Gastro-Intestinal Nematodes in Ontario sheep flocks: An Epidemiological Study of Overwintering and Anthelmintic Resistance

by

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ABSTRACT

GASTRO-INTESTINAL NEMATODES IN ONTARIO SHEEP FLOCKS:
AN EPIDEMIOLOGICAL STUDY OF OVERWINTERING AND ANTHELMINTIC RESISTANCE

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This thesis was conducted to evaluate important epidemiological features of Gastro-Intestinal Nematode (GIN) infections in Ontario sheep flocks; namely, the PeriParturient Egg Rise (PPER), overwintering of GIN free-living stages on pasture, and Anthelmintic Resistance (AR). Three main studies were carried out: a longitudinal study was conducted on six sheep farms to evaluate the PPER in ewes lambing in different seasons and to determine whether total plasma protein (TPP) levels and packed cell volume (PCV) were associated with increased fecal GIN-egg shedding. Secondly, a pilot-study was conducted on three farms to describe pasture-level environmental conditions and over-wintering survival and infectivity of free-living GIN larvae, especially Haemonchus contortus. Lastly, a cross-sectional study was conducted on 47 sheep farms in Ontario, to evaluate the frequency of AR, compare different diagnostic tests for AR, and evaluate management practices associated with AR. In the longitudinal study, the PPER was observed in winter, spring and autumn lambing ewes, though the magnitude and distribution of the PPER varied with season. Lower TPP and PCV values were associated with increased fecal GIN-egg counts. The pilot-study suggested that H. contortus larvae did not overwinter successfully on pasture, while other GINs, such as Teladorsagia sp.,
"Trichostrongylus" spp. and "Nematodirus" spp., were able to overwinter on pasture, and were infective the following spring. Resistance to ivermectin, fenbendazole and levamisole was demonstrated on 97% (28/29), 95% (19/20) and 6% (1/17) respectively of the farms tested; most of the resistance observed was found in "Haemonchus" sp. The Fecal Egg Count Reduction percentage following treatment was influenced by which mean (i.e. arithmetic vs. geometric) was used in the formula; use of pre-treatment in addition to post-treatment faecal egg counts was not influential. Both the fecal egg count reduction test and the larval development assay diagnosed resistance, but there was poor agreement between the two tests, as indicated by the Kappa test. The prior use of benzimidazoles on farms was associated with higher levels of fenbendazole resistance. The information generated in this thesis will be used to develop a parasite control program for sheep flocks in Ontario and to guide future research on GIN parasitism.
ACKNOWLEDGEMENTS

People often laugh when I tell them that, when I first found out I was coming to Guelph, I looked at a world map and drew a horizontal line across Canada. I saw that Guelph was at the same level as the south of France, and thought to myself “Oh, then it can’t be too bad!” Little did I know then about the lake effect and snow squalls, though Paula explained it very clearly and exhaustively on my first day here! Yet, even less did I know then that my experience in this distant land of snow and polar bears (or so I thought!) was going to surpass all of my expectations and enrich my life, both through the lessons learnt and, more importantly, through every encounter made along the way.

“Every time I asked a question, that magnificent teacher, instead of giving the answer, showed me how to find it. She taught me to organize my thoughts, to do research, to read and listen, to seek alternatives, to resolve old problems with new solutions, to argue logically.”

Isabelle Allende

I would like to start by thanking my Advisory Committee – it has been an extreme privilege and honour working with each one of you. Thank you to Paula for listening to my song, and for your boundless passion and staunch dedication to the small ruminant industry. Thank you to Andria, for believing in me, and for infusing me with enthusiasm for epidemiology. A special thank you to Andrew, for all the time spent with me in lab meetings, journal clubs and lectures; for your encouraging notes and for painstakingly going through everything I write, and helping me improve; you are a true teaching inspiration. Thank you to John, for being constantly present despite the distance, and for
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“... I am part of a large family... and it’s enough for me...”

Great Lake Swimmers

Coming to school every morning has always felt like coming home. Thank you to Cate for keeping the department such a friendly and warm place. Thank you to all the lovely ladies that work in the main office; everyday brings a smile to my face as I call
“Buenos días” to Karla before checking in on Julie’s latest pictures; then, I discuss Halloween costumes or the coffee club with Sally before chatting to Linda about the weather. Thank you to William for all our random conversations, sometimes about statistics, but often hitting a complete tangent, and discussing the latest “Big Bang Theory” episode, or listening to his constant pun about Andrew and myself writing the “Peregrine-Falcon” paper... I’m afraid it hasn’t happened yet!

“... true friendship withstands time, distance, and silence...”

Isabelle Allende

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“... the world was an imperfect place – but within that vale of tears there were many sites and times of quietude and contentment...”

Alexander McCall Smith

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“... for families who delight in being together...”

Anonymous

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CHAPTER 1

Introduction and Objectives

1.1 The sheep industry in Canada

Within Canada, in the last decade there has been a growing demand for lamb products and, consequently, an opportunity for flock expansion (Fleming, 2008). A recent census by Statistics Canada indicated that the national sheep flock responded to this opportunity by increasing sheep inventory numbers in recent years, with an 11% increase in the number of breeding sheep reported in the last three years. Ontario currently has the largest sheep flock within Canada, with 364,000 sheep reported in July 2012, followed by Quebec (277,000 sheep) and Alberta (201,000) (Statistics Canada, 2012).

Sheep management in Ontario differs from that in other important sheep-raising countries due to its climate. Ontario is considered to have a humid continental climate, with cold snowy winters and warm-to-hot summers (World Maps of Köppen-Geiger Climate Classification, 2012). As suggested by Morgan and van Dijk (2012), climate often dictates management practices, and most sheep producers in Ontario house their flock indoors during the winter season, then put it out to graze during the summer months.

Ontario producers often practice lambing out-of-season (i.e. in seasons other than spring) – in addition to the traditional spring lambing. A recent survey conducted by the Ontario Sheep Marketing Agency (OSMA) showed that 32% (148/461) of all responding sheep producers, and 65% (34/52) of the respondents with flocks larger than 300 ewes, practiced out-of-season lambing (Ontario Sheep Industry Survey – Composite Report,
2009). This practice is mostly driven by economics, i.e. to increase the profitability of the industry and meet the current demand for lamb meat. Lamb consumption in Canada has increased in the past decade, and this is often attributed to the change in human demographics in the country, and the increase in ethnicity (Menzies, 2006; Fleming, 2008). However, despite the fact that many producers are practicing out-of-season lambing, the Canadian sheep industry is still not supplying enough lamb meat to meet the demand, indicating that there is potential for the sheep industry to keep growing (Ontario Sheep Industry Survey – Composite Report, 2009).

The Ontario Sheep Marketing Agency recently conducted a survey to identify challenges that might be hindering the growth of the sheep industry. The results indicated that high mortality rates in young lambs, predation, and endemic flock health problems such as gastro-intestinal nematodes (GINs), were among the most common concerns reported by producers (Ontario Sheep Industry Survey – Composite Report, 2009). Moreover, in a recent workshop for sheep producers, the majority of the attendees indicated GINs were one of the major concerns on their sheep farms (New Liskeard parasite workshop, 2012; unpublished data).

1.2 Gastro-intestinal nematodes

Parasitism due to GINs is often described as one of the most important production-limiting diseases for grazing sheep worldwide (van Dijk et al., 2010; Stear et al., 2011; Knox et al., 2012). GINs cause both acute infections with a rapid onset and high mortality levels, and chronic infections which are commonly sub-clinical, and may lead to insidious and important economic losses (Taylor, 2009) via reduction of
liveweight gain, reduced wool and milk production, and poor reproductive performance (Sutherland and Scott, 2010).

While there are several nematode genera, *Haemonchus* sp., *Teladorsagia* sp. and *Trichostrongylus* spp. are often described as the most production-limiting nematodes in temperate climates (O’Connor *et al*., 2006; Sargison, 2012). A recent study on the epidemiology of GINs in Ontario sheep flocks confirmed that these are also the three most common genera found in sheep in this region (Mederos *et al*., 2010).

1.2.1 Life cycle of gastro-intestinal nematodes

All three important GIN genera belong to the nematode order *Strongylida* and the superfamily *Trichostrongyloidea* (Zajac, 2006). Furthermore, they share a common direct life-cycle, with larval stages occurring in the environment, and the adult stage within sheep (Hansen and Perry, 1990).

Adult parasites within the abomasum or small intestine lay eggs which are passed out with feces. The eggs are 60-80 µm long, oval, and morphologically indistinguishable between different nematode genera (Taylor *et al*., 2007). If environmental conditions are favourable (discussed below), the eggs embryonate and hatch into first-stage larvae (L1), which then develop into second-stage (L2) and third-stage (L3) larvae within the fecal pellet. The latter stage is the infective stage, and moves out of the fecal pellet and onto herbage, where it can be ingested by sheep. L3s have a protective cuticle which increases their resistance to desiccation (Ellenby, 1968); however, the cuticle also inhibits nutrition. As a result, the L3 is reliant on its own energy stores for survival (Zajac, 2006). Development from L1 to L3 depends on temperature and humidity levels; under optimal
conditions (discussed below) it can be completed in as short as five days, but may take weeks or months in cooler conditions (Taylor et al., 2007).

After ingestion, the L3 moves down the alimentary tract of sheep and moult into L4 and L5 within the abomasal gastric glands (T. circumcincta) or mucosa (T. axei), before emerging as mature adults which produce eggs. Unless hypobiosis occurs (see below), the prepatent period is approximately 2-3 weeks long (Taylor et al., 2007).

While Haemonchus contortus, Teladorsagia circumcincta and Trichostrongylus spp. all share a similar life-cycle, they exhibit important differences in their pathogenicity, fecundity and environmental requirements (Taylor et al., 2007), as described below.

1.2.2 Haemonchus contortus

Haemonchus contortus is the largest GIN, measuring 20-30 mm, and the female parasite is a prolific egg layer, producing thousands of eggs each day (Zajac, 2006; van Dijk et al., 2010). The adults are found attached to the abomasal mucosa and the female parasite is hematophagous, causing a loss of both blood and protein (Taylor et al., 2007). Due to this hematophagous activity, H. contortus is considered the most pathogenic GIN, and clinical signs of haemonchosis include anemia and sub-mandibular edema (Sargison, 2008).

