) (Coles et al., 1992) with 95% CrIs. Each point represents the mean FECR, with colour indicating the resistance status classified for the entire strongyle population: green, susceptible/normal; red, resistant; pink, low resistant/suspected resistant; orange, inconclusive/suspected susceptible.

Using the categories for each modelling / statistical method based on the revised guideline and treatment results, with the assignment of suspected resistance equal to low-resistance, both statistical methods agreed for 10/11 datasets (Table 3.3). With one dataset classified as susceptible by *eggCounts* and classified as low resistance by *bayescount*. This resulted in a Cohen's κ value of 0.656, corresponding to substantial agreement.

Table 3.3 The inter-rater agreement between eggCounts and bayescount results based on the revised guidelines for the faecal egg count reduction test (Kaplan et al., 2023) for the entire strongyle population.

bayescount	***eggCounts***			
	Susceptible	**Inconclusive**	**Low resistant**	**Resistant**
Susceptible	-	-	-	-
Inconclusive	-	-	-	-
Low resistant	1	-	3	-
Resistant	-	-	-	7
	Cohen's k = 0.656 (substantial agreement)			

Comparing the interpretations of the FECRT results between the original and revised guidelines was conducted using only egg count data. The interpretation of results using both guidelines agreed for 8/11 datasets (Table 3.4). Three datasets classified as low-resistant by the revised guidelines were classified as normal (susceptible) by the original guidelines. This results in the same level of agreement as between both modelling packages and the revised guidelines.

Table 3.4 Inter-rater agreement between the original guidelines (Coles et al., 1992) and the revised guidelines (Kaplan et al., 2023) based on the faecal egg count reduction test for the entire strongyle population analysed using eggCounts.

Original guidelines	**Revised guidelines**			
	Susceptible	**Inconclusive**	**Low resistance**	**Resistant**
Normal	1	-	3	-
Suspected susceptibility	-	-	-	-
Suspected resistance	-	-	-	-
Resistance	-	-	-	7
	Cohen's k = 0.656 (substantial agreement)			

When comparing the inter-rater agreement of the statistical models using the revised guidelines' interpretations of the FECRT for the interpolated *Os. ostertagi* and *C. oncophora* populations. The *Os. ostertagi* populations were classified as the same statuses as the entire strongyle populations for all treatments (Appendix G). This resulted in a Cohen's κ value of 0.656, corresponding to substantial agreement. The inter-rater agreement for the *C. oncophora* populations was perfect, with a Cohen's κ value of 1, consistently assigning ten populations as resistant and one as low resistant.

Comparing the inter-rater agreement of the original and revised guidelines' interpretation using the *eggCounts* model of the interpolated species datasets, there was fair agreement between the guidelines when assigning the resistant status to the *Os. ostertagi* populations with a Cohen's κ value of 0.333, where three populations were initially classified as normal by the original guidelines but were classified as low-resistant by the revised guidelines, and one population classified as normal was also classified as resistant. The inter-rater agreement for the *C. oncophora* populations was lower, with a Cohen's κ value of 0.19, consistently assigning resistance to eight populations; however, it also classified two resistant populations as normal by the original guidelines, as well as a low-resistant population and a normal population, corresponding to only slight agreement between the guidelines.

3.3.5. Egg hatch test dose-response

Sufficient numbers of eggs were collected from all organic farms and pre-treatment FECRT populations to conduct the egg hatch test. The mean number of eggs added to each well was 207, while the mean proportion of egg hatching in the control wells was 92.6%. The dose-response curves for all populations (all strongyle, *Os. ostertagi*, and *C. oncophora*) are presented in Appendix H and the effective concentrations are shown in Figure 3.6. The interpolated effective concentrations for *Os. ostertagi* ranged from EC_{50} (0.017 to 0.157 µg/ml) and EC_{95}(0.045 to 1.293 µg/ml) and for *C. oncophora*, EC_{50} (0.025 to 0.111 µg/ml) and EC_{95} (0.416-0.859 µg/ml). The relative resistance ratio was also calculated for each species, with farm FECRT_3 used as the reference sensitive isolate. The RR ranged from 4.24 to 28.71, and 1.70 to 12.87 for *Os. ostertagi* and *C. oncophora,* respectively. For the specific EC_{50}, EC_{95} and RR for each population, refer to Appendix E.

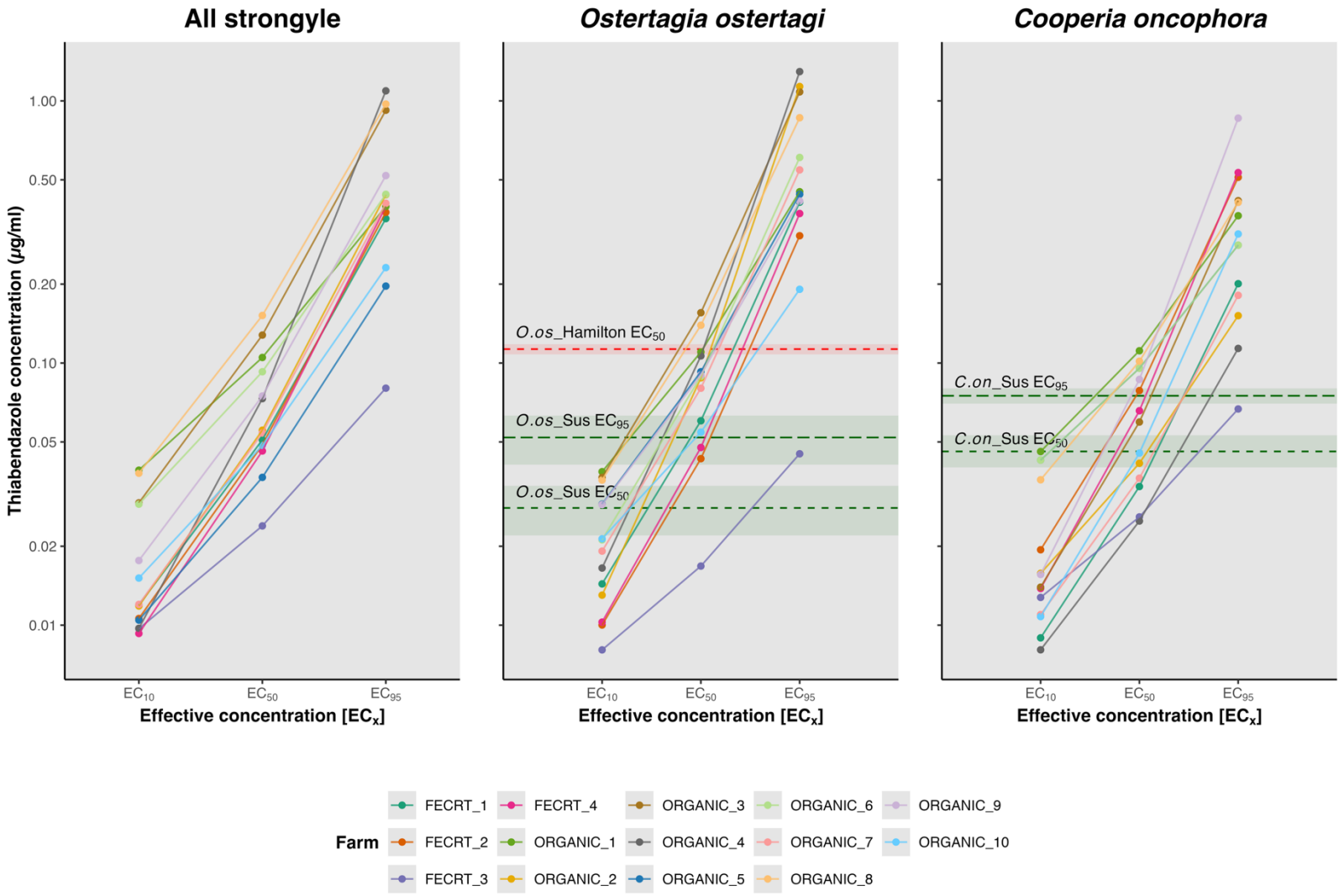

Figure 3.6 | Nematode effective concentration estimates (µg/ml thiabendazole) for each strongyle and interpolated species population.

The effective concentrations [E_C] were estimated from the respective LL.4 dose-response curve model. The green dashed line represents the EC_{50} and EC_{95} of a fully susceptible *Ostertagia ostertagi* and *Cooperia oncophora* isolate, with the standard error of the mean. The red line represents the EC_{50} of a resistant *Os. ostertagi* population ± SEM.

3.3.6. Amplicon sequencing

Sufficient numbers of larvae were obtained from all treatment groups pre- (range 20,000 to 83,000) and post-treatment (range 2,300 to 5,100) to conduct mixed amplicon sequencing of all loci. Ten populations were subjected to the mixed amplicon sequencing marker panel based on the abundance of larvae, parasite management practices, and the likelihood of resistance being present, as determined by either or the FECRT or EHT. Four GIN populations from organic dairy farms and six paired pre- and post-treatment FECRT populations (FBZ, IVM, MOX) were selected.

3.3.6.1. Nemabiome

We used ITS-2 nemabiome metabarcoding to determine the species composition of the GIN populations and identified 105 ASVs, classified into eleven taxa, either at the genus (3.7%) or species level (96.3%). A minority of ASVs (n = 5) could not be classified to the species level but were identified as belonging to the genera *Cooperia* (n = 3) and *Trichostrongylus* (n = 2). The most prevalent species identified were *Os. ostertagi* and *C. oncophora*, observed in every population (see Figure 3.7; Appendix I). *Trichostrongylus axei* was observed on every farm but was absent in both the post-IVM and MOX populations, while *Tr. colubriformis* was observed on six farms. *Oesophagostomum* spp. were only observed in GIN populations from organic farms (n = 4), and *Haemonchus contortus* was observed on two farms. In terms of relative abundance, *Os. ostertagi* was the most abundant species in the majority of populations (8/10) and the most abundant species on all farms except farm FECRT_3. Comparing the changes in species abundance pre- and post-BZ-treatment, *Os. ostertagi* and *Teladorsagia circumcincta* increased from 53.1% to 85.2% and 1.5% to 3.9% respectively, while *C. oncophora* decreased from 29.7% to 7.9%. Comparing the effect of macrocyclic lactone products, *Os. Ostertagia* was observed to decrease from 67.2% to 51.7% and from 42.6% to 28%, post-IVM and MOX treatment, respectively, while *C. oncophora* increased from 21.3% to 39.9% and from 45.8% to 71.1%, respectively.

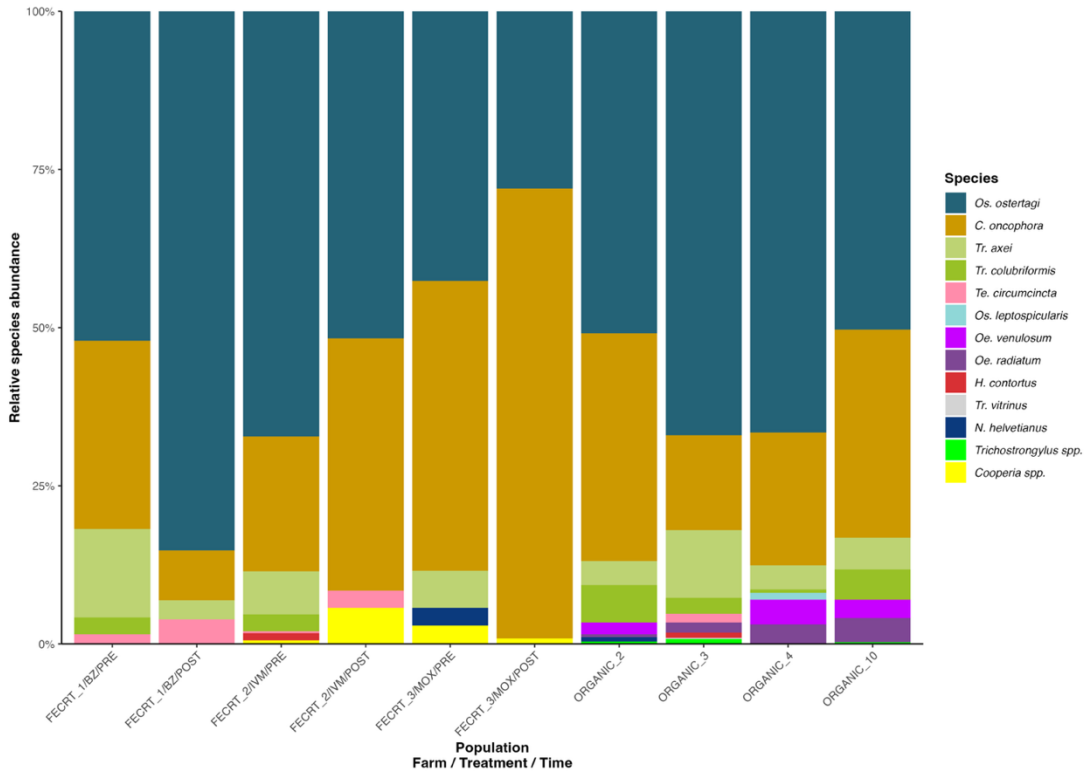

Figure 3.7 | Gastrointestinal nematode composition determined by ITS-2 nemabiome metabarcoding.

Relative species abundance of gastrointestinal nematode communities, determined by ITS-2 rDNA nemabiome metabarcoding, of pools of L_3 harvested from individual coprocultures of pre- and post-faecal egg count reduction test populations and free-catch pasture samples from organic farms. A minority of ASVs could only be identified to the genus level: *Cooperia* spp. and *Trichostrongylus* spp. groups.

3.3.6.2. Frequency of β-tubulin isotype-1 resistance-associated polymorphisms

Between 4,385 and 6,211 (mean = 5,381) sequence reads for the *β-tubulin isotype-1* loci were generated from each GIN population using the mixed amplicon sequencing panel and screened for BZ-resistance polymorphisms at codons 167, 198 and 200. Sequences mapped to *Os. ostertagi*, *Tr. axei*, *Tr. colubriformis*, *Tr. vitrinus*, *C. oncophora*, *Te. circumcincta*, *H. contortus* C. *curticei*, *Os. leptospicularis*, and *Oesophagostomum* spp.. *Ostertagia ostertagi* and *C. oncophora B-tubulin* were identified in all samples (Figure 3.8). Benzimidazole resistance alleles were present to some degree in all strongyle populations from organic farms and were detected in seven of the ten sequenced populations. The majority of *Os. ostertagi* resistance alleles detected were F200Y (TTC > T**A**C), as well as two resistance alleles at codon 198, E198A (GAA > G**C**A), E198L (GAA > **TT**A). However, the F167Y (TTC > T**A**C) polymorphism was also present in organic farms 3 and 10 at very low frequencies of 1.3% and 2.6%, respectively. All *C. oncophora* resistance alleles were the F200Y variant, present at a mean frequency of 11.2%, but showed high variability in frequency between farms, ranging from 2% to 65.8%. No non-synonymous polymorphisms were detected at codons 167 or 198. *Trichostrongylus* spp. (*Tr. axei*, *Tr. colubriformis*, *Tr. vitrinus*) alleles were detected on all farms but were absent in the post-macrocyclic lactone treatment populations. The F200Y polymorphism was detected at a mean frequency of 13.9% in *Trichostrongylus* spp., while the E198L polymorphism was present at a highly variable frequency, with a mean of 11.5%, ranging from 1.1% to 43.4%. Comparing the effect of fenbendazole treatment on allele frequency, the total frequency of resistance alleles for all strongyles increased from 12.7% to 43.8%, while for *Os. ostertagi*, *C. oncophora*, and *Trichostrongylus* spp., the frequencies increased from 20.8 to 35.6%, 2 to 65.8%, and 22.6 to 64.6%, respectively.

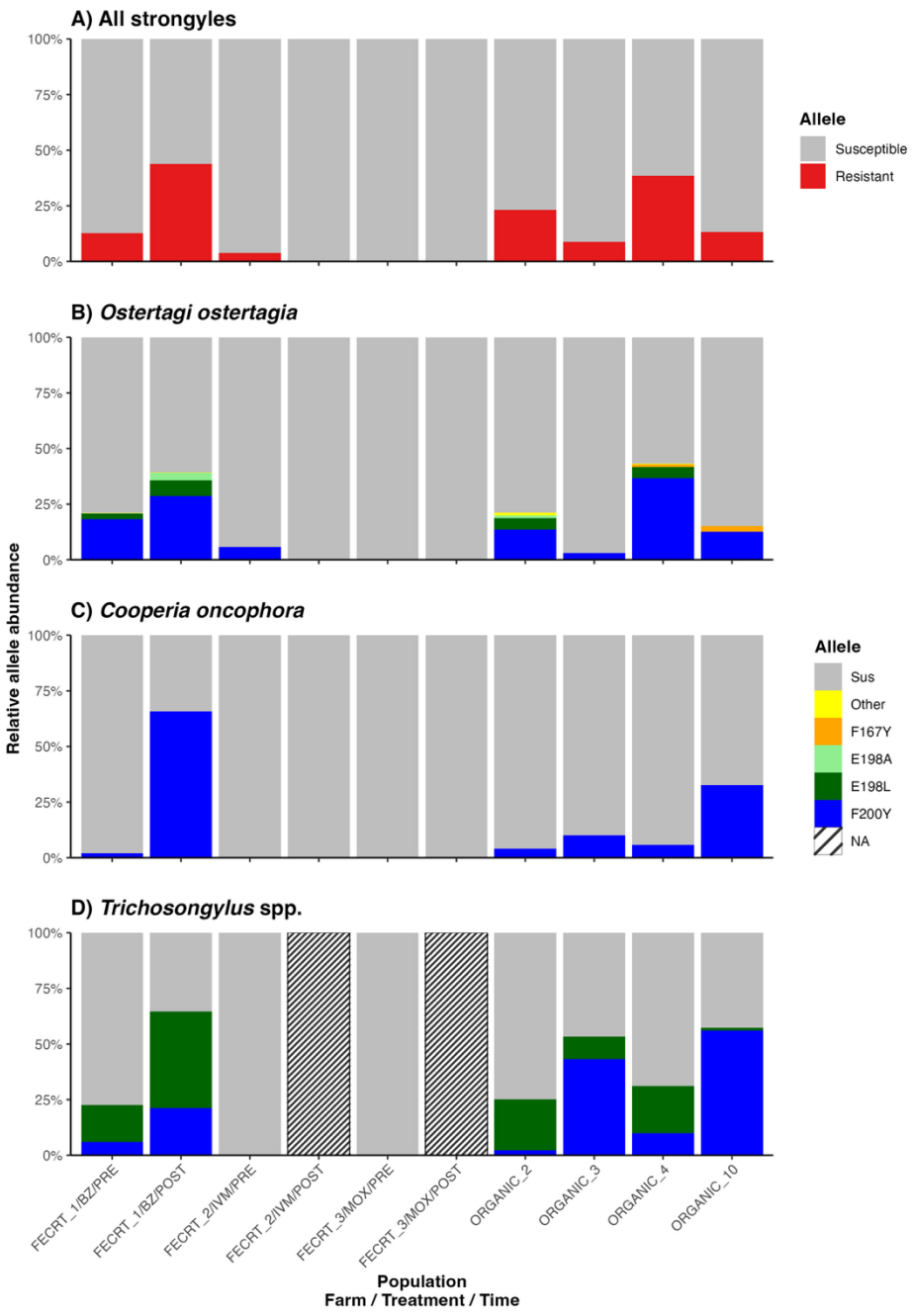

Figure 3.8 | Frequency and prevalence of β-tubulin isotype-1 gene resistance alleles.

