Prevalence, burden and anthelmintic resistance of gastrointestinal nematodes in the south west region of Western Australia dairy herds

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This thesis is presented for the degree of Bachelor of Science Honours, School of Veterinary and Life Sciences, of Murdoch University

June 2021

Dedication

I dedicate this thesis to my family, partner, close friends for their patience and support towards my never-ending journey. I would also like to dedicate this thesis to Josh Aleri, for without his guidance, I would still be lost in the abyss of academic research.

Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

The thesis is less than 25,000 words, inclusive of tables and figures.

Mikayla Elizabeth Mauger

Abstract

Anthelmintic resistance in gastrointestinal nematodes (GIN) of dairy cattle is of global importance. The objective of this study was to determine the prevalence of GIN among post-weaned replacement heifers and bull calves aged between 4 - 12 months old in Western Australia dairy farms and quantify the level of anthelmintic resistance. A secondary objective of this study was to explore pooling faecal samples for cost effective diagnostic purposes of faecal egg counts (FECs). Pre-treatment FECs were monitored on 14 dairy farms, anthelmintic resistance was assessed on 11 of the farms based on FEC of ≥500 eggs per gram (epg) in at least 10 - 15% of the samples. Control FECs were compared with anthelmintic FECs at 14 days post-treatment with doramectin (injectable), levamisole (oral), fenbendazole (oral) and, a levamisole/abamectin combination (Eclipse® combination pour-on). The results demonstrate a high level of anthelmintic resistance, with at least one class of anthelmintic failing to achieve a 95% reduction in FEC in one or more GIN species. Doramectin was fully effective against *Ostertagia*, but *C. oncophora* displayed resistance to it on 91% of the farms. Conversely, levamisole was fully effective against *C. oncophora*, but *Ostertagia* displayed resistance in 80% of the farms. Fenbendazole resistance was present in both *C. onocphora* and *Ostertagia* in 64% and 70% of the farms respectively. *Trichostrongylus* showed low resistance, only occurring in doramectin (14%) and levamisole/abamectin combination (14%) on the farms sampled. A high level of correlation between pooled groups of 5, 10 and 20 samples was recorded (R=0.947, 0.987, 0.972 and *P*=0.015, 0.002, and 0.006) respectively. This study confirms that anthelmintic resistance within Western Australian dairy farms is common and regular faecal egg count reduction testing is recommended to monitor and guide decision-making for appropriate anthelmintic usage. Utilisation of pooled FECs provides a potential cost-effective method for farmers to regularly monitor FECs.

Preface

All the researched work presented was conducted in commercial dairy farms in Western Australia, with the approval of the Animal and Human Ethics Approval Committees from Murdoch University and in accordance with the Australian Code of Practice for the Care and Use of animals for scientific purposes.

Chapter 2 has been submitted for peer review in the Veterinary Parasitology journal in June 2021. My role as lead author of this publication included study design, data collection and analysis as well as manuscript and thesis composition. Josh Aleri was the supervisory author involved throughout the project in concept formation, study design, data collection and analysis, manuscript composition and edits. The contributions of my co-authors (Gareth Kelly, Cornelius Henry Annandale, Ian Robertson and Frank Waichigo) involved manuscript edits.

Publications and Presentations

Publications

1. M. Mauger, G. Kelly, C. H. Annandale, I. D. Robertson, F. W. Waichigo and J. W. Aleri, 2021. Prevalence, burden and anthelmintic resistance of gastrointestinal nematodes in the south west region of Western Australia dairy herds. Submitted to Veterinary Parasitology journal.
2. Murdoch University, 2020. Young gun Murdoch dairy researcher wins scholarship. [online] Murdoch University Intranet. Available at: < https://murdochuniversity.sharepoint.com/sites/intranet-news-and-events/SitePages/news-Young-gun-Murdoch-dairy-researcher-wins-scholarship.aspx?utm_campaign=SHEE+NEWS+%7C+7+October+2020&utm_medium=email&utm_source=newsletter>
3. Gill, M., 2020. Mikayla loves everything about dairy. [online] Farm Weekly. Available at: <https://www.farmweekly.com.au/story/6919922/mikayla-loves-everything-about-dairy/>.

Presentations

4. Production Animal Department: Project Proposal Presentation. 22nd May 2020.
5. Dairy Innovation Day: Western Dairy Honours Students Panel. 6th May 2021. Yoongi Downs, Sabina River, WA.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my primary supervisor Josh Aleri, without whose strong encouragement and phenomenal assistance I would not have finished my honours to the standing achieved. I truly appreciate the effort and time he dedicated towards ensuring I was on the right track and aiming to perform at the best of my abilities, unlocking my full potential. I am greatly indebted for allowing me the opportunity to undertake an amazing project and always providing assistance in all aspects of my study despite his busy schedule. I was truly lucky to have him as my supervisor throughout my honours thesis and I hope there will be future opportunities to continue working alongside him.

I further express my gratitude to my co-investigators Cornelius Henry Annandale and Ian Robertson (Murdoch University), and Frank Waichigo (Brunswick Veterinary) for their guidance, feedback and encouragement during stressful times. Special thanks to my external supervisor Gareth Kelly (Boehringer Ingelheim) for his timely and valuable feedback of my drafted manuscript, as well as organising donations towards the study.

I am indebted to the support and funding provided to the study by Murdoch University, Dairy Australia, Western Dairy and Boehringer Ingelheim. I thank Western Dairy and Dairy Australia for the generous scholarship and opportunity to present, network and collaborate with dairy farmers throughout my project. I thank Boehringer Ingelheim for their generous funding and donation of their products. I also thank the technical staff at the Department of Primary Industries and Regional Development (DPIRD) for their assistance within the laboratory aspect of this study.

I express my gratitude to many staff of Murdoch University, with special thanks to John Abbott and Aprille Jack for technical assistance and valuable on-farm help. I am hugely indebted to

all the farmers and their staff for allowing me to use their stock and assistance despite their busy schedules. Special thanks to Tavis Hall, Andrew Mahaffey and his sons.

I would like to thank my parents (Tracey Newman, Jason Mauger), siblings (Isabella and Cassidy), stepfather (Paul Newman), late stepmother (Kate Jolley, forever in our hearts, may you rest in peace), friends and partner for their support throughout my honours and encouraging me to shoot for the moon. I thank them deeply for support during difficult times of stress, grief and injury. Special thanks towards my friends Bailey Richardson, Chin-Ning (Safyre) Chong and Kirsty Cunningham.

Finally, I would like to thank my partner Taylor Decosta for his phenomenal support throughout this endeavor. Your support and assistance on-farm despite no previous experience was truly invaluable and was my saving grace on many occasions. Thank you for your encouragement, love, and patience with me and I hope you will continue to stand by me throughout future endeavors.

"Shoot for the moon. Even if you miss, you'll land among the stars."

- Norman Vincent Peale

List of Abbreviations

ADG	Average Daily Gain
BJD	Bovine Johne's disease
BZ	Benzimidazole
CI	Confidence Intervals
DPIRD	Department of Primary Industries and Regional Development
EHA	Egg Hatch Essay
EPG	Eggs Per Gram
FEC	Faecal Egg Count
FECR	Faecal Egg Count Reduction
FECRT	Faecal Egg Count Reduction Test
GIN	Gastrointestinal Nematodes
LDT	Larval Development Test
LMIT	Larval Mitigation Inhibition Test
LZ	Levamisole
ML	Macrocyclic Lactones
PCR	Polymerase Chain Reaction
SC	Subcutaneous
TST	Targeted Selective Treatment
TT	Targeted Treatment

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Chapter 1. Literature Review

1.1 Overview of the Australian dairy industry

Dairy farming is the third-largest agricultural industry in Australia (behind wheat and beef), contributing a farmgate value of A$4.4 billion dollars in 2018-19 (Dairy Australia, 2021a). In 2008, Australia's dairy exports accounted for 4% of the world's production and 11% of the global trade (Walsh, 2009). Currently, the industry ranks fourth in terms of world dairy trade, with a 5% market share behind United States, European Union and New Zealand (Dairy Australia, 2020). As a result of deregulation in 2000, the number of dairy farms in Australia has fallen from 12,500 to 5,055 registered dairy farms in 2020 (Dairy Australia, 2020, 2021b).

The majority of dairy farms are located in Australia's coastline and depend on rainfall, whereas a quarter of Australian dairy rely on irrigation and are situated in Northern Victoria and Southern New South Wales (Dairy Australia, 2019, 2020). It's characterised with an extensive pasture-based feeding system and with variations on grain supplementation (Dairy Australia, 2019; Walsh, 2009).

Within the Australian dairy herd there is an approximate 1.41 million cows, with an average herd size of 279 cows (Dairy Australia, 2020). Western Australia currently accounts for 4% of the national production, with Holstein-Friesian and their crosses as the predominate breed (Dairy Australia, 2019).

1.2 Common health issues

Within Australia, the livestock industry is heavily reliant on the usage of year-round pasture feeding however, due to the intensification of these systems, transmission and exposure of gastrointestinal parasites has effectively increased (Barger, 1993; Bullen et al., 2016). Gastrointestinal nematodes (GIN) are a common liability of pasture production herds, leading

to a decrease in productivity, animal health and farm profitability (Velde et al., 2018). GINs are of most importance to dairy calves, especially weaned dairy calves with the potential to cause mortality (Charlier et al., 2020; Eguale et al., 2011; Hutchinson, 2009).

Several practices are used to control GIN populations, with a main practice of anthelmintic drug usage (Barger, 1993; Bullen et al., 2016; Sutherland and Leathwick, 2011). Heavy reliance on anthelmintic drugs has shown a positive association with anthelmintic resistance. Studies have been conducted globally with resistance being documented extensively. These studies have focused mainly against the three main nematode species of economic and welfare importance; *Trichostrongylus*, *Cooperia* and *Ostertagia* species.

1.3 Epidemiology of gastrointestinal parasite in Australia

Many species of nematodes have evolved towards parasitic life in plants or animals. All nematodes have the same basic development with parasitic species containing stages which occur outside the definitive host. All the major species of GIN are transmitted through a direct lifecycle, by ingestion of infective third stage (L3) larvae from pastures. The prepatent period from ingestion of infective L3 larvae to mature adult worms is generally 3 weeks (Hutchinson, 2009). GIN eggs are generally resistant to environmental changes, but first and second stage (L1 and L2) larvae remain within the dung pats where they are susceptible to adverse conditions, whereas infective L3 are more resistant but survival on pasture is dependent on moisture and temperature (Charlier et al., 2020; Hutchinson, 2009; Navarre, 2020). Larvae can become arrested (dormant/hypobiotic) for several months, emerging when environmental conditions are favourable. Hypoboitic larvae usually occur after the autumn and winter period, emerging during the next spring.

The distribution of cattle GIN in Australia is generally climate related, with *Cooperia* species being the most prevalent species occurring Australia-wide, followed by *Haemonchus* and

Oesophagostomum species in summer rainfall dominant regions and *Ostertagia* and *Trichostrongylus* species in winter rainfall regions. *Ostertagia ostertagi* is considered the most pathogenic and important species in temperate zones, occurring in highest proportion within southwest Western Australia (Taylor and Hodge, 2014). *O. ostertagi* is not reported as endemic in the subtropic or tropical zones of Australia, with the exception of occasional infections from imported dairy cattle (Hutchinson, 2009). Type I Ostertagiosis is known to occur in young grazing herds where calves become infected with *Ostertagia* for the first time. This manifests with marked weight loss, profuse watery diarrhea, inappetence and mortality (Charlier et al., 2020; Hoglund et al., 2009; Myers and Taylor, 1989). Type II Ostertagiosis is related to a high emergence of inhibited larvae with clinical signs identical to type I disease in older calves and adult cattle, with a primary clinical sign of inappetence in less severely infected animals (Kaplan, 2020; Myers and Taylor, 1989). This may manifest as acute outbreaks due to larval emergence or as chronic cases (Berghen et al., 1993; Myers and Taylor, 1989; Rinaldi and Geldhof, 2012). Fatalities from *Ostertagia* are known to be frequent in both old and young cattle.