Haemonchus contortus is described as a tropical parasite (Gordon, 1948; Waller et al., 2004), and is very susceptible to desiccation (O’Connor et al., 2007; Reynecke et al., 2011). Development of eggs to L1 and hatching occurs at an optimal temperature of 15°C or higher, but may still occur at a slower rate at 5°C; however, development
completely ceases below -3°C (Troell et al., 2005; van Dijk et al., 2010). Optimal larval development from L1 to L3 occurs between 25-37°C, and L3s do not survive below -10°C or freeze/thaw cycles (O’Connor et al., 2006).

1.2.3 *Teladorsagia circumcincta*

*Teladorsagia circumcincta* (formerly known as *Ostertagia circumcincta*) is a smaller parasite, measuring 8-10 mm (Taylor et al., 2007). While the adult parasites are found on the abomasal mucosal surface, the developmental stages occur within the gastric glands of the fundic region of the abomasum, leading to the formation of visible nodules on the abomasal mucosa (Zajac, 2006). The resulting hyperplastic gastritis disrupts the secretion of gastric enzymes and alters the gastric pH, which in turn leads to clinical signs of diarrhea, dehydration and weight loss (Sutherland and Scott, 2010).

*Teladorsagia circumcincta* is more cold-tolerant than *H. contortus* (Smith and Archibald, 1965; Uriarte et al., 2003; van Dijk et al., 2010; Waghorn et al., 2011), and optimal development to the infective L3 stage occurs between 16-30°C (O’Connor et al., 2006). L3s can survive longer than *H. contortus* and *Trichostrongylus* spp. on pasture, which may be because they remain in the fecal material for longer periods of time, where they are protected from adverse climatic conditions, and also because they are more resistant to freeze-thaw cycles (O’Connor et al., 2006).
1.2.4 *Trichostrongylus* spp.

*Trichostrongylus* spp. are the smallest GINs, measuring 5-6 mm. The adult parasites burrow into the mucosal epithelium of the abomasum or small intestine, causing extensive desquamation of the epithelium and sub-epithelium (Taylor et al., 2007). This leads to a chronic inflammatory process which compromises nutrient absorption, and may lead to severe diarrhea (colloquially called ‘black scours’) and weight loss (Zajac, 2006).

*Trichostrongylus* spp. L3s can survive at lower temperatures of -10°C, compared to *H. contortus* (Gordon, 1948; Ayalew and Gibbs, 1973; Familton and MacAnulty, 1994), but are not as cold-tolerant as *Teladorsagia* sp. (Smith and Archibald, 1965); optimal development from L1 to L3 occurs between 22-33°C (O’Connor et al., 2006).

1.2.5 Clinical signs

Both *T. circumcincta* and *H. contortus* may manifest as a Type I or Type II syndrome, though Type II teladorsagiosis is uncommon in sheep (compared to Type II ostertagiosis in cattle) (Taylor et al., 2007). Type I teladorsagiosis and haemonchosis is observed in lambs in late summer/early autumn, and is a consequence of infection with large numbers of L3s on pasture and their development into adult parasites within the same grazing season. This syndrome is typically associated with a high morbidity, but mortality is low to high depending on the level of parasites present in the animal. Type II haemonchosis is commonly observed in adults, especially yearling ewes, in late winter/early spring, and results from the resumption of activity and maturation of hypobiotic larvae (discussed below). Though Type II haemonchosis may only affect a
small proportion of the flock, mortality may be high unless the ewes are treated early (Taylor et al., 2007).

1.2.6 Hypobiosis

When climatic conditions are unfavourable, GINs may undergo a temporary cessation of development within the host at the L3 (*Trichostrongylus* spp.) or L4 stage (*H. contortus* and *T. circumcincta*) (Taylor et al., 2007). While it is yet unknown what triggers this hypobiotic stage, Ayalew and Gibbs (1973) suggested that it may be a consequence of physiological changes or acquired immunity of the host. More recently, van Dijk et al. (2010) suggested that environmental factors related to temperature, photoperiod and drought may also be responsible for the arrested development observed in GINs.

During hypobiosis, the larvae have a lower metabolic rate, which allows them to survive longer within the host when environmental conditions are unfavourable (Sargison et al., 2007). This mechanism is particularly important for *H. contortus* as it allows this parasite to survive the winter within the host (Blitz and Gibbs, 1972; Ayalew and Gibbs, 1973; McKenna, 1974; Uriarte et al., 2003; Waller et al., 2004; Waller et al., 2006; Morgan and van Dijk, 2012; Taylor, 2012a). When environmental conditions become favourable or when host immunity wanes, the parasites complete their development and start producing eggs (Michel, 1974; Michel, 1976); this leads to pasture contamination, and therefore represents an important source of infection. As discussed earlier, resumption of parasite activity may also lead to an acute Type II syndrome, resulting in a sudden onset of clinical signs in late winter and early spring (Taylor et al., 2007).
1.2.7 Periparturient Egg Rise

The periparturient egg rise (PPER) is a term used to describe an increase in fecal egg shedding observed in ewes around lambing time and lactation (Radostits et al., 2007; Sargison, 2008) and, as mentioned above, is one of the most significant contributors to pasture contamination with GIN eggs (Donaldson et al., 1998; Barger, 1999; Zajac, 2006; Morgan and van Dijk, 2012).

Initial studies of the PPER suggested that it was due to the increased availability of larvae on pasture in the spring (Zawadowsky and Zyjaguintzev, 1933). However, Taylor (1935) criticized this theory, indicating that if the PPER phenomenon was merely linked to pasture availability, the fecal egg shedding would increase exponentially over the grazing season as pasture contamination built up. In contrast, the PPER is often described as a transient phenomenon, peaking at 6-8 weeks after lambing, and then decreasing. Taylor (1935) therefore suggested that other factors related to immunity and nutrition were more likely the causative factors of the PPER, as these influence the egg production of the parasites, and consequent fecal egg shedding.

Dunsmore (1965) described the PPER as an interaction between environmental and physiological factors, whereby seasonal stimuli triggered changes in hypobiotic larvae, while the animals’ productivity stage influenced the fecundity of the worms. Michel (1974, 1976 and 1978) also suggested that the PPER occurred due to a combination of both environmental factors that stimulated the resumption of parasitic activity, and endocrine and metabolic changes that occurred during the parturient and
lactation stage which increased the longevity of worms and resulted in higher fecal egg counts (FECs).

Following these studies on seasonal and physiological factors, several authors investigated the possible role of nutrition (Coop and Holmes, 1996; Donaldson et al., 1998; Houdijk, 2008); all studies indicated that dietary protein was an important factor in determining the PPER. More recently, a study by Beasley et al. (2012) showed that pregnant or lactating ewes on low-protein diets had higher FECs compared to pregnant or lactating ewes on high-protein diets. A study by Houdijk (2012) also indicated that protein plays a more important role in the PPER compared to metabolizable energy. These studies suggest that limited protein availability invariably increases the PPER, and are in accordance with the nutrient partitioning framework described by Coop and Kyriazakis (1999) and Houdijk et al. (2001). This framework indicates that different bodily functions are given different priorities, depending on the age and type of animal; while maintenance of body protein remains top priority in all age groups, reproducing animals prioritize reproductive performance over expression of anti-parasite immunity.

While the role of metabolizable protein in determining the PPER has been generally accepted, more research is required to elucidate the link between dietary protein levels, immune changes and consequent PPER. Jeffcoate and Holmes (1992) suggested that local IgA may play a role in the PPER since it is synthesized in the gut, but moves to the plasma in late gestation to be secreted in the milk, resulting in a temporary decline of IgA in the gut. Beasley et al. (2010) also showed that changes consistent with a reduction in local immunity expression, such as lower antibody levels and fewer mast cells, globule leucocytes and goblet cells in the intestinal tissue, occurred in both pregnant and lactating
ewes. These observations all suggest that changes in local immunity conditions, particularly IgA levels, may enhance parasite egg shedding, and are in accordance with work by Stear et al. (1997, 1998 and 2011), which indicated that parasite-specific IgA negatively impacts parasite fecundity. Consequently, a decrease in local IgA concentrations during gestation and parturition would result in the increased fecal egg shedding observed during this period.

1.2.8 Overwintering on pasture

As described earlier, the GIN life-cycle involves free-living stages which are susceptible to several environmental factors (Gordon, 1948; O’Connor et al., 2006; van Dijk et al., 2010); this can lead to seasonal changes in both the genera and number of larvae present on pasture (Waghorn et al., 2011). Several influential environmental factors have been described, which include temperature (Veglia, 1915; Krecek et al., 1992; Stromberg, 1997; van Dijk et al., 2010; Reynecke et al., 2011), moisture (Callinan and Westcott, 1986; Familton and MacAnulty, 1994; O’Connor et al., 2007), barometric pressure (Stromberg, 1997), soil type (Krecek et al., 1992), sward height and type (Veglia, 1915), snow cover (Smith and Archibald, 1965; Troell et al., 2005) and, more recently, ultraviolet irradiation (van Dijk et al., 2009). Of these, temperature and moisture are often described as the most influential factors (van Dijk et al., 2009).

Temperature influences both the development of eggs to the infective larval stage, and the migration and survival of L3 on pasture; however, optimal temperatures are genera specific (O’Connor et al., 2006). Additionally, while warm temperatures accelerate the rate of development from eggs to infective larvae, above a certain genera-
specific threshold (discussed above), high temperatures are detrimental as the L3s rapidly deplete their energy reserves and die (Zajac, 2006). Moisture is required to allow for the migration of larvae on herbage (Callinan and Westcott, 1986; Hansen and Perry, 1990; Santos et al., 2012), and can therefore become a limiting factor for completion of the parasite cycle, especially in hot conditions (Familton and MacAnulty, 1994; O’Connor et al., 2006; Reynecke et al., 2011).

1.3 Anthelmintics and anthelmintic resistance

Anthelmintic drugs have traditionally been used to control GIN infections as they are simple to use, cheap and highly effective (Sargison, 2012; Taylor, 2012b). Since the first introduction of phenothiazine in the early 1940s, anthelmintics have been employed for both therapeutic and prophylactic purposes worldwide (Jackson & Miller, 2006).