The relative proportions of the *β-tubulin isotype-1* gene resistance allele frequencies of 10 populations from seven different farms for all strongyles and three nematode species. (A) All strongyles; (B) *Ostertagia ostertagi*; (C) *Cooperia oncophora*; (D) *Trichostrongylus* spp. Susceptible alleles (F167, E198 and F200) are displayed in grey, while previously described resistance-associated polymorphisms (F167Y (TTC > T**A**C), E198A (GAA > G**C**A), E198L (GAA > **TT**A) and F200Y (TTC > T**A**C)) are displayed in red for all strongyles and as orange, green, light green and blue respectively for the resistance allele frequency per species. All other identified non-synonymous polymorphisms were grouped and are displayed in yellow. If no alleles were identified, this is represented as a diagonal stripe (NA).

3.4. Discussion

The results of this study are consistent with the global trends of increasing resistance to all anthelmintic compounds and conclusively demonstrate that resistance to the main anthelmintic compounds exists in multiple nematode populations and species infecting Scottish dairy cattle. The control of GIN infections relies primarily on metaphylactic and therapeutic treatment with anthelmintics. In the UK and Europe, resistance in parasitic nematodes of cattle is currently not considered widespread and reported resistance levels have generally been low to moderate. The FECRT remains the most important field test for evaluating the susceptibility or resistance status of GIN populations (Kotze et al., 2020). However, the original WAAVP guidelines for the FECRT (Coles et al., 1992) had not been updated in more than 30 years, until a recent revision by Kaplan et al., 2023. These revised guidelines not only update the recommendations for study design and reporting but also change the criteria (cut-offs) for identifying resistant populations. In addition, several different statistical approaches have been suggested to analyse FECRT data and to account for sources of variation (Denwood et al., 2023; Torgerson et al., 2014). It is essential to understand that the application of different guidelines and statistical approaches can yield varying interpretations of the same FECRT data. Ehnert et al., 2025, conducted a similar study and observed levels of agreement between the two statistical models consistent with this study, as well as low levels of agreement between the original and revised guidelines. In the current study, it should be emphasised that many of the interpolated species populations considered resistant by the revised guidelines were classified as susceptible when applying the original guidelines. This makes it difficult and unreliable to compare findings between studies using the original and revised guidelines, as it is likely that the original guidelines underestimate the prevalence of resistant strongyle populations.

The nemabiome analysis revealed that *Os. ostertagi* and *C. oncophora* were the most prevalent and abundant species in this study. This finding aligns with other UK and European studies, which have shown that these species dominate. The number of species identified in pre-treatment populations ranged from four to eight, totalling 11 species from six genera identified in the study. Despite the limitations of nemabiome analysis, cost, PCR bias, and egg shedding variation. It represents the best technique to characterise strongyle nematode communities. It is the only method that

determines the species profile of strongyle nematode communities without making assumptions about their composition, while the volume of data generated enables the detection of rare species that are rarely studied. Correlation factors somewhat limit the impact of copy number variation between species; however, these limitations apply to all metabarcoding techniques. In most studies, read counts are used as a proxy for abundance.

After treatment with macrocyclic lactones, all farm populations showed an increase in the proportion of *Cooperia* spp. The generally low pathogenicity of this parasite could be a reason why resistance to the drug is rarely recognised by the farmers. In New Zealand, where most cases of GIN resistance to MLs have been reported, there are still no case reports of clinical parasitism due to this species (Jackson et al., 2006). Furthermore, the limited sensitivity of the FECRT, requiring at least 25% of a population to be resistant (Martin et al., 1989), in combination with the higher fecundity of *Cooperia* spp., likely underestimates efficacy against the less fecund *Os. ostertagi*. Additionally, the IVM concentration in the abomasal mucosa that *Os. ostertagi* experiences is higher than that of the intestine, the target site of *C. oncophora* (Lifschitz et al., 2000). This may be one reason why *Cooperia* spp. are the dose-limiting GIN. It was therefore expected that *Cooperia oncophora* would be the first species showing ML resistance in cattle (Coles, 2002b).

In this study, the standardised egg hatch test protocol using thiabendazole was used to compare *in vitro* assay data, BZ-treatment FECRT data, and BZ-resistance allele frequency data. It is commonly accepted that the cut-off value for BZ-resistance is 0.1 µg TBZ/ml (Coles et al., 2006). The EC values reported in this study (EC_{50}: 0.023 to 0.15 µg TBZ/ml, EC_{95}: 0.08 to 1.07 µg TBZ/ml) were significantly higher than those obtained in a similar European study, where pre-treatment EC_{50} values of 0.027 to 0.038 µg TBZ/ml were observed for a mixed species population (Demeler et al., 2012). However, only one publication has given EC_{50} values for known susceptible *Os. ostertagi* and *C. oncophora* populations, which were reported at 0.022 to 0.034 µg and 0.04 to 0.052 µg, respectively. An EC_{50} value (0.108 to 0.118 µg) for a known resistant *Os. ostertagi* population (*O.o.*Hamilton, 2010) has been published (Demeler et al., 2013), but no efficacy values from an FECRT or controlled efficacy test have been reported. The extremely high EC_{50} and EC_{95} values for some populations are undoubtedly suggestive of a resistant population or subpopulation.

The mixed amplicon sequencing approach was successful in identifying known BZ-resistance alleles that were present in 0 to 43.8% of all *β-tubulin isotype-1* reads and observed to significantly increase after fenbendazole treatment on farm FECRT_1. The F200Y variant was the most commonly observed resistance allele in *Os. ostertagi,* and the only resistance allele observed in *C. oncophora*. However, of the *Trichostrongylus* spp. resistance alleles, the E198L variant was the most commonly observed allele in 5/6 populations and was only more abundant on farm ORGANIC_10.

3.5. Conclusion

This study has examined the anthelmintic efficacy of the main anthelmintic compounds used to treat cattle in the UK, utilising both *in vivo* and *in vitro* assays, and highlighted the variance in anthelmintic efficacy between farms and strongyle species. The present study highlights the emergence of anthelmintic resistance to MLs and BZs in cattle nematodes, as well as the reliance of both conventionally and organically managed farms on these products. The variation in the efficacy of such treatments is evident, as well as the impact of differing statistical methodologies and diagnostic criteria on the assignment of resistance status. The revised FECRT guidelines complicate the comparability of results under previous guidelines and emphasise the need for methodological consistency. The higher-than-expected EC values for *Os. ostertagi* and *C. oncophora* are concerning and warrant further investigation, as there is a lack of empirical data and guidance on how to interpret such data and assign a resistance status. The variable frequency and high abundance of BZ-resistance alleles in some populations are noteworthy because, if the increasing trend for using BZ products against GIN infection in UK cattle continues, then selection for AR will likely rise. Consequently, continued and improved testing of efficacy to encourage remedial action would be very beneficial.

Data Availability

Sequence data generated by this project were submitted to the SRA section of GenBank and are available under the SRA accession numbers within BioProject PRJNA. Additional information, data, and analysis code are available from GitHub (https://github.com/pau1campbe11/Characterising-AR-against-BZs-MLs-in-GINs.git)

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Credit Authorship Contribution Statement

Paul Campbell: Conceptualisation, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Jennifer McIntyre:** Conceptualisation, Formal analysis, Writing – review & editing. **Kerry O'Neill:** Investigation. **Andrew Forbes:** Conceptualisation, Formal analysis, Writing – review & editing. **Roz Laing:** Conceptualisation, Formal analysis, Supervision, Writing – review & editing. **Kathryn Ellis:** Conceptualisation, Formal analysis, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Chapter 4

4. Genome-wide analysis of macrocyclic lactone treatment response by field populations of *Ostertagia ostertagi*

Abstract

Ostertagia ostertagi is a common and pathogenic gastrointestinal nematode of cattle in temperate regions, and it is a major contributor to parasitic gastroenteritis. Despite widespread use of macrocyclic lactones (MLs) such as ivermectin and moxidectin, the genetic basis of resistance in *Os. ostertagi* remains poorly understood. In this study, we describe a genome-wide approach to detect evidence of selection by ivermectin and moxidectin in field populations of *Os. ostertagi* from Scotland using gastrointestinal nematode populations collected from the same animals pre- and post-treatment. Using pooled whole-genome sequencing, we assessed changes in nucleotide diversity, allele frequency, and genetic differentiation across the genome. Our results revealed low overall genetic differentiation between pre- and post-treatment populations, but identified a consistent peak of differentiation on chromosome 5 (51 – 53.5 Mb), suggesting a putative quantitative trait locus for ML resistance. These findings indicate that ML resistance in the field population may be driven by selection on standing genetic variation, with resistance alleles segregated across diverse genetic backgrounds. This study provides foundational insights into the genome-wide effects of ML treatment and the genomic architecture of ML resistance in *Os. ostertagi* and highlights candidate regions for further investigation and molecular marker development.

4.1. Introduction

The nematode *Ostertagia ostertagi* is one of the most common parasites of cattle and one of the most pathogenic gastrointestinal nematodes (GIN) in temperate climates. Together with *Cooperia oncophora*, *Os. ostertagi* infections are the primary cause of clinical disease (parasitic gastroenteritis), a significant health and welfare concern, particularly in young animals. Additionally, the ubiquity of subclinical infections is a significant constraint on productivity in cattle of all ages (Charlier et al., 2020). Infections cause hyperplasia of the abomasal mucosa, impaired pepsinogen activation and increased abomasal permeability, leading to reduced protein digestion and loss of plasma protein to the gastrointestinal tract (Taylor et al., 1989). This pathophysiological disruption, combined with reduced feed intake by infected animals, leads to reduced animal performance, which can impact the efficiency and economics of cattle farms (Forbes, 2008; Högberg et al., 2019). Anthelmintic treatment is the primary means by which GIN infections are controlled, and the macrocyclic lactone (ML) drug class accounted for the largest market share (41%) in 2024, for all livestock parasiticides sold globally (Faizullabhoy and Wani, 2024). Reports from some European countries indicate that MLs account for ~85% of all anthelmintic treatments prescribed for cattle, with ivermectin (IVM) representing 72% of all prescribed MLs (Peña-Espinoza et al., 2016). Anthelmintic resistance in cattle GIN, however, is being increasingly diagnosed, reflecting a steadily growing issue spanning several decades (Kaplan and Vidyashankar, 2012; Hannah Rose Vineer et al., 2020; Sutherland and Leathwick, 2011), highlighted by the emergence of simultaneous resistance to all three classes of anthelmintics available for the treatment of cattle GIN (Sauermann et al., 2024).

Despite IVM's widespread use in both human and veterinary medicine, our understanding of the mechanisms by which resistance evolves and the mode of action of the macrocyclic lactone (ML) class of anthelmintics is lacking. Numerous candidate genes have been proposed over the decades as putative drivers of IVM resistance in *Haemonchus contortus* (summarised in Doyle et al., 2019). Still, strong evidence for the involvement of many of these genes remains elusive. This lack of knowledge about the fundamental mechanisms of resistance and their mode of action inhibits our ability to monitor resistance development in the field and limits our capacity to administer anthelmintic treatments sustainably and effectively.

Genome-wide association studies (GWAS) of genetic variation provide a comprehensive and unbiased framework towards identifying the genomic region(s) associated with quantitative, phenotypic traits of interest, i.e., quantitative trait loci (QTL). Such approaches have been instrumental in confirming known and identifying novel drug

resistance-conferring loci in helminths (Beesley et al., 2023; Doyle et al., 2022, 2019, 2017; McIntyre et al., 2025).

This study is motivated by the success of QTL mapping and GWAS in identifying signatures of selection around candidate genes for benzimidazole, levamisole, and IVM resistance in multiple nematode species. Additional motivation comes from the recent assembly of a reference-quality *Ostertagia ostertagi* genome through the Darwin Tree of Life initiative (The Darwin Tree of Life Project Consortium, 2022) and the continuing need for validation in other nematode species. We describe an approach to detect and characterise ML-mediated selection in field populations of *Os. ostertagi*. Performing pooled whole-genome sequencing of L_3 from the same populations pre- and post- ivermectin or moxidectin treatment, we measure the changes in nucleotide diversity and genetic differentiation throughout the genome in response to treatment.

4.2. Methods

4.2.1. Sample collection and FECRT resistance phenotype

The GIN populations used in this study were from the pre- post-treatment FECRT populations described in Chapter 3. Paired pre- and post-IVM treatment samples from two farms (Farm 2 and Farm 3) and pre- and post-MOX treatment samples from one farm (Farm 2) were selected for whole-genome sequencing. Sample choice was based on a previous history of frequent ML use, reduced faecal egg count reduction post-treatment, and a high enough proportion of *Os. ostertagi* to provide a representative sample pre- and post-treatment, yielding sufficient gDNA. The species composition of the samples, predicted by ITS2 PCR, ranged from 37.6 to 70.2% *Os. ostertagi* and 25.8 to 62.4% *Cooperia oncophora* (Table 4.1). The interpolated *Os. ostertagi* faecal egg count reductions for the samples were: Farm 2 post-IVM (mean reduction of 86.7%), Farm 3 post-IVM (mean reduction of 82.5%), Farm 3 post-MOX (mean reduction of 91.7%)

Table 4.1 Species composition of pre- and post-treatment samples for whole-genome sequencing

Farm	Treatment	Population	*Ostertagia ostertagi* (%)	*Cooperia oncophora* (%)	Other species (%)
Farm 2	IVM	Pre-	70.2	25.8	4
		Post-	57.9	42.1	0
Farm 3	IVM	Pre-	60.3	38.7	1
		Post-	51.1	48.9	0
	MOX	Pre-	58.7	40.5	0.8
		Post-	37.6	62.4	0

IVM; ivermectin, MOX; moxidectin

4.2.2. Sample preparation and whole-genome sequencing

Genomic DNA from pools of 1,000 unidentified L_3 was isolated using the Monarch® Spin gDNA Extraction Kit (T3010) following the manufacturer's instructions, with a final elution in 30 µl buffer (10 mm Tris-HCl, pH = 9.0, 0.1 mm EDTA). Genomic DNA was stored at 4 °C and shipped to Azenta GENEWIZ (Oxford, UK) on dry ice for library preparation and sequencing. Illumina sequencing libraries were prepared using the NEBNext® Ultra™ II FS DNA Library Prep Kit (E7805) and sequenced on the Illumina NovaSeq 6000 platform using 150 bp PE chemistry. We aimed to generate sufficient sequencing to achieve a minimum 100-fold coverage of the ~407 Mbp *Os. ostertagi* genome for each pooled sample.

4.2.3. Sequencing data analysis

Raw sequence data were first inspected for quality using *FASTQC* v0.12.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and visualised using *MultiQC* v1.17 (Ewels et al., 2016). The sequencing reads for all six populations were mapped to the reference quality genome nxOstOste4.1 (BioProject PRJEB78849; https://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/964/213/955/GCA_964213955.1_nxOstOste4.1/GCA_964213955.1_nxOstOste4.1_genomic.fna.gz) using the following workflow. Briefly, indexed FASTQ files of trimmed reads were aligned to the reference genome using *BWA* v0.7.17 *bwa mem* (-Y -M -C) (Li and Durbin, 2009). Mapped reads were merged using *SAMtools* v1.21 *merge* (-c -p) (Danecek et al., 2021), and sorted, duplicate reads were marked using Picard v2.5.0-2 *MarkDuplicates* (http://broadinstitute.github.io/picard), and mapping statistics were generated using *samtools flagstats* and *stats* and summarised using *MultiQC*.

4.2.4. Comparison of coverage between samples.

To compare the coverage between pre- and post-treatment samples, the depth per site was calculated using *samtools depth* (-q30 -Q30 -a -g SECONDARY,QCFAIL -G DUP -s), which requires both the base and mapping quality to be at least 30 and all sites to be included in the output, even if they have zero coverage. Coverage was then normalised by the respective chromosomal mean coverage, and the $\log_2$(ratio) of post-treatment:pre-treatment coverage was calculated for each position and averaged for each 10 kbp window along each chromosome using *awk.*

4.2.5. Determining *Ostertagia ostertagi* sample proportion

Samples were previously selected based on the proportion of *Os. ostertagi* predicted by individual worm PCR of 94 L_3. To better determine the true proportion of *Os. ostertagi* in the samples sequenced, the raw reads from each sample were searched with a custom blast database (*-evalue 1e-10*) (Altschul et al., 1990) generated from GIN ITS2 rDNA sequences retrieved from (https://www.nemabiome.ca/its2-database.html) (Workentine et al., 2020).

4.2.6. Within and between samples analysis of genetic differentiation.

To calculate between-sample genetic differentiation, we used *Grenedalf* v0.6.3 (Czech et al., 2024) using mapped bam files as input. To calculate an unbiased-Nei measure of F_{ST}, sliding windows of set distance (50 kb) or a set number of SNPs (5,000) were used, keeping

the remaining run parameters consistent (--method unbiased-nei --sam-min-map-qual 30 --sam-min-base-qual 30 --pool-sizes --filter-sample-min-count 2 --filter-sample-min-coverage 20 --filter-sample-max-coverage 300 --window-type interval --window-sliding-width 5000/50000 --window-average-policy valid-loci \ --write-pi-tables). To account for the varying *Os. ostertagi* estimated pool sizes of each sample; -pool-size was adjusted to equal the number of haploid genomes per sample (e.g., 100 diploid individuals = pool size 200). Empirical thresholds of greater than 3 and five standard deviations above the mean F_{ST} were applied to each dataset. To capture a broader set of candidate loci and loci under moderate selection, a threshold of three standard deviations above the mean is used, which would account for 0.13% of observations if loci are normally distributed. A more stringent threshold of five standard deviations above the mean is used to highlight the strongest outliers, representing approximately 1 in 1.7 million loci if the distribution is normally distributed. The rolling average F_{ST} values were calculated using a sliding window of 5x the smaller window size and with a sliding window width equal to 1x the smaller window.

4.2.7. Within-sample diversity analysis

Within-sample genetic diversity, theta π and Tajima's D were calculated using *Grenedalf* v0.6.3 using mapped BAM files as input. To calculate diversity, sliding windows of a set distance of 50kb were used with the following parameters (--method diversity --sam-min-map-qual 30 --sam-min-base-qual 30 --pool-sizes --filter-sample-min-count 2 --filter-sample-min-coverage 20 --filter-sample-max-coverage 300 --window-type interval --window-sliding-width 50000 --window-average-policy valid-loci). All graphs were generated using the R package *ggplot2()* and the LOESS function applied to generate a curve through the datapoints.

4.3. Results

4.3.1. Whole genome sequencing of pre- and post-treatment populations

Macrocyclic lactone resistance was confirmed on both farms by performing a FECRT as described in Chapter 3, Section 3.2.5. Pooled larvae collected pre- and post-treatment were sequenced, of which 43.2% to 63.8% of reads per sample mapped to the *Os. ostertagi* genome. After processing with deduplication, an average genome-wide coverage of 148x for each population (excluding mtDNA) was achieved.