Trichostrongylus are recorded in small numbers mixed with larger numbers of *Cooperia*. The *Cooperia* species vary geographically in that *C. oncophora* is found in temperate areas (Hutchinson, 2009; Taylor and Hodge, 2014), whereas the more pathogenic *Cooperia punctata* and *Cooperia pectinata* are found in the subtropics and tropical areas (Lyndal-Murphy et al., 2010). *C. oncophora* occurs mainly in cooler southern regions of Australia, playing a significant role in GIN parasitism of dairy cattle (Hutchinson, 2009; Taylor and Hodge, 2014). Furthermore, parasitic gastroenteritis in young calves is caused by *C. oncophora* and *O. ostertagi*, typically occurring during the young calves first season when they are heavily stocked (Charlier et al., 2020).

1.4 Classes of anthelmintics and administration methods

Anthelmintic drugs are grouped by their mechanism of action, with 3 broad spectrums registered for use in Australian dairy cattle: benzimidazole's ('white'), levamisole's ('clear') and macrocyclic lactones (MLs). Additionally, within sheep amino-acetonitrile derivatives (AADs) and spiroindoles have become commercially available (Knox et al., 2012). Anthelmintics with greater efficacy against early or inhibited stages of parasitic worm are ideal in treating cattle. These treatments include third generation benzimidazole carbamates (albendazole, fenbendazole, oxfendazole) and macrocyclic lactones (ivermectin, abamectin, moxidectin, doramectin, eprinomectin).

Benzimidazole resistance was first discovered in the 1960s from overuse leading to a rapid increase in resistance predominately within *Ostertagia, Cooperia, Haemonchus* and *Trichostrongylus* species. Administration of these treatments is generally oral with little to no residual effect from the treatment. Efficacy of fenbendazole, oxfendazole and albendazole can be attributed to a slower excretion rate (Prichard et al., 1980). Levamisole has been in use for over 5 decades and studies have reported ineffectiveness towards *O. Ostertagia,* which could be attributed to the rapid replacement of adults by larval stages during treatment intervals (Anderson, 1977; Williams et al., 1991), alongside resistance in other *Ostertagia* and *Trichostrongylus* species. Administration methods of levamisole are oral or pour-on formulations with minimal residual effects post treatment.

The first macrocyclic lactones commercially available was an ivermectin in the 1980's. Macrocyclic lactones tend to have consistently high efficacy, including against inhibited larval stages, and ongoing activity against ingested L3 larvae while also performing as highly effective anthelmintic (Prichard et al., 1980). Administration methods of MLs include oral, pour-on and injectable options, offering a high residual activity from being stored in the body fat post-administration, slowly releasing into the blood (Prichard et al., 1980). Combination

drenches containing two or more different classes of anthelmintics have shown effectiveness in controlling gastrointestinal parasites, usually involving a macrocyclic lactone in combination with benzimidazole and/or levamisole. A combination anthelmintic is likely to be most effective against GIN which have developed resistance to one or more classes (Soutello et al., 2007; Waghorn et al., 2006a), to both maintain animal health and keep resistant genes as scarce as possible (Dobson et al., 2001). Adopting combination treatments prior to development of resistance is important to maintain their efficacy (Leathwick et al., 2012). Within Australia, there are currently several different registered combination anthelmintics for use in cattle, including Trifecta® (abamectin/levamisole/oxfendazole, MSD Animal Health Australia), Eclipse® (abamectin/levamisole, Boehringer Ingelheim), and Cydectin Platinum® (moxidectin/levamisole, Virbac), but their use by farmers is very low, especially compared to New Zealand farmers.

1.5 Anthelmintic resistance

Anthelmintic resistance is defined as being present when "a greater frequency of individuals within a population are able to tolerate doses of a anthelmintic than in a normal population of the same species and is heritable" (Prichard et al., 1980). The rate of resistance development is associated with several factors: biological fitness, parasite genetics and livestock management and husbandry practices (Sangster, 2001). Successful dissemination of resistant genetics relies upon competition of the GIN lifecycle and is believed to be accelerated in species with high fecundity, such as *Cooperia* spp, in comparison to species such as *O. ostertagia* (Coles, 2005; Van Zeveren et al., 2007). Studies exploring anthelmintic resistance have been conducted for several decades from the 1970s, to benzimidoles and levamisole in the 1980s and ivermectin in the 1990's (Charlier et al., 2009). Heavy reliance on the usage of anthelmintic drugs has shown a positive association with anthelmintic resistance to all available classes, occurring

globally within parasite species of most economic importance (Barger, 1993; Bullen et al., 2016; Sutherland and Leathwick, 2011).

With the US, parasite control programs utilising anthelmintics are thought to led to an increase in resistant selective pressures but at the same time have proved to be very effective (Gasbarre, 2014). Resistance to ivermectin is highly reported internationally within the *Cooperia* species by studies in the US (Edmonds et al., 2010; Gasbarre, 2014), south-western England (Stafford and Coles, 1999) with approximately 60% in Europe (Sweden, Germany and Belgium, (Demeler et al., 2009) and Argentina (Mejia et al., 2003; Suarez and Cristel, 2007), and over 90% in New Zealand (Waghorn et al., 2006a), where 74% of the farms also presented simultaneous Albendazole resistance.

Failure by macrocyclic lactones were also reported in case reports from USA (Edmonds et al., 2010), UK and 38% of farms from a survey in Sweden (Areskog et al., 2013). Fenbendazole and levamisole were reported to be fully effective against *Cooperia* in Argentina (Suarez and Cristel, 2007) and Europe (Demeler et al., 2009). Within New Zealand, benzimidazole resistance was evident in 76% of tested farms (Waghorn et al., 2006a).

Approximately half of the farms surveyed within New Zealand (Waghorn et al., 2006a) showed a resistance to both levamisole and benzimidazole by *O. ostertagi*. Suarez and Cristel (2007) showed no reported levamisole resistance, or benzimidazole resistance in European surveys (Demeler et al., 2009). Additionally, macrocyclic lactone resistance of *O. ostertagi* was only detected on a small proportion of properties surveyed in Sweden (Areskog et al., 2013) and New Zealand (Waghorn et al., 2006a). Limited evidence of the possible emergence of ivermectin resistance but not eprinomectin in New Zealand was also reported by Mason (2012).

Within Australia, anthelmintic resistance has been recognised as a significant limitation to the effective control of GIN in sheep (Besier and Love, 2003), but resistance within cattle has only

recently reported (Cotter et al., 2015; Lyndal-Murphy et al., 2010; Rendell, 2010). Resistance was first detected in the benzimidazole derivative known as thiabendazole, within *H. contortus* in the Northern Tablelands, NSW (Smeal et al., 1968). *Cooperia* was reported by Rendell (2010) in 62% of beef cattle properties surveyed in south-western Victoria. Geographical macrocyclic lactone resistance to two suptropical *Cooperia* species was indicated in a case study in eastern subtropical Queensland (Lyndal-Murphy et al., 2010). A recent study by Bullen (2016) of Victorian dairy cattle reported resistance of all three anthelmintic classes detected in three farms. Additionally, resistance to doramectin on 70% of farms, levamisole on 25% and fenbendazole on 80% of farms.

A study in the south-west region of Western Australian in beef cattle (Cotter et al., 2015) found failure of at least one anthelmintic group on all surveyed properties for both *O. ostertagi* and *C. oncophora*. Ivermectin resistance was present within 59% of farms for *Cooperia*, but fully effective against *O. ostertagi*. Levamisole and fenbendazole resistant *O. ostertagi* were present on 67% and 50% respectively but were fully effective against *C. onophora*. However, there were no reported cases where resistance occurred simultaneously to all anthelmintics within any major worm species. There is limited data within the Western Australia dairy farms.

1.6 Use of anthelmintics in different age groups within the dairy industry

1.6.1 Calves

Gastrointestinal nematodes (GIN) parasitism is a significant health concern and production liability within weaned dairy calves. Calves are known to be more susceptible than adult cattle due to the development of exposure-based immunity (Charlier et al., 2020; Coles, 2002). The practice of raising successive groups of calves on permanent pastures allows for an increased cycle rate of natural infection resulting in an increased development of anthelmintic resistance and large worm burdens over the summer season (Leathwick and Besier, 2014; Parkinson et al., 2010). Dung pats from the calves provide the ideal moist, damp environments required to

survive the unfavourable conditions, where with high rates of contamination, infection rates can rapidly increase (Gasbarre, 2014; Hutchinson, 2009; Lean et al., 2008).

The main source of prevention regarding GIN infections in dairy calves is through use of frequent anthelmintic treatments. Within Australia, the recommendations on GIN parasite control have included frequent treatments from the time of weaning at intervals of 4-6 weeks (Leathwick and Besier, 2014; Parkinson et al., 2010). The trade-off to this highly reliant practice is the potential for an increase in anthelmintic resistance. Treatment is usually administered early in the first grazing to prevent the winter recycle of larvae. Due to the increased productivity and milk withholding period (0 days), subsequent treatments in the secondary and later seasons are also being administered (Charlier et al., 2009). Practices which utilise frequent treatments and permanent calf paddocks have significantly increased potential for resistant genomes within GIN to develop throughout populations, passing onto subsequent generations (Demeler et al., 2009; Leathwick and Besier, 2014).

1.6.2 Replacement heifers

The importance of replacement heifers for farmers is represented by the economic investment of potential returns to the enterprise. The cool, moist rainfall season over the winter/spring period provides environmental conditions optimal for the growth of lush green pastures, which are heavily relied on for nutritional requirements. However, these environmental conditions are also optimal for nematode development, survival, and transmission. A study in Holstein-Friesian heifers (Forbes and Rice, 2000) showed cattle infected by GIN demonstrated a marked difference in development and behaviours compared to uninfected control heifers. Infected individuals had a lower dry matter intake with less grazing time (-105min/day) and lower mean body weight (-158g/day). Effects of GIN on the reproductive performance within dairy is limited (Mason et al., 2012; Walsh et al., 1995). Studies in beef cattle have demonstrated that treated cattle have increased conception and calving rates, alongside reductions in calf

mortality and breeding intervals (Charlier et al., 2009; Gross et al., 1999). In Australia, a clinical trial on 5 dairy herds (430 cows), found that cows treated with ivermectin during the dry period had a 4.8-day shorter calving-to-conception interval (Walsh et al., 1995).

1.6.3 Milking Herd

Over the past few decades, subclinical effects of parasitic infestations have been studied and reported extensively. A review by (Gross et al., 1999) reviewed 87 trials which were divided into categories based on the timing of treatments. Observations from these trials showed that milk production increased post anthelmintic treatment with an average of 0.6kg/cow per day. Furthermore, a study by Walsh *et al* (1995) conducted in south-western Victoria found that seasonally calved dairy herds which were treated with injectable ivermectin in the dry period produced 74L and 86L of milk within the first 100 days and entire lactation respectively. Charlier *et al* (2009) reported that first lactation is related to body weight at calving, with GIN infections within the first two years negatively impacting on weight gain, time to first breeding and milk production. Significant interactions ($P = 0.02$) between treatment and pre-calving optical density ratios (ODR) on milk production after treatments of endectocides have been reported in an Canadian study (Sanchez et al., 2005). Contradictory to the above studies, a trial in New Zealand reported on one of three pasture-based herds, despite a high level of parasitism indicated by *O. ostertagia* optical density levels (ODR >0.5) within the bulk tank, presented an increase in milk production (Mason et al., 2012).