The benzimidazoles were introduced to the market in the 1960s, and have ovicidal, larvicidal and adulticidal properties (Sargison, 2012). In susceptible parasites, benzimidazoles bind to beta-tubulin, inhibiting microtubule polymerization and consequent cellular metabolism and cellular processes such as mitosis (Adams, 2001). They are relatively safe for sheep, although albendazole has been shown to have teratogenic effects, and should therefore not be used in ewes in early gestation (Zajac, 2006).

The imidazothiazoles, such as levamisole, were introduced in the 1970s. Levamisole is a cholinomimetic, causing sustained nematode muscle contraction and paralysis (Adams, 2001). While this drug class kills adult parasites, a study by Grimshaw et al. (1994) indicated that levamisole may not be effective against hypobiotic larvae.
Also, levamisole has a narrower therapeutic safety index compared to the benzimidazoles, and should therefore be used with caution in pre-lambing ewes (Sargison, 2012).

Macrocyclic lactones were introduced in the 1980s, and include both avermectins such as ivermectin, and milbemycins such as moxidectin (Adams, 2001). While both of these drug sub-classes act on glutamate- and gamma-amino-butyric acid-gated chloride channels, the milbemycins are more lipophilic, and therefore have a longer residual activity, compared to the avermectins (Sargison, 2011a). Macrocyclic lactones have activity against both internal (nematodes) and external (arthropod) parasites, and have a wide safety margin in sheep as they do not readily cross the blood-brain barrier (Adams, 2001).

In the past five years, two new broad-spectrum anthelmintic drug classes were introduced for the control of GIN parasitism in sheep: the amino-acetonitrile derivatives (AADs) (Kaminsky et al. 2008) and the spiroindoles (Ruiz-Lancheros et al., 2011). The AADs bind to unique nematode-specific acetylcholine receptor sub-units, and are therefore very specific for nematodes, reducing toxic side effects (Kaminsky et al., 2008). The spiroindole derquantel also binds to acetylcholine receptors, and is marketed as a combination product with abamectin (Sargison, 2012).

Lastly, the narrow-spectrum salicylanilide derivative drugs closantel and nitrooxynil are protein ionophores and act by uncoupling the oxidative phosphorylation process within the parasite mitochondria (Martin, 1997). These drugs bind strongly to plasma proteins, which allows them to concentrate within hematophagous parasites
(Sutherland and Scott, 2010). These drugs, therefore, have a limited spectrum of activity and are only effective against the hematophagous *H. contortus* (Waller *et al.*, 2006).

In Canada, only ivermectin is licensed for use in sheep (Compendium of Veterinary Products, Canada, 2012). Thiabendazole was the first benzimidazole to be marketed in Canada in the early 1960s (Adams, 2001), but was subsequently replaced with other structurally similar, but improved drugs, such as fenbendazole and albendazole. Fenbendazole and albendazole are licensed for use in Canada in cattle (Compendium of Veterinary Products, Canada, 2012), but are often used in sheep in an extra-label manner. Levamisole has not been licensed for use in sheep in Canada for the past 10 years (Health Canada – Drug Product Database Online Query, 2012).

1.3.1 Anthelmintic resistance

Anthelmintic resistance (AR) is defined as “the heritable ability of the parasite to tolerate a normally effective dose of the anthelmintic” (Abbott *et al.*, 2009), which implies that the parasite can survive exposure to the standard recommended dose of the anthelmintic, and pass on this ability to its offspring. Anthelmintic resistance is a common cause of drench failure (i.e. inadequate control of parasite FECs after anthelmintic treatment) (McKenna, 1990); however, other confounding factors may also lead to treatment failure, such as dosing animals with insufficient anthelmintics (Sangster and Gill, 1999), or use of an inappropriate anthelmintic for the parasite present (Taylor *et al.*, 2002; Abbott *et al.*, 2009).

Anthelmintic resistance is an escalating problem in many countries (Kaplan, 2004). It is a threat to sheep welfare (Wolstenholme *et al.*, 2004), and has important
economic consequences as it leads to sub-optimal growth (Coles, 2001; Sargison, 2011b), and reduces both carcass and fleece weight (Miller et al., 2012). Anthelmintic resistance is widespread in New Zealand (Waghorn et al., 2006; Hughes et al., 2007), Australia (Love et al., 1992; Besier and Love, 2004), and in several South American countries, such as Brazil and Uruguay (Waller et al., 1996; Cezar et al., 2010). In recent years, AR has also been described in the United States (Howell et al., 2008; Kaplan and Vidyashankar, 2012) and in several European countries including Greece (Gallidis et al., 2009; Gallidis et al., 2012), Italy (Cringoli et al., 2009), Spain (Calvete et al., 2012) and the United Kingdom (Jackson and Coop, 2000). Moreover, reports of triple-class resistance are now common (Sargison et al., 2010; Knox et al., 2012 Taylor, 2012a; Voigt et al., 2012), indicating the urgency to improve control strategies for AR (Taylor, 2012a). In 2007, the first case of AR in Canada was described in a sheep flock in Ontario (Glauser et al., 2007). However, as indicated recently by Torres-Acosta et al. (2012), information on the current state of AR in a number of countries, including Canada, is lacking.

The insurgence and rate of AR development is attributed to various risk factors related to the mode of genetic inheritance and number of genes involved, the parasite biology and epidemiology (Sargison, 2012), and selection pressure for resistance. Each of these factors is further described below.
1.3.1.1 Mode of inheritance and number of genes involved

Anthelmintic resistance is an expression of certain parasite genes that enable parasites to survive the anthelmintics’ mechanism of action (Prichard et al., 1980). Benzimidazole resistance occurs as a result of an alteration to the parasites’ beta-tubulin genes, with the result that the drugs cannot bind to the intended target (Lacey and Gill, 1994). The mechanisms behind levamisole resistance are still being investigated, yet studies in resistant strains of *H. contortus* seem to suggest that resistance is associated with the presence of low-affinity acetyl-choline-gated cation channel binding-sites for levamisole (Sangster et al., 1998b). Macrocyclic lactone resistance likely involves multiple mechanisms, including an increase in the number of low affinity glutamate-chloride receptors and mutations in the P-glycoprotein genes which allow for an increased drug efflux (Jabbar et al., 2006; Sutherland and Scott, 2010).

The rate of AR development is inevitably influenced by the number of genes involved and their heritability (Vidyashankar et al., 2012). Depending on the parasite species and anthelmintic involved, genes encoding resistance may be recessive or dominant, autosomal or sex-linked, and single or multigenic (Sutherland and Scott, 2010). For instance, studies on avermectin resistance have indicated that it is a dominant, autosomal trait, controlled by a single gene in both *H. contortus* and *T. circumcincta* (Le Jambre et al., 2000; Leathwick et al., 2001), while in *Trichostrongylus colubriformis*, it appears to be a multigenic, partially dominant trait (Gill and Lacey, 1998). Levamisole resistance is described as a recessive, autosomal, multigenic trait in *H. contortus* (Sangster et al., 1998a), but as a sex-linked recessive trait in *T. colubriformis* (Martin and McKenzie, 1990). Finally, benzimidazole resistance in both *H. contortus* and *T.*
*colubriformis* is described as an autosomal, multigenic trait, either incompletely recessive or semi-dominant (Le Jambre et al., 1979; Dobson et al., 1996).

### 1.3.1.2 Parasite biology and epidemiology

Anthelmintic resistance has been shown to develop faster in *H. contortus* compared to *T. circumpincta* and *Trichostrongylus* spp. (Sangster and Gill, 1999; Mejía et al., 2003), which may be a result of both genetic and biological differences between the nematode genera. However, the genera-specific prevalence of AR in a specific country or region also depends on the prevalence of the different genera of GIN on farms, which, in turn, is influenced by climate (Vidyashankar et al., 2012). In New Zealand, resistance has been described as common in *Nematodirus* spp., *Teladorsagia* spp. and *Trichostrongylus* spp. (Waghorn et al., 2006), while in the United Kingdom, the majority of AR cases have been associated with *Teladorsagia* spp. (Bartley et al., 2003). The latter finding is in agreement with a recent survey conducted by Burgess et al. (2012) that determined the nematode species present on United Kingdom sheep farms; *T. circumpincta* was the only parasite present on all farms surveyed. Both New Zealand and the United Kingdom are described as having temperate climates, which favours the survival of the more cold-tolerant *T. circumpincta* (O’Connor et al., 2006). In contrast, a study conducted in the southeastern United States (Howell et al., 2008) indicated that *H. contortus* was both the most common parasite, and the parasite most commonly associated with resistance, on the sheep farms surveyed. However, the climate in the southeastern United States is generally warm and humid, with mild winters, favouring the survival of the more tropically adapted *H. contortus* (Troell et al., 2005).
1.3.1.3 Selection pressure for resistance

Selection pressure for AR depends on a number of factors, such as drug efficacy and dose administered, frequency and timing of anthelmintic treatment, and the proportion of the parasite population in refugia (Sargison, 2011a).

Sub-optimal dosing may select for AR (Calvete et al., 2012), as it allows both homozygous resistant and heterozygous resistant worms to survive treatment, increasing the reproductive advantage of heterozygous-resistant worms (which would normally be killed with a full anthelmintic dose) over homozygous-susceptible worms (Abbott et al., 2009). Sub-optimal dosing may occur due to incorrect calibration of the anthelmintic drench gun, poor delivery of the drench into the back of the mouth, and under-estimation of the animal’s live weight (Scott and Sutherland, 2010). A recent survey by Burgess et al. (2012) in the United Kingdom found that many producers were either under-estimating the weight of individual animals or using the average weight of the flock for dosing, leading to sub-optimal dosing of animals.

The frequency of treatment has often been incriminated as one of the most important factors in determining AR (Prichard et al., 1980; Sangster, 1999; Cabaret et al., 2009; Calvete et al., 2012), as it removes susceptible worms, and proffers a reproductive advantage to any remaining resistant worms. However, recent work has indicated that factors other than the frequency of treatment may be more important determinants of AR, as they impact the number of worms that are left in refugia (Sutherland and Scott, 2010). These include which groups of animals (i.e. which age group and productivity stage) are treated (Leathwick et al., 1995; Leathwick et al., 2006; Leathwick et al., 2008; Waghorn...
et al., 2010), and management practices associated with anthelmintic treatment, such as drench-and-shift (i.e. treating the animals with an anthelmintic and immediately moving them onto pastures with low numbers of parasite larvae) (Waghorn et al., 2009).