4.3.2. Genome-wide genetic diversity within pre- and post-treatment populations

To understand the effect of ML treatment on genetic diversity within each group, nucleotide diversity (π) (Table 4.2) and Tajima's D were calculated for each pool in 50 kbp windows throughout the genome (Supplementary File 1). No significant difference was observed between the π value distributions for all autosomes between pre- and post-treatment pools for any of the three treatments. However, the average π for each X chromosome was approximately half that observed for its respective autosomes (for both pre- and post-pools), consistent with observations from other similar studies (Doyle et al., 2020). The mean Tajima's D values for the autosomes of all pre- and post-treatment samples were negative (range: -1.634 to -2.123), and no significant differences were observed between the pre- and post-treatment pools (p-value > 0.05; two-sample Kolmogorov-Smirnov test). However, the mean Tajima's D values were significantly higher on Farm 2 than Farm 3 (p-value < 0.05; K-sample Anderson–Darling test). No large-scale differences were observed between any pre- and post-treatment group pools for any autosome.

Table 4.2 Mean genetic diversity (π) and Tajima's D estimates across all autosomes and the X chromosome per 50kbp window

Group	Treatment pool	Mean autosomal diversity (π)	Mean X chromosome diversity	Mean autosomal Tajima's D	Mean X chromosome Tajima's D
Farm 2 IVM	Pre-	0.03572	0.02369	-1.634	-2.083
	Post-	0.03391	0.01941	-1.519	-1.982
Farm 3 IVM	Pre-	0.03486	0.02292	-2.053	-2.447
	Post-	0.03365	0.01868	-1.984	-2.444
Farm 3 MOX	Pre-	0.03664	0.02177	-1.987	
	Post-	0.03593	0.02023	-2.123	

4.3.3. Window-based measure of genetic differentiation

To identify treatment-induced changes in the distribution of genetic variation throughout the genome, pairwise genetic differentiation (F_{ST}) was estimated between the two treatment pools in 50 kbp windows across the genome for each treatment. Genetic differentiation between populations of larvae sampled before and after ML treatment reflect a change in the adult population directly exposed to drug selection, with genotypes of sensitive adults removed (either by death or by extended suppression of egg output). Pairwise F_{ST} analysis revealed a low degree of genetic differentiation between each treatment pool (mean autosome-wide F_{ST} ranged from 0.0492 to 0.0443; Figure 4.1., Table 4.3). Comparison of genetic differentiation between pre- and post-treatment samples in all treatment groups revealed a single, small, overlapping peak of differentiation on chromosome 5 (peak coordinates: ~51-53.5 Mbp), suggesting a common genetic response to ML treatment. Very few outlier windows were above 5 SD in any treatment group, but of those that were above this cut off for significance, almost half were on chromosome 5 (Farm2:IVM 13/27; Farm3:IVM 9/23; Farm3:MOX 11/24). Over the same overlapping chromosome 5:51-53.3 Mbp peak, both Farm2:IVM and Farm3:IVM treatment groups had three and four consecutive 50 kbp windows above the mean F_{ST} + 5SD, respectively.

Table 4.3 Mean autosome genetic differentiation and autosome-wide level of significance using 50 kbp windows along the genome

Group	Mean autosome-wide F_{ST}	Mean F_{ST} +3 SD	No. of outlier windows above 3SD	Mean F_{ST} +5 SD	No. of outlier windows above 5SD
Farm2: IVM	0.0492	0.0955	78	0.1263	27
Farm3: IVM	0.0443	0.0860	104	0.1138	23
Farm3: MOX	0.0443	0.0873	100	0.1161	24

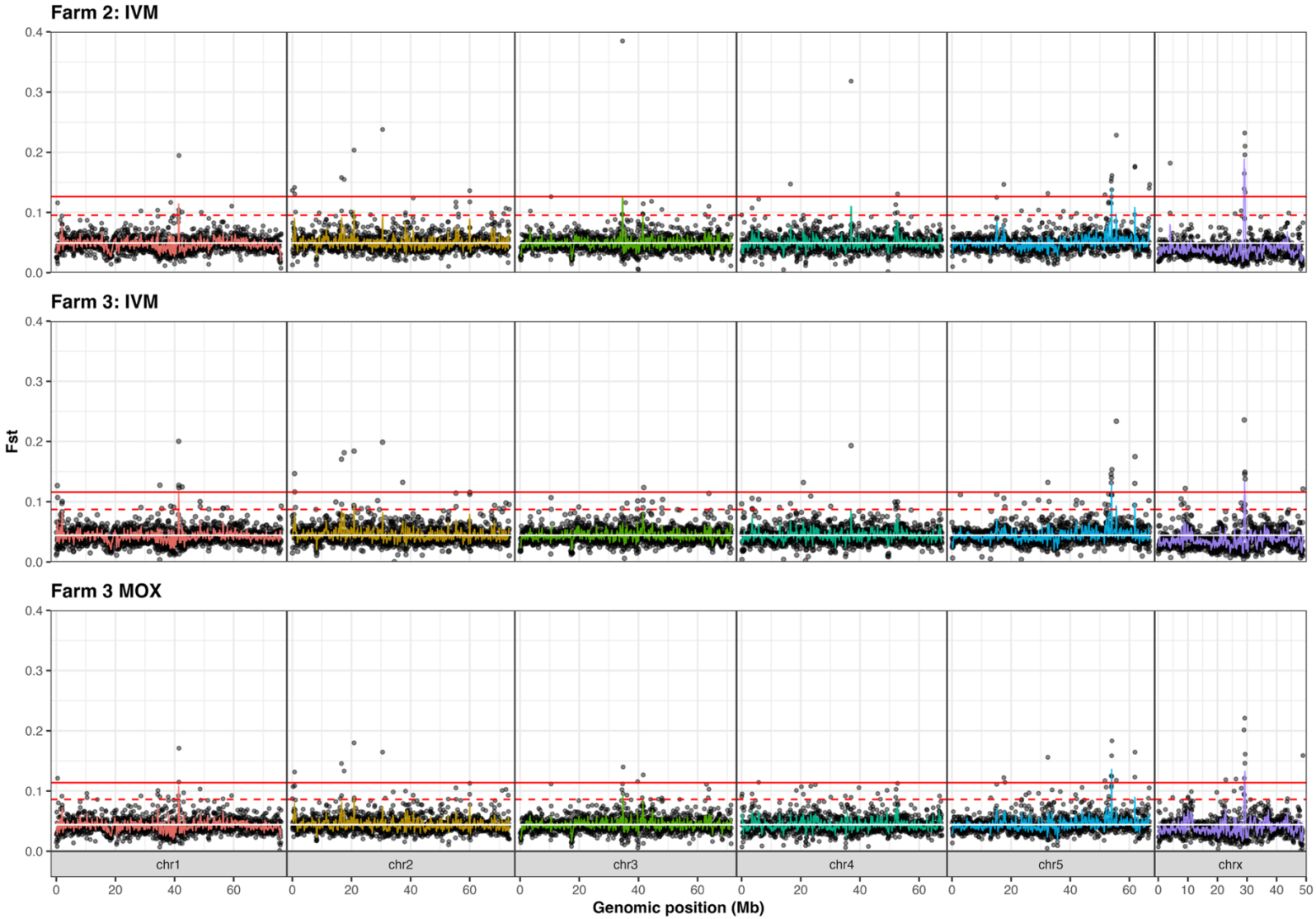

Figure 4.1 | Genome-wide genetic differentiation associated with macrocyclic lactone treatment.

In all plots, each point represents the genetic differentiation (F_{ST}) calculated between each pre- and post-treatment sample in 50 kbp windows along the genome, with each coloured line representing the rolling average. The solid white line represents the mean autosome F_{ST} estimate. The dashed red line represents a genome-wide level of significance, defined as the mean +3 standard deviations of the autosome-wide F_{ST}. The solid red line represents a level of significance defined as the mean +5 standard deviations. IVM = ivermectin, MOX = moxidectin.

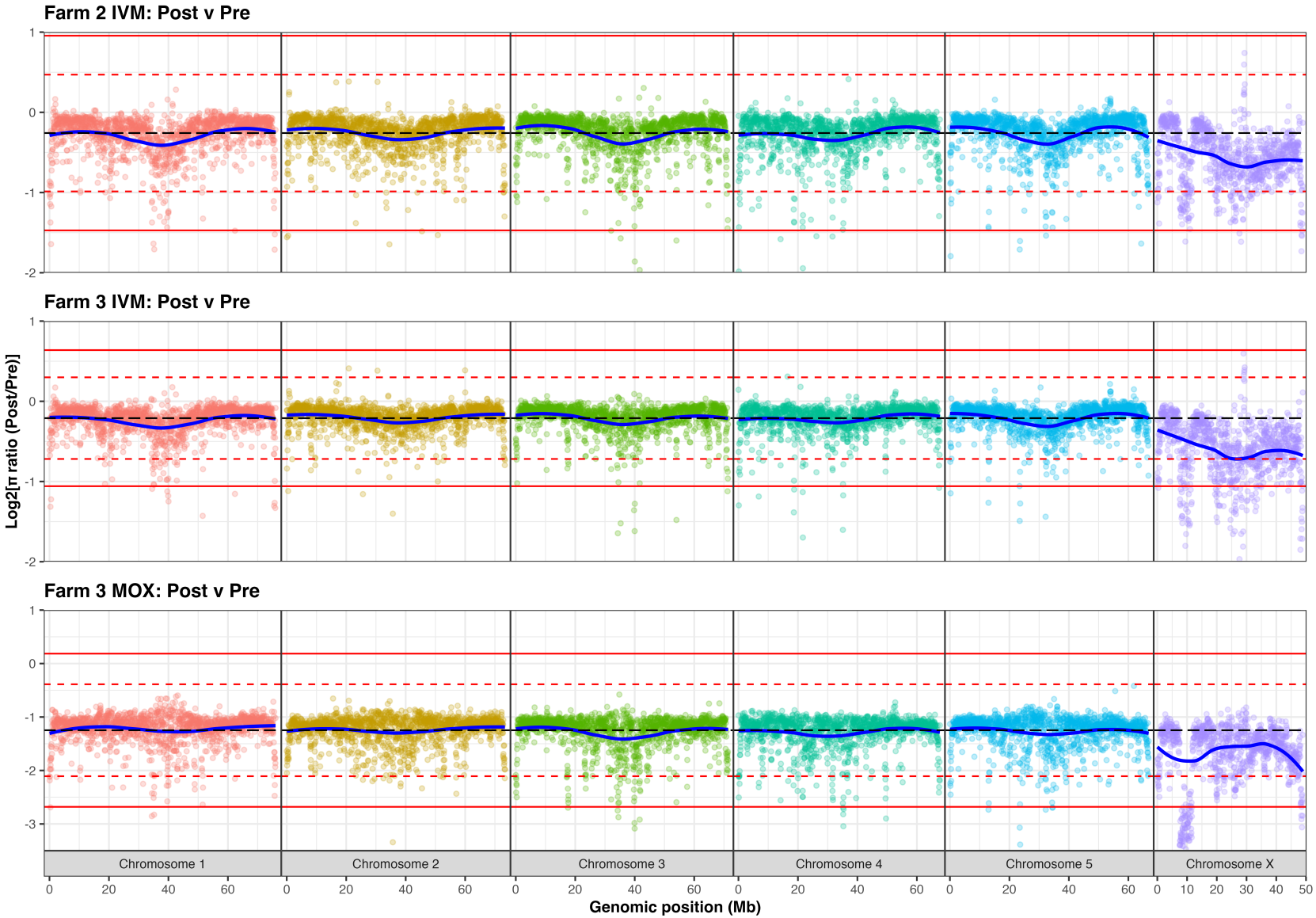

Figure 4.2 | The fold change of nucleotide diversity (π) post-/pre-treatment across the genome.

The change in genome-wide nucleotide diversity is estimated per 50 kbp window and log2-transformed across the genome, where zero indicates neutrality. Dashed black line = autosomal average. The level of significance is indicated by the dashed red line (mean ± 3SD) and the solid red line (mean ± 5 SD). The blue lines represent the LOESS function drawn through the dataset.

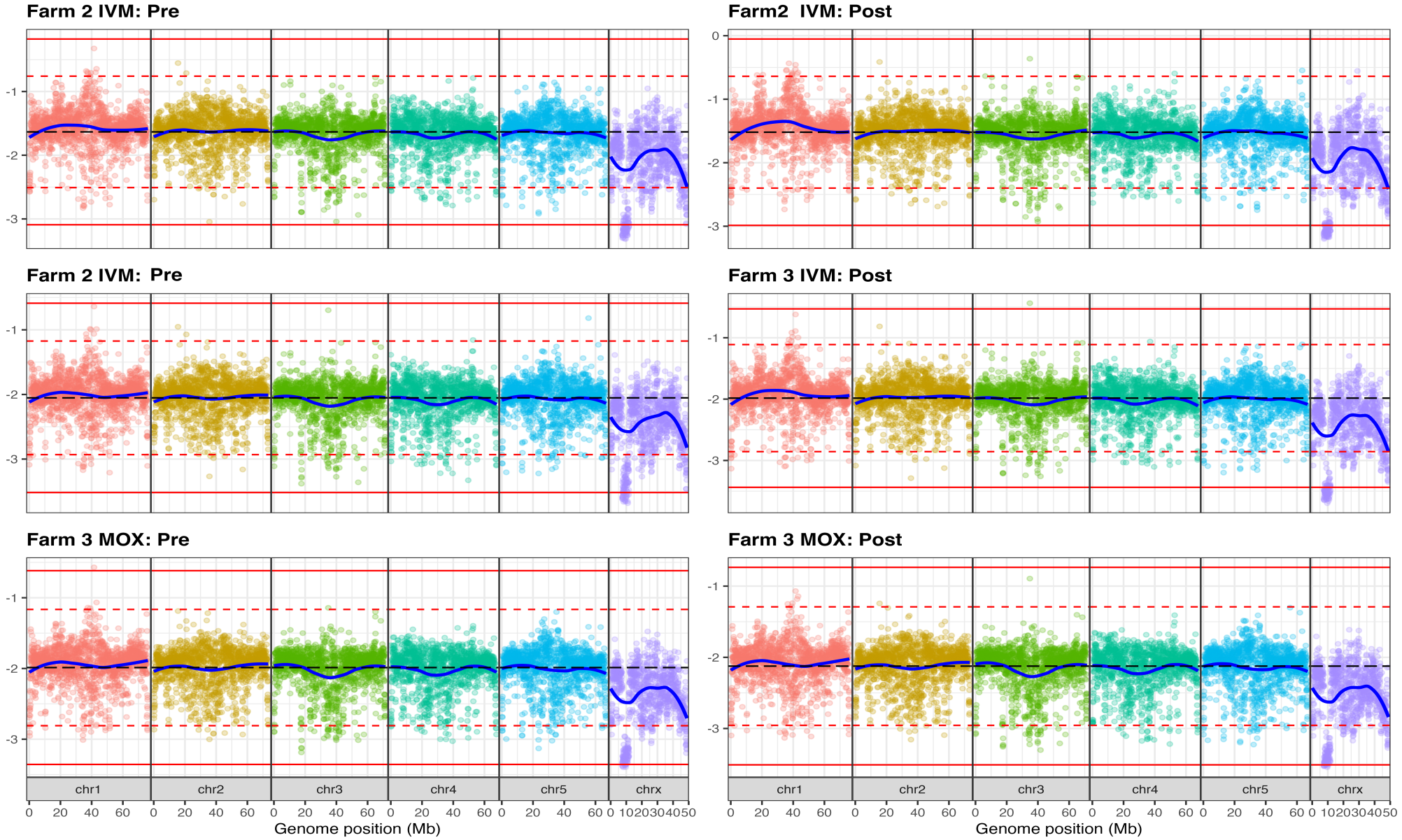

Figure 4.3 | The Tajima's D estimates pre- post-treatment across the genome

Within treatment group, Tajima's D estimates per 50kbp window across the genome. Dashed black line = autosomal average. The level of significance is indicated by the dashed red line (mean ± 3SD) and the solid red line (mean ± 5 SD). The blue lines represent the LOESS function drawn through the dataset.

4.3.4. SNP-based measure of genetic differentiation

In order to avoid bias from heterogeneous SNP density and normalise the number of informative sites per window, pairwise genetic differentiation was also estimated for 5,000 SNP windows. Pairwise F_{ST} analysis revealed a low level of differentiation between each treatment pool, with a mean autosome-wide FST ranging from 0.0435 to 0.0485 (Figure 4.4, Table 4.4). Comparing the genetic differentiation using this method for all treatment groups revealed an overlapping peak on chromosome 5, which also overlaps with the locus revealed by the window-based estimate. An overlapping, distinct peak on chromosome 2, spanning 10 – 20 Mbp, can also be observed for each treatment group. A peak on chromosome 1 at approximately 40.2 Mbp, present in all window estimates, was not observed when assessed by SNP density.

Significantly more outliers were observed by the SNP-based measure above 3SD (range: 201 to 269) than for the 50 kbp window method. A total of 5 consecutive windows were observed above the 5SD from Farm3:MOX over the same Chromosome 5:51-53.5 Mbp locus previously identified by the 50 kbp window measurement. All groups also showed between 3 and 5 consecutive 5,000 SNP windows above 3 SD on chromosome 4, over a subtle peak at around 50.5 to 52.5 Mbp.

Table 4.4 Mean autosome genetic differentiation and autosome-wide level of significance estimated per 5k SNP window.