1.7 Control methods of gastrointestinal parasites

1.7.1 Drench usage

Control of GIN in cattle is typically managed using two main strategies: anthelmintic drugs and grazing strategies. A survey study of beef farmers reported the type and frequency of treatments of anthelmintic drugs used on yearlings. In this study, a majority of the farmers

utilised macrocyclic lactones (59%) followed by benzimidole (16%) and levamisole/bezimidole (16%) combination drenches, varying in annual drenching frequency (1-22%, 2-29%, 3-12%, +4-29%)(Jackson et al., 2006). Majority of farmers reported to only using macrocyclic lactones or combination-based drenches in the last four years. From the 59 farmers who completed the questionnaire; one third routinely treated their calves around 6-8 weeks at marking, one fifth treated mixed-age cows, and approximately half treated 2-year old heifers prior to calving (Jackson et al., 2006). Calves which were sourced and reared from dairy farms were treated at least once by the age of 9-10 weeks by 32% of the farms. Sustainable usage of drenches for long-term reliability will rely on the usage of quarantine drenches alongside a routine drench program, including the rotation of anthelmintic classes to reduce the rate of anthelmintic resistance development. Additionally, within sheep amino-acetonitrile derivatives (AADs) and spiroindoles have become commercially available (Knox et al., 2012). Monepantel (AAD) is effective against GIN in cattle has been commercialised for use in cattle (Zolvix™ Plus (monepantel and abamectin) by Elanco). Within these newly available drugs available to sheep (monepantel and derquantel), first cases of resistance have already been reported (Velde et al., 2018). Therefore, reliance of the use of frequent anthelmintic treatment alone cannot be relied upon as a method of control.

1.7.2 Pasture management

GIN impact on the ability to efficiently utilise pasture is firstly influenced by the effects on metabolic and physiological processes of the infected stock, then secondly by the management decisions to reduce the extent of pasture infection (Waller, 2006). Traditionally, pasture management involves a variety of practices including, resting, rotation, moving, and late turn-out of stock, etc. However, these practices require substantial effort and can be limited due to lack of resources and limited knowledge of nematode epidemiology (Leathwick and Besier, 2014; Velde et al., 2018). Grazing management strategies for the control of gastrointestinal

nematodes infection in ruminant livestock has been classified into three categories: preventative, evasive and diluting (Michel, 1985). Preventative strategies rely on moving worm-free animals onto clean pasture or supressing the degree of eggs being shed until the initial infective population declines to safe levels via anthelmintic treatment. Evasive strategies rely on the movement of livestock to alternate pastures before larvae from the contamination are likely to appear in significant numbers. Finally, diluting strategies exploit concurrent grazing of susceptible animals with a greater population of animals with natural resistance of the same or different livestock species, with the intention of reducing herbage infestation.

Within a New Zealand study (Jackson et al., 2006), farmers were surveyed about grazing and management practices, findings showed no clear pattern regarding the placement of cattle after anthelmintic treatment. There was strong indication that previously grazed pastures were preferred. Responses indicated a poor understanding of parasite survival capabilities during the rest-period required for a pasture to become 'safe' over the summer-autumn period. Common grazing management involved the shifting of stock every 2-10 days, whereas set-stocking, daily shifts, and co-grazing as uncommon practices. (Jackson et al., 2006). Practices which utilise the movement of uninfected animals onto uncontaminated pasture intend to keep re-infection rates low and prolong the suppressive effect of anthelmintic treatment for months, rather than weeks as seen on contaminated pastures (Waller, 2006). Farms where grazing cattle and calves is restricted to one part of the farm, especially when using successive permanent pastures, have implications regarding parasite control and the persistence of larvae in refugia (Leathwick and Besier, 2014). Larval challenge in a permanent pasture-based system would be expected to have a higher degree of generation turner and resistance development when compared to more extensive pasture conditions (Barger and Southcott, 1975; Jackson et al., 2006; Leathwick and Besier, 2014).

Highly host-specific infective GIN larvae are destroyed when ingested by a different herbivore host species. As a result, multi-species, or alternate grazing strategies present alternatives for pasture decontamination. In Australia, contamination of *H. contortus* and *T. colubriformis* were reduced in pasture used by sheep after 6, 12 or 24 weeks of subsequent cattle grazing (Barger and Southcott, 1975). Similar results were found in scenarios where cattle and sheep were grazed alternatively in intervals of 6 months. *H. contortus* can prove difficult to control as the species cycles in calves within temperate regions. However, as the calves mature, they acquire natural immunity and become perverse to infection by 12 months of age (Barger and Southcott, 1975; Coles, 2002). A similar study conducted in Brazil found that pasture contamination was considerably reduced after 96 or 192 days of cattle grazing. Cross-infection of GIN between the two species were not significant, suggesting that integrated grazing between these species could be utilised for pasture decontamination (Rocha et al., 2008). Contaminated pastures which were grazed with frequently drenched cattle, spelled, or grazed with sheep, were able to effectively limited *O. ostertagi* in susceptible cattle which subsequently grazed those pastures (Barger and Southcott, 1975). Conventionally, pasture contamination in cow-calf enterprises substantially arises from adult cattle shedding eggs and susceptible calves becoming infective, with suggestion that refugia worms in non-treated animals play an important aspect in delaying the onset of resistance (Barger and Southcott, 1975; Stafford and Coles, 1999).

1.7.3 Nutrition

Nutrition can be used as a short-term alternative to anthelmintic drugs, influencing the development and consequences of host-parasite relationship. Outlined by Coop and Kyriazakis (2001), nutrition can become an influence in three different ways. Firstly, nutrition can improve host ability to overcome and contain parasitism (resistance) through limiting establishment, fecundity, and growth rate of the population. Secondly, it can increase the hosts ability of cope with the adverse consequences (resilience) from parasitism. Parasitic populations can also be

directly affected through antiparasitic compound intake. Nutrition should be carefully monitored as intake of nutrients such as fats containing immunosuppressive properties can positively influence parasite populations. Several studies have explored the use of protein supplementation in parasite suppression (Bown et al., 1991; Coop and Kyriazakis, 2001; Donaldson et al., 1998). Protein is expected to first be allocated to the hosts maintenance functions and highest priority as it guarantees survival short-term. Growth and reproduction are expected to also have a high priority to ensure the hosts genetic material is preserved (Coop and Kyriazakis, 2001).

A study regarding twin-bearing ewes experimentally infected with GIN during late pregnancy and early lactation, were offered rations supplemented with varying amounts of mainly undegradable proteins. It was observed that supplementation with higher levels of protein reduced worm burdens and faecal egg count (Donaldson et al., 1998). Similar results were observed in a trial regarding *Trichostrongylus colubriformis* infection and body composition in lambs through comparative slaughter technique (Bown et al., 1991). Furthermore, the study concluded that a major limiting factor in the feed efficiency of GIN infected animals is through a parasite-induced protein possibly resulting from increases in endogenous protein losses in the gastrointestinal tract. Similar positive effect of protein supplementation of nematode resilience has been recorded in studies using goats (van Houtert and Sykes, 1996). Similarly, sheep which were fed an increased phosphorus content and experimentally infected with *Trichostrongylus vitrinus*, presented a reduced worm burden by 89% and an overall faecal egg count reduction by 55% (Coop and Field, 1983).

Apart from research in mineral and protein supplementation, grazing of tanniferous forages are being investigated as a sustainable alternative. Tannins are a form of non-biodegradable complexes with protein in the rumen, dissociating at a low pH found in the rumen, releasing more protein in the small intestines for metabolism (Mueller-Harvey and Caygill, 1999; Waller,

2006). This indirectly improves the hosts resilience and resistance to nematode infections and potentially possess a direct anthelmintic effect towards nematode populations. Tannins are found in a variety of plants where concentration vary from 5% in some temperate legume fodder to 50% in dry matter of some tropical plants (Mueller-Harvey and Caygill, 1999). Anthelmintic benefits of condensed tannins can be utilised by occasional grazing through a short-term period on a 'deworming' paddock or forages such as hay or silage (Coop and Kyriazakis, 2001; Thamsborg et al., 1999). Suggestions have been made that tannins and/or metabolites in dung may also directly affect the viability of nematode free-living stages (Waller, 2006). Adversely, a negative consequence of tannins is the reduction of digestibility and feed intake due to enzymatic and microbial inhibition (Dawson et al., 1999).

1.7.4 Control programs

Several sustainable worm control strategies have been developed to assist farmers in controlling GIN populations and slowing the development of anthelmintic resistance. These programs include Sustainable Control of Parasites in Sheep (SCOPS), Control of Worms Sustainably (COWS) in the UK, FAMACHA©, and Wormkill and Wormboss for small ruminants within Australia, to name a few. These initiatives are generally a collaboration between parities and stakeholders to develop and promote recommendations for 'best practice' control towards the preservation of future and current anthelmintics (Velde et al., 2018). A study in the UK evaluated the use of SCOPS over a 3-year period and found significant reduction in anthelmintic usage without performance loss within the herd (Anderson, 1977; Learmount et al., 2015; Learmount et al., 2016). Similarly, in Brazil, farmers using the FAMACHA© program used to assess the ocular membrane colouration as an indicator of hemonchosis in small ruminants, reported less usage of anthelmintic drugs (Velde et al., 2018). Morgan *et al* 2012 surveyed 600 sheep farmers to understand the current practices and identify potential factors correlated with perceived anthelmintic failure. Keys outcomes of the survey

indicated that farmers had considered worm burdens, yet half were concerned about anthelmintic resistance, with considerably less believing their herd was compromised. Only a minority were aware of the SCOPS program with current anthelmintic use influenced by experience and perceived reality. Furthermore, treatment failure was not considered as a farmer consequence (Morgan et al., 2012; Velde et al., 2018). Knowledge is an important factor in the adoption of control program. A survey in Scottish farmers regarding SCOPS found that confirmation of anthelmintic resistance by diagnosis or external advisors had the largest effect of farmers perception (Jack et al., 2017).

The need to understand farmer behaviour towards parasite control, uptake of these control programs and applicable advice is increasing. Understanding farmer intention and barriers is necessary for creating and promoting sustainable control strategies. Barriers towards adoption include factors which are both general and specific to each farmer's respective herd and enterprise (i.e complexity and compatibility, time requirements, ability to trial practices). Studies recommend that for farmers to abandon historical practices, economic analysis and scientific evidence will be invaluable. Furthermore, parasite control programs which integrate several control methods that are financially, economically, and practically feasible are the most ideal way to ensure long-term sustainability.

1.7.5 Vaccine

GIN epidemiology is majorly affected by the development of immunity, regulating worm development, establishment, survival, fecundity, and development of arrested (hypobiosis) L4 larvae (Besier and Love, 2003). Vaccines can influence the overall manifestations of the immune response in the reduction in nematode transmission within herds (Charlier et al., 2018; Hein et al., 2001; Rinaldi and Geldhof, 2012). In an experimental setting, these reductions can reach a range of 50-90% (Charlier et al., 2020). Currently the only vaccine against GIN within the market is a subunit vaccine in sheep for *Haemonchus contortus*, available in Australia

(Barbervax®) and South Africa (Wirevax®), administered at monthly intervals for protection maintenance (Charlier et al., 2018). Providing future research can overcome and understand the immune effector mechanisms and production of protective antigens, vaccines can begin to be considered a viable control method (Charlier et al., 2020; Hein et al., 2001).

1.8 Diagnostic methods of anthelmintic resistance

1.8.1 Conventional methods

Faecal egg count reduction tests (FECRT) were the first tests developed for anthelmintic evaluation and are the most widely used methods of diagnosis in commercial settings. Aside from controlled efficacy (slaughter) trails, the technique allows for multiple anthelmintic classes to be examined simultaneously (Cabaret and Berrag, 2004; El-Abdellati et al., 2010). Standardised FECRT guidelines have been proposed by Coles (2002) and Coles (2006) however, a there is a wide variation in the literature on the methods for comparisons (El-Abdellati et al., 2010; Levecke et al., 2012; Rendell, 2010). The test provides an estimation on the anthelmintic efficacy through the reduction of FEC counts before and after treatment or against a control. Consensus among studies is that a reduction in FEC less than 95% with a lower 95% confidence interval of 90% is representative of anthelmintic resistance. Carbaret and Berrag (2004) compared individual counts through both geometric and arthritic means in the anticipation of reflecting what was occurring in the herd, rather than influence based on problematic individuals. In general, individual-based counts presented lower FECR efficacy than average counts, leading to discrepancies in the presence of anthelmintic resistance.