*Refugia* has been described as the most important concept in selection for AR (van Wyk, 2001), and describes the proportion of the parasite population that is not exposed to anthelmintic drugs, either because they are within untreated hosts, or are free-living on pasture (Kenyon et al., 2009). Preserving a proportion of the susceptible parasites on a farm in *refugia* increases the likelihood that these parasites may mate with resistant ones; this, in turn, reduces the proportion of homozygous-resistant parasites present on pasture and slows the development of resistance (van Wyk et al., 2006).

The concept of *refugia* has been investigated in several studies. A study by Martin et al. (1981) showed that resistance built up faster when fewer parasites were left in *refugia*, and a modelling study by Barnes et al. (1995) indicated that leaving a proportion of the lambs untreated slowed the development of resistance. More recently, several clinical trials have been carried out in New Zealand to test the concept of *refugia*, either by leaving adult ewes (Leathwick et al., 2006), or a proportion of the lambs in a flock (Waghorn et al., 2008), untreated. Both studies indicated that leaving 10-15% of the flock untreated slowed the development of resistance by increasing the number of susceptible parasites in *refugia*.

Selective treatment is a practical application of *refugia* theory (Jackson and Miller, 2006), and is defined as treatment of individual animals when GIN parasitism is suspected; as opposed to targeted treatment, i.e. treatment of the whole flock when GIN
parasitism is suspected (Kenyon and Jackson, 2012). Selective treatment is based on the idea that, within a flock, the parasite population is over-dispersed and the majority of the parasites are found within a small proportion of the animals (Srèter et al., 1994; Stear et al., 1998). Consequently, identifying and treating the high-shedder animals should effectively reduce the parasite burden on the farm, while also allowing for a reduction in the treatments given (Kenyon and Jackson, 2012).

Several parameters have been suggested to correctly identify which animals should be treated, and include parasitological parameters such as FECs, pathophysiological parameters, such as FAMACHA® (van Wyk and Bath, 2002) and DISCO diarrhoea scores (Bentounsi et al., 2012), and performance-based parameters (Bath and van Wyk, 2009). Each of these is further discussed below.

Fecal egg counts are very accurate in identifying infected animals. Two recent field studies that evaluated the efficacy of using FECs as a decision parameter for targeted selective treatment compared to whole-flock systematic treatment, showed that targeted treatment of animals with high FECs decreased the mean flock FECs, while also reducing the number of treatments administered (Gallidis et al., 2009; Cringoli et al., 2009). However, using FECs as an indicator is currently not very practical, as analysis requires a laboratory setting and is time-consuming and expensive (Gallidis et al., 2009); ideally, a faster test should be developed which allows for a rough estimation of the FEC with the opportunity to take an immediate decision regarding treatment of the animal.

Patho-physiological parameters that are used for targeted selective treatment include the FAMACHA® score (van Wyk and Bath, 2002) and the diarrhea DISCO score.
The FAMACHA© score is based on the notion that *H. contortus* causes anaemia, which can be assessed by evaluating the colour of the conjunctivae (van Wyk and Bath, 2002; Jackson and Miller, 2006). While this system is effective in areas where *H. contortus* is the most prevalent parasite, such as South Africa (Malan *et al*., 2001) and southeastern United States (Kaplan *et al*., 2004), FAMACHA© may be less reliable in temperate areas where non-haematophagous parasites such as *T. circumcincta* and *Trichostrongylus* spp. are more prevalent (Bentounsi *et al*., 2012). Recent work conducted in Ontario indicated that FAMACHA© was poorly correlated with FECs and packed cell volume (Mederos, 2010). This may be due to the low prevalence of *H. contortus* in certain flocks, which lowers the predictive ability of the FAMACHA© test (Dohoo *et al*., 2009). Therefore, in temperate regions, the DISCO diarrhea score, based on the state of the feces at the moment of collection, may be of more use in identifying heavily parasitized animals (Bentounsi *et al*., 2012). However, both the FAMACHA© and DISCO scores have important limitations: firstly, both anaemia and diarrhea can be multi-factorial, therefore reducing the specificity of these tests in diagnosing GIN infections (van Wyk and Bath, 2002; Bentounsi *et al*., 2012). Secondly, they are based on signs of clinical disease and may thus result in a delayed response, increasing the risk of irreversibly compromising the animals’ productivity and welfare (Kenyon *et al*., 2009). Lastly, targeting the treatment towards those animals that only show overt clinical signs may result in missing those more resilient animals that may not be showing any clinical repercussions, but are still shedding eggs and contributing to pasture contamination.

Performance-based parameters that can be used to direct selective treatment include body condition scoring (Osoro *et al*., 2007), live weight gain (Stafford *et al*.,
2009) and milk production (Gallidis et al., 2009). While all three parameters have been shown to be effective within certain settings, the application of these parameters may be limited either by the subjectivity of the assessment (for body condition scoring), or the costs and practicality associated with measurement of live weight gain and milk production on multiple occasions (Bath and van Wyk, 2009).

While targeted selective treatment may be a promising option for helping control AR, several factors require further elucidation. Leaving a certain proportion of the flock untreated may increase pasture contamination, with consequent negative effects on the animals’ productivity and welfare (Waghorn et al., 2008; Kenyon and Jackson, 2012). It is therefore important to determine the proportion of the flock that should be left untreated without consequent adverse effects on the rest of the flock (Cabaret et al., 2009). While Waghorn et al. (2008) indicated that leaving 10% of the population untreated should be sufficient to slow down the development of resistance, this proportion may vary as it is influenced by drug efficacy and the overall pasture contamination (Kenyon and Jackson, 2012). Secondly, refugia is an elusive concept, difficult to explain and put into practice (Jackson and Miller, 2006), and more research is required to identify extension methods that will appeal to different target audiences (Woodgate and Love, 2012) and increase the uptake of targeted selective strategies (Besier, 2012).
1.4 Tests for determining anthelmintic resistance

1.4.1 Fecal Egg Count Reduction Test

The standard and most commonly employed field test for diagnosing AR is the Fecal Egg Count Reduction Test (FECRT) (Coles et al., 1992; Taylor et al., 2002; Dobson et al., 2012). The FECRT is an indirect measure of an anthelmintic’s efficacy, and measures the FEC reduction 10-14 days after treatment with an anthelmintic (McKenna, 2006); a threshold of <95% is recommended as indicative of resistance (Coles et al., 1992). The FECRT is easy to perform (Maingi et al., 1998), can be used to test multiple anthelmintics simultaneously (Martin et al., 1989), and is therapeutically relevant (Sangster and Gill, 1999). However, it is laborious and time-consuming (Craven et al., 1999), and can only detect resistance when at least 25% of the parasite population is expressing the resistance genes (Coles et al., 1992; Martin et al., 1989).

When carrying out a FECRT, it is important to appreciate that multiple factors can influence the outcome of the test. Studies have shown that the FECRT is less reliable at diagnosing AR when the drug efficacy ranges around 90-95%, compared to very low drug efficacies (Cabaret and Berrag, 2004; Miller et al., 2006). The parasites’ fecundity and density-dependent effects of worm numbers on egg production may also impact the FECRT outcome, as they increase the variability of the FECs (Mejía et al., 2003; Papadopoulos et al., 2012). Several authors have also discussed the impact of low (Maingi et al., 1998; Miller et al., 2006; Knox et al., 2012) or over-dispersed (Dobson et al., 2009; Dobson et al., 2012) pre-treatment FECs on the Fecal Egg Count Reduction (FECR) percentage, as these parameters increase the variance of the estimate, making it
less reliable. Over-dispersion of the parasite population has led to several discussions on whether arithmetic (Dash *et al.*, 1988; McKenna, 1990; Coles *et al.*, 1992; McKenna, 1997) or geometric (Presidente, 1985; Wood *et al.*, 1995; Smothers *et al.*, 1999) means should be used when calculating the FECR percentage.

Another important factor when estimating the FECR is which treatment data are included in the FECR formula. The formula endorsed by the World Association for the Advancement of Veterinary Parasitology (Coles *et al.*, 1992) recommends the inclusion of a control group when calculating the FECR, to adjust for any changes in FECs that may occur in untreated animals (e.g. when parasites become hypobiotic or the animals’ immune status changes). However, recent publications have questioned whether such a group is really necessary (Coles *et al.*, 2006), and whether it is ethically correct to leave part of the flock untreated, especially if parasite infection levels are high (McKenna, 2006).

Another important consideration is whether both pre- and post-treatment FECs should be included in the FECR calculation, or whether post-treatment FECs suffice to evaluate the efficacy of the treatment. Dash *et al.* (1988) suggested including both, to adjust for low pre-treatment counts, while Coles *et al.* (1992) described post-treatment FECs as sufficient to calculate the drug efficacy. McKenna (2006) found that, using arithmetic means, there was no significant difference in the estimate when both pre- and post-treatment FECs were used, compared to only post-treatment FECs, and therefore suggested that the latter simpler method could be used as this would reduce the number of fecal samples required, reducing the overall cost of the FECRT.
More recently, authors have discussed the analytical sensitivity of the tests used to estimate FECs, and the effect this may have on the overall FECR estimate (El-Abdellati et al., 2010; Levecke et al., 2011; Levecke et al., 2012b). A diagnostic method commonly used for determining FECs is the modified McMaster technique, since it is fast and easy to perform (Ministry of Agriculture, Fisheries and Food, 1986). However, this test has a minimum detection limit ranging from 10 to 50 eggs per gram (epg) (Levecke et al., 2012a), which may introduce a bias when calculating FEC reductions (El-Abdellati et al., 2010), especially when the baseline FEC is low (Levecke et al., 2012a). Therefore, the use of more sensitive tests such as the FECPAK techniques (detection limit = 10 epg) or FLOTAC® technique (detection limit = 1-2 epg) is often recommended (El-Abdellati et al., 2010; Torgerson et al., 2012) to improve test accuracy, while repeated FEC measurements could improve the test precision (Kaplan and Vidyashankar, 2012).

Since the FECRT outcome may be influenced by all the aforementioned factors, there is a need to standardize both the FEC diagnostic methods and the FECR calculations used (Coles et al., 2006; Denwood et al., 2010; Knox et al., 2012), to avoid misclassification of the farm resistance status (Torgerson et al., 2005).