Group	Mean autosome-wide F_{ST}	Mean F_{ST} +3 SD	No. of outlier windows above 3SD	Mean F_{ST} +5 SD	No. of outlier windows above 5SD
Farm2: IVM	0.0485	0.0989	269	0.1326	50
Farm3: IVM	0.0435	0.0893	202	0.1199	31
Farm3: MOX	0.0438	0.0894	201	0.1197	42

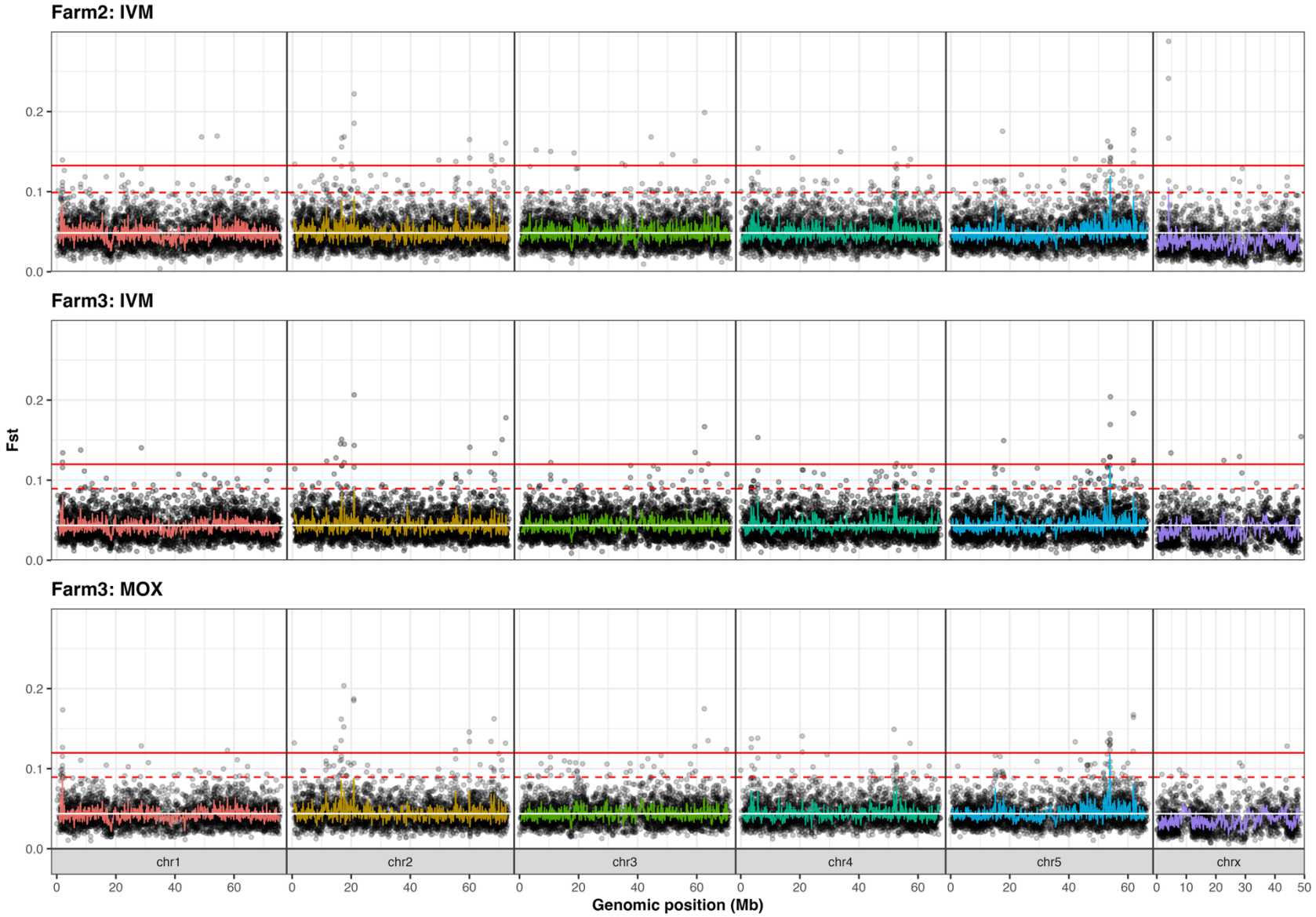

Figure 4.4 | Genome-wide variation associated with macrocyclic lactone treatment 5k SNP window.

In all plots, each point represents the genetic differentiation (F_{ST}) calculated between each pre- and post-treatment sample in 5k SNP windows along the genome. The solid white line represents the mean autosome F_{ST} estimate. The coloured variable line represents the rolling mean of 25k SNPs, moving in 5k SNP intervals. The dashed red line represents a genome-wide level of significance, defined as the mean +3 standard deviations of the autosome-wide F_{ST}. The solid red line represents a level of significance defined as the mean +5 standard deviations.

4.4. Discussion

To understand macrocyclic lactone resistance in *Os. ostertagi*, it is necessary to define the relevant regions in the genome and the underlying genetic changes within them that are responsible for the development of the resistant phenotype. Here, we assessed the impact of anthelmintic treatment on *Os. ostertagi* from Scottish farm populations by sampling infective-stage larvae (L_3) recovered from the same animals before and after treatment with either IVM or MOX, and characterised changes in genome-wide genetic diversity through whole-genome sequencing. We hypothesised that a significant change in the adult GIN population after ML treatment would likely correspond to a substantial change in the allele frequency in the larval populations, with these changes most pronounced in regions of the genome associated with resistance.

The key finding from this analysis is evidence of selection within the 51–53.5 Mbp interval on chromosome 5. No discrete or treatment-specific peaks attributable separately to IVM or MOX exposure were resolved; however, pairwise genetic differentiation also revealed a broad region of elevated differentiation on chromosome 2 spanning 20–30 Mbp. The populations selected for genome-wide analysis exhibited reduced macrocyclic lactone (ML) efficacy as diagnosed by FECRT and contained substantial proportions of *Ostertagia ostertagi* both before and after treatment. Both farms showed detectable IVM resistance, and Farm 3 additionally displayed MOX resistance, although differences in IVM efficacy were apparent between sites.

Genome-wide patterns indicated low genetic differentiation between pre- and post-treatment groups, coupled with high nucleotide diversity, suggesting that most genetic variation is partitioned within samples rather than between treatment conditions. Tajima's D values were consistently negative across all populations, reflecting an excess of low-frequency alleles compatible with population expansion following recent and repeated bottlenecks. Such demographic dynamics are expected in field populations exposed to long-term, annual ML treatments, as was the case on both farms for more than seven years. Comparisons of Tajima's D among treatment groups did not reveal strong treatment-related shifts, and rare alleles remained abundant.

Technical factors may further contribute to this skewed site-frequency spectrum. Although overall coverage was high (~160×), this depth may remain

insufficient to capture the full diversity of large, genetically heterogeneous nematode populations. An underrepresentation of intermediate-frequency variants could accentuate the apparent excess of rare alleles. Examination of relative changes in Tajima's D showed consistent increases near chromosome centres, regions expected to have reduced recombination, suggesting that alleles may persist at higher frequencies. Conversely, the chromosome arms, characterised by higher recombination rates, may harbour diversity that is not fully captured at the available sequencing depth.

Estimates of pairwise differentiation F_{ST} revealed little deviation between treatment pools, with only a small number of windows exceeding stringent thresholds. The maintenance of genome-wide genetic diversity in post-treatment populations implies that resistance alleles segregate on multiple genetic backgrounds, consistent with standing genetic variation and the action of soft selective sweeps. In such scenarios, pre-existing alleles increase in frequency under drug pressure and subsequently recombine through admixture between resistant and susceptible genotypes.

In addition to amplifying the pool of standing variation, the introduction of resistance alleles through animal movement can substantially influence the evolutionary and epidemiological dynamics of ML resistance. Gene flow from other farms can increase the diversity of resistance-associated haplotypes, enabling multiple soft selective sweeps and accelerating the rise of resistance allele frequencies beyond what local selection alone would produce. Recombination between introduced and locally circulating genotypes may generate fitter resistant backgrounds or multilocus resistance profiles, promoting the durability and persistence of resistance even in the absence of ongoing drug pressure. Such introductions can erode spatial genetic structure, complicate efforts to identify the origins of resistance, and undermine the effectiveness of farm-specific management strategies, including refugia-based approaches. Furthermore, the arrival of resistance alleles at intermediate frequencies can obscure genomic signatures of selection, reducing the clarity with which selected loci can be resolved. These processes highlight that resistance evolution in field populations is shaped by both local selection and continuous gene flow, with animal movement acting as a major driver of resistance propagation and genomic complexity.Such a process contrasts with the signature of a hard selective sweep, expected from a single de novo mutation that rapidly increases in frequency across the

population. Nonetheless, the two small overlapping peaks (Farm 2: IVM and Farm 3: IVM) identified by pairwise genetic differentiation on chromosome 5 align with the simultaneous increase in π and decrease in Tajima's D after treatment. This pattern indicates that genetic diversity may be re-accumulating at the locus through new mutations, which are currently present only at low frequencies.

While significant progress and success have been made in mapping drug-resistance-associated variation using genetic crosses (Beesley et al., 2023; Doyle et al., 2019; McIntyre et al., 2025; Niciura et al., 2019), the validation of these loci in field populations remains critically important for the development of diagnostic molecular resistance markers (Kotze et al., 2020). However, analysing genetic variation in the field, even on a genome-wide scale, presents substantial challenges, as demonstrated here. Field populations are characterised by large effective population sizes, high standing genetic variation, and complex underlying population structures. Field isolates invariably harbour extensive genetic variation relating to traits other than resistance (Gilleard and Beech, 2007), which is why laboratory backcrossing experiments are often required to control for these confounding factors. However, the benefits of using field populations include: high levels of recombination, which are expected to result in narrower regions detected as under selection, aiding in the detection of variants driving resistance and in the identification of loci/variants relevant in natural populations selected under 'real-life' conditions.

Collectively, our data suggest that, despite a significant reduction in faecal egg counts post-treatment—consistent with resistance—the genetic diversity of these *Os. ostertagi* field populations has been maintained across the genome. Importantly, evidence of a moderate level of genetic differentiation at chromosome 5:51-53.5Mbp likely represents a novel QTL associated with ML resistance. While we have not yet identified causal variants or genes, the detection of this QTL in a major cattle parasite is the first major step in elucidating the genetic basis of ML resistance and will be used to advance our understanding of resistance in the field.

Data Availability

Sequence data generated by this project were submitted to the SRA section of GenBank and are available under the SRA accession numbers within BioProject PRJNA. Additional information, data, and analysis code are available from GitHub.

Ethical Approval

The University of Glasgow MVLS College Ethics Committee (Project No: 200210097) approved all research procedures involving animal use.

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Credit Authorship Contribution Statement

Paul Campbell: Conceptualisation, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Jennifer McIntyre:** Conceptualisation, Formal analysis, Supervision, Writing – review & editing. **Andrew Forbes:** Conceptualisation, Formal analysis, Supervision, Writing – review & editing. **Kathryn Ellis:** Conceptualisation, Formal analysis, Supervision, Writing – review & editing. **Roz Laing:** Conceptualisation, Formal analysis, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Chapter 5

5. Inefficacy of ivermectin and moxidectin treatments against *Dictyocaulus viviparus* in dairy calves

Author Declaration & Contribution

Full paper citation, incl. authors and their affiliation (or link/ref to preprint server for papers not published)	Campbell P, Forbes A, McIntyre J, Bartoschek T, Devine K, O'Neill K, Laing R, Ellis K. Inefficacy of ivermectin and moxidectin treatments against *Dictyocaulus viviparus* in dairy calves. Vet Rec. 2024;e4265. https://doi.org/10.1002/vetr.4265

Please state your author contribution below for each category in accordance with the principles of the Contributor Roles Taxonomy system (CRediT). Please refer to the descriptors of each category before completion (https://credit.niso.org/)

Conceptualisation	PC, AF, JM, RL, KE
Data Curation	PC
Formal Analysis	PC
Investigation	PC, TB, KD, KO
Methodology	PC, AF, JM, RL, KE
Project Administration	PC, RL
Visualisation	PC
Writing – original draft	PC
Writing – review & editing	PC, AF, JM, RL, KE

We declare that the author contribution statements, in accordance with the CRediT system, are an accurate representation for this chapter/paper:

Author signature	
Primary supervisor signature (or corresponding author if different)	

Abstract

Background: The bovine lungworm *Dictyocaulus viviparus* negatively impacts bovine health and leads to substantial economic losses. Lungworm infections can be difficult to manage due to the unpredictable and severe nature of clinical outbreaks. Despite the widespread use of macrocyclic lactones (MLs) in grazing cattle in the UK, there have been no confirmed reports of resistant lungworms to date, with only one case of anthelmintic-resistant (ML) lungworm confirmed worldwide.

Methods: Lungworm Baermann filtrations were conducted on first-season grazing dairy calves as part of a wider study investigating anthelmintic resistance in gastrointestinal nematodes in Scotland using the faecal egg count reduction test.

Results: Clinical signs and significant numbers of lungworm larvae in faeces were observed after treatment with either ivermectin or moxidectin.

Limitations: There are no established guidelines for the diagnosis of resistant lungworms in the field. Currently, resistance can only be diagnosed after a controlled efficacy test has been conducted. This limits the conclusions that can be drawn; however, they are highly suggestive of resistance.

Conclusion: This short report describes the inefficacy of ivermectin and moxidectin against *D. viviparus* and is highly suggestive of ML resistance.

5.1. Introduction

Parasitic bronchitis is caused by the bovine lungworm *Dictyocaulus viviparus*. Lungworm infections can cause mild to severe respiratory distress, inappetence and, in severe cases, death (Forbes, 2018; May et al., 2018). *Dictyocaulus viviparus* is regarded as one of the most pathogenic endoparasites of cattle in the UK and is of severe welfare and economic concern. When exposed to a high level of challenge, youngstock with limited immunity are at significant risk of clinical disease (Morgan, 2020). In the UK, lungworm outbreaks have been increasing since 2009 and are most pronounced in Scotland and northern England, with a shift in seasonality from late summer/autumn to any time of year and a wider temporal distribution of reported clinical cases, although there is still a peak incidence in September (McCarthy and van Dijk, 2020).

Despite the availability of a vaccine (Bovilis Huskvac, MSD Animal Health), there has been a decrease in its use since the advent of anthelmintic products belonging to the macrocyclic lactone (ML) class (Bain, 1999; van Dijk, 2004). Reliance on a single anthelmintic class would be expected to select for resistance, as has rapidly occurred in gastrointestinal nematode (GIN) populations (Kaplan, 2020). However, to our knowledge, only one case of confirmed resistance has been reported worldwide, (Jewell et al., 2019; Molento et al., 2006) although two reports of lack of efficacy of eprinomectin in lactating dairy cows were reported in 2018 and 2020 (APHA, 2020; Jewell et al., 2019). This short report describes the inefficacy of ivermectin (IVM) and moxidectin (MOX) treatment against *D. viviparus* and is highly suggestive of ML resistance.

5.2. Materials and methods

5.2.1. Farm information

The study was undertaken on a commercial dairy unit located in central Scotland. The region has average annual maximum/minimum temperatures of 12.7 ◦C / 5.7 ◦C and an average annual rainfall of 1370.2 mm over 181.2 days. The monthly meteorological data for 2023 varied appreciably from those of the previous 30 years. On average, June was 3.2 ◦C warmer, experienced 13.7 mm less rainfall and 87.8 more sunshine hours. On average, July was 0.4 ◦C warmer, experienced 39.6 mm more rainfall and 10.9 fewer sunshine hours (Appendix J).

The herd comprised 170 Holstein-Friesian milking cows and followers, totalling 400 individuals; no other stock was grazed on the holding. The herd had been closed for more than 10 years, with service by artificial insemination and an all-year-round calving pattern. Dairy X Beef male calves were reared and sold to slaughter at 24 to 32 months of age, with dairy heifers retained as replacements. The farm was recruited as part of a study investigating anthelmintic inefficacy in GIN using the faecal egg count reduction test (FECRT).

5.2.2. Study design

Thirty-three spring-born Holstein-Friesian heifer and Dairy X Beef first grazing season (FGS) calves were turned out onto 4.4 ha of permanent pasture on 2 May 2023 at a stocking density of 7.5 individuals/ha. The pasture had not been previously grazed that year and was only used for turnout of FGS calves. The calves are usually set-stocked from turnout in late April/early May until mid-September and then moved to new grazing until housing in mid-October. For seven years prior to the 2023 grazing season, all FGS calves were treated with MOX (Cydectin 10% LA Solution; Zoetis) at turnout and IVM (Enovex 0.5%, w/v, Pour-on Solution; Norbrook Laboratories) at housing. In previous years, calves were reported to have maintained good body condition and growth rates, there were no confirmed diagnoses of parasitic bronchitis on the farm and lungworms were not a significant concern to the farmer.

The 33 calves were not wormed at turnout, and faecal egg counts (FEC) and body conditions were monitored fortnightly from early July 2023 until the FEC reached a group average of ∼100 eggs per gram (epg). In addition, opportunistic faecal

samples were collected for lungworm detection using a modified Baermann filtration technique with a reported sensitivity of one patent female adult (Eysker, 1997). Freshly voided faeces were collected from pasture for this monitoring phase and Baermann filtrations were performed within 4 hours of collection by adding 30 g of faecal material to a 12-ply gauze, forming a pouch and suspending it in a 500 mL glass beaker of tepid water overnight. The following morning, the faecal material and most of the suspension were removed without disturbing the sediment. The sediment and washings were added to a Petri dish, and larvae were identified under a stereo microscope at 10× magnification (see Figure 5.1). High larvae counts were estimated by evenly distributing the sediment onto a scored Petri dish and counting the larvae present in one-quarter of its surface area. Multiple individuals were confirmed as *D. viviparus* by capillary sequencing of the ITS2 region using generic strongyle primers (Bisset et al., 2014).

Figure 5.1 | *Dictyocaulus viviparus* L_1 recovered by Baermann filtration of a per-rectum faecal sample collected after moxidectin treatment.

On 21 July 2023, the group mean FEC reached 94 epg (range 68−178 epg). Fourteen days later (day 0 in the FECRT), half of the group (16 individuals) were treated with MOX (Cydectin 10% LA Solution for Injection; Zoetis) by subcutaneous ear injection and the other half (17 individuals) were treated with subcutaneous IVM (IVOMEC Classic Injection for Cattle and Sheep; Boehringer Ingelheim) at the manufacturers' recommended dose rates of 1.0 and 0.2 mg/kg bodyweight, respectively. Individual animal weight was estimated by dairy calf weight band (AHDB) according to the manufacturer's instructions with the calves' weight ranging from 190 to 230 kg. All dose calculations and administration of the anthelmintics were undertaken by the researchers. Individual per-rectum samples were collected from all animals for FEC on days 0 and 15. From the post-treatment groups, nine samples per group were randomly selected and processed for Baermann filtration.

5.3. Results

Fifteen Baermann filtrations were performed on a random subset of samples collected 21 and 14 days prior to the FECRT (faeces collected from pasture), and no larvae were detected. On these visits, the calves were in good body condition and displayed no clinical signs of parasitic bronchitis or gastroenteritis. On the day of treatment, the calves were in good body condition and displayed no clinical signs, and the farmer was happy with their performance.

On day 15 post-treatment, several calves displayed clinical signs of parasitic bronchitis: intermittent coughing with increased frequency after they were moved to the holding pen. The farmer noted that some of the calves had begun to develop diarrhoea and that the group was not as 'bright'. Of the 18 per-rectum samples that were chosen for Baermann analysis, 12 were positive for lungworm: eight from the IVM treatment group and four from the MOX treatment group (Table 5.1). The larval counts of the lungworm-positive individuals varied greatly, ranging from 2 to 340.

Table 5.1 *Dictyocaulus viviparus* larvae counts of per-rectum samples collected 15 days after ivermectin or moxidectin treatment.

Ivermectin		Moxidectin	
Animal ID	**Larvae counted**	**Animal ID**	**Larvae counted**
1	40	10	2
2	340[a]	11	2
3	12	12	300[a]
4	250[a]	13	210[a]
5	16	14	0
6	33	15	0
7	2	16	0
8	51	17	0
9	0	18	0

[a] Estimated larvae count per 30 g faeces.

Following the identification of lungworm larvae in both IVM- and MOX-treated groups, the case was reported to the Veterinary Medicine Directorate and relevant pharmaceutical companies as an adverse event with a suspected lack of efficacy. All calves were treated with levamisole (Levacide Low Volume 7.5% Oral-Solution; Norbrook Laboratories) and a supportive treatment of flunixin (Finadyne 50 mg/mL Solution for Injection; MSD Animal Health). They were moved from the high-risk pasture to low-risk grazing: silage aftermath that heifers had grazed. Fourteen days after levamisole treatment, a Baermann filtration was conducted on faecal samples from 10 randomly selected individuals, and no larvae were recovered. The calves had maintained good body condition, although a minority still coughed intermittently.