The advantage of using FECRT such as the McMaster method is its simplicity, cost-effectiveness, and practical use under field conditions. In contrast, a disadvantage is the potential for some drugs (i.e ivermectin) may temporarily suppress nematode egg laying (Demeler et al., 2010). Most modified McMaster FEC techniques imply a lower detection limit of 50 eggs per gram. Sensitivity of this method can be poor due to lack of ability to detect

resistance below 25% of the population (Martin et al., 1989). FECRT's can only be reliably interpreted when there is a high pre-treatment faecal egg count therefore, when counts are low, a higher analytical sensitivity is required (Coles et al., 2006). FECPAK is a modified McMaster method with a lower detection limit of 30 eggs per gram due to is large initial faecal aliquot (Coles et al., 2006). A study comparing FECPAK (lower detection limit 30 eggs per gram) and mini-FLOTAC (lower detection limit 5 eggs per gram) found better diagnostic performance with mini-FLOTAC in terms of measurement error and precision, with the tendency to under-estimate FEC using FECPAK at densities lower than 500 eggs per gram (Godber et al., 2015).

A study by Levecke *et al* (2012) compared the bias, accuracy, and precision of three FECRTs; modified McMaster (10 eggs/g), Cornell-Wisconsin (1 egg/g) and FLOTAC (1 egg/g). No significant difference regarding bias, accuracy and final efficacy was found between the three methods despite the increased sensitivity and labour output of FLOTAC and Cornell-Wisconsin. Sensitivity in this study to detect the true drug efficacy became sub-optimal when FECs were low. Therefore, a modified McMaster technique with a lower egg detection limit such as 25-33 eggs per gram with less labour intensity would be more appropriate (El-Abdellati et al., 2010), as it uses few resources, easily performed, and can be utilised under field conditions.

1.8.2 Alternative and future techniques

Other diagnostic techniques include controlled slaughter tests and *in vitro* tests such as larval migration inhibition test (LMIT) and larval development test (LDT). The controlled slaughter test is the gold standard for detecting anthelmintic efficacy however, its field ability is impeded by the labour and animal usage expense (Coles et al., 2006). *In vitro* methods are cost-effective, incubating one of more free-living species in a range of drug concentrations (Demeler et al., 2010). For example, the egg hatch assay (EHA) is used for the detection of benzimidazole resistance where nematode eggs are incubated in a solution of thiabendazole. In sheep, a larval

development assay, DrenchRite® (Microbial Screening Technologies, Kemps Creek, NSW, Australia) was developed to determine benzimidazole, levamisole and macrocyclic lactone resistance in gastrointestinal nematodes of sheep and goats by utilising drug-impregnated agar (Demeler et al., 2010). A study by Demeler *et al.* (2010) was able to improve the limited success rate in cattle by implementing an increased well size and higher incubation temperature. Thus far, this method has not become commercially available for use in cattle application. ELISA has been used to measure *O. ostertagi* antibodies since the 1980's. An antibody ELISA on *O. ostertagia* in milk (SVANOVIR® *O.ostertagi*-Ab ELISA, Svanova Biotech Ab, Uppsala, Sweden) was developed and *C. oncophora* ELISAs have been evaluated, showing promising results in calves (Ploeger et al., 1994).

Nemabiome metabarcoding is a recently developed approach which "involves short-read next generation sequencing of the internal transcribed spacer (ITS-2) rDNA amplicons for nematode species identification and relative quantitation" (Avramenko et al., 2015). ITS-2 rDNA is analogous to the 16S rDNA sequencing of bacterial communities; therefore, it was chosen as the target due to the appropriate level of species-specific variation for discrimination (Avramenko et al., 2015; Queiroz et al., 2020). The method was initially developed for cattle GIN species quantification using larval L3 coproculture. ITS-2 rDNA nemabiome metabarcoding allows for large-sample sets, pooling hundreds of samples in a single sequence run, generating thousands to million readings from hundreds or thousands of eggs/larvae per sample. Without the need of species-specific primers, the readings are then compared against a database for species identification (Avramenko et al., 2015; Queiroz et al., 2020). This method presents a copious amount of advantages including, versatility, scalability, specificity, sensitivity and a high cost-efficiency (Avramenko et al., 2015; Queiroz et al., 2020; Redman et al., 2019).

Methods which are available to identify nematode species include coproculture combined with L3 morphology/morphometry, conventional PCR, real-time PCR, digital droplet PCR and pyrosequencing (Queiroz et al., 2020). Multiplex-tandem PCR (MT-PCR) kits are another method available for use in Australia. MT-PCR has been found to be an advanced method of specific diagnosis within cattle GIN with a high sensitivity and specificity (both >90%) in comparison of traditional larval culture (Roeber et al., 2017). Furthermore, the study identified through primer modification, additional kits could be developed for diagnosis of anthelmintic resistance markers in nematode populations.

Pyrosequencing is a recently introduced strategy which offers reproducible, accurate and high-throughput allele-specific single nucleotide polymorphism quantification opportunities. The method uses quantitative detection of light signals formed through multi-enzyme reactions. In comparison to alternative methods such as minisequencing, real-time PCR and MALDI-TOF mass spectrometry, pyrosequencing presented higher precise average allele frequencies for the single nucleotide polymorphisms (von Samson-Himmelstjerna, 2006).

1.9 Alternative methods of anthelmintic usage and gastrointestinal nematode control

1.9.1 Quarantine and combination drench usage

Combination usage of effective drenches has been recommended for improving the sustainability and perseverance of all anthelmintic classes, especially the macrocyclic lactone group (Dobson et al., 2001) and newly marketed anthelmintics (Jackson et al., 2006). As few worms are unlikely to be simultaneously resistant to serval anthelmintics, the rotation and combination use of anthelmintic classes within an enterprise can extend the usability and reduce the development rate of anthelmintic resistance. Additionally, the use of an effective quarantine protocol can reduce the risk of introducing existing resistant nematodes to introduced stock (Dobson et al., 2001; Learmount et al., 2015; Leathwick and Besier, 2014).

Treating imported animals with a highly effective combination of anthelmintic groups (either through multiple or combination drenches), a period of isolation or withholding stock off pasture and the release onto a high contaminated pasture (Dobson et al., 2001; Leathwick and Hosking, 2009; Woodgate and Besier, 2010).

1.9.2 Refugia

The term refugia defines the proportion of parasite populations which is not exposed to a particular control measure, avoiding the selection for resistance (Greer et al., 2020; van Wyk, 2001). The importance of not overlooking refugia and maintaining a source of susceptible parasites is the most important factor concerning anthelmintic resistance development (van Wyk, 2001). Parasites which were not exposed to the last anthelmintic treatment create the refugia subpopulation which arise from three sources: free-living on pasture, inhibited larvae not susceptible to anthelmintics or untreated animals (Kenyon et al., 2009; van Wyk, 2001). The theory of effective refugia in GIN management is the proportion of susceptible alleles will be maintained and therefore will dilute the anthelmintic-resistance alleles from the nematodes which survived treatment, slowing the rate resistance selection (Greer et al., 2020; Kenyon et al., 2009; van Wyk et al., 2006). One of the main challenges regarding refugia is finding the optimal proportion of susceptible nematode population while maintaining acceptable animal performance (Velde et al., 2018).

In general, infective L3 in temperate climates best develop and move onto pasture in the warmer summer months, during periods of rain. In cool-temperate areas, L3 of *Cooperia*, *Trichostongylus* and *Ostertagia* develop in within 1-2 weeks of infected faecal deposition in summer and 6 weeks during winter, but presence can be delayed for several months, persisting on the pasture for up to a year (Charlier et al., 2020; Hutchinson, 2009). Although infective larvae are predisposed to desiccation when ambient temperatures are high, the protection offered by the cattle dung pats allows for larvae to persist. Mortality of larvae in winter is low

and increases further into spring. Dung pats deposited in spring or summer contain a large proportion larvae capable of moving onto pasture after a rain event (Young and Anderson, 1981). In irrigation areas, the movement of larval migration appears earlier despite the lack ideal development conditions.

On dairy farms, larger the proportion of animals requiring anthelmintic treatments to achieve control combined with the lush, productive pastures is expected to produce a large population of parasites which can be utilised for refugia. The intensive drenching regime used in replacement heifers, often with limited diagnosis or monitoring of parasites, constitutes a risk in the development of anthelmintic resistance (Coles, 2002). Consideration should be given to the use of injectable and pour-on macrocyclic lactones. This class of anthelmintic is known for its degree of persistence of activity which can lead to a prolonged advantage to resistant genotypes already established, suppressing the susceptible genotypes for the duration of post-treatment activity (Leathwick and Besier, 2014).

The evaluation of refugia benefits for optimal strategies has mostly been conducted *in silico* (using computer modeling), rather than *in situ* (actual field trials). There are many limitations to computer monitoring however, it provides a timely and useful platform to evaluate strategies for many different environments, highlighting the rate of resistance development reduction can be influenced by factors such as environment and drug efficacy (Cornelius et al., 2016; Greer et al., 2020). There are two main concepts of treatment regime which have been proposed as a way of maintaining refugia. Methods include target treatment (TT) where animals are treated based in a risk assessment of parasitism severity and targeted selective treatment (TST) where treatment is administered to individuals appearing to suffer parasitic consequences or would presumably benefit from treatment (Greer et al., 2020; Leathwick and Besier, 2014; van Wyk et al., 2006). Delaying anthelmintic resistance may not be enough incentive for farmers to engage in refugia-based strategies. Combining strategies such as TST with electronic tagging

allows farmers to identify animals with less resistance for replacement while providing the opportunity to market products produced sustainably the responsible chemical usage (Greer et al., 2020).

1.9.3 Targeted treatment (TT) and targeted selective treatment (TST)

Targeted treatment strategies involved leaving complete groups of animals untreated unless historical and/or epidemiological information suggests an incoming period of high risk for GIN parasitism (Leathwick and Besier, 2014). There have been several examples of successful strategies in sheep such as the movement away from 'summer drenching' of the whole flock to 'summer-autumn drenching' where only susceptible individuals are drenched in the summer period. Support tools in cattle are less common however, Meat and Livestock Australia developed "Cattle Parasites Atlas" as a decision support tool to TT specific to the imputed parameters of the respective herd. The application of bulk tank anti-*O. ostertagia* antibody ELISA has been suggested with a study reporting that treatment based on bulk milk ODR could expect an overall milk yield increase of 0.35kg/cow per day (Sanchez et al., 2005). However, further exploration in commercial pastured based system treatment thresholds and associated production responses is required (Mejia et al., 2011).

Targeted selective treatment involves only treating individuals in a grazing group based on single a or combination of treatment indicators. Indicators can include production parameters (e.g. weight gain, body condition scoring), morbidity parameters (e.g. FAMCHA©, serum pepsinogen concentration) and parasitological parameters (e.g. FEC) (Kenyon et al., 2009; O'Shaughnessy et al., 2014; van Wyk et al., 2006). Several models developed have predicted performance indicators to be an effective TST criteria in sheep and cattle (Berk et al., 2016; Merlin et al., 2017) however, a major drawback is that they can be influenced by additional factors such as nutrition and genotype which need to be considered (Greer et al., 2020).