1.4.2 Larval Development Assay

Larval Development Assays (LDAs) are in vitro tests used to determine anthelmintic susceptibility (Taylor, 1990; Coles et al., 2006; Howell et al., 2008). These tests determine the effect of anthelmintics on the GIN development process, by exposing GIN eggs or L1s to different anthelmintic concentrations in separate test wells, and
counting the number of L3s that develop within each well after 7-10 days (Jabbar et al., 2006).

LDAs are more sensitive diagnostic tests compared to the FECRT, as they can detect AR when 10% of the parasite population expresses resistance (Jabbar et al., 2006; Papadopoulos, 2008), therefore providing early warning of an impending resistance problem (Roush and Tabashnik, 1990; Taylor et al., 2009). Moreover, LDAs are rapid (Taylor et al., 2002), can be easily replicated and standardized (Sangster and Gill, 1999), and only require one set of fecal samples (Diéz-Baños et al., 2008). On the other hand, LDAs require a high level of technical expertise (Kaplan and Vidyashankar, 2012), and, given that they are in vitro tests, they ignore the pharmacodynamics and pharmacokinetics of the anthelmintic within the host (Sangster and Gill, 1999; Sargison, 2011a).

An ideal laboratory bioassay should generate data that correlate closely with the standard FECRT field test (Roush and Tabashnik, 1990; Coles et al., 2006), thereby providing an easier and faster way to diagnose AR on sheep farms. While a few studies have been performed to compare different in vitro tests (e.g. egg hatch assay, larval mobility tests and LDAs) and the FECRT (Craven et al., 1999; Königová et al., 2003; Wolstenholme et al., 2004; Diéz-Baños et al., 2008), these have provided discordant results, and therefore more research is required to investigate the correlation between in vivo and in vitro tests (Coles et al., 2006).
1.5 Thesis objectives

The overall goal of this thesis was to elucidate important epidemiological features of GIN parasitism in Ontario sheep flocks, including the PPER, overwintering of GIN on pasture, the prevalence of AR, and risk factors associated with AR.

The specific objectives of this thesis were:

1. To determine: (a) whether ewes that lamb out-of-season experience a PPER, (b) whether ewes either not bred or in early gestation during the spring season experience an increase in fecal egg shedding at that time, related to seasonal effects, and (c) whether total plasma protein and packed cell volume levels are associated with the PPER (Chapter 2).

2. (a) To describe the environmental factors that may affect the over-wintering survival of GIN on three commercial sheep farms in south-western Ontario; and (b) to determine if *Haemonchus contortus* larvae are able to over-winter on pasture and/or soil under central Canadian winter conditions, and are capable of establishing a patent infection in naïve tracer lambs the following spring (Chapter 3).

3. (a) To determine the frequency of ivermectin treatment failure, and the frequency of resistance to ivermectin, fenbendazole and levamisole using a FECRT; and (b) to assess the frequency of resistance to thiabendazole and levamisole using a LDA (Chapter 4).

4. To: (a) compare the FECR percentages obtained using different formulae, for resistance to ivermectin, fenbendazole, and levamisole; and (b) compare categorized
results obtained with the FECRT and LDA for resistance to benzimidazoles and levamisole (Chapter 5).

5. (a) To describe parasite control and farm management practices commonly used on Ontario sheep farms; and (b) to determine whether any of these practices are associated with the presence of resistance to ivermectin, fenbendazole or levamisole (Chapter 6).
1.6 References


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CHAPTER TWO

A longitudinal study on the effect of lambing season on the periparturient egg rise in Ontario sheep flocks

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Abstract

The epidemiology of the periparturient egg rise (PPER) of gastrointestinal nematodes (GIN) in sheep remains unclear, and may be influenced by the lambing season. This longitudinal study was performed to determine the effect of out-of-season lambing on the PPER in ewes in Ontario, and whether Total Plasma Protein (TPP) and Packed Cell Volume (PCV) were associated with the PPER. Six farms that practiced out-of-season lambing were enrolled, and sampled for three consecutive lambing seasons (winter, spring, and autumn). For each lambing season, all farms were visited five times. On the first visit for each lambing season, 15-20 pregnant ewes and 15-20 non-pregnant/early gestation ewes were randomly selected. At each visit, fecal samples were collected from all selected animals and processed individually to measure GIN fecal egg counts (FEC). Blood samples were collected on three visits in each lambing period and processed to measure TPP and PCV. The ewes were classified into one of five production stages (maintenance [i.e. not pregnant], early or late gestation [<120d and ≥120d, respectively], and early or late lactation [<40d and ≥40d, respectively]) based on information collected during farm visits. Linear mixed models were developed for the TPP, PCV and logarithmic-transformed FEC (lnFEC). During the winter and spring lambing season, the FECs increased gradually over the gestation period and peaked during lactation, with
these increases being larger in ewes with a low PCV (three-way interaction in the final model). In the autumn lambing season, the FECs started off higher in early gestation, and increased rapidly to peak in late gestation, particularly for animals with low PCV levels. In the TPP model, PCV and lnFEC were positively associated with TPP. During both autumn and winter lambing seasons, the TPP decreased from maintenance throughout gestation and early lactation, followed by an increase in late lactation, except for when there were high FECs. During the spring lambing season, TPP peaked at early gestation, and then decreased in late gestation, to increase more gradually over lactation. In the PCV model, PCV increased with TPP and decreased exponentially with increases in lnFEC. The PPER occurred during all three lambing seasons, and its magnitude and distribution varied with the lambing season, suggesting that the PPER in ewes depends on both environmental and animal physiological factors, an important consideration when implementing preventive parasite control strategies on sheep farms that practice out-of-season lambing.

**Keywords:** Gastro-intestinal nematodes; periparturient egg rise; out-of-season lambing

2.1 Introduction

Gastrointestinal nematodes (GINs) are a leading cause of clinical disease and death in grazing sheep worldwide, hindering both sheep production and profitability (Sutherland and Scott, 2010). In Ontario, Canada, the most predominant GIN genera are *Teladorsagia circumcincta*, *Haemonchus contortus* and *Trichostrongylus* spp. (Mederos et al., 2010).
The periparturient egg rise (PPER) observed in the spring in temperate climate countries is a major source of GIN pasture contamination for both lambs and ewes (Barger, 1999). Moreover, it may sometimes result in an acute Type II syndrome, whereby parasites that survived the winter in the host as arrested larvae resume development, resulting in a sudden onset of clinical signs in late winter and early spring (Taylor et al., 2007).

There are conflicting ideas on the cause, and occurrence, of increased fecal egg shedding in ewes. Some authors consider this egg rise to be a seasonal phenomenon, observed only during the spring months, and independent of the ewes’ productivity stage (Zawadowsky and Zvjaguintzev, 1933; Brundson, 1964; Gibbs, 1967; Gibbs and Barger, 1986). This so-called “spring rise” (Cvetkovic et al., 1971) has been related to the increased availability of parasites on pasture in favourable ambient conditions (Zawadowsky and Zvjaguintzev, 1933), while others have suggested that this could be due to reactivation of hypobiotic parasites. The arrested development of nematodes has been described in several studies (Blitz and Gibbs, 1972; Michel, 1974), and the resumption of parasite development may occur spontaneously or be triggered by seasonal changes in photoperiod or ambient temperatures (Blitz and Gibbs, 1972).

Other authors believe the fluctuation in egg shedding observed during the periparturient period is linked to the ewes’ productivity stage, and the endocrine, immunological, and metabolic changes that ensue (Taylor, 1935; Crofton, 1954; Brunsdon, 1970; Michel, 1976; Jeffcoate and Holmes, 1990; Coop and Holmes, 1996; Donaldson, 1998; Beasley et al., 2010b). Crofton (1954) suggested that prolactin may play a role in the PPER, since it also increased during parturition and lactation, but other
studies have indicated that this is most likely an incidental finding (Jeffcoate and Holmes, 1990; Beasley et al., 2010a). Brunsdon (1970) suggested that the PPER may be caused by a relaxation in immunity during the periparturient period, and work conducted by Beasley et al. (2010b) showed that changes consistent with a reduction in immunity expression occur in both pregnant and lactating ewes. These changes in immunity may facilitate the parasites’ establishment within the host, enhance their prolificacy, and increase their longevity (Michel, 1976). Houdijk et al. (2001) suggested that a lack of metabolizable protein may also be a determinant factor in the PPER since both gestation and lactation are nutritionally demanding periods (Houdijk, 2008), and compete with the hosts’ immune system for available protein. Total plasma protein (TPP) is a useful indicator of the protein available to the animal, while low Packed Cell Volumes (PCV) are suggestive of blood and protein loss, which could be a consequence of a parasitic infection (Radostits et al., 2007). Both TPP and PCV could therefore be useful diagnostic indicators of the PPER.

Since its first description (Taylor, 1935), the PPER in ewes has been observed in both temperate (Brunsdon, 1970; Cvetkovic et al., 1971; Beasley et al., 2010b) and tropical climates (Tembely et al., 1998; Ng’ang’a et al., 2006). More recently, Mederos et al. (2010) reported an increase in fecal egg shedding in ewes that lambed during the spring, prior to pasture exposure, on conventional farms in Ontario, Canada. However, a recent survey conducted by the Ontario Sheep Marketing Agency (OSMA) showed that 32% (148/461) of all respondents, and 65% (34/52) of the respondents with flocks larger than 300 ewes, practiced out-of-season lambing (Ontario Sheep Industry Survey, 2009). Consequently, in many flocks in Ontario, ewes lamb throughout the year and not all ewes
are pregnant during the spring time. Dunsmore (1965) suggested that both environmental and physiological factors might be important contributors to the PPER. It is therefore important to elucidate the epidemiology of fecal egg shedding patterns on Ontario sheep farms that practice out-of-season lambing, and to identify clinical parameters that might be associated with the PPER. Ultimately, this would enable us to devise improved preventive strategies for parasite control on these farms.

The objectives of this study were therefore to determine whether: (i) ewes that lamb out-of-season experience a PPER; (ii) ewes either not bred or in early gestation during the spring season experience an increase in fecal egg shedding at that time, related to seasonal effects, and (iii) TPP and PCV are associated with the PPER. Our hypothesis was that all ewes lambing out-of-season would experience a PPER, and that ewes not bred or in early gestation during the spring would experience an increase in fecal egg shedding related to seasonal effects. Furthermore, we hypothesized that low concentrations of TPP and PCV would be associated with the PPER.