5.4. Discussion

MLs are active against both larval and adult *D. viviparus*, with an efficacy of more than 99% in susceptible populations (Benz et al., 1989; Rehbein et al., 2012). The claimed period of protection from reinfection by lungworms is 28 and 120 days for IVOMEC and Cydectin 10% LA, respectively. Therefore, the larvae that were recovered on day 15 post-treatment were well within this period of activity. The expected speed of action of MLs against *D. viviparus* in order to eliminate adult worms from the lungs is estimated to be ~24 hours (Forbes, 2018), so the presence of larvae indicates the presence of patent adults. With a pre-patent period of 21 to 28 days, the females producing these larvae must have been present at some early developmental stage during the time of treatment. We therefore conclude that both treatments were

ineffective, with the findings highly suggestive of an ML-resistant population of *D. viviparus*.

Although there are established guidelines for the detection of anthelmintic resistance in GIN based on the FECRT (Geurden et al., 2022; Kaplan et al., 2023), there are currently no guidelines for diagnosing resistance in *D. viviparus*. In the present study, the lack of a pre-treatment (day 0) larvae count meant that a larvae count reduction could not be conducted; however, the feasibility of calculating a reliable percentage reduction in this species is unclear.

We hypothesised that this selection of an ML resistant *D. viviparus* population arose from repeated and prolonged exposure to MOX over multiple years. As this field was only used for FGS calves, which were always treated with a long-acting MOX product at turnout, refugia for lungworms would be severely limited. Furthermore, the long half-life of MOX provides an extended 'tail' where the drug remains in the host but at a sub-therapeutic concentration (Le Jambre et al., 1999); this period and eventual end of protection from reinfection likely coincided with increasing lungworm challenge in late autumn on this farm. In GIN, MOX resistance confers high-level cross-resistance to IVM (Kaplan et al., 2007), and while the lungworm larval counts reported here are consistent with this finding, we are limited in the conclusions that can be drawn from this small study, where IVM was also used at housing. The FECRT results for GIN infections in the same calves, collected as part of a wider study, identified both IVM and MOX resistance (88.9% efficacy [lower confidence interval (LCI) = 86.7%, higher confidence interval (HCI) = 92.2%] and 92.8% efficacy [LCI = 91.5%, HCI = 94%] respectively), again suggesting that continued reliance on MLs is not sustainable.

The previous climatic conditions may go some way to explain why a patent lungworm infection was not detected on this farm prior to the FECRT, but bovine lungworm epidemiology is relatively poorly understood. During the 2 months prior to the FECRT, climate conditions differed from those of the 30-year average; June was significantly warmer and drier, whereas July was wetter. It is hypothesised that these dry conditions would have been detrimental to the survival of any free-living nematodes on pasture and would have limited dispersal from the pat (Rose, 1956). However, the humid conditions of July would have promoted the dispersal of larvae that would have accumulated during this period while also promoting survival (Rose, 1956). Given this sequence of events, the sudden onset of disease in mid-August fits

nicely with clinical signs expected to develop 22–26 days after the expected high parasite abundance on pasture (McCarthy and van Dijk, 2020).

The prolonged period of protection offered by MLs, especially long-acting products, coupled with their ease of application, make them a popular choice for controlling lungworms and GIN in calves. However, it is imperative that producers and veterinary practitioners consider the possibility of anthelmintic resistance in lungworms and implement sustainable parasite control strategies (COWS, 2023). In particular, vaccination against lungworm infection in calves can be used successfully alongside other management strategies to reduce anthelmintic usage.

Chapter 6

6. General discussion

The central aim of this thesis was to advance understanding of the GIN communities infecting cattle on Scottish dairy farms, with a particular focus on characterising species composition and assessing anthelmintic resistance status. This was addressed using a combination of *in vivo* (FECRT) and *in vitro* methods (EHT), as well as molecular diagnostics.

Chapter 2 presented a longitudinal study of FECs and GIN species composition across 23 dairy farms, examining the influence of management practices and anthelmintic treatment. This study demonstrated that many of the established patterns of the most clinically important GIN species of cattle remain consistent when assessed using modern molecular techniques, despite the passage of fifty to sixty years and substantial changes in dairy farming practices since the original empirical studies.

Chapter 3 presented the assessment of anthelmintic resistance on 14 dairy farms, using the FECRT, EHT and mixed amplicon sequencing. This work highlighted the complexities involved in interpreting resistance tests, particularly with mixed species communities, and underscored the need for clearly defined criteria and thresholds if these methods are to be applied reliably in the field.

Chapter 4 presented the genome-wide analysis of the response to ML treatment by field populations of *Os. ostertagi* identifying a QTL on chromosome 5, providing a novel insight into the location of ML selection within the genome.

In Chapter 5, a case study of suspected ML-resistant *D. viviparus* provides further evidence of the consequences of long-term anthelmintic treatment in a low refugia environment.

In this discussion chapter, I place this body of work within the context of previous studies and address the challenges of investigating cattle parasitism and integrating parasite research and control.

6.1. The importance of parasite species and population composition.

A pervasive theme throughout this thesis is the significance of mixed-species infections, both in terms of their clinical relevance and their implications for methodology and interpretation of results. Empirical studies on the composition of

GIN in Scotland / UK largely focused on single management practices or farms (Armour et al., 1979; Eysker and Van Miltenburg, 1988; Lancaster and Hong, 1987; Michel, 1969d, 1969b, 1969c; Rose, 1970). These studies relied on morphological identification, which is inherently limited in its accuracy for differentiating nematode species, and were conducted prior to the introduction of macrocyclic lactones. Observations from the longitudinal study presented in Chapter 1 confirmed, unsurprisingly, that *Os. ostertagi* and *C. oncophora* remain the most abundant species. Their seasonal fluctuations in abundance continue to follow the patterns originally described in earlier studies, with influences from anthelmintic treatment evident. Consistently, anthelmintic treatments were shown to have selected for particular species. Generally, even in the presence of resistance in both species, BZ treatments increased the proportion of *Os. ostertagi*, while the opposite was observed after ML treatment. The identification of a wide range of less common but clinically significant genera such as *Trichostrongylus*, *Nematodirus* and *Oesophagostomum* spp. occurred more frequently than previously reported. This likely, however, reflects the greater accuracy and higher throughput of assigning species identity using molecular methods.

Failure to consider the pathogenicity of different species when interpreting FECs may lead to misdiagnoses or inappropriate treatment strategies. For instance, reliance on FECs alone cannot differentiate between species. This can result in under-treatment of clinically significant infections or over-treatment of benign ones, both of which have implications for animal health, productivity, and the development of resistance. However, this scenario is overly simplistic, as FECs are not a reliable indicator of worm burden, and parasite management decisions should be made based on a holistic approach that accounts for management history and growth rates.

In the context of anthelmintic resistance research, accurate species identification is crucial for understanding the dynamics of resistance and developing effective control strategies. Resistance does not occur uniformly across all nematode species; rather, it tends to emerge in specific taxa under particular selection pressures. For example, *C. oncophora* has shown high levels of ML resistance, whereas *Os. ostertagi* may remain susceptible in the same population.

Accurate identification enables researchers to monitor species-specific resistance trends, assess the efficacy of various anthelmintics, and evaluate the impact of different treatment protocols. Amplicon sequencing has been increasingly replacing

traditional morphological methods, offering greater sensitivity and specificity. These tools enable the detection of resistant alleles, the quantification of species composition in mixed infections, and the tracking of resistance spread across regions and management systems.

Moreover, species-level data are essential for modelling parasite population dynamics, predicting treatment outcomes, and designing integrated parasite management programs. Without this granularity, research findings may be confounded by interspecies variation, leading to inaccurate conclusions and ineffective recommendations.

6.2. How farmers manage parasitism and implications for resistance

Gastrointestinal nematode infections remain a persistent challenge, particularly among first-season grazing calves. Farms typically employ a range of strategies to manage these infections. On non-organic farms, control is primarily achieved through the use of anthelmintic treatments, supplemented to some extent by grazing rotation and pasture management. These farms predominantly rely on ML products, the rationale for which will be outlined in the following section. In contrast, organic farms focus on pasture-based strategies to limit exposure to helminth infections, using anthelmintics (BZs or LEV) only when necessary and based on evidence of worm burdens that negatively impact growth rates or pose a health and welfare concern. The management strategies employed by dairy farmers vary widely, influenced by factors such as farm size, labour availability, resistance and specific parasite concerns. Despite the availability of best practice guidelines, such as those provided by the ‘Control of Worms Sustainably’ (COWS) group, implementation remains inconsistent.

To date, faecal egg counts have been seldom employed by dairy farmers as a diagnostic or monitoring tool (TASAH, 2023), and only four of the 23 farms participating in the longitudinal study (Chapter 2) reported using FECs as a decision-making tool. An exception to this are organic dairy farms, which more readily employ FECs as a decision-making tool, as anthelmintic treatments must be based on an indicator of worm burden. One of the principal reasons for the limited use of FECs in cattle is their limited diagnostic power. diagnostic power. The predominant species,

Os. ostertagi, and *C. oncophora* typically produce relatively low FECs. Consequently, FECs often underestimate the true parasite burden and provide a poor proxy for production-limiting infections (Murrell et al., 1989; Ploeger and Eysker, 2000), thereby reducing farmer confidence in their value. In addition, the logistics of FEC testing present barriers to adoption: sample collection, submission, and analysis impose additional labour and costs, which many farmers perceive as unjustified in the absence of clear economic returns. The ready availability of broad-spectrum anthelmintics has further reinforced a "treat rather than test" culture, in which routine whole-group treatments are favoured over targeted approaches based on monitoring. This behaviour is compounded by the limited emphasis on parasite diagnostics in cattle compared to sheep, with veterinarians and advisors historically prioritising treatment over surveillance. As a result, although FECs can contribute to sustainable parasite control, their application in cattle production systems has remained sporadic, highlighting the need for more sensitive and practical diagnostic tools, combined with stronger knowledge transfer and incentives to shift farmer practices and perspectives.

Importantly, while the convenience of long-acting formulations often encourages simultaneous treatment of entire groups, such practices can conflict with sustainable parasite control principles. Even with the application of diagnostics such as FEC or targeted-selective-treatment strategies, the use of persistent formulations risks undermining the efficacy of MLs as a class. By contrast, short-acting anthelmintics, such as BZs or LEV, when strategically rotated or applied in targeted, selective treatment schemes, may better maintain refugia and apply less intense selection pressures.

Pour-on anthelmintic formulations are widely used for treating cattle due to their ease of application, which addresses the real-world challenges of managing large herds. This was evident in the high proportion of pour-on formulations across all non-organic farms surveyed. Notably, the only non-pour-on formulations used by any participating farm were for products that do not currently have a pour-on equivalent. The primary driver for the adoption of pour-on treatments is the significant reduction in labour requirements and handling stress. Injectable formulations necessitate individual animal restraint, precise dosing, and sterile technique, procedures that become prohibitively time-consuming and labour-intensive when managing large groups. In contrast, pour-on formulations enable a single operator to treat cattle quickly by applying the product along the backline from the withers to the tailhead.

This can reduce treatment time from several minutes per animal to mere seconds, making it feasible to treat entire groups efficiently. The application process requires minimal restraint, thereby reducing stress for both cattle and handlers. This is particularly beneficial when calves are grazed away from the main holding, where handling facilities are limited. Additionally, pour-on formulations eliminate many safety hazards associated with injectable anthelmintics: no risk of needle-stick injuries, no need for sharps disposal, and reduced risk of injection site reactions.

One of the primary concerns associated with pour-on formulations is the variability in drug absorption. Unlike injectable or oral treatments, pour-on products rely on transdermal absorption, which can be influenced by environmental conditions (e.g., rainfall, humidity), animal behaviour (e.g., licking or grooming), and application technique. These factors can lead to sub-therapeutic dosing, a well-established driver of resistance selection. It was for these reasons that only injectable or oral formulations were used for the FECRT in Chapter 3, and a long-acting formulation of MOX was employed, as it is the only injectable formulation available.

The availability of long-acting injectable MOX formulations has been an important development in cattle parasite management. Their principal advantage lies in their ability to provide sustained protection against clinically significant GINs. This persistent efficacy reduces the need for repeated treatments, which is especially beneficial in youngstock systems where animal handling can be infrequent and labour-intensive. Long-term suppression of worm burdens translates into improved weight gain, feed efficiency, and overall productivity. In addition, the broad-spectrum activity of MOX, including efficacy against ectoparasites, provides further practical utility.

However, these benefits are counterbalanced by considerable risks to sustainability. From a population genetics perspective, long-acting formulations exert the strongest possible selection pressure for resistance. Prolonged drug exposure can create a gradient of subtherapeutic concentrations within the host after the end of the period of protection, a scenario that disproportionately favours the survival and amplification of resistant genotypes. The near-elimination of an otherwise expanding refugia at the start of the grazing season exacerbates this problem, as long-acting drugs effectively suppress the establishment of susceptible larvae, leaving little opportunity for susceptible alleles from unexposed parasites to dilute resistance alleles. The consequence is an accelerated trajectory toward anthelmintic resistance, which has been repeatedly demonstrated in field populations of *Cooperia* spp., and more

recently, *Os. ostertagi*. The severity of which is highlighted in Chapter 5, where the prolonged and repeated exposure to long-acting MOX products has resulted in ML resistance in all clinically significant nematode parasites (*Os. ostertagi*, *C. oncophora*, and *D. viviparus*).

Given the observed lack of effective quarantine measures for parasite control on the surveyed farms, this is likely a key point at which anthelmintic-resistant GIN could be introduced. These findings align with those of Brennan and Christley (2012), who reported that effective biosecurity measures were either infrequently applied or not implemented at all.

Currently, the UK quarantine guidelines recommended by the COWS group suggest best practice involves sequential treatment with oral BZs or LEV products. This approach was considered appropriate due to the presence of ML-resistant *C. oncophora*. However, no LEV products are licensed for use in dairy cattle producing milk for human consumption, and withdrawal periods range from 60 hours to 120 days for BZs. The use of BZ and LEV as quarantine treatments is further complicated by the perceived lack of efficacy of fenbendazole and the reported inefficacy of LEV against inhibited *Os. ostertagi*. Given the requirement of two anthelmintic treatments with perceived questionable efficacy against *Os. ostertagi*, it is perhaps unsurprising that implementation of this best practice remains limited. Analysis of the questionnaire and discussions with farmers revealed a lack of adherence to COWS guidelines, which include developing parasite control plans, reducing reliance on anthelmintics, and using them only when necessary. These findings raise important questions about farmers' awareness of the guidelines and their practicality. The current COWS Technical Manual spans 57 pages, which is arguably too lengthy and likely deters farmers and their advisors from engaging with the material in search of clear, accessible advice.

Farms, however, have to take an integrative approach, managing not only multiple helminth species but also ectoparasites, an inherently complex undertaking. Bovine lungworm remains a significant concern for many farms; however, the cost of vaccination is often perceived as prohibitively expensive and difficult to incorporate into existing management practices. Anthelmintics, which are effective against a range of parasites, inevitably exert unintended selection pressures; yet, such trade-offs are often unavoidable in practical settings.

While integrative parasite management has long been an aspirational goal for farmers, its implementation has met with varying degrees of success. The effectiveness of integrated strategies depends heavily on accurate and timely diagnosis and monitoring, which offer a sustainable path forward. Tools such as the FECRT, *in vitro* assays, and molecular diagnostics facilitate evidence-based decision-making. These methods enable species-specific identification, detection of resistance alleles, and assessment of treatment efficacy. When incorporated into integrative parasite management frameworks, they support adaptive responses and early intervention, ultimately enhancing the long-term sustainability of parasite control.

6.3. Diagnosing anthelmintic resistance: implications of method choice

The diagnosis and monitoring of anthelmintic resistance in GIN are essential for developing sustainable parasite control strategies. However, no single method offers a comprehensive or definitive assessment. Each available approach, from the on-farm FECRT to modern genomic tools, measures different aspects of resistance development while being limited by its own methodological and interpretive constraints.

6.3.1. *In vivo* methods: Biological relevance, but limited sensitivity

The faecal egg count reduction test (FECRT) is widely regarded as the gold standard method for field-based detection of anthelmintic resistance in livestock, as it provides a direct measurement of drug efficacy under real-world conditions, integrating host, parasite, and pharmacokinetics factors. However, it is also a blunt instrument and a late-stage diagnostic tool, poorly suited for surveillance, as it identifies resistance phenotypes only once resistance alleles have reached a moderate frequency (>25%) (Martin et al., 1989) and are already impacting treatment efficacy. By this stage, resistance alleles are well established, and management options for slowing their spread are limited.

While the FECRT can detect the presence of all resistance phenotypes, it provides no information about the underlying mechanisms, preventing the development of targeted intervention strategies and limiting understanding of how

resistance might evolve or spread within populations. Consequently, the test cannot distinguish between different resistance mechanisms, each with different evolutionary dynamics, stability, and potential management implications:

Proper implementation of the FECRT is not a trivial task; the recently updated WAAVP guidelines (Kaplan et al., 2023) have introduced stricter requirements and statistical modelling to increase the reliability of the test. They require substantial buy-in from farmers to undertake, necessitating significant time and effort, including handling, treating, and sampling animals, as well as the costs of individual FECs, which may seem as an unnecessary expenditure of finite resources when there is little to no concern regarding anthelmintic efficacy.

As its name suggests, the FECRT relies on calculating the reduction in FECs pre- and post-treatment; however, as already discussed, FECs are inherently a poor proxy for the true worm burden, requiring assumptions about egg count distributions, which makes them a less reliable indicator of anthelmintic resistance when used in isolation. Faecal egg counts are further compromised by variable egg excretion, exemplified by the great variation in egg counts illustrated in Chapter 2, where, in some instances, individual FECs from a group of animals varied from 64 - 304 epg. While this represents a more extreme example of FEC variation, it highlights how FEC can vary between host immune status, nutritional status, and age. In older cattle with acquired immunity, worm fecundity is often suppressed despite the presence of a substantial worm burden. Moreover, faecal consistency and sample inconsistency introduce additional noise, leading to within- and between-animal variability that is unrelated to the true egg output or worm burden. As this thesis demonstrates, significant effort must be invested to undertake a FECRT reliably. The participating farmers first had to refrain from treating their FGS calves prior to the test, which involved monitoring FECs and body condition to ensure that growth rates were not affected by parasite burden. This also required waiting until FECs were sufficiently high for a statistically significant reduction, which necessitated at least three months of grazing. Since the FECRT was conducted in a research setting, the participating calves were also blocked by sex and age to limit host variability, likely requiring changes in stock management and grazing patterns.

These biological and technical limitations directly affect the use of FECs for detecting anthelmintic resistance, particularly in cattle, where low baseline egg counts reduce the statistical power of the test, making it more difficult to distinguish between

normal variation and a genuine reduction in drug efficacy. In some cases, low post-treatment egg counts fall below detection limits, particularly when methods such as the McMaster technique, which has a sensitivity of 25 - 50 epg, are used, preventing meaningful interpretation altogether. Moreover, since natural infections comprise mixed species, they introduce additional complexity, as FECs cannot distinguish between species with differing drug susceptibilities, leading to under- or overestimation of resistance depending on the dominant species present.

Nevertheless, the FECRT will likely continue to play a central role in anthelmintic resistance research and detection due to its established track record, standardised protocols, and practical applicability in diverse settings.