There are important differences in parasite epidemiology and host-parasite interactions when comparing sheep and cattle, so differences in TST/TT methodology is expected. Within cattle, there is an over-dispersion of parasites arising from a small percentage of hosts due to genetic differences in an individual's ability to mount an immune response to parasitism, through resilience or resistance (Gasbarre, 2014; van Wyk et al., 2006). In small ruminants, performance-based strategies have been effective in reducing selection for anthelmintic resistance (Besier et al., 2010; Kenyon et al., 2013). Kenyon *et al* (2013) compared the anthelmintic efficiency of ivermectin over a five-year period on lamb body weight, drug efficacy, and nematode contamination. Initial efficacy rate was 95-95% in all treatment groups, efficacy declined in the blanket 4-weekly treated group to 62% (CI 55%, 68%) and maintained an average of 86% (CI 81%, 92%) in TST groups. Lambs were only treated if they failed to reach individual target growth rates, though no associated effects on lamb body weight in the TST group was observed. A study involving computer modelling for refugia-based nematode control strategies in Western Australian merino ewes explored a variety of factors such as environment, percentage of untreated flock, FEC, and treatment timing, efficacy, and frequency. Results confirmed that to significantly delay resistance, a proportion of untreated flock could be as low as 10% (Cornelius et al., 2016). Additional results found within low rainfall environments, treatment in autumn rather than summer can effectively delay resistance development.

Swedish studies in first grazing season calves have suggested measurements of midseason (6-8 weeks post-turnout) average daily weight gain (ADG) can identify whether an individual animal should be given a treatment (Hoglund et al., 2009). They reported that ADG at that time provided the best indication of final liveweight, with an optimal treatment threshold of <0.75kg per day at midseason, with a specificity of 0.5 and sensitivity of 0.7. Reductions in both animal performance and anthelmintic use was reported in a subsequent three-year field study where

TST was administered to calves with an ADG poorer than one-quarter of the monthly blanket treatment animals (Hoglund et al., 2013). Reductions of both anthelmintic use by 92% and growth from 0.39 to 0.61kg per day in the blanket group to 0.36 to 0.50kg per day in the TST group. These results were consistent of a New Zealand study on two pasture-based farm which set ADG targets using breed-specific predetermined live weight gains, ranging in winter and summer from 0.30 to 0.68kg per day (Greer et al., 2010). Compared with the monthly blanket treatment group, on each farm, the number of treatments administered to TST animals decreased by 84% and 65% respectively. They reported that there was no difference in mean calf live weight between treatment groups, however, mean cumulative weight was reduced by 6% in TST animals. Overall, the use of ADG as a decision criterion seems to provide a means of reducing anthelmintic usage with only a small compromise in calf growth regarding parasite control in first season grazing dairy calves. Recent studies have looked at TST methods through implementing parasitological indicators such as FEC and pepsinogen thresholds (Anderson, 1977; O'Shaughnessy et al., 2015a, b; O'Shaughnessy et al., 2014). In each study there was no difference in live weight gain and O'Shaughnessy *et al* (2015a) reported a 50 % reduction in anthelmintic usage with 1.5 treatments required per calf.

Berk *et al* (2016) used computer modelling to compare consequences and identify sustainable and effective methods of TST. Indicators which were evaluated were FEC, plasma pepsinogen, ADG, combined FEC and plasma pepsinogen against random individual selections. The model assessed success in terms of benefit per R (BPR) where "the ratio of average benefit in weight gain to change in frequency of resistance alleles R (relative to an untreated population)" (Berk et al., 2016). For fixed treated calf percentages in terms of BPR, plasma pepsinogen was the optimal indicator with ADG being the worst. The study reported significant support of TST with all simulated TST regimens improving weight gain and most measures of parasitism at 3,

8- and 13-weeks post-turnout. The developed simulation model appears to be capable of predicting the consequences of TST methods amongst calf populations.

1.9.4 Breeding and genetic manipulation

Growth and reproduction traits are in general correlated, and frequently integrated into breeding programs for livestock (Abreu et al., 2018; Ribeiro et al., 2021; Santana Jr et al., 2018). Several studies have explored the ideas of breeding and identifying genes, models can quantify different traits. One study breed line of sheep and parasites to test for reproductive fitness over 30 generations within *H. contortus* and *T. colubriformis*. After 14 of the planned 30 generations, there was no significant interaction between sheep and parasite lines, indicating that worms passaged in resistant sheep are no more successful in reinfecting sheep than worms passaged through susceptible sheep (Woolaston et al., 1992). Heritability for infection resistance is moderate (h2=0.23-0.44), like other traits such as fleece weight, FEC and weight gain. After 10 years of selective breeding, sheep resistant to *T. colubriformis* showed sufficient refractoriness to infection, no longer needing anthelmintic treatment (Barger, 1993). This resistance appears to be evident in periparturient ewes, young and adult lambs (Woolaston et al., 1992). Reliance is a less heritable trait than resistance but appears to be correlated positively genetically, allowing for concentrated breeding on resistance. Furthermore, as the assessment for reduced treatment cost is subjective, the heritability is low (h2=0.05-0.14) (Woolaston and Baker, 1996).

A recent study in beef cattle identified several genes which could become functional candidate genes (FCG) for production and parasite resistance (Ribeiro et al., 2021). Examples of these genes include: the DUSP10 gene (dual specificity phosphatase 10) on chromosome 16 on chromosome 20, MAP3K1 (Mitogen-activated protein kinase 1) was found to be a FCG for parasite burden and growth traits of cattle. The study concluded there were several FCG (SLC16A4, KCNA2, LAMTOR5, DUSP10, MAP3K1, TPMT, and KIF13A) which control

and correlate to the genetic values of parasite burden, growth and productive traits in beef cattle.

1.9.5 Plant-based anthelmintics

Medicinal plants can prove to be a promising and sustainable alternative to the use of synthetic drugs. Medicinal plants offer a lower toxicity and higher biodegradability in comparison while offering an organic option of parasite control. A variety of studies have been undertaken in equids, proving significant anthelmintic effect within the respective plant's tests. One study in Iran presented the anthelmintic activity of *Trachyspermum ammi* on GIN in vivo. The results showed anthelmintic activity with increasing dose, in both extract and powder forms (Imani-Baran et al., 2020). The effects of *T. ammi* could be related to its highly abundant compound thymol. Another study in equids using crude extracts of plants from both Ethiopia and the UK, serially diluted and screened of anthelmintic activity using egg hatch test and larval inhibition test (Peachey et al., 2015). Three of the five extracts from Ethiopia and all four UK extracts showed significant anthelmintic activity. An *In vitro* anthelmintic activity of five medicinal plant crude extracts against egg-hatching land larval development on *Haemonchus contortus*, found that all aqueous and hydro-alcoholic extracts presented statistically significant dose dependent egg hatching inhibition. Furthermore, most of the plants showed larval development inhibition (Eguale et al., 2011). The overall findings of these studies demonstrate the potential use of medicinal plants for anthelmintic control, further evaluation and testing of these plants will be imperative.

1.9.6 Biological control

There is evidence to suggest that dung beetles are capable of significant reductions in the number of free-living stages of parasites, through mechanical damage to the dung pats during feeding, brood ball production and deep burial of dung, preventing nematode migration to the soil surface (Forgie et al., 2018; Waller and Faedo, 1996). A study in New Zealand found that

infective L3 larvae recovery from foliage across the three trials varied considerably however, dung beetle activity reduced overall nematode numbers around the dung pats by 71% (Forgie et al., 2018). Similar results were recovered from an Australian study within the Southern Tablelands of NSW in bovine nematode ecology. Larval recoveries from pasture were decreased during periods of high dung beetle activity, attributed to the native species *O. granulatus* and *O. australis (Waller and Faedo, 1996)*. Dispersal activity of dung beetles can be liable due to the dependence of optimal weather conditions, adversely affecting the ability to utilising these organisms for reliable, cost-effective control. In cool, moist regions, earthworms take over the main role of dung beetles for dung degradation (Waller, 2006). In Northern Europe, earthworms play an important role, responsible for significant reduction of infective L3 larvae and removal of cattle faeces from pastures (Grønvold et al., 1996).

Another form of biological control is the use of nematophagous fungi which entrap the free-living stages of nematodes found in faeces (Coop and Kyriazakis, 2001). There exists a large array of fungi including, endoparasitic fungi, fungi that invade nematode eggs, fungi that produced metabolites toxic to nematodes and predacious fungi (Waller and Larson 1993). Various studies have been conducted using the nematode-destroying microfungus, *Duddingtonia flagrans* (eg. BioWorma® and Livamol with BioWorma®). This species has three important attributes, making it a potentially viable methods of biological control. The species has the ability of survive gut passage, predisposition for rapid growth in fresh dung pats and processes a voracious nematophagous capacity (Waller, 2006; Waller and Faedo, 1996). Under laboratory conditions, where monocultured fungal isolates on nutrient poor media and provided with nematode prey with cannot escape, can produce results where all nematodes are captured and killed within hours (Waller and Faedo, 1996). However, efficacy is only reached when the chlamydospores (resting spores) are fed in a frequency of at least every second or third day. Additionally, efficacy in cattle was impaired when rainfall caused

degradation of dung pats, coinciding with high FEC counts (Charlier et al., 2018). The above-mentioned control methods will never be a complete substitute for anthelmintic usage however, they should be incorporated into integrated management systems or programs to provide sustainable and efficient nematode control in livestock.

Chapter 2. Prevalence, burden and anthelmintic resistance of gastrointestinal nematodes in the south west region of Western Australia dairy herds

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2.1 Abstract

Anthelmintic resistance in gastrointestinal nematodes (GIN) of dairy cattle is of global importance. The objective of this study was to determine the prevalence of GIN among post-weaned replacement heifers and bull calves aged between 4 - 12 months old in Western Australia dairy farms and quantify the level of anthelmintic resistance. A secondary objective of this study was to explore pooling faecal samples for cost effective diagnostic purposes of faecal egg counts (FECs). Pre-treatment FECs were monitored on 14 dairy farms, anthelmintic resistance was assessed on 11 of the farms based on FEC of ≥500 eggs per gram (epg) in at least 10 - 15% of the samples. Control FECs were compared with anthelmintic FECs at 14 days post-treatment with doramectin (injectable), levamisole (oral), fenbendazole (oral) and, a levamisole/abamectin combination (Eclipse® combination pour-on). The results demonstrate a high level of anthelmintic resistance, with at least one class of anthelmintic failing to achieve a 95% reduction in FEC in one or more GIN species. Doramectin was fully effective against *Ostertagia*, but *C. oncophora* displayed resistance to it on 91% of the farms. Conversely, levamisole was fully effective against *C. oncophora*, but *Ostertagia* displayed resistance in 80% of the farms. Fenbendazole resistance was present in both *C. onocphora* and *Ostertagia* in 64% and 70% of the farms respectively. *Trichostrongylus* showed low resistance, only occurring in doramectin (14%) and levamisole/abamectin combination (14%) on the farms sampled. A high level of correlation between pooled groups of 5, 10 and 20 samples was recorded (R=0.947, 0.987, 0.972 and *P*=0.015, 0.002, and 0.006) respectively. This study confirms that anthelmintic resistance within Western Australian dairy farms is common and regular faecal egg count reduction testing is recommended to monitor and guide decision-making for appropriate anthelmintic usage. Utilisation of pooled FECs provides a potential cost-effective method for farmers to regularly monitor FECs.

Keywords: Anthelmintics; dairy calves; doramectin; fenbendazole; levamisole; macrocyclic lactones

2.2 Introduction

Trichostrongylus axei, *Ostertagia ostertagi* and *Cooperia oncophora* are the predominant and important gastrointestinal nematodes (GIN) of cattle (Berghen et al., 1993; Bullen et al., 2016; Waghorn et al., 2006a). Among these, *O. ostertagia* is the most pathogenic species, characterised by severe burdens in the first season of grazing (FSG) among calves, causing type I Ostertagiosis. This manifests with marked weight loss, profuse watery diarrhea, inappetence and mortality (Charlier et al., 2020; Hoglund et al., 2009; Myers and Taylor, 1989). Type II Ostertagiosis is related to a high emergence of inhibited larvae with clinical signs identical to type I disease in older calves and adult cattle, with a primary clinical sign of inappetence in less severely infected animals (Kaplan, 2020; Myers and Taylor, 1989). This may manifest as acute outbreaks due to larval emergence or as chronic cases (Berghen et al., 1993; Myers and Taylor, 1989; Rinaldi and Geldhof, 2012). Despite being less harmful than *T. axei* and *O. ostertagi*, *Cooperia* species have been associated with increased production losses such as marked weight loss in young stock (Leathwick and Besier, 2014), clinical disease in adult cattle (Lyndal-Murphy et al., 2010) and as parasitic gastroenteritis (PGE) due to mixed infections with *O. ostertagi* (Charlier et al., 2020).