2.2 Materials and methods

2.2.1 Number and selection of sheep farms

A longitudinal study was conducted between December 2009 and June 2011 in which six farms were purposively selected in south-western Ontario; the sample size was dictated by logistical and financial constraints. The farms were selected based on their willingness to participate in the study, distance from University of Guelph (within a 200 km radius) due to a requirement for frequent sampling, compliance to withhold routine use of anthelmintics, and known history of GIN parasitism on the farm. The latter was
based on the producers’ indication that their local veterinarian had confirmed the presence of clinical parasitism in their flock. Other inclusion criteria were that farms had to (i) practice out-of-season lambing, and (ii) have more than 50 ewes in their flock. During the first year, one farm was lost to follow-up due to personal reasons; another farm was enrolled as a replacement and a full dataset was collected on that farm.

2.2 Study design

The six farms were visited following a specific schedule, which was set around the predicted date when 50% of ewes scheduled to lamb that season would have lambed ("50L"). The 50L was estimated as: date of ram introduction +148 days, based on the average gestational length in ewes (Senger, 2003). The farms were visited five times for every lambing period, for three consecutive lambing seasons (winter, spring, autumn). Specifically, they were visited: six and three weeks before 50L, at 50L, and three and eight weeks after 50L (Appendix I). These time-points were selected since previous work has suggested that the PPER occurred between two to four weeks before lambing, and six to eight weeks after lambing (Abbott et al., 2009). During the winter lambing season, animals were housed indoors on all six farms. During the spring lambing season, animals were either housed indoors (Farm E), put on pasture (Farm D and F), or housed indoors for the first half of the lambing season, and put on pasture for the second half of the lambing season (Farms A, B and C). In the autumn lambing season, animals were on pasture for the first half of the lambing season, then housed indoors for the second half of the lambing season.
2.2.3 Animal selection

On the first farm visit of each lambing season, 15 pregnant animals that were due to lamb that season, and 15 animals that were open or had just been bred, were randomly selected from the entire sheep flock. The selection was either simple random using a random number generator, or systematic random, depending on the handling facilities available. The sample size was based on a recommendation that 10-15 animals per group are sufficient to detect differences in fecal egg counts (FEC) between groups (Coles et al., 2006). Animals were identified using ear-tag numbers, and remained in the study for a lambing season, unless individual ewes were culled or lost their ear tag; lost animals were not replaced since repeated measurements on the same animal were necessary. Different animals were selected for each lambing season. After the first lambing season, the number of animals was increased to 20 animals per group in order to meet or exceed the recommendation of 10-15 animals per group, even with animals lost to follow-up.

2.2.4 Data collection

At each visit, fecal samples were collected directly from the rectum of each selected ewe, and the FEC was determined for these individual samples. Blood samples were collected on three of the five farm visits, per lambing season (3 weeks before 50L, at 50L, and 8 weeks after 50L). These were obtained from each individual ewe via jugular venipuncture into 10ml vacutainer tubes containing 15% ethylene-diamine-tetraacetic acid (BD Franklin Lakes, NJ, USA) to determine the blood PCV and TPP. Blood and fecal samples were stored with ice-packs both at the farm and during transport.
Data on the animals’ nutrition on each farm were collected by the researchers during every farm visit using a short questionnaire. The questionnaire gathered information on quantity and protein content of forage and concentrate being fed to the sheep, minerals or supplements being given to the sheep, and whether the animals had access to pasture since the previous farm visit. A copy of the questionnaire can be obtained from the authors upon request (Appendix II).

For every lambing season, a forage sample was collected from each farm in the study. This was obtained using a drill hay-corer, and composite samples were taken from a minimum of five different hay bales located in different storage areas. Forage samples were submitted to the Agri-Food Laboratory (Guelph, ON N1H 6T9, Canada) for crude protein analysis based on laboratory nitrogen analysis. Both questionnaire data and forage analysis data were incorporated in a calculation to determine the Crude Protein (CP) ingested by each animal, as explained in Section 2.2.6.

For each lambing season, data were obtained from the producers on lambing dates, ewe parity, litter size, lamb birth-weights, number of lambs weaned, and lamb 50-day weights, for the lambs born from the ewes enrolled in the study.

All the animal work was approved by the Animal Care Committee (University of Guelph, Animal Utilization Protocol Approval No. 09R090), while the method for selection of farmers, structure and implementation of the questionnaire was approved by the Research Ethics Board (University of Guelph, Protocol Approval No. 09DC005).
2.2.5 Laboratory methods

Fecal and blood samples were processed at the Parasitology Laboratory, Department of Pathobiology, Ontario Veterinary College, University of Guelph. GIN (trichostrongyle-type) FECs were performed on individual fecal samples using a modified McMaster concentration method (Ministry of Agriculture, Fisheries and Food, 1986), with a lower detection limit of 50 eggs per gram (epg). The flotation fluid used was a saturated sodium chloride solution with a specific gravity of 1.20. PCV was determined using a Baxter Canlab microhematocrit centrifuge (Baxter Corporation, Mississauga, ON L5N 0C2, Canada), and TPP was determined using a hand refractometer (ATAGO SPR-NE®, Bellevue, WA 98005, U.S.A.). A hand refractometer was used for logistic and financial reasons – there was no budget for a direct laboratory method.

Blood samples were tested 24-48 hours after collection, and fecal samples were tested 3-7 days after collection. All samples were refrigerated at 4°C before being tested.

2.2.6 Data entry and management

All data were entered into an Excel spreadsheet (Microsoft Office Excel©, 2007) and data cleaning was performed manually. Data were compared with information on all available lambing dates obtained from the producers, and were used to classify animals into one of five different production stages. Ewes that were not bred at the time of sampling were classified as ‘maintenance’. Pregnant ewes that were less than 120 days in gestation were classified as ‘early gestation’. Pregnant ewes that were at, or beyond, 120 days in gestation were classified as ‘late gestation’. Ewes less than 40 days after lambing
were classified as ‘early lactation’. Ewes at, or beyond, 40 days lactation were classified as ‘late lactation’. Animals for which no lambing data were available were assumed to be in ‘maintenance’.

The CP ingested daily by each animal was estimated using the estimated daily Dry Matter Intake (DMI), expressed as a percentage of body weight (National Research Council of the National Academies, 2007), for different production stages and prolificacy. The estimated DMI percentage was multiplied by the estimated weight of the animal (based on the average expected weight for the main breed present on the farm), and then divided by 100 to obtain total daily feed consumption, in kg/d. A constant term was obtained by dividing 100 by the total consumption of feed in kg/d. This constant was then used to calculate the total estimated CP ingested (expressed as the percentage of protein ingested/day) as:

\[(\text{Quantity forage fed} \times \text{forage CP content} \times \text{constant}) + (\text{Quantity concentrate fed} \times \text{concentrate CP content} \times \text{constant})\]

For this calculation, three assumptions were made: (i) unless otherwise specified, all the ewes on the same farm received the same quantity and type of nutrition; (ii) unless otherwise specified, the forage and concentrate fed were constant over the same lambing season; and (iii) weight and prolificacy were estimated at the group level, based on the main breed present (i.e. ≥75% of the sheep flock) on each farm.

2.2.7 Data analysis

All statistical analyses were conducted using SAS® 9.3 (SAS Institute Inc., Cary, NC, U.S.A) and the significance level was set at alpha≤0.05. Causal diagrams were
designed for each of the three outcomes FEC, TPP and PCV, to guide the model-building process. In the FEC model, production stage, lambing season, PCV, litter size, total birth weight, number of lambs weaned and total 50-day weights were considered explanatory variables; CP and TPP were considered intervening variables between production stage and FEC, and were therefore not included in the multivariable model. Similarly, access to pasture was considered an intervening variable between season and FEC, and was not considered in the multivariable model. In the TPP model, FEC, lambing season, PCV and production stage were considered as explanatory variables; CP was considered an intervening variable between production stage and TPP, and was therefore not included in the multivariable model. In the PCV model, lambing season, FEC, TPP and production stage were considered explanatory variables. Summary statistics were generated using PROC MEANS (SAS 9.3), and collinearity between predictor variables (Pearson correlation coefficient >0.8) was assessed by testing pair-wise correlations.

Three general linear mixed models (GLMM) were fit using PROC MIXED (SAS 9.3). The first model had the natural logarithm of FEC (lnFEC) as the response variable; different transformations (e.g. square root, natural logarithm, and logarithm to base 10) were performed in an attempt to achieve a normal distribution of the FEC data, which were assessed by plotting histograms, normal probability plots, and four different tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling). For the logarithmic transformations of the FEC, the addition of a constant term to the zero FECs was investigated, with the options being counts from 1 to 49 epg (50 was the lowest number of eggs per gram of feces that the test detected). A constant term of 25 was selected for the zero FECs since it was associated with the best normality and
the natural logarithm was found to be the best transformation. The second and third models had TPP and PCV as the response variables, respectively.

The dependence of the data was modeled by a random intercept parameter at the farm level (to account for between-sheep clustering within flocks), and repeated measures parameters at the sheep level (to account for within-sheep auto-correlation). While accounting for the farm random effect and repeated measurements, each fixed effect variable was examined on its own to screen for variables to start the modeling process. A liberal alpha value for model entry of ≤0.20 was used to select variables eligible to be entered in the model. A final GLMM was built by first including all variables that were significant in the univariable analysis, and keeping those variables with an alpha value ≤0.05 in the final model. Next, predictors of interest that were not significant were forced into the model to assess potential statistical confounding and conditional effects. All possible and biologically plausible two-way and three-way interactions were also tested for significance (alpha≤0.05). The linearity of associations between continuous predictor variables and outcomes was assessed graphically by plotting lowess smoother curves, and by testing a quadratic term in the model, as described by Dohoo et al. (2009).

The model assumptions were assessed by plotting residuals against the predicted outcomes and explanatory variables, to look for homoscedasticity, non-linearity and outliers. Normality was visually assessed with histograms of the residuals and normal quantile plots, and assessed statistically using the four normality tests mentioned earlier. Observations that were identified as outliers or influential were cross-checked with the original data sheets for any peculiarity in the data to explain its influential behavior. The model was repeated without the influential observations, and differences in coefficients
and goodness-of-fit tests were noted. If the assumptions of linearity or homoscedasticity were not met, different data transformations were performed and the residuals were re-assessed (as noted below).