6.3.2. *In vitro* assays: Greater sensitivity, constrained applicability

Laboratory-based assays, such as the Egg Hatch Test (EHT) and Larval Development Test (LDT), provide a more controlled and sensitive means of detecting anthelmintic resistance phenotypes. Unlike the FECRT, which relies on indirect measurements of drug efficacy *in vivo*, these *in vitro* assays directly expose eggs or larvae to anthelmintic compounds under controlled conditions. This approach reduces host-level variability by pooling eggs from multiple hosts and allows earlier detection of resistance, with some studies demonstrating that resistance can be identified when alleles are present at frequencies as low as 5 to 10% in the population (Dolinská et al., 2023; Várady et al., 2007). Such sensitivity makes *in vitro* assays valuable for surveillance and proactive management of resistance.

However, these assays are technically demanding and constrained by practical limitations. High-quality samples with large numbers of viable eggs/larvae are required, which are often difficult to obtain and maintain. Faecal samples must be fresh, appropriately stored, and processed within narrow time frames to preserve egg viability, and the concentrations and purification procedures necessary to recover sufficient eggs are labour-intensive. For example, conducting the EHT on pre-treatment cattle samples with an average FEC of ~100 epg was just feasible, requiring ~3,000 eggs per assay. By contrast, post-treatment populations with mean FECs ≤10 epg were unsuitable for testing. Similarly, the DrenchRite® assay provides a comprehensive resistance profile against all three major classes of anthelmintics from a single pooled sample, but demands ~10,000 eggs (Gill et al., 1995). While this is

achievable in small ruminants, where pooled samples of 200 - 350 epg are significantly more obtainable, it is impractical in cattle.

Another major limitation is the lack of standardisation across laboratories. Egg age, incubation temperature, water pH, and mineral content can all introduce significant variability (von Samson-Himmelstjerna et al., 2009), hindering the establishment of widely accepted thresholds for resistance classification. This problem is compounded in GIN of cattle, where empirical data remain scarce. *Os. ostertagi*, for example, has only two isolates that have been characterised, and the extent of resistance in the reference population is poorly defined. Such gaps in knowledge restrict the diagnostic utility of these assays for routine use and limit their value in comparative studies across regions and species.

Finally, while *in vitro* assays reduce host-level variation and can detect resistance at lower allele frequencies, their relevance to *in vivo* efficacy is not always straightforward. Collectively, these constraints—large sample requirements, labour intensity, lack of standardisation, and uncertain translation of field efficacy—explain why *in vitro* assays remain useful complementary research tools but are currently poorly suited for widespread application at the farm level, particularly in cattle.

6.3.3. Molecular diagnostics: Precision, but incomplete coverage

Deep amplicon sequencing represents a significant advancement in detecting anthelmintic resistance, offering exceptional sensitivity for identifying low-frequency resistance alleles. The approach provides a better measure of resistance allele frequencies, using the relative read abundance as a proxy, with the caveat that exact allele frequencies cannot be calculated and certain sequences may be over- or under-represented within the data. It can reliably detect resistance allele frequencies as low as 0.1%, making it particularly valuable for screening for early stages of resistance (Avramenko et al., 2019). This high sensitivity at scale far exceeds traditional molecular methods, providing early warning capabilities that are crucial for implementing effective resistance management strategies before resistance becomes widespread. Deep amplicon sequencing delivers estimates of resistance allele frequencies within populations. This quantitative capability is essential for monitoring the dynamics of resistance over time, assessing the effectiveness of management interventions, and understanding the population genetics of resistance development.

The ability to track subtle changes in allele frequencies can provide invaluable insights into the early stages of resistance evolution. Deep amplicon sequencing offers an optimal balance between comprehensive genetic analysis and practical implementation. It requires higher sequencing depth but considerably less sequencing overall compared to WGS, making it significantly more cost-effective than whole-genome approaches while maintaining high analytical precision.

The scalability with multiplexing allows for high-throughput screening of multiple samples simultaneously, making it suitable for large-scale surveillance programs. Amplicon sequencing panels can be built to target resistance loci across multiple anthelmintic drug classes (Francis et al., 2024). A mixed amplicon metabarcoding and sequencing approach for the surveillance of resistance to LEV and BZs in *Haemonchus* spp. demonstrates the potential for more comprehensive resistance profiling. This multitarget capability, while remaining focused on drug targets, is more cost-effective than genome-wide methods. Incorporating species-specific or pan-nematode primers enables the simultaneous identification of species and resistance profiling in mixed GIN infections. This capability addresses one of the major limitations of phenotypic assays, such as the FECRT, which cannot distinguish between species with different resistance profiles. The fundamental limitation of any molecular approach, including deep amplicon sequencing, is the assumption that genotypes accurately predict phenotype. Resistance mechanisms may be polygenic, involving multiple genes and regulatory elements that may not be fully captured in target amplicon panels.

Additionally, host genetics and parasite genetic background can influence the phenotypic expression of resistance, potentially leading to discordance between molecular predictions and true drug efficacy. The most significant limitation of amplicon sequencing approaches compared to genome-wide approaches is their restricted coverage of the genome. While this targeted approach is cost-effective and sensitive for known resistance loci, it inherently limits the potential for discovery. Genome-wide studies can identify novel resistance genes, regulatory variants, and complex structural rearrangements that amplicon-based approaches would completely miss.

Deep amplicon sequencing is ideally positioned as a bridge between discovery-oriented genome-wide studies and practical field applications, providing the

sensitivity and throughput needed for effective resistance monitoring while remaining cost-effective and accessible for surveillance.

6.3.4. Genome-wide association studies: Discovery power, but not practical

Genome-wide association studies (GWAS) are crucial for identifying novel genetic variants that underlie anthelmintic resistance. The inherent limitation of candidate gene studies is that they rely on very specific and narrow assumptions about the likely identity of resistance-associated genes. In contrast, forward genetic approaches do not make such assumptions, enabling the systematic interrogation of genetic variation across the entire genome. Genome-wide approaches will significantly enhance the sensitivity of experiments searching for the genetic basis of anthelmintic resistance, helping to unravel the contributions of both known and novel genes to treatment failure. The hypothesis-free nature of GWAS does not require prior knowledge of specific resistance mechanisms, allowing the discovery of unexpected or novel loci associated with drug resistance. Moreover, GWAS data can be used for population genetic analyses, providing insight into allele frequency shifts, selective sweeps, and the evolutionary dynamics of resistance in natural parasite populations. Such information is critical for understanding how resistance arises and spreads within and between host populations. The results of such studies require extensive functional validation to confirm that identified genetic variants actually cause resistance rather than being linked to causal variants. This validation process often requires the same phenotypic assays that GWAS aims to replace, creating a circular dependency. These genetic forward approaches offer an excellent discovery framework but remain resource-intensive, requiring high-quality reference genomes, sufficient sequencing depth, and advanced bioinformatics. Currently, they serve as research tools rather than practical diagnostics, although their outputs are crucial for developing the next generation of molecular markers.

6.3.5. The case for the integration of methodology

For the reasons outlined, it is evident that no single method is sufficient to diagnose and monitor anthelmintic resistance across all contexts. Each methodology contributes unique insights, but their combined use offers a comprehensive framework

for resistance research. The FECRT provides field-level and biologically relevant efficacy data. *In vitro* assays offer drug and species-specific sensitivity, while deep amplicon sequencing enables species- and allele-level resolution. Genome-wide association studies uncover the broader genetic context. Together, these tools support evidence-based decision-making, inform sustainable treatment protocols, and guide the development of policy.

In conclusion, the integration of phenotypic, molecular, and genomic methodologies is not only scientifically justified but operationally necessary for advancing anthelmintic resistance research. A multidisciplinary approach will be crucial to safeguarding drug efficacy, enhancing diagnostic precision, and ensuring the long-term viability of parasite control strategies across all livestock systems.

6.4. Challenges in researching gastrointestinal nematodes of cattle compared to sheep

Research into GIN of cattle presents greater challenges than comparable work in sheep, largely due to differences in parasite biology, host–parasite dynamics, and practical constraints. Cattle GIN species, particularly *Os. ostertagi* and *C. oncophora*, tend to present as low-level infections characterised by relatively low faecal egg outputs (often <200 epg). In contrast, the predominant ovine GIN, such as *H. contortus*, are highly fecund, often producing very high FEC, especially in a research setting, which facilitates diagnosis, experimental infection, and genomic analysis. Consequently, the sensitivity of widely used resistance diagnostics such as the FECRT is inherently reduced in cattle, compared with sheep, increasing the likelihood of underestimating resistance in field populations. Similarly, *in vitro* assays such as the egg hatch or larval development test are more easily applied to sheep parasites because of higher egg yields, whereas low recovery from cattle faeces can limit assay reliability.

Experimental infections, which are relatively tractable in sheep, are logistically more challenging and costly in cattle due to their size, and husbandry requirements, limiting replication and sample size. These practical barriers, combined with the low parasite yields, restrict opportunities for controlled genomic studies in cattle compared to sheep. From a molecular perspective, the sheep GIN *H. contortus*, has become a model system to study anthelmintic resistance in GIN due to its high fecundity,

comparatively short life cycle, and the availability of good genomic resources as early as 2013. In contrast, genomic resources for cattle parasites remain relatively underdeveloped, and field populations often present as mixed-species infections, which complicates both sequencing and the interpretation of resistance signals. This has contributed to a historical research bias towards ovine GINs, resulting in a relative knowledge gap for cattle parasites, despite their considerable economic importance.

Collectively, these factors explain why ovine parasites have disproportionately driven advances in anthelmintic resistance research, while progress in cattle GINs has lagged. Translating diagnostic and genomic approaches developed in sheep parasites to cattle systems, therefore, requires methodological adaptation to overcome challenges posed by lower parasite burdens, complex management systems, and less developed genomic infrastructure.

6.5. Drivers of anthelmintic resistance in gastrointestinal nematodes of cattle

Much is known about the dynamics of selection for resistance in GIN of sheep, including which management practices are highly selective for resistance and how its progression can be delayed or mitigated (Kenyon et al., 2009; Leathwick et al., 2012). In contrast, relatively little is known about the selection for resistance in GIN of cattle, with few comparable studies or detailed information available. Differences in parasite biology, host immunity, grazing behaviour, and treatment frequency between sheep and cattle may influence selection pressure. Consequently, although recommendations for sustainable parasite control in cattle are often extrapolated from sheep-based research, these may not always be entirely appropriate. However, many aspects of selection are likely to be similar, including the indiscriminate or overuse of anthelmintics, the importance of maintaining a refugia, and underdosing.

As mentioned, anthelmintics with persistent activity extend the selection period, and when these compounds fall below therapeutic thresholds in the animal, increase the likelihood that resistant individuals will establish. The same principles of underdosing can apply to the pour-on formulations, where serious questions arise about the ability of these formulations to deliver high efficacy consistently.

The lack of refugia is particularly relevant to *C. oncophora*, as GIN populations are concentrated in youngstock, and immunity to *Cooperia* spp. is

typically acquired after a single grazing season, earlier than for *Os. ostertagi*. Consequently, infections in older animals are predominantly caused by *Os. ostertagi*, with only low levels of *C. oncophora* persisting. Because anthelmintic treatments are most frequently applied to youngstock, selection pressure disproportionately affects *C. oncophora*, as little refugia remains in older cattle to dilute resistant alleles.

6.6. Concluding remarks and future work

Assessing anthelmintic resistance is challenging, particularly in GIN of cattle. However, this thesis has successfully achieved its aims by

- Illustrating that the established patterns of the most clinically relevant GINs remain unchanged using modern molecular techniques.
- Application of *in vivo*, *in vitro*, and molecular methods to detect resistance phenotypes and genotypes, highlighting the complexities of their interpretation.
- Identifying novel insights into the genome-wide response to ML-treatment in *Os. ostertag*i, revealing a QTL on chromosome 5 associated with treatment.

The recent availability of a high-quality chromosome-scale genome assembly for *Os. ostertagi* (nxOstOste4.1) has allowed for novel insights into the genome-wide effects of ML-treatment and identified a single locus that is consistently strongly differentiated on chromosome 5, a promising finding given that similar studies in *H. contortus* and *Te. circumcincta* have identified regions on the same chromosome (Doyle et al., 2022; McIntyre et al., 2025). To fully interpret the genome-wide analysis in this study, the genome assembly must be annotated, and this resource is expected to be available in the near future.

Re-analysis of WGS data using both *Os. ostertagi* and *C. oncophora* genome assemblies will be beneficial and hopefully reveal insights into selection in the latter. The detection of this region under ML selection for both species will be the basis for further investigation into the mechanism(s) of ML resistance and for molecular marker development.

Reflecting on the current dilemma of anthelmintic resistance, it is evident that while farmers bear some responsibility, academia, government policy, and industry

should also be held accountable. There has been a lack of commercialisation of molecular approaches; consequently, surveillance of resistance has relied on traditional diagnostic tools, for which I have outlined the limitations, and the reporting of it is only passively collected. Commercialisation of amplicon sequencing represents a significant opportunity to modernise anthelmintic resistance surveillance. Its success will depend on balancing sensitivity and scalability, as well as affordability and validation. If these barriers are addressed, amplicon sequencing can transition from a research tool to a routine diagnostic platform, transforming how resistance is monitored and managed. Providing valuable information for research and a decision-making tool for livestock producers.

The lack of diagnostic markers for ML resistance currently limits the applicability of the amplicon sequencing approach and underscores the need for the research outlined in this thesis. While current amplicon sequencing makers - ITS-2, *β-tubulin isotype 1*, and *acr-8* – remain valuable, the industry will likely continue to rely on MLs in the future, and the development of such marker(s) is urgently needed.

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Appendix

Appendix A: Primer sequences for ITS2 species identification used in two-round PCR (Chapter 2)

Table S1: Primer sequences for the ITS2 region used for gastrointestinal nematode identification, as developed by Bisset et al., 2014.

Target species/genus	Primer name	Sequence (5′–3’)	Melting temperature (T_m)	Expected amplicon size (bp)
ITS2 Generic	ITS2GF	CACGAATTGCAGACGCTTAG	54°C	370-398
	ITS2GR	GCTAAATGATATGCTTAAGTTCAGC	54°C	
Cooperia oncophora	COONRV1	CTATAACGGGATTTGTCAAAACAGA	53°C	173
Haemonchus spp.	HACOFD3	CATGTATGGCGACGATGTTCTT	55°C	90
Oesophagostomum venulosum	OEVERV2	CGACTACGGTTGTCTCATTTCA	54°C	323/327/329
Ostertagia leptospicularis	OSL3FD2	CATGCAACATAACGTTAACATAATG	52°C	196
Ostertagia ostertagi	OSOSRV1	CAATGTTAACGTCATGTTGCATTTCA	55°C	207
Teladorsagia circumcincta	TECIFD3	GTACATTCAAATAGTAGCAATACGC	53°C	295
Trichostrongylus axei	TRAXFD1	CAAATATTGTGATAATTCCCATTTTAGTTT	53°C	236
Trichostrongylus colubriformis	TRCORV1	ACATCATACAGGAACATTAATGTCA	52°C	232

The first four letters of each primer name indicate the target species: OSOS = *Ostertagia ostertagi*. FD denotes forward primer
RV denotes reverse primer

Appendix B: Farm management questionnaire completed through a semi-structured interview

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Farm Management Questionnaire: University of Glasgow Dairy Worms Project – Sampling Summer 2022

Section 1: Milk Recording

1. Does your farm milk record?

   ☐ Yes
   ☐ No

2. If yes, could we have access to your CIS/NMR records?

   ☐ Yes
      ☐ Vet login
      ☐ Farmer login
   ☐ No

Section 2: Anthelmintic Treatments

Section one refers only to the group of first grazing season calves that we will be sampling from this summer.

3. If your first grazing season calves have received an anthelmintic (wormer) treatment. What have they received and when?

Product	When	Number of animals

.

4. When do you next plan to treat these calves for worms and with what products?

Product	When

.

Section 3: General Farm & Farm Management

5. How many years has your farm held organic status for?

   _____ Years

☐ Not applicable

6. What is your current herd size? Please fill in how many animals of each age range do you have.

   a. All milking cows _____
   b. Heifers 12 months to 1st calving _____
   c. Weaned calves (up to 12 months)_____
   d. Unweaned calves _____
   e. Bulls _____

7. Do you have any beef cattle or sheep on the farm?

   ☐ Beef
   ☐ Sheep
   ☐ Neither

   If yes, are the animals:
   a. Only there for winter grazing? ☐ Beef ☐ Sheep
   b. Always on farm ☐ Beef ☐ Sheep

8. Within the last 12 months, how many animals have been bought in?

   a. Cows _____
   b. Calves _____
   c. Heifers _____
   d. Bulls _____
   e. ☐ No animals bought in

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9. At what age do you aim to calve your heifers?

_____ Months

10. What is your average milk yield (L/Cow/Annum)?

_____

11. How many first grazing season calves did you turn out to pasture last year?

_____

12. What were your first grazing season calf's turnout and housing dates last year?

    a. Turned out _____

    b. Housed _____

13. When does your herd calve? Please fill in how many cows calved in each month of the past 12 months.

Month	March	Feb	Jan	Dec	Nov	Oct	Sept	Aug	July	June	May	April
Number Calved												

14. How are your first grazing season calves grazed?

    a. What pasture types is used?

        ☐ Permanent pasture
        ☐ Temp leys

    b. What pasture management system is used?

☐ Mob / Strip grazing
☐ Rotational grazing
☐ Set stocked

15. Do you turn out first grazing season calves onto the same pasture every year?

☐ Yes
☐ No

16. Do you move your first grazing calves to different pasture during the summer?

☐ Yes
☐ No

If yes, please tell us when and where?

_____
_____
_____
_____
_____
_____

17. What animals have been grazed on the first grazing season calves' pasture in the last year?

_____
_____
_____

18. Complete the table by answering the following questions and marking it on the table?

a. Date on which you turned out calves to pasture
b. Number of calves turned out on these dates

c. Dates you wormed your calves

d. Type of wormer was used on these dates

e. Number of calves treated on these dates

f. Dates the group of the first turned-out calves changed pasture

QUESTION	JAN				FEB				MAR				APR				MAY				JUN				JUL				AUG				SEP				OCT				NOV				DEC			
a																																																		
b																																																		
c																																																		
d																																																		
e																																																		
f																																																		

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Section 3: Wormer (Anthelmintic) Use

19. How do you determine how much wormer (anthelmintic product) to use?

☐ According to estimated weight of each animal
☐ According to estimated average weight of the group
☐ According to estimated weight of the heaviest animal in group
☐ Weigh individual animals and treat accordingly

20. Do you worm bought in animals on arrival?

☐ Yes
☐ No

If yes, what do you use?
_____

21. Are all bought in animals kept separate (isolated) from the herd on arrival?

☐ Yes
☐ No

22. Has there been any reports of resistance to wormers (anthelmintic products) in your herd?

☐ Yes
☐ No

a. If yes, what product was the resistance to? _____
b. What was the resistant worm? (If known) _____
c. How was it diagnosed?
   ☐ Product had no or little effect
   ☐ Diagnosed by lab
   ☐ Other _____

23. Are you currently taking any measures to reduce the amount of wormer (anthelmintic drugs) used on your farm?

☐ Yes
☐ No

If yes, please specify what measures

_____
_____
_____
_____
_____
_____
_____
_____

24. Do you or your vet conduct regular faecal egg counts on your farm?

☐ Yes
☐ No

25. When and why do you usually treat your first grazing season calves with an anthelmintic (wormer) product and what with?