In cattle production systems, the major risk factors for GIN parasitism includes parasite characteristics (fecundity, hypobiotic larvae, transmission, morphology), host factors (genetic resistance, physiological status, immune immunity), and environmental factors (nutrition, husbandry practices, management, climate) (Navarre, 2020; Odoi et al., 2007; Zulfikar et al., 2019). FSG calves are at major risk as they are the most susceptible to clinical disease due to an underdeveloped immune system (Charlier et al., 2009; Navarre, 2020), especially when

raised on permanent pastures (Leathwick and Besier, 2014). GIN species with increased fecundity and hypobotic capabilities that are able to survive unfavorable environmental and host conditions, allows accelerated infection rates and the successful dissemination of resistant alleles to subsequent GIN generations (Charlier et al., 2020; Demeler et al., 2009). Animal husbandry and management practices that can cause an increase in anthelmintic resistance include early weaning onto a pasture-based diet (Bullen et al., 2016), failure to provide quarantine treatments in new stock (Dobson et al., 2001; Leathwick and Hosking, 2009; Woodgate and Besier, 2010) and intensive anthelmintic treatment regimens (Coles, 2002; Jackson et al., 2006).

Anthelmintic resistance is defined as being present when, "there is a greater frequency of individuals within a population that are able to tolerate doses of a compound than in a normal population of the same species and is heritable" (Prichard et al., 1980). Several studies have reported anthelmintic resistance both globally (Edmonds et al., 2010; Stafford and Coles, 1999; Suarez and Cristel, 2007) and within New Zealand and Australia (Cotter et al., 2015; Waghorn et al., 2006b). In Australia, a recent study in the eastern states reported anthelmintic resistance in 20 commercial dairy farms among replacement heifers (Bullen et al., 2016). Anthelmintic resistance was detected against doramectin, levamisole and fenbendazole anthelmintics.

There is limited information on the anthelmintic resistance profiles in the south west region of Western Australia dairy farms. Dairy farming in Western Australia is characterized by a predominantly pasture-production based system under Mediterranean conditions characterised by hot summers and relatively mild winter temperatures (Kassam et al., 2012). Data and information on anthelmintic resistance profiles will provide evidence-based medicine management strategies (Jack et al., 2017; Learmount et al., 2015; Velde et al., 2018). The primary objective of this study was to determine the prevalence of GIN among post-weaned

replacement heifers and bull calves aged between 4 - 12 months old in Western Australia dairy farms and quantify the level of anthelmintic resistance. A secondary objective was to explore the viability of pooling faecal samples for cost effective diagnostic purposes of FECs.

2.3 Materials and Methods

2.3.1 Study area and approval

The study was conducted in the south west region of Western Australia. The region has a temperate Mediterranean climate with an annual rainfall of approximately 730 mm. The study was conducted between June and December 2020 in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The study was approved by the Animal and Human Ethics Committees of Murdoch University (Approval No. R3213/20 and 2020/006 respectively).

2.3.2 Study design and sampling

A convenience sample of 14 dairy herds were included in the study with a total of 1271 animals. The selection criteria for inclusion into the study was calf availability, good animal identification methods, physical restraint facilities and willingness to participate in the study. On each farm, approximately 75 - 100 post-weaned replacement heifer and bull calves aged between 4 - 12 months old were considered and enrolled into the study. A total of 11 farms were enrolled into the second part of the trial which involved assessing for anthelmintic resistance based on FEC of ≥500 eggs per gram (epg) in at least 10 - 15% of the samples. The secondary visit was conducted 10 - 14 days post-anthelmintic treatment, and thereafter, faecal samples were analysed for FECs, larval culture and differentiation and anthelmintic resistance quantification.

A questionnaire template (**Appendix 1**) was used to capture farm data and anthelmintic management strategies such as pasture management, frequency, type and decisions on

anthelmintic use.

2.3.3 General data collection

A minimum of two farm visits were conducted for each farm sampled. The activities included collection of faecal samples, estimation of body weights and allocation of the sampled individuals into four respective anthelmintic treatment groups and a control group. Faecal samples from the calves on the initial farm visit were used to determine individual FEC load whereas faecal samples from the second visit were used to determine faecal egg count reduction (FECR), larval culture, and the quantification of anthelmintic resistance. Briefly, faecal samples were collected directly from the rectum, animals weighed, and assigned into the respective treatment groups. Samples were stored at 4ºC until processing to prevent faecal worm eggs from further development and hatching. Calves were evenly spilt into groups (15 minimum per group) and weighed using an electronic cattle scale (W110 Livestock Weighing System, Gallagher Group Limited) and allocated into one of five treatment groups. These were (1) untreated controls; (2) ML (doramectin) 0.2 mg/kg SC (Dectomax®, Zoetis Australia); (3) BZ (fenbendazole) 7.5 mg/kg Oral (Panacur 100®, MSD Animal Health Australia); (4) LV (levamisole hydrochloride) 8 mg/kg Oral (Nilverm LV®, MSD Animal Health Australia); and (5) LV/ML (10 mg/kg levamisole, 0.5 mg/kg abamectin) 1ml/20kg (Eclipse Combination Pour-on®, Boehringer Ingelheim).

2.3.4 Laboratory analysis

2.3.4.1 FEC, larval differentiation and quantification of anthelmintic resistance

Initial FEC samples were examined using the Modified McMaster Technique (Hutchinson, 2009). Slides were observed under a light microscope at 10x magnification and all faecal worm eggs were recorded. All FECs were performed by a single operator using 2g of faeces in 60ml of saturated sodium chloride (NaCl), where 2 chambers were counted, and one egg equated to 50 epg. *Strongylodies*, *Strongyle* and *Nematodirus* species were pooled into a single count for

total farm FEC. Post-anthelmintic treatment faecal samples were submitted to the Department of Primary Industries and Regional Development (DPIRD) for larval cultures, differentiation and anthelmintic resistance quantification.

2.3.4.2 Estimation of herd FEC using pooled samples

Five farms had a secondary analysis of the initial samples comparing FEC from individual animals to pooled samples. Two grams from each individual sample were homogenized into the respective groups before processing as per initial individual FECs. Pooled groups of 5, 10, 20 and 40 samples were conducted with the respective arithmetic mean compared to the individual FEC.

2.3.5 Statistical data analysis

Data were analysed using SPSS Statistics software version 22.0, 2013 (SPSS Inc., Chicago 111). FECR was calculated by comparing the post-treatment arithmetic mean FECs, 100 (1-[$\bar{x}t/\bar{x}c$]) where $\bar{x}$ is the mean, *t* is the treated group FEC and *c* is the control group (Coles et al., 1992). Anthelmintic resistance was defined as <95% reduction in FEC with a lower 95% confidence interval (CI) of <90% (Coles et al., 2006). Descriptive statistics were generated and thereafter tests of associations between body weight and FEC performed. Paired t-test was used to assess the correlations and mean difference between individual and pooled FEC counts.

2.4 Results

2.4.1 General descriptions

A total of 1271 animals from 14 dairy herds were sampled. The median age of animals sampled was 6 months (range, 4 - 11 months). A total of 68% animals were ≤ 6 months and 32% ≥ 6 months of age. The distribution of females to males was 62.5% (794/1271), and 37.5% (477/1271) respectively. Animal body weight had a median weight of 152kg (range, 50 - 430kg) across all individuals sampled **Figure 1**.

2.4.2 FEC and larval differentiation

The median FEC count across all farms was 100 epg (range, 0 – 6700 epg) (**Table 4**). A total of 38% (489/1271) animals recorded a FEC of zero. There was a significant difference in FEC between the farms, $t(13) = 364.22$, $P=0.0001$. The larval differentiation was 60% *Cooperia oncophora*, 25% *Ostertagia*, 8% other *Cooperia* species, 5% *Trichostrongylus*, and 2% *Haemonchus* respectively.

2.4.3 Anthelmintic resistance

Anthelmintic resistance was highest within the doramectin class 91% (10/11) of the farms, followed by the fenbendazole class in 80% (8/10) of the farms. Anthelmintic resistance was lowest in the levamisole class in 10% (1/10) of the farms, followed by the levamisole/abamectin combination in 31% (4/11) of farms. Average overall FECR was highest in levamisole with a reduction of 96%, followed by levamisole/abamectin combination of 83% overall reduction. The lowest overall FECR was present in doramectin with a 59% reduction, followed by an overall reduction of 64% in fenbendazole. A summary of anthelmintic resistance within each farm is outlined in **Table 1**.

C. oncophora showed highest resistance against doramectin on 91% (10/11) of the farms, and to fenbendazole on 64% (7/11) of the farms. *Ostertagia* showed highest resistance to fenbendazole on 80% (8/10) of the farms and in 70% (7/10) of the farms to levamisole. *Trichostrongylus* showed resistance, in doramectin and levamisole/abamectin combination in 14% (1/7) of the farms. The proportion of resistance for each anthelmintic at a species level is outlined in **Table 2**.

2.4.4 Estimation of herd FEC using pooled samples and other associations

There was a high correlation on the individual FEC with the pooled samples of 5 (R=0.947, $P=0.015$), 10 (R=0.987, $P=0.005$) and 20 (R=0.972, $P=0.006$). There was moderate correlation between FEC from individual samples and the pooled group of 40 feacal samples (R=0.258,

P=0.676) (**Table 3**). There was a negative correlation between FEC and body weight (R= -0.119, *P*=0.0001).

2.4.5 Questionnaire

A total of 12 out of 14 questionnaires were completed **(Tables 5, 6)**. All farms utilised rotational grazing. Of these, only 42% (5/12) treated their animals with anthelmintics prior to moving their stock on to another paddock. A total of 92% (11/12) of the farms used anthelmintics. Of the farms which used anthelmintics, only 50% (6/12) used animal quarantine when treating new stock. All farms reported using macrocyclic lactones or combination anthelmintics on their cattle.

The methods utilised for estimating stock weight at treatment varied amongst farms. No farms reported using weighing scales to determine the weight. Of the farms, 66% (8/12) reported treating their weaners twice a year. One farm reported treating weaners with anthelmintics once per year and the remaining two reported treating three and four times a year. Previous problems regarding anthelmintic resistance were reported from one farm. Cattle death due to GIN burden was reported on 25% (3/12) of the farms.