2.3 Results

2.3.1 Study population

The six farms were selected purposively to represent different areas in southwestern Ontario (latitude from 42.7°N to 44.4°N; longitude from 78.7° to 81.8°W) and different flock sizes (range from 50 to 1500 ewes; arithmetic mean=300 ewes). Farms were visited five times each lambing season, for a total of 3043 observations. Table 2.1 presents the number of observations for each production stage in each lambing season, for each farm. Farms A, C, D and E were sampled for three consecutive lambing seasons (winter, spring and autumn), while Farms B and F were only sampled for two consecutive lambing seasons (winter and spring). Farm B dropped out of the study after two lambing seasons, and Farm F did not have sufficient ewes lambing in the autumn. The 50L date ranged from January 26 to March 10, April 29 to June 18, and October 1 to November 8 for the winter, spring and autumn lambing seasons, respectively.

2.3.2 Fecal egg counts

There were a total of 2581 FEC observations; 462 of the 3043 total animal observations (15%) had missing FECs. Most missing FEC observations were due to fecal samples not obtained (i.e. no feces in the rectum at time of sampling) or a sheep was not present in the flock at the time of sampling. Losses to follow-up in the ewes were mainly due to culling or ear tag loss.
For all fecal samples, the arithmetic mean epg was 690 (range = 0 – 13,000) and the standard deviation was 1225. Table 2.1 shows the arithmetic mean, standard deviation and median epg for each farm, by season and production stage; with the lowest mean being 10 among the maintenance ewes on Farm B in winter, and the highest mean being 2289 among late gestation ewes on Farm C in autumn.

2.3.3 Packed cell volume and total plasma protein

With a possible maximum total of 1875 blood samples collected over 3 of the 5 farm visits per lambing season, there were actually 1662 (89%) and 1670 (89%) PCV and TPP observations, respectively. The missing observations were due to loss to follow-up of ewes or insufficient blood for processing.

For all samples, the arithmetic mean PCV was 30.5% (range = 14 – 45%) and the standard deviation was 4.08, while the arithmetic mean TPP was 7.1 g/dl (range = 4.8 – 10.4 g/dl) and the standard deviation was 0.67 (Table 2.2). Both PCV and TPP observations were normally distributed and did not require any transformations.

2.3.4 Final lnFEC model

A total of 25% (645/2581) of the fecal samples had zero or undetectable levels of GIN eggs, and 54% (1394/2581) of the fecal samples had ≤250 epg, causing the data to be right-skewed.

Table 2.2 shows the arithmetic means, standard deviations, and ranges, as well as coefficients and p-values, of all the continuous variables included in the univariable analyses. None of these variables showed pair-wise collinearity. The final model of
lnFEC is shown in Table 2.3, while Figures 2.1a, b and c show the three-way interaction between production stage, lambing season and PCV. Three levels of PCV were used in the figure to demonstrate the predicted effect of a low (PCV=24), medium (PCV=30.5) and high PCV (PCV=37) for the three different lambing seasons (i.e. winter, spring and fall) and five production stages (i.e. maintenance, early and late gestation, early and late lactation).

The final model explained 30% of the variation in the outcome when taking into account both fixed and random effects. The farm random effect (p = 0.0681) accounted for 11.5% of the variation in lnFEC. The final model met assumptions of both homoscedasticity and normality. Residual analyses revealed one major outlier. The model was rerun without the observation, but this did not change the final fit of the model, and there was no reason to omit the observation; hence, it was kept in the analysis.

2.3.5 Final TPP model

The final model of TPP is shown in Table 2.4. Although the main fixed effect of lnFEC was not statistically significant in the final model, it was retained in the model since it was part of a significant interaction. Both lnFEC and PCV showed a linear relationship with the outcome TPP, and were left as continuous variables in the model. After adjusting for the other variables in the model, every 1 unit increase in PCV predicted a 0.018 (95% CI = 0.01 – 0.026) increase in TPP.

Figure 2.2 shows the interaction effect between production stage and season on TPP, after adjusting for the other variables in the model. Figure 2.3 illustrates the interaction effect between production stage and lnFEC on TPP; four levels of FEC were
used in the figure to demonstrate the predicted effect of lnFECs for the different production stages.

The final TPP model explained 20% of the variation in the outcome when taking into account both fixed and random effects. The farm random effect \( p = 0.0678 \) explained 9.3% of the variation. Residuals for the final model showed homoscedasticity and normality. Residual analyses revealed three outliers. The model was fit omitting the three outliers, and this improved the fit of the model. Further investigation of the raw data revealed possible transcription errors for one observation, and it was therefore removed from the final dataset. No errors were found with the other two observations, and they were retained.

### 2.3.6 Final PCV model

The final model of PCV is shown in Table 2.5. The model coefficient for TPP indicated that, after adjusting for the other variables in the model, every 1 unit increase in TPP was associated with a 0.8% (95% CI = 0.51 – 1.1) increase in PCV.

Figure 2.4 illustrates the curvilinear relationship between PCV and lnFEC, where low lnFECs were associated with high PCV, but PCV decreased exponentially with increases in lnFEC. Figure 2.5 illustrates the interaction between lambing season and production stage and its relationship with PCV, after adjusting for the other variables in the model.

The final model explained 20% of the variation in the outcome when taking into account both fixed and random effects. The farm random effect \( p = 0.0727 \) explained 5.9% of the variation. Assumptions of both homoscedasticity and normality were met in
the final model. Residual analyses revealed two outliers; their removal did not improve the fit of the final model, and there was no justification to omit the observations, hence they were retained.

2.4 Discussion and conclusions

2.4.1. Final lnFEC model

The effects of lambing season, production stage and PCV on lnFEC were each dependent upon one another. During both the winter and spring lambing seasons (Figures 2.1a and 2.1b, respectively), the FECs were consistently lower than the autumn lambing season for all production stages except late lactation (Fig. 2.1c). During the winter and spring seasons, the ewes on all farms were housed indoors for most of the time; therefore, their exposure to parasites was limited. During the summer months, the ewes were kept on pasture, increasing their exposure to parasites. This would have resulted in higher fecal egg shedding during the autumn, regardless of production stage.

During the winter and spring lambing seasons, the FECs were significantly higher in ewes classified as late gestation (spring only), early lactation and late lactation, compared to ewes classified as maintenance or early gestation. This suggests that the increase in fecal egg shedding may be explained by a decline in immunity experienced during parturition and lactation, which would allow hypobiotic parasites to resume development and egg shedding (Michel, 1978). There was no significant difference in the FECs of ewes in maintenance or in early gestation in the winter and spring lambing season, indicating that ewes that were not pregnant (i.e. in maintenance) or in early gestation in the spring did not experience an increase in fecal egg shedding related to
seasonal effects. This finding is in disagreement with the suggestion that the PPER always occurs in the spring time, regardless of the pasture exposure or ewes’ production stage (Cvetkovic et al., 1971). In the autumn lambing season, animals in late gestation had a significantly higher FEC compared to ewes in early gestation (especially when PCV was low). In contrast, the FECs of ewes in early and late lactation was significantly lower compared to ewes in late gestation, especially in animals with low PCV values. The observed trend in the autumn may have occurred because of the increased availability of GIN third-stage larvae during the early gestation period, while the sheep were grazing on pasture. In ewes lambing in the autumn, late gestation usually occurred in October-November and coincided with a drop in ambient temperatures, and consequent parasite hypobiosis (Blitz and Gibbs, 1972). This hypobiosis could explain the decrease in FECs observed after late gestation and during lactation. Also, most animals were housed in October-November, resulting in a reduced exposure to GINs and, consequently lower parasitic burdens and a decrease in fecal egg shedding. These findings suggest that physiological changes associated with down regulation of immunity occurred in periparturient ewes regardless of the lambing season, leading to a gradual increase in fecal egg shedding during late gestation and lactation. However, during the early autumn months, these physiological effects, while still present, were over-shadowed by environmental factors.

The observations in FEC trends are somewhat in accordance with Southcott et al. (1972) who investigated the PPER in ewes lambing at different times of the year in New South Wales, Australia. These authors also observed a PPER in all lambing seasons, although in that study, the PPER was greatest in the summer, when pasture infectivity
was at its highest. Dunsmore (1965) suggested that the PPER may be influenced by an interaction between parasites and host animals, whereby environmental factors related to changes in ambient temperature and photoperiod allow the parasite to resume its life cycle within the sheep, while changes in the hormonal and immunological status experienced by pregnant ewes facilitate the establishment and propagation of the parasite. Our results are consistent with this theory, with both environmental and immunological factors likely contributing to the observed changes in fecal egg shedding patterns. However, in our study, the ewes’ exposure to, and the availability of, parasites on pasture seemed to be the most influential environmental factors.

During all three lambing seasons, animals with lower PCVs experienced a more marked increase in fecal egg shedding. While this effect was fairly constant throughout the spring lambing season (Fig. 2.1b), in the winter lambing season (Fig. 2.1a) the effect of low PCV on fecal egg shedding was greatest during the lactation period. On the other hand, in the autumn lambing season (Fig. 2.1c), ewes with a low PCV experienced a more marked increase in fecal egg shedding during late gestation.

The reference range for PCV in sheep is 27-45% (Radostits et al., 2007). Our results suggest that when the PCV is within this normal range, slight changes in PCV have little to no effect on the FEC. However, below a certain threshold of PCV, animals may be more susceptible to parasite infection and, hence, increased fecal egg shedding. Low PCVs may reflect sub-optimal energy and protein nutrition, leading to reduced immunity against the parasites (Radostits et al., 2007); this reduction in immunity may be further exacerbated by the strain of parturition and lactation (Crofton, 1954). Based on a causal diagram, CP and TPP were considered to be intervening variables between
production stage and lnFEC, and were therefore not included in our final lnFEC model. Also, we were unable to draw comparisons between CP ingested and PCV since the former was determined at the group-level, while the latter was determined at the individual level. Low PCV values are also caused by blood and protein loss, suggestive of an ongoing parasitic infection (Radostits et al., 2007); one must therefore be careful when interpreting cause and effect between PCV and FEC.

Houdijk (2008) showed that, at a constant nutrition level, the ewes’ litter size affected the PPER. However, in our study, none of the lamb productivity variables (ewe parity, litter size, number of lambs weaned, total birth weights and total 50-day weights) were significantly associated with FEC. The discordance in our results may be attributed to a lack of statistical power to detect differences in FEC by litter size or other productivity measures. Since our study was conducted on commercial farms, we had to rely on data collected by the producers; not all producers had an efficient data-recording system, leading to some missing productivity data. Also, for those who kept records, most either measured birth weights or 50-day weights; few measured both. The limited number of observations might therefore have precluded any observable differences in FEC by productivity measures.