_____
_____
_____
_____
_____
_____
_____
_____

26. Did you treat individual first grazing season calves due to a suspected worm problem in 2021?

☐ Yes
☐ No

a. How many animals? _____
b. What product did you use? _____
c. Why did you treat? _____

27. Did you treat all first grazing season calves due to a suspected worm problem in 2021?

☐ Yes
☐ No

a. When did you treat? _____
b. What product did you use? _____
c. Why did you treat? _____

28. What anthelmintic products have you given to your first grazing season calves up to the last 5 years?

Year	Products used
2021	1.
	2.
	3.
2020	1.
	2.
	3.
2019	1.
	2.
	3.
2018	1.
	2.
	3.
2017	1.
	2.
	3.

29. Are there any parasites you believe to be a problem for your herd?

(e.g., lungworm, lice)

_____
_____
_____
_____

Appendix C: Primer sequences for ITS2 species identification (Chapter 3)

Table S2: Primer sequences for the ITS2 region used for gastrointestinal nematode identification, as developed by Bisset et al., 2014.

Target species	**Primer name**	**Sequence (5′–3’)**	**Melting temperature (T_m)**	**Expected amplicon size (bp)**
ITS2 Generic	ITS2GF	CACGAATTGCAGACGCTTAG	54°C	370-398
	ITS2GR	GCTAAATGATATGCTTAAGTTCAGC	54°C	
Cooperia oncophora	COONRV1	CTATAACGGGATTTGTCAAAACAGA	53°C	173
Haemonchus spp.	HACOFD3	CATGTATGGCGACGATGTTCTT	55°C	90
Ostertagia ostertagi	OSOSRV1	CAATGTTAACGTCATGTTGCATTTCA	55°C	207
Trichostrongylus axei	TRAXFD1	CAAATATTGTGATAATTCCCATTTTAGTTT	53°C	236

The first four letters of each primer name indicate the target species: OSOS = *Ostertagia ostertagi*. FD denotes forward primer
RV denotes reverse primer

Appendix D: Primer sequences for *Ostertagia ostertagi* anthelmintic marker panel

Target species	Target loci	Primer name	Sequence (5′–3’)	Melting temperature (T_A)	Cycles	Extension time (Seconds)	Expected amplicon size (bp)	Source
Pan-nematode	ITS2	NC1	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACGTCTGGTTCAGGGTTGTT		40			(Avramenko et al., 2015)
		NC2	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TTAGTTTCTTTTCCTCCGCT					
Pan-nematode	Beta-tubulin isotype-1	Oos_tbb1_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACGCACTCTTTGGGAGGAGG		40			(Avramenko et al., 2019)
		Con_tbb1_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TACGCATTCTCTTGGAGGAGG					
		Oos_tbb1_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGTGAGTTTTAGTGTGCGGAAG					
		Con_tbb1_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGTGAGCTTCAATGTGCGGAA					
		Con/Hco_tbb1_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CGCATTCWCTTGGAGGAGG					
		Con/Hco_tbb1_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GTGAGYTTCAAWGTGCGGAAG					
		Tci/Tcol_tbb1_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CGCATTCYTTGGGAGGAGG					
		Tci/Tcol_tbb1_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GTGAGTTTYAAGGTGCGGAAG					
Ostertagia ostertagi	Beta-tubulin	Oos_tbb2_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	51	40	15		This thesis, Chapter 3

	isotype-2	Oos_tbb2_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	cky-1	Oos_cky1_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_cky1_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	acr-8	Oos_acr8_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_acr8_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	pgp-9	Oos_pgp9_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_pgp9_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	unc-29	Oos_unc29_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_unc29_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	unc-63	Oos_unc63_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_unc63_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	chk	Oos_chk_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_chk_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					

Ostertagia ostertagi	avr-14	Oos_avr14_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_avr14_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	avr-15	Oos_avr15_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	30		This thesis, Chapter 3
		Oos_avr15_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	Neutral loci 1	Oos_nloci_1_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_nloci_1_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	Neutral loci 2	Oos_ nloci_2_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_ nloci_2_RV	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**					
Ostertagia ostertagi	Neutral loci 3	Oos_ nloci_3_FW	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**	56	40	15		This thesis, Chapter 3
		Oos_ nloci_3_RV	**GTCTCGTGGGC.AGATGTGTATAAGAGACAG**					

The first three letters of each primer name indicate the target species: Oos = *Ostertagia ostertagi*.
FD denotes forward primer
RV denotes reverse primer

Appendix E: Individual population egg hatch test results

The results of the egg hatch test performed with thiabendazole (TBZ) in 2023 on each study farm are presented at the extrapolated species and total strongyle level. Presented are the mean maximum effective concentrations (EC), EC_{10}, EC_{50}, and EC_{95} values, with their 95% confidence intervals (CI).

Farm	Population	EC_{10} µg TBZ/ml (CI)	EC_{50} µg TBZ/ml (CI)	EC_{95} µg TBZ/ml (CI)	RR EC_{10}	RR EC_{50}	RR EC_{95}	AIC	Mean control hatch %
FECRT 1	All strongyles	0.0119337 (0.0012125)	0.0509084 (0.0024112)	0.3556748 (0.0423910)	1.23113761	2.12894778	4.43518633	144.0472	92.7
	Os. ostertagi	0.0143691 (0.0030346)	0.0602162 (0.0059686)	0.4107894 (0.0993258)	1.78542495	3.58378565	9.11692289	53.64525	92.8
	C. oncophora	0.0089455 (0.0018214)	0.0338034 (0.0032040)	0.2007497 (0.0481958)	0.7011003	1.30589479	3.00535245	52.7031	91.3
FECRT 2	All strongyles	0.0106009 (0.0013033)	0.0487082 (0.0027461)	0.3759052 (0.0552840)	1.09363958	1.80552657	4.68745495	150.9229	91.2
	Os. ostertagi	0.0100143 (0.0018932)	0.0431746 (0.0037479)	0.3059468 (0.0716763)	1.24432157	4.67221349	6.79008121	51.41523	93.9
	C. oncophora	0.0193927 (0.0046827)	0.0785044 (0.0085789)	0.5112810 (0.1385647)	1.51989579	3.03278625	7.65420625	55.15238	89.9
FECRT 3	All strongyles	0.00969323 (0.00073598)	0.02391247 (0.00070135)	0.08019388 (0.00664311)	NA*	NA*	NA*	131.1158	96.4
	Os. ostertagi	0.0080480 (0.0011563)	0.0168024 (0.0011492)	0.0450579 (0.0081578)	NA*	NA*	NA*	51.24016	97.6
	C. oncophora	0.01275923 (0.00112194)	0.02588524 (0.00077946)	0.06679739 (0.00679301)	NA*	NA*	NA*	40.5817	96.2
FECRT 4	All strongyles	0.0092948 (0.0013621)	0.0461786 (0.0028377)	0.3957303 (0.0760966)	0.95889605	1.9311514	4.93466958	154.5357	92.5
	Os. ostertagi	0.0102716 (0.0030392)	0.0476184 (0.0058641)	0.3719197 (0.1512479)	1.27629225	2.83402371	8.25426174	56.85585	93.8
	C. oncophora	0.0138007 (0.0021740)	0.0657557 (0.0047907)	0.5327762 (0.1002479)	1.08162483	2.54027778	7.97600325	48.10137	91.9
Organic 1	All strongyles	0.0390151 (0.0021380)	0.1050033 (0.0025842)	0.3957252 (0.0244986)	4.02498445	4.3911524	4.93460598	123.4612	92.7

	Os. ostertagi	0.0385068 (0.0045677)	0.1101367 (0.0057687)	0.4503321 (0.0577333)	4.78464215	6.55481955	9.99452038	46.29631	92.7
	C. oncophora	0.0459672 (0.0031150)	0.1113913 (0.0034601)	0.3647367 (0.0299381)	3.60266254	4.30327476	5.46034359	39.56164	92.7
Organic 2	All strongyles	0.0118376 (0.0010963)	0.0554326 (0.0022466)	0.4388187 (0.0503764)	1.22122347	2.31814614	5.47197242	135.5862	92
	Os. ostertagi	0.0130175 (0.0022133)	0.0878500 (0.0060138)	1.1348755 (0.2236040)	1.6174826	5.22841975	25.1870482	45.5948	91.3
	C. oncophora	0.0157589 (0.0017693)	0.0414654 (0.0018623)	0.1516112 (0.0222332)	1.23509804	1.60189359	2.26971742	44.12307	91.3
Organic 3	All strongyles	0.0292810 (0.0029930)	0.1278455 (0.0057728)	0.9214350 (0.1115102)	3.0207681	5.34639458	11.4900913	143.9322	91.4
	Os. ostertagi	0.0365974 (0.0067762)	0.1556091 (0.0122075)	1.0823918 (0.2383759)	4.54739066	9.26112341	24.0222425	51.28981	94
	C. oncophora	0.0139624 (0.0017768)	0.0595655 (0.0034937)	0.4161831 (0.0621494)	1.09429801	2.30113764	6.23052937	45.36691	93.5
Organic 4	All strongyles	0.0097278 (0.0018272)	0.0731594 (0.0056526)	1.0927365 (0.2320984)	1.00356641	3.05946646	13.6261832	163.7521	92.4
	Os. ostertagi	0.0165157 (0.0069141)	0.1064716 (0.0177297)	1.2936106 (0.6024265)	2.0521496	6.33669	28.7099621	61.46076	89.3
	C. oncophora	0.0080526 (0.0012904)	0.0249623 (0.0016390)	0.1136916 (0.0222276)	0.63111959	0.96434493	1.70203656	49.38255	91.6
Organic 5	All strongyles	0.01045391 (0.00077104)	0.03662733 (0.00116865)	0.19656662 (0.01791606)	1.07847539	1.53172508	2.45114241	128.2538	92.6
	Os. ostertagi	0.0289504 (0.0027579)	0.0926374 (0.0038906)	0.4402493 (0.0503271)	3.5972167	5.51334333	9.77074608	47.59801	92.9
	C. oncophora	0.0426220 (0.0019649)	0.0955249 (0.0019842)	0.2817013 (0.0164751)	3.34048371	3.69032313	4.21725011	35.4871	92.9
Organic 6	All strongyles	0.0289504 (0.0027579)	0.0926374 (0.0038906)	0.4402493 (0.0503271)	2.98666182	3.87402054	5.48981169	144.7402	90.8
	Os. ostertagi	0.0211555 (0.0019548)	0.0888857 (0.0035527)	0.6084787 (0.0662158)	2.62866551	5.29005975	13.5043733	39.98248	94.4
	C. oncophora	0.0426220 (0.0019649)	0.0955249 (0.0019842)	0.2817013 (0.0164751)	3.34048371	3.69032313	4.21725011	33.51425	91.3
Organic 7	All strongyles	0.0119998 (0.0011622)	0.0540808 (0.0022209)	0.4066997 (0.0454871)	1.2379568	2.26161496	5.07145558	137.4504	94.2
	Os. ostertagi	0.0191599 (0.0033876)	0.0801495 (0.0060025)	0.5454631 (0.1120522)	2.38070328	4.77012213	12.1058261	49.73166	93.3

	C. oncophora	0.0109699 (0.0017339)	0.0363695 (0.0023311)	0.1812554 (0.0359333)	0.85976191	1.4050285	2.71351021	48.02988	92.7
Organic 8	All strongyles	0.0379902 (0.0082538)	0.1518750 (0.0127264)	0.9726588 (0.2210851)	3.91925086	6.35128868	12.1288408	174.2375	91.5
	Os. ostertagi	0.0357684 (0.0074248)	0.1393750 (0.0112714)	0.8624724 (0.1924796)	4.4443837	9.0388873	19.1414247	51.37389	91.5
	C. oncophora	0.0358603 (0.0042376)	0.1016091 (0.0052023)	0.4102727 (0.0521597)	2.81053794	5.38434258	6.14204687	45.6656	91.5
Organic 9	All strongyles	0.0176462 (0.0016217)	0.0748474 (0.0030827)	0.5189282 (0.0584032)	1.82046645	4.24920972	6.47092022	139.2358	92.9
	Os. ostertagi	0.0288277 (0.0023112)	0.0900963 (0.0033897)	0.4148617 (0.0410180)	3.58197068	4.45456601	9.20730216	40.7812	94.3
	C. oncophora	0.0156165 (0.0021274)	0.0865862 (0.0051030)	0.8595706 (0.1422484)	1.22393749	3.48060516	12.8683261	44.33899	91.9
Organic 10	All strongyles	0.0151232 (0.0013673)	0.0485001 (0.0019124)	0.2311808 (0.0242190)	1.56018169	2.02823464	2.8827736	140.2134	91.7
	Os. ostertagi	0.0213525 (0.0027061)	0.0544768 (0.0028198)	0.1911193 (0.0281091)	2.65314364	3.24220349	4.24163798	46.68144	91,9
	C. oncophora	0.0107845 (0.0029378)	0.0453442 (0.0057131)	0.3107092 (0.1028604)	0.84523126	1.7517396	4.65151707	57.15371	91.7

EC, Maximum effective concentration; TBZ, Thiabendazole; CI, Confidence interval; RR, Resistance ratio; AIC, Akaike Information Criterion; FECRT, faecal egg count reduction test

* Susceptible population used to calculate relative ratio

Appendix F: Comparison of the eggCounts and bayescount faecal egg count reduction estimates

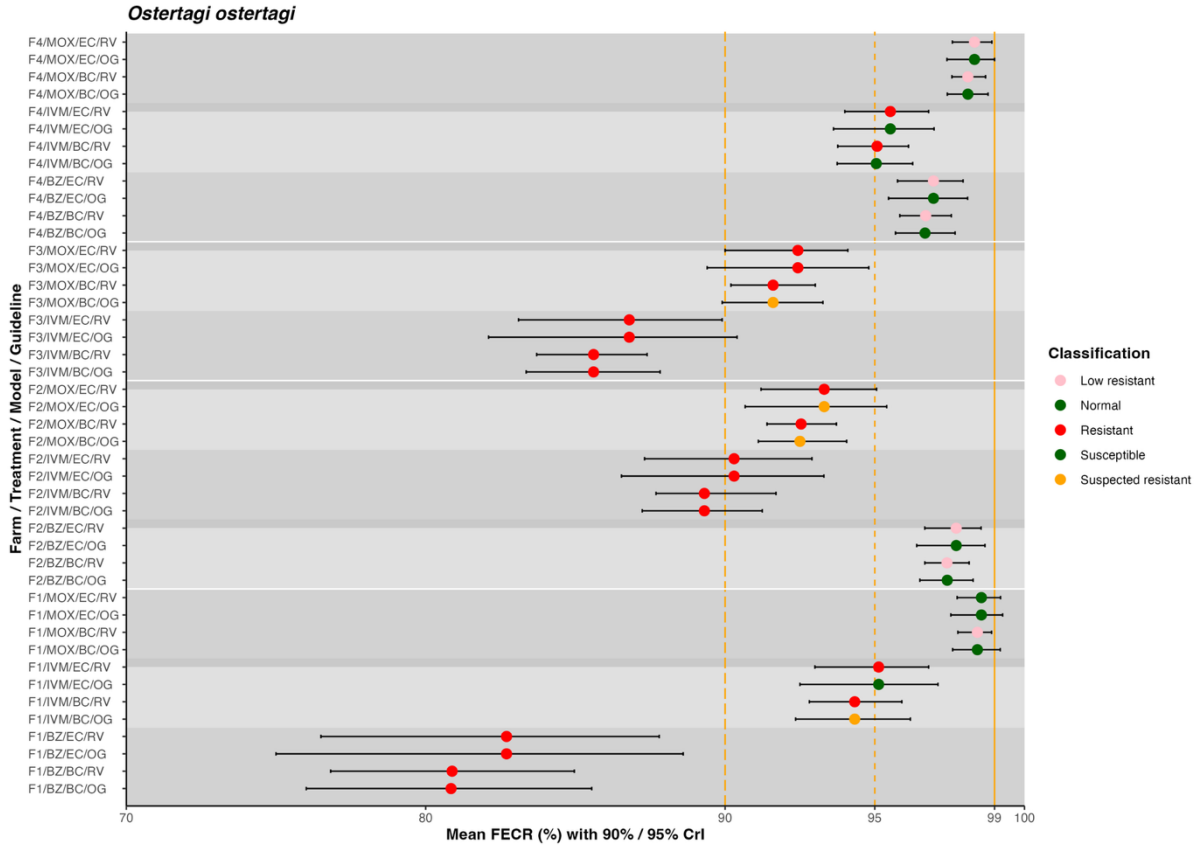


Figure S1: Comparison of the *eggCounts* and *bayescount* faecal egg count reduction estimates for the interpolated *Ostertagia ostertagi* population

Faecal egg count reductions (FECR) with the credible intervals (CrIs) for anthelmintic treatment against the interpolated *Ostertagia ostertagi* population. The CrIs were calculated using either *eggCounts* (EC) or *bayescount* (BC) models and interpreted based on either the revised guidelines (RG) for the faecal egg count reduction test (Kaplan et al., 2023) with corresponding 90% CrIs, or on the original guidelines (OG) (Coles et al., 1992) with 95% CrIs. Each point represents the mean FECR, with colour indicating the resistance status classified to the strongyle population: green, susceptible/normal; red, resistant; pink, low resistant / suspected resistant; orange, inconclusive / suspected susceptible.