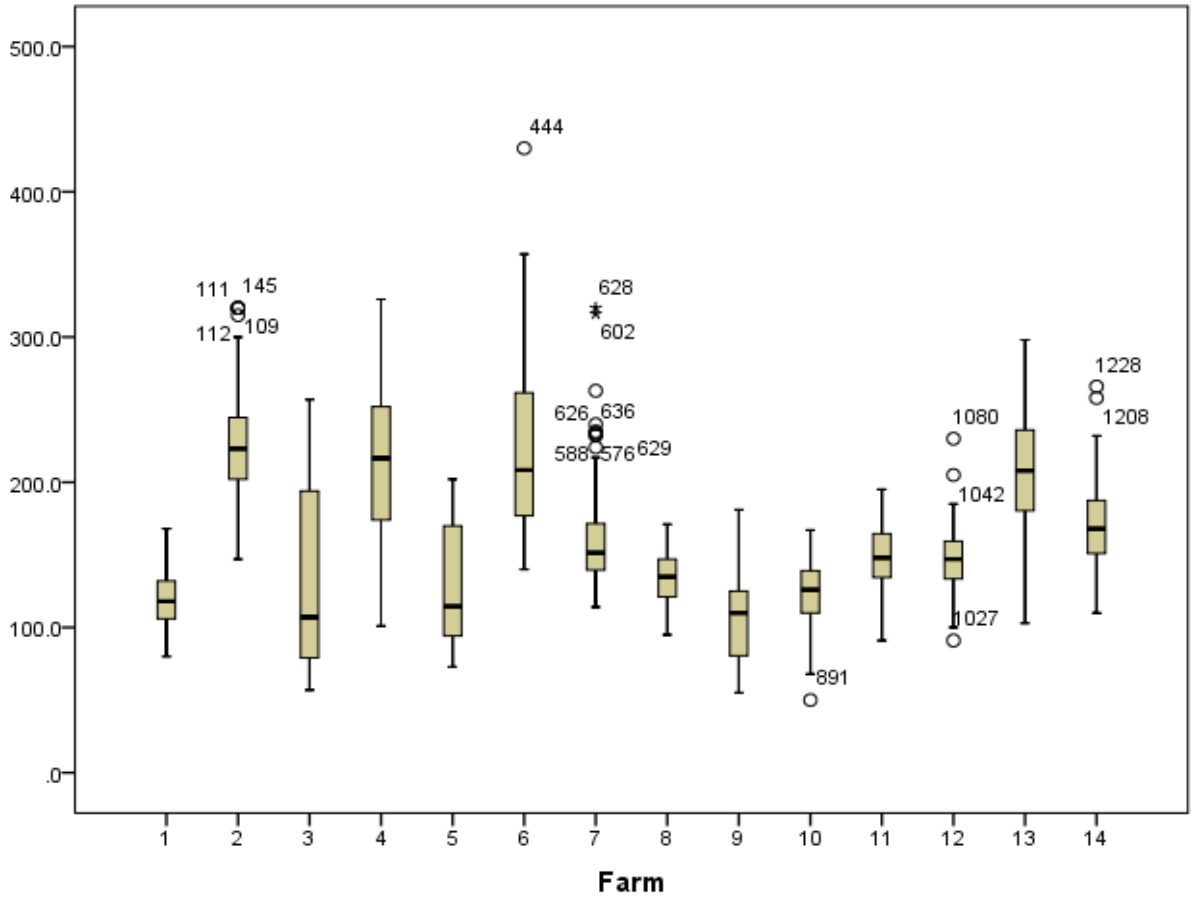

Figure 1: The distribution of median body weight for animals aged between 4 – 12 months, sampled across 14 dairy farms in the south west region of Western Australia between June – December 2020. Values displayed represent the animal identification number with FECs that was either a high or low outlier.

Table 1: Percentage reductions in strongyle species for each anthelmintic group tested on 11 dairy farms in the south west region of Western Australia between June – December 2020.

	Doramectin	Levamisole/Abamectin	Levamisole	Fenbendazole
Farm no.	FECR (%)	FECR (%)	FECR (%)	FECR (%)
2	15	44	95	-50
3	32	55		
5	90	98	95	42
6	76	96	97	98
7	89	99	99	96
8	77	30	90	65
10	46	100	96	86
11	-1	100	100	83
12	94	92	99	94
13	99	99	97	92
14	35	98	96	35

Table 2: Proportion of properties with anthelmintic resistance (<95% faecal egg count reduction) at species level across 11 farms in the south west region of Western Australia between June – December 2020.

Active Ingredient	*Ostertagia*	*Trichostrongylus*	*Cooperia oncophora*	*Cooperia* spp.	At least one spp.
Doramectin (ML)	0/10 (0%)	1/7 (14%)	10/11 (91%)	7/8 (88%)	10/11 (91%)
Abamectin/ Levamisole (ML/LV)	5/10 (50%)	1/7(14%)	4/11 (36%)	3/8 (38%)	6/11 (55%)
Levamisole (LV)	7/10 (70%)	0/7 (0%)	0/11 (0%)	0/8 (0%)	7/10 (70%)
Fenbendazole (BZ)	8/10 (80%)	0/7 (0%)	7/11 (64%)	6/8 (75%)	9/10 (90%)

Table 3: Summary of paired t-test results comparing individual faecal egg count means with respective pooled sample groups (G) of 5, 10, 20 and 40, including 95% confidence intervals, correlations (R) value and significance (P <0.05).

		Mean	95% Confidence Intervals	Correlation (R)	Sig. (p-value)
Pair 1	Individual	367	261, 475	0.947	0.015
	G5	377	286, 470		
Pair 2	Individual	367	261, 475	0.987	0.002
	G10	398	281, 516		
Pair 3	Individual	367	261, 475	0.972	0.006
	G20	380	237, 523		
Pair 4	Individual	367	261, 475	0.258	0.676
	G40	380	322, 438		

Table 4: Faecal egg count (FEC) descriptive results across 14 dairy farms sampled in the south west region of Western Australia between June – December 2020.

Farm	Median FEC*	Minimum FEC	Maximum FEC
1	0	0	300
2	100	0	5000
3	700	0	4700
4	0	0	400
5	100	0	2900
6	200	0	3300
7	100	0	2800
8	1250	100	6700
9	0	0	400
10	300	0	5300
11	100	0	1000
12	400	0	3400
13	100	0	2500
14	100	0	1100

*Median FEC was used as FEC failed to fit within a normal distribution

Table 5: Herd characteristics and pasture management practices of dairy farms sampled in the south west region of Western Australia.

	Dairy Enterprise							Pasture Management						
Farm	Herd Size	Calving Pattern	Predominant Breed	Grazing	BJD Strategy[a]	Introduced Stock	Bio-security	Rotational Grazing	Rest Period	Seasonal Differ?	Rest Period	Drench Period[d]	Cattle Death due to GIN	Experienced Anthelmintic Resistance
1	700	Split	Holstein-Friesians	Both	Yes	No	Yes	Yes	Yes	Yes	Season dependent	Prior	No	No
2	350	Split	Holstein-Friesians	Dry	Yes	Not Often	Yes	Yes	Yes	Yes		Prior	No	No
3	95	Year Round	Aussie Red	Dry	No	No	Yes	Yes	Yes	Yes	17-35 days	Variable	No	No
4	600	Year Round	Crossbreed	Both		Yes[b]	Yes	Yes	Yes	Yes	Season dependent	Other	No	No
5	180	Year Round	Holstein-Friesians	Both	Yes	No	Yes	Yes	Yes	Yes	Season dependent		No	No
6	300	Year Round	Holstein-Friesians	Dry	Yes	Yes[b]	Yes	Yes	Yes	No			No	Yes
7	360	Year Round	Holstein-Friesians	Both	Yes	Sires[c]	Yes	Yes	Yes	Yes	3-6 weeks	Prior	No	No
8	570	Split	Crossbreed	Both	Yes	Sires[c]	No	Yes	Yes	Yes	Leaf emergence Rate	Variable	Yes	No
10	3200	Split	Crossbreed	Both	No	Sires[c]	No	Yes	Yes	Yes	20-40 days	Prior	Unsure	Unsure
11	150-300	Spring	Holstein-Friesians	Both	Yes	Yes[b]		Yes	Yes	Yes	Variable	Variable	Yes	No
12	550	Split	Holstein-Friesians	Both	Yes	No	Yes	Yes	Yes	Yes		Prior	No	Unsure
13	830	Split	Crossbreed	Dry		No		Yes	Yes	No			Yes	No

[a] Implement a Bovine Johne's Disease (BJD) strategy where calves are isolated from adult cattle for the first 12 months of age

[b] Yes: >10% of stock is introduced to the property

[c] Sires: bulls were only introduced cattle to the property

[d] Drenching period for cattle regarding moving pastures

Table 6: Anthelmintic usage and worm control practices of dairy farms sampled in the south west region of Western Australia

	Anthelmintic Usage and Worm Control							
						Annual Anthelmintic Treatment Frequency		
Farm	Quarantine Drench	Anthelmintic Class	Estimate Weight at Treatment	Estimation Method	Treated Group	Weaners (0-12 months)	Heifers (12-24 months)	Milking Herd (>24 months)
1	Yes	ML	Yes	Herd Average	Individual groups	2	2	1
2	No	ML	Yes	Guess Individual Weight	Individual groups	2	1	Individuals
3	No		Yes	Guess Individual Weight	Individual groups	2	1	1
4	Yes	ML	Yes	Overestimating	Individual groups	2	4	2
5	Yes	ML	No		Individual groups	1	1	1
6	Yes	ML	Yes	Guess Individual Weight	Select Individuals	2	1	Individuals
7	Yes	ML	Yes	Guess Individual Weight	Individual groups	2	1	1
8	No	ML	Yes	Herd Average	Individual groups	2-3	1-2	0
10	No		Yes	Overestimating		3	1	0
11	No	ML	Yes	Overestimating	Individual groups	2	1	0
12	Yes	Combination and ML	Yes	Heaviest cow's weight	Individual groups	4	3	0
13	This section was left blank as enterprise does not use anthelmintic treatment and relies on rotational grazing							

2.5 Discussion

The primary objective of this study was to determine the prevalence of GIN among weaned replacement heifers and bull calves aged between 4 - 12 months in Western Australia dairy farms and to quantify the level of anthelmintic resistance. A secondary objective was to explore the viability of pooling faecal samples for cost effective diagnostic purposes. The results of this investigation clearly indicate a high level of anthelmintic resistance, with at least one class of anthelmintic failing to achieve a 95% reduction in FEC in one or more GIN species per farm.

C. oncophora was the most prevalent species with significant anthelmintic resistance to doramectin. The finding of resistance to doramectin by injection in *C. oncophora* in 91% of the farms is consistent with prevalence figures reported in previous studies (Bullen et al., 2016; Rendell, 2010; Waghorn et al., 2006a) confirming predisposition for macrocyclic lactone resistance in this genus. The level of resistance in this survey is concerning as all farms reported use of macrocyclic lactones in the treatment of stock. The lack of production impact recognised by farmers could be attributed to the prevalence of *C. oncophora*, generally considered of low pathogenicity (Gibbs and Herd, 1986), though high numbers of parasitism can result in clinical disease and significant production losses. A recent New Zealand report cited a liveweight loss of 14kg in calves at 12 months of age has been associated to resistant *C. oncophora* (Sutherland and Leathwick, 2011). In addition, a study examining the effects of experimental infections of macrocyclic lactones resistant *C. punctata* in steers reported a decrease in liveweight gain of 7.5% (P = 0.02), and a reduction in dry matter intake of 680 grams per day (P = 0.02) (Stromberg et al., 2012).

Resistance to macrocyclic lactones in *Ostertagia* was not detected on any of the farms tested, although resistance has been detected in Victorian cattle (Bullen et al., 2016; Rendell, 2010). Cotter *et al* (2015) reported a lower efficacy of macrocyclic lactones in *Ostertagia* through

pour-on formulations where 25% of the farms tested had <95% FECR at day 14, compared to the injectable formulation which was fully effective. Additionally, within *C. oncophora*, there was little difference between injectable and pour-on at day 14, with mean reductions of 85% and 93%, respectively. Prevalence of resistance in *Ostertagia* towards macrocyclic lactones has not been reported in Western Australia. However, with the popularity of pour-on formulations, there is a risk of resistance.

To the authors knowledge this is the first report of fenbendazole resistance within *Cooperia* in Western Australian dairy cattle. Resistance to fenbendazole was common in this study with 80% of farms failing to achieve ≥95% FECR. Resistance was highest in *Ostertagia* (80%) and *C. oncophora* (64%). Previous resistance has been reported to *Ostertagia* and *C. concophora* (Bullen et al., 2016; Cotter et al., 2015; Rendell, 2010). Unfortunately, there is little information regarding the management of anthelmintic resistance in cattle (Sutherland and Leathwick, 2011). A study in New Zealand beef cattle reported rare benzimidazole usage, occurring in combination with either levamisole or macrocyclic lactones (Jackson et al., 2006). Similar results were reported in this study, where no farmers reported use of benzimidazoles in their properties. These results suggest that before further usage, fenbendazole efficacy should be tested within the property or used in combination with additional anthelmintics for broad-spectrum coverage.

Levamisole remained highly effective against nematodes of cattle, with resistance only found within *Ostertagia* species in this study. However, reduced efficacy in *Ostertagia* could be attributed to the rapid replacement of adults by larval stages during treatment intervals where there was failure to remove inhibited and developing larvae (Anderson, 1977; Williams et al., 1991).

The mixed nature of worm infection in all cattle herds and the contrasting efficacies of anthelmintics reported in this study creates challenges for effective worm control. Levamisole was highly effective in the control of *Cooperia* species, yet performed poorly against *Ostertagia.* Macrocylic lactones, on the other hand, had high efficacy against *Ostertagia*, but performed poorly to control *Cooperia*. A combination anthelmintic is likely to be most effective (Soutello et al., 2007; Waghorn et al., 2006a) to both maintain animal health and keep resistant genes as scarce as possible (Dobson et al., 2001). Adopting combination treatments prior to development of resistance is important to maintain their efficacy (Leathwick et al., 2012). Within Australia, there are currently several different registered combination anthelmintics for use in cattle, including Trifecta® (levamisole/abamectin/oxfendazole MSD Animal Health Australia), Eclipse® (levamisole/abamectin, Boehringer Ingelheim), and Cydectin Platinum® (moxidectin/levamisole, Virbac), but their use by farmers is very low, especially compared to New Zealand farmers.

In this study, the levamisole/abamectin combination on 69% of the farms produced a high ≥95% FECR. However, on some farms the FECR was less than levamisole alone. These results are significantly different to previous studies, where a levamisole/abamectin combination was fully effective against GIN (Leathwick et al., 2016; Rendell, 2010). The decreased efficacy could be as a result of inaccurate doses of anthelmintics due to the effect of weather conditions on anthelmintic performance (Forsyth et al., 1983; Sargent et al., 2009) and potential licking behaviour (Bousquet-Melou et al., 2004; Leathwick and Miller, 2013). Furthermore, outside of an experimental setting, inaccurate dosages can also be attributed to farmers using unconventional methods of estimating cattle weight instead of calibrated scales to determine the appropriate dosage.

Sustainable control of GIN requires additional strategies to just anthelmintics. Refugia is a key asset in the sustainable control of GIN and viability of future anthelmintic treatments (Leathwick and Besier, 2014; van Wyk, 2001). Pasture contaminated with susceptible GIN larvae from free-living stages or untreated animals form a prime source of refugia, allowing for a decreased rate of resistance development providing there is not heavy anthelmintic reliance (Coles, 2002; Navarre, 2020; van Wyk, 2001). The lack of anthelmintic usage and reliance on grazing management and refugia for GIN control was reported on one farm within this study, with resistance only evident in the fenbendazole group (FECR 92%). Reliance on refugia and grazing management is a key factor in minimising the development of resistance without compromising stock production.

The challenge exists in finding the optimal proportion of refugia to minimise anthelmintic resistance development, whilst maintaining animal performance. Two approaches are considered to optimise anthelmintic treatments (Kenyon and Jackson, 2012); targeted treatment (TT; whole groups treated after diagnostic information) and targeted selective treatment (TST; selected individuals treated within a group based on individuals diagnostic information). TST approaches have shown to be effective in sheep using various criteria for selection of individual treatment, including liveweight or liveweight gain (Leathwick et al., 2006a; Leathwick et al., 2006b; Stafford et al., 2009). The Happy Factor™ TST utilises individual animal weight predictions to determine required treatment, based on single animal failures to reach a predicted weight threshold (McBean et al., 2021). This method of TST has shown to slow the development of resistance (Greer et al., 2009; Kenyon et al., 2013), where the standard threshold is transferable between farms, allowing for refinement using local data in cases where farm and animal specific characteristics are required (McBean et al., 2021). Other individual-animal treatment decisions such as "FAMCHA" in the control of *H. contortus* have also proved feasible (Kenyon et al., 2013; van Wyk et al., 2006). Attempts of implementing TST concepts

for cattle have been made (Greer et al., 2010; Hoglund et al., 2013) with studies showing substantial reduction in anthelmintic treatments, however small production losses have been associated to the TST.

It is recommended that farmers conduct regular FECRT to assess the efficacy of anthelmintics used on their farms, however the test is seldom used as most producers have not perceived resistance on their farm and the expense of conducting FECRT is seen as uneconomical (George et al., 2017). Reducing the cost of FECRT may facilitate an increase in testing (Rinaldi et al., 2014). Results from this study found a high level of correlation between pooled groups of 5, 10 and 20 samples but not within pooled group of 40 samples. Several studies have also reported a high correlation and substantial level of agreement in FEC and FECR between individual and pooled sampling methods in both sheep (Rinaldi et al., 2014) and cattle (George et al., 2017; Rinaldi et al., 2019), confirming the validity of pooled sampling. Furthermore, George *et al* (2017) reported a reduction in the number of samples to evaluate FEC or anthelmintic efficacy by 79.2%, significantly reducing the expense of testing. Therefore, pooled sampling can significantly reduce the cost and labour associated with FECRT.

2.6 Conclusion

The results of the current study revealed anthelmintic resistance in the major species of cattle to all available anthelmintics is widespread in dairy farms of south west Western Australia. Furthermore, pooled FEC could prove as a practical, cost-effective method for farmers to monitor FECs, as routine FECR testing is recommended to guide decision-making of appropriate anthelmintics with adequate efficacy and optimal productivity on farm.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

This project was jointly funded by Western Dairy, Dairy Australia, Boehringer Ingelheim, and Murdoch University. Special thanks to Jess Andony, Esther Jones, and Jenelle Bowles of Western Dairy, Stephanie Bullen and John Penry of Dairy Australia. The authors would like to acknowledge the support of producers who contributed their time and livestock to the study. Boehringer Ingelheim for the donation of products. Staff at DPIRD for conducting faecal egg counts, larval cultures, and differentiation. Fellow peers (Chin-Ning Chong, Dawn Chung and Paige Ludbey), staff (John Abbott and Aprille Jack) and Taylor Decosta for valuable on-farm technical assistance and support.

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Appendix 1: Survey template for determining farm details, pasture management and anthelmintic usage in tested farms.

Dairy enterprise
Milking herd size? (approx.): _____

Calving pattern? *Split/Year round/Spring/Other (Please specify)* _____

Pre-dominant breed in herd? *Holstein-Friesians/Crossbred/Other* _____

What form of grazing is used for the cattle on the property? *Dry/Irrigated/Both*

Does your farming enterprise implement a Bovine Johne's Disease (BJD) control strategy on farm? (*Calves and young stock are isolated from adult animals until at least 12 months of age*) *Yes/No*

Do you introduce stock into your farming enterprise? *Yes (<10% of herd is introduced)/No/Sires/ Other____*

Do you have a farm biosecurity plan? *Yes/No*

Worming control practices
Do you drench your new cattle within two days of arrival on the property? (*Quarantine drench*) *Yes/No*

Regarding your quarantine drench: What drench do you currently use? *Please circle one or more: Combination/ML/Bezimidazole/Levamisole*

If possible, can you recall which specific drench product is used? _____

When drenching, do you estimate the weight of your cattle? *Yes/No*

Which method do you use? *Overestimating/Herd average/Scales/Heaviest Cow's Weight/Guess Individuals' Weight/Other*

When drenching, which cattle do you drench? *All at once/Individual groups/Select Individuals/Other*

Please specify: _____

What is your annual treatment frequency within your enterprise? *Weaners (0-12 Months) _____/Heifers (12-24 Months) _____/Milking Herd (>24 Months) _____*

Pasture management and drenching

Do you use rotational grazing? *Yes/No*

Does your pastures have a rest period? *Yes/No*

Does it differ seasonally? *Yes/No*

If so, how long? _____

Which method(s) best describes your drenching period? *Prior to moving pasture/Immediately after moving pasture/ After having been on the pasture for an extended time/Variable/Other* _____

In the last 1 – 2 years, have any of your calves or cows died because of a worm burden/ problem? *Yes/No*
If yes, approximately how many calves _____ and cows_____

In the last 1 – 2 years, have you experienced a drench resistance problem in any of your animal mobs? *Yes/No*

Appendix 2: Strongyle faecal egg counts, larval differentiations and drench efficacy percentage at species level for 11 dairy farms which underwent anthelmintic resistance testing in the south west region of Western Australia between June – December 2020.

Farm	Treatment	EPG (Mean)	Larval %						Anthelmintic Efficacy %						
			Ostertagia	Trichostrongylus	Haemonchus	C. Oncophora	Cooperia spp.	Oesophagostomum	Overall	Ostertagia	Trichostrongylus	Haemonchus	C. Oncophora	Cooperia spp.	Oesophagostomum
2	Control	82	42	2		56									
	Doramectin	70				100			15	100	100		-52		
	Levamisole/Abamectin	46	11			89			44	85	100		11		
	Levamisole	4	100						95	89	100		100		
	Fenbendazole	124	24			76			-50	14	100		-104		
3	Control	666	16	1	15	68									
	Doramectin	453				100			32	100	100	100	32		
	Levamisole/Abamectin	297	13		2	79	6		55	64	100	94	58		
	Levamisole	Drenches not tested due to inadequate calf numbers													
	Fenbendazole														
5	Control 1 (ML-LV/BZ) *	499	57	4		23	16								
	Control 2 (ML/LV) *	242	10			79	11								
	Doramectin	25				88	12		90	100			88	89	
	Levamisole/Abamectin	10	93			7			98	97	100		99	100	
	Levamisole	13	100						95	48			100	100	
	Fenbendazole	288	25			71	4		42	75	100		-78	86	
6	Control	393	6		2	92									
	Doramectin	94			2	98			76	100		76	75		
	Levamisole/Abamectin	15	28		6	66			96	82		88	97		
	Levamisole	11	64			35			97	71		100	99		
	Fenbendazole	6	22		53	25			98	94		60	100		
7	Control	293				90	10								
	Doramectin	32				81	19		89				90	79	
	Levamisole/Abamectin	3				36	64		99				100	94	
	Levamisole	2				67	33		99				99	98	
	Fenbendazole	11	2			89	9		96				96	97	
8	Control	1332	4	8		84	4								
	Doramectin	305		4		92	4		77	100	89		75	77	
	Levamisole/Abamectin	928	5	2		81	12		30	13	83		33	-109	
	Levamisole	128	100						90	-139	100		100	100	
	Fenbendazole	467	28			64	8		65	-146	100		73	30	
10	Control	119	10	2		81	7								
	Doramectin	65				81	19		46	100	100		46	-48	
	Levamisole/Abamectin	0	36			36	28		100	100	100		100	100	
	Levamisole	5	100						96	58	100		100	100	
	Fenbendazole	16	18			56	26		86	76	100		91	49	
11	Control	150	3			84	13								
	Doramectin	151				92	8		-1	100			-10	38	
	Levamisole/Abamectin	0							100	100			100	100	
	Levamisole	0	80			20			100	100			100	100	
	Fenbendazole	26	6			84	10			65			83	87	
12	Control	363	6	1		87	6								
	Doramectin	21				83	17		94	100	100		94	84	
	Levamisole/Abamectin	29	8			67	25		92	90	100		94	67	
	Levamisole	4	100						99	84	100		100	100	
	Fenbendazole	21	32			52	16		94	69	100		97	84	
13	Control	99	21	44		29	1	5							
	Doramectin	1	15	62		12		12	99	99	98		99	100	97
	Levamisole/Abamectin	1		60		40			99	100	98		98	100	100
	Levamisole	3	43	57					97	95	97		100	100	100
	Fenbendazole	8	9	2		88		1	92	97	100		77	100	98
14	Control	202	7			70	22	1							
	Doramectin	130				95	5		35	100			12	85	100
	Levamisole/Abamectin	3				100			98	100			98	100	100
	Levamisole	8	83			14	3		96	51			99	99	100
	Fenbendazole	132	8			81	11		35	25			24	67	100

*Two controls were used as two separate visits were conducted to facilitate calf numbers required. Control 1 (Levamisole/Abamectin and Fenbendazole), Control 2 (Doramectin and Levamisole)

Appendix 3: Photos from study experience.