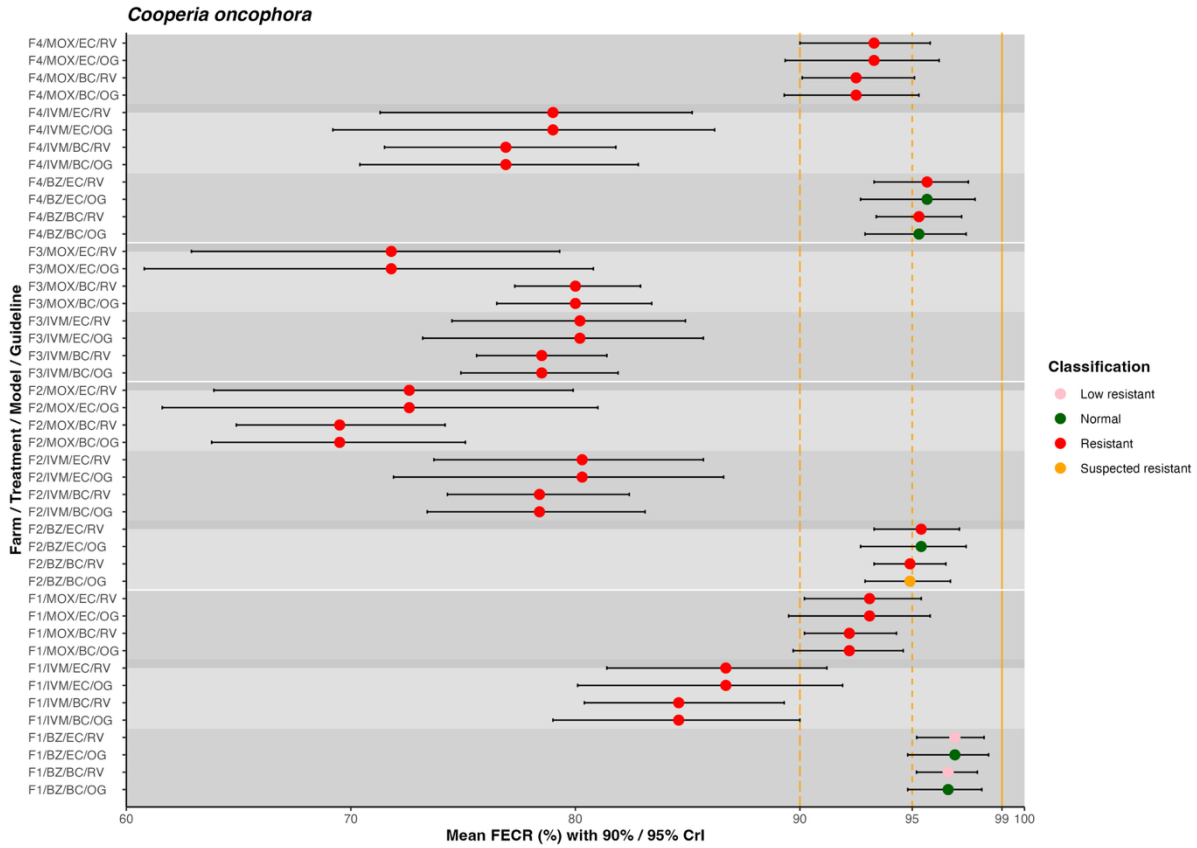


Figure S2: Comparison of the *eggCounts* and *bayescount* faecal egg count reduction estimates for the interpolated *Cooperia oncophora* population

Faecal egg count reductions (FECR) with the credible intervals (CrIs) for anthelmintic treatment against the interpolated *Cooperia oncophora* population. The CrIs were calculated using either *eggCounts* (EC) or *bayescount* (BC) models and interpreted based on either the revised guidelines (RG) for the faecal egg count reduction test (Kaplan et al., 2023) with corresponding 90% CrIs, or on the original guidelines (OG) (Coles et al., 1992) with 95% CrIs. Each point represents the mean FECR, with colour indicating the resistance status classified to the strongyle population: green, susceptible/normal; red, resistant; pink, low resistant / suspected resistant; orange, inconclusive / suspected susceptible.

Appendix G: Inter-rater agreement between methodologies and guidelines

Table S4. The inter-rater agreement between *eggCounts* and *bayescount* results based on the revised guidelines for the faecal egg count reduction test (Kaplan et al., 2023) for the entire strongyle population.

bayescount	***eggCounts***			
	Susceptible	**Inconclusive**	**Low resistant**	**Resistant**
Susceptible	-	-	-	-
Inconclusive	-	-	-	-
Low resistant	1	-	3	-
Resistant	-	-	-	7
	Cohen's k = 0.656 (substantial agreement)			

Table S5. The inter-rater agreement between *eggCounts* and *bayescount* results based on the revised guidelines for the faecal egg count reduction test (Kaplan et al., 2023) for the interpolated *Ostertagia ostertagi* population.

bayescount	***eggCounts***			
	Susceptible	**Inconclusive**	**Low resistant**	**Resistant**
Susceptible	-	-	-	-
Inconclusive	-	-	-	-
Low resistant	1	-	3	-
Resistant	-	-	-	7
	Cohen's k = 0.656 (substantial agreement)			

Table S6. The inter-rater agreement between *eggCounts* and *bayescount* results based on the revised guidelines for the faecal egg count reduction test (Kaplan et al., 2023) for the interpolated *Cooperia oncophora* population.

bayescount	***eggCounts***			
	Susceptible	**Inconclusive**	**Low resistant**	**Resistant**
Susceptible	-	-	-	-
Inconclusive	-	-	-	-
Low resistant	-	-	1	-
Resistant	-	-	-	10
	Cohen's k = 1 (perfect agreement)			

Table S7. Inter-rater agreement between the original guidelines (Coles et al., 1992) and the revised guidelines (Kaplan et al., 2023) based on the faecal egg count reduction test for the entire strongyle population analysed using *eggCounts*.

Original guidelines	**Revised guidelines**			
	Susceptible	**Inconclusive**	**Low resistance**	**Resistant**
Normal	1	-	3	-
Suspected susceptibility	-	-	-	-
Suspected resistance	-	-	-	-
Resistance	-	-	-	7
	Cohen's k = 0.656 (substantial agreement)			

Table S8. Inter-rater agreement between the original guidelines (Coles et al., 1992) and the revised guidelines (Kaplan et al., 2023) based on the interpolated faecal egg count reduction test for *Ostertagia ostertagi* analysed using *eggCounts*.

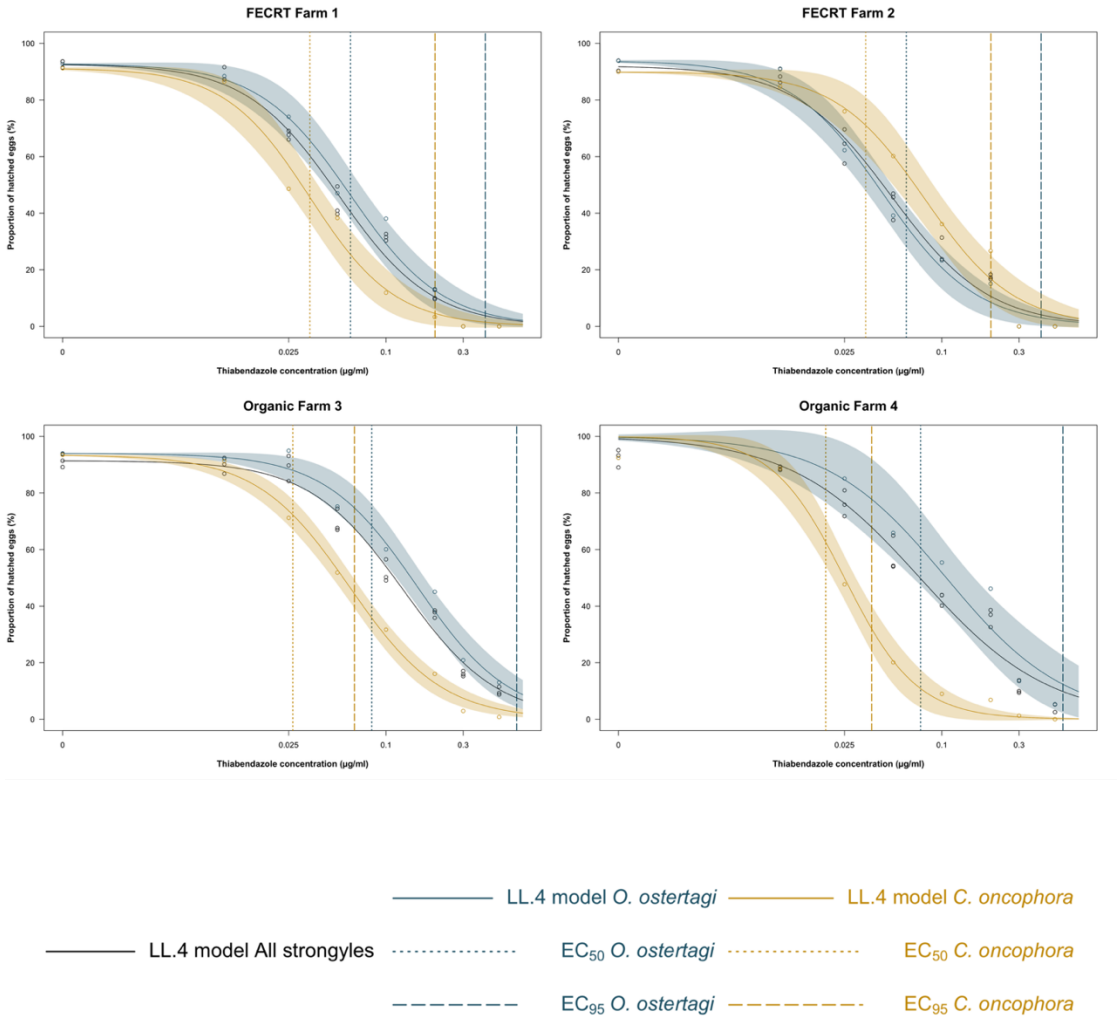
Original guidelines	**Revised guidelines**			
	Susceptible	**Inconclusive**	**Low resistance**	**Resistant**
Normal	1	-	3	2
Suspected susceptibility	-	-	-	-
Suspected resistance	-	-	-	1
Resistance	-	-	-	4
	Cohen's k = 0.33 (fair agreement)			

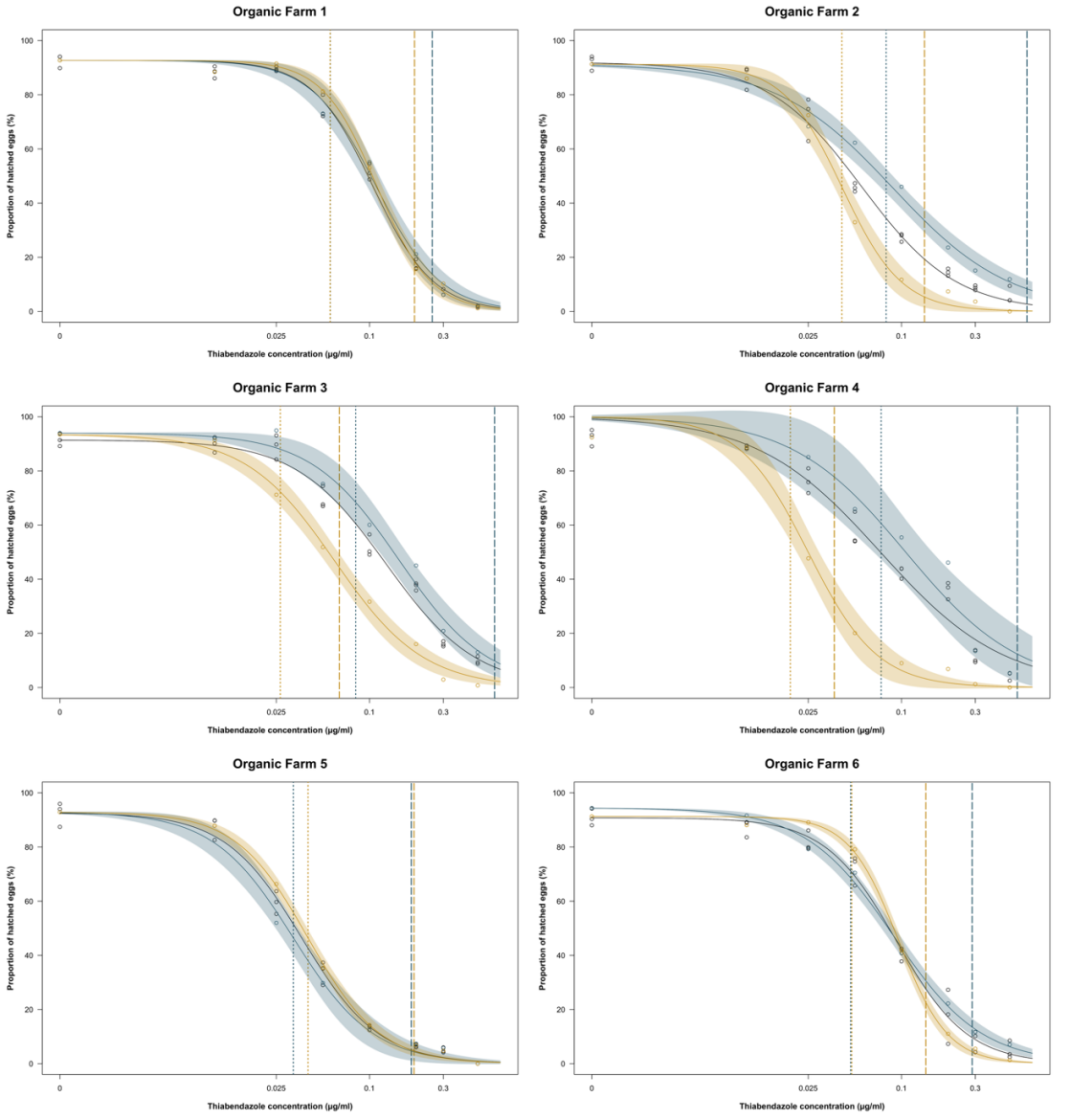
Table S9. Inter-rater agreement between the original guidelines (Coles et al., 1992) and the revised guidelines (Kaplan et al., 2023) based on the interpolated faecal egg count reduction test for *Cooperia oncophora* analysed using *eggCounts*.

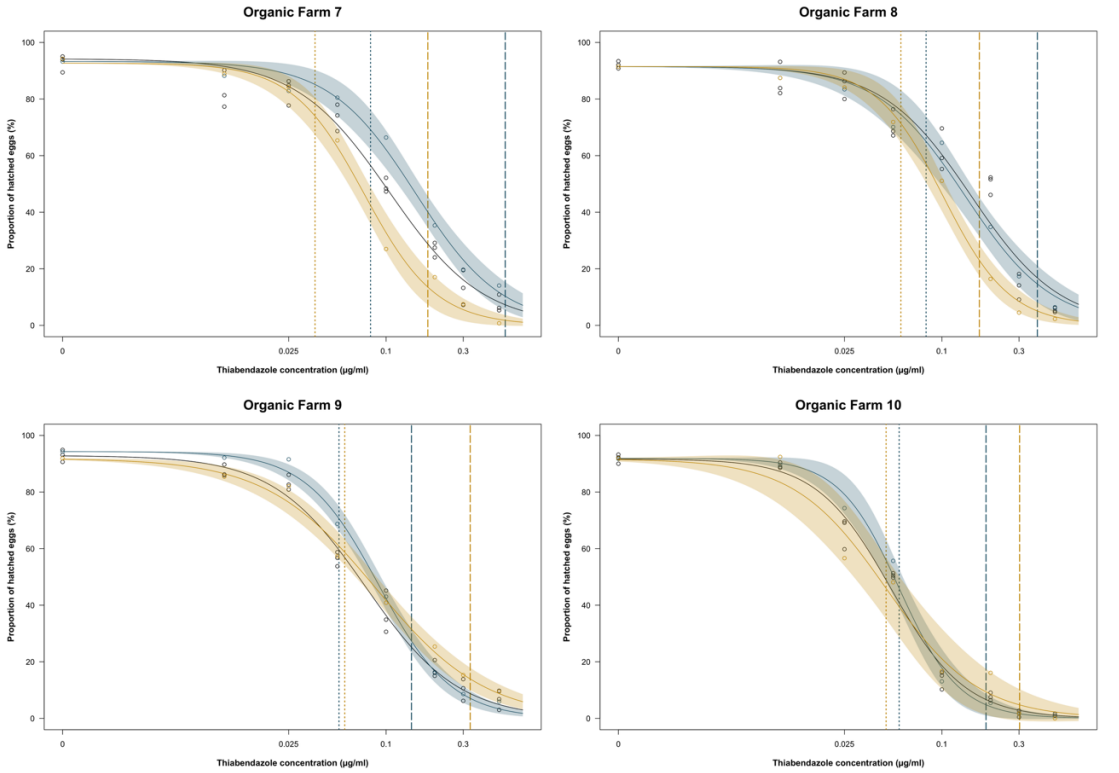
Original guidelines	**Revised guidelines**			
	Susceptible	**Inconclusive**	**Low resistance**	**Resistant**
Normal	-	-	1	2
Suspected susceptibility	-	-	-	-
Suspected resistance	-	-	-	-
Resistance	-	-	-	8
	Cohen's k = 0.19 (slight agreement)			

Appendix H: Egg hatch test dose-response curves

The results of the egg hatch test performed with thiabendazole (TBZ) in 2023 on each study farm are presented at the extrapolated species and total strongyle level. Presented are the LL.4 model dose-response curves and the maximum effective concentrations (EC), EC_{50} and EC_{95} values.




Organic Farm 1
Organic Farm 2
Organic Farm 3
Organic Farm 4
Organic Farm 5
Organic Farm 6
Proportion of hatched eggs (%)
Thiabendazole concentration (µg/ml)
0
0.025
0.1
0.3
LL.4 model O. ostertagi
LL.4 model C. oncophora
LL.4 model All strongyles
EC50 O. ostertagi
EC50 C. oncophora
EC95 O. ostertagi
EC95 C. oncophora


Organic Farm 7
Organic Farm 8
Organic Farm 9
Organic Farm 10
Proportion of hatched eggs (%)
Thiabendazole concentration (µg/ml)
0
0.025
0.1
0.3
LL.4 model All strongyles
LL.4 model O. ostertagi
LL.4 model C. oncophora
EC50 O. ostertagi
EC50 C. oncophora
EC95 O. ostertagi
EC95 C. oncophora

Appendix I: Farm prevalence of strongyle species identified by deep amplicon sequences in ten field populations

Table S10. Prevalence of gastrointestinal nematode species composition identified by mixed amplicon sequencing of the ITS-2 region in ten populations from seven Scottish dairy farms.

ID	*Os. ostertagi*	*C. oncophora*	*Tr. axei*	*Tr. colubriformis*	*Te. circumcincta*	*Os. leptospicularis*	*Oe. venulosum*	*Oe. radiatum*	*H. contortus*	*Tr. vitrinus*	*N. helvetianus*	*Trichostrongylus* spp.	*Cooperia* spp.
FECRT_1/BZ/PRE	52.1	29.7	14	2.7	1.5	0	0	0	0	0	0	0	0
FECRT_1/BZ/POST	85.2	7.9	3	0	3.9	0	0	0	0	0	0	0	0
FECRT_2/IVM/PRE	67.2	21.3	6.8	2.7	0.3	0	0	0	1.1	0	0	0	0.6
FECRT_2/IVM/POST	51.7	39.9	0	0	2.7	0	0	0	0	0	0	0	5.7
FECRT_3/MOX/PRE	42.6	45.8	5.9	0	0	0	0	0	0	0	2.8	0	2.9
FECRT_3/MOX/POST	28	71.1	0	0	0	0	0	0	0	0	0	0	0.9
ORGANIC02	50.9	36	3.8	5.9	0	0	1.9	0.4	0	0	0.7	0.4	0
ORGANIC03	67	15	10.7	2.5	1.4	0	0	1.6	0.8	0.2	0	0.8	0
ORGANIC04	66.6	21	3.8	0.5	0	1.1	3.9	3.1	0	0	0	0	0
ORGANIC10	50.3	32.9	5	4.8	0	0	2.9	3.8	0	0	0	0.3	0

Appendix J: Monthly meteorological data for 2023

Table S11: Monthly meteorological data for 2023 and long term monthly mean values over the past 30-year climate period (1991-2020) for the region (UK Meteorological Office data).

	Monthly mean temperature (°C)		Monthly mean rainfall (mm)		Monthly mean sunshine hours	
Year / Month	2023	1991-2020	2023	1991-2020	2023	1991-2020
April	9.20	8.45	48.1	58.4	167.4	136.56
May	13.30	11.22	31.7	63.9	195.9	182.63
June	17.15	13.92	56.6	70.3	242.2	154.42
July	16.05	15.63	125.4	85.8	138.5	149.40
August	16.50	15.33	72.9	94.7	147.3	138.81