2.4.2 Final TPP model

The reference range for TPP in sheep is 6.0-7.9 g/dl, and TPP was measured as an indicator of protein available to animals, since several studies have implicated a lack of dietary protein in the PPER (Coop and Kyriazakis, 1999; Houdijk et al., 2001; Houdijk, 2008). We found that the association of production stage with TPP was dependent on
During the autumn and winter lambing seasons, the TPP decreased throughout gestation, and was lowest during early lactation. This was then followed by an increase in TPP during late lactation. The first few weeks of lactation are a nutritionally demanding period for the ewes, since milk production is at its highest, while food intake is only gradually increasing after parturition (National Research Council of the National Academies, 2007). Also, blood volume increases during pregnancy, due to an increased production of aldosterone and estrogens, and increased fluid retention by the kidneys (Guyton, 2006), leading to an apparent decrease in TPP during gestation. During the spring, TPP seemed to peak at early gestation, after which it followed the same trend observed in the autumn and winter lambing seasons, decreasing substantively in late gestation and increasing during lactation. The apparent increase in TPP observed in early gestation is likely a consequence of the low TPP observed in the maintenance animals. However, the maintenance group in the spring only had 33 observations from one farm, and therefore the predicted TPP might not be representative.

A significant interaction effect was also observed between FEC and production stage on TPP (Fig. 2.3). At all the different levels of FECs, TPP decreased during the gestation period, and remained low between late gestation and early lactation. This is in accordance with the nutrient partitioning framework proposed by Coop and Kyriazakis (1999) which suggests that different bodily functions are given different priorities, depending on the age and type of animal - while maintenance of body protein remains top priority in all age groups, reproducing animals prioritize reproductive performance over expression of anti-parasite immunity.
In the present study, the change in TPP in ewes after early lactation depended on the FEC. The TPP increased in ewes with low (FEC=200 epg) or no (FEC=0 epg) egg shedding, although ewes in the latter group experienced a greater increase in TPP. On the other hand, the TPP in ewes with moderate (FEC=800 epg) and heavy (FEC=1200 epg) egg shedding did not change. Gastrointestinal nematodes cause a reduction in the host’s protein levels by reducing voluntary feed intake, increasing the endogenous loss of protein (Coop and Holmes, 1996), and triggering chronic inflammatory responses in the gut (Sutherland and Scott, 2010). Therefore, the presence of internal parasites in ewes with moderate or heavy parasite burdens likely prevented a recovery in TPP after early lactation.

2.4.3 Final PCV model

*Haemonchus contortus* is a hematophagous parasite, causing protein loss and anaemia in infected animals (Taylor *et al.*, 2007). PCV was used to measure anaemia in animals, which is a clinical consequence of the feeding activity of *H. contortus*.

The effect of production stage on PCV was dependent on lambing season (Fig. 2.5). During the autumn lambing season, the PCV decreased gradually over the gestation and lactation periods. In the winter, the PCV increased during the gestation period, peaked at late gestation, and then declined during lactation. In the spring lambing season, the PCV decreased during early gestation, then increased during late gestation, after which it remained constant during lactation.

Interpretation of these results is complicated by the fact that parasite cultures were not performed, and therefore we were unable to speciate the GIN eggs. However, during
the spring lambing season, a few farms on the study reported clinical signs of Type II haemonchosis in some of their pregnant ewes (sub-mandibular oedema and pale mucous membranes); this might explain the drop in PCV observed during early gestation in the spring. In a study examining the arrested development of *H. contortus* in sheep (Blitz and Gibbs, 1972), it was shown that *H. contortus* overwintered in ewes as arrested fourth-stage larvae and resumed development in the spring. The consequent accumulation of adult *H. contortus* within the abomasum during early gestation would potentially lead to blood loss and low PCVs. Compensatory erythropoiesis may then allow for a recovery in the haematocrits in the following weeks (Taylor *et al.*, 2007).

2.4.4 Study limitations

While the sample size was small, the objectives of the study were at the individual level (i.e. to determine whether ewes that lamb out-of-season experienced a PPER, and to determine whether ewes not bred or in early gestation during the spring season experienced an increase in fecal egg shedding, related to seasonal effects). Moreover, the target population was Ontario sheep farms that practice out-of-season lambing, and most of these farms have small acreage and little variation in farm topography. Therefore, we believe that the sample size was appropriate for the study; however, we recognize that the external validity of the findings may be limited.

We recognize that pasture sampling may have been beneficial to estimate the level of pasture larval contamination (Couvillion, 1993; Stromberg, 1997). However, due to financial constraints, we evaluated the additional information this procedure would have provided, and the limited sensitivity of the procedure to assess pasture
contamination, compared to the use of tracer lambs (O’Connor et al., 2006), and decided to focus on sheep FECs, as these represent the parasitic infection the animals acquire from pasture (Stromberg, 1997).

Parasite cultures were not performed due to logistic and financial constraints; however most of these farms reported lambs dying of haemonchosis in the summer. Furthermore, farm B participated in a study on the epidemiology of GIN parasites in Ontario sheep flocks between May 2006 and March 2008 (Mederos et al., 2010), and larval cultures available monthly during the grazing season and twice during the winter (January and March), indicated that *Haemonchus* sp. was present on that farm during the grazing season.

While we recognize that the limited farm variability observed in this study (11.5% of the total variability) may be due to the low number of farms and the fact that the farms were not selected randomly, the flocks were purposively selected from geographically diverse regions and had different flock sizes. Moreover, the target population in this study was farms that practice out-of-season lambing, which are often more similar as they share common management practices.

The farms were followed for three consecutive lambing seasons, except for Farms B and F, which were not sampled in the autumn, leading to fewer overall observations for the autumn lambing season than the other two seasons. Also, few of the ewes sampled during the entire study, and none of the ewes sampled during the autumn season, were in the maintenance group. This might have reduced the statistical power to detect differences between maintenance and other production groups, especially during the
autumn. For the classification of ewes into production stages we used owner-recorded data, which is not always reliable; therefore misclassification bias might have occurred.

2.4.5 Conclusion

In summary, the PPER was observed during all three lambing seasons, although the magnitude and timing of maximum fecal egg shedding for each production stage varied between seasons. Ewes that were open or in early gestation during the spring did not experience a spring rise in fecal egg shedding. In contrast, ewes lambing in the autumn experienced a rise in fecal egg shedding over the gestation period, which peaked at late gestation and then decreased. While both TPP and PCV were associated with FEC, this association varied between production stages and lambing seasons. Therefore, the usefulness of these clinical parameters as potential diagnostic indicators of PPER should be investigated further. Collectively, these findings show that both seasonal and animal physiological factors play an important role in determining fecal egg shedding, and need to be considered when implementing preventive parasite control strategies on Ontario sheep farms that practice out-of-season lambing.

2.5 Acknowledgements

This research was supported by the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) – New Directions Research Program, with additional financial assistance from the Ontario Sheep Marketing Agency (OSMA), the Nova Scotia Agricultural College – Organic Science Cluster, and the University of Guelph. The study sponsors were not involved in the study design; collection, analysis and interpretation of the data; in the writing of the manuscript and in the decision to submit the manuscript for
publication. The authors are very grateful to William Sears for statistical advice and to Brad De Wolf, Steve Roche, Grazyna Adamska-Jarecka, Katie Sippel, Kirstie Puskas, Lee Siertsema and Hasani Stewart for laboratory and field assistance. We especially acknowledge the sheep producers that participated in the study.
2.6 References


Table 2.1. Arithmetic mean, standard deviation and median of the gastro-intestinal nematode (trichostrongyle-type) fecal egg counts (eggs per gram) from 2581 repeated ewe observations, from six farms in south-western Ontario, Canada, presented by season and production stage (December 2009 to June 2011)

<table>
<thead>
<tr>
<th></th>
<th>Farm A</th>
<th>Farm B</th>
<th>Farm C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>winter</td>
<td>spring</td>
<td>autumn</td>
</tr>
<tr>
<td>Maintenance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>161</td>
<td>65</td>
<td>-</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(152)</td>
<td>(99)</td>
<td>(35)</td>
</tr>
<tr>
<td>Median</td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>N(^2)</td>
<td>38</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Early Gestation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>224</td>
<td>231</td>
<td>630</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(156)</td>
<td>(289)</td>
<td>(626)</td>
</tr>
<tr>
<td>Median</td>
<td>200</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>N(^2)</td>
<td>41</td>
<td>100</td>
<td>101</td>
</tr>
<tr>
<td>Late Gestation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>337</td>
<td>485</td>
<td>459</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(257)</td>
<td>(318)</td>
<td>(614)</td>
</tr>
<tr>
<td>Median</td>
<td>300</td>
<td>450</td>
<td>100</td>
</tr>
<tr>
<td>N(^2)</td>
<td>15</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td>Early Lactation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>323</td>
<td>776</td>
<td>807</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(311)</td>
<td>(606)</td>
<td>(818)</td>
</tr>
<tr>
<td>Median</td>
<td>250</td>
<td>675</td>
<td>450</td>
</tr>
<tr>
<td>N(^2)</td>
<td>31</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>Late Lactation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>350</td>
<td>523</td>
<td>616</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(244)</td>
<td>(401)</td>
<td>(804)</td>
</tr>
<tr>
<td>Median</td>
<td>325</td>
<td>450</td>
<td>300</td>
</tr>
<tr>
<td>N(^2)</td>
<td>15</td>
<td>20</td>
<td>21</td>
</tr>
</tbody>
</table>

\(^1\)SD = standard deviation; \(^2\)N = number of animal observations with a fecal egg count

- = no data available
Table 2.1. (continued)

<table>
<thead>
<tr>
<th></th>
<th>Farm D</th>
<th>Farm E</th>
<th>Farm F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>winter</td>
<td>spring</td>
<td>autumn</td>
</tr>
<tr>
<td><strong>Maintenance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>-</td>
<td>366</td>
<td>-</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(638)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(^2)</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Early Gestation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>233</td>
<td>885</td>
<td>604</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(464)</td>
<td>(1266)</td>
<td>(892)</td>
</tr>
<tr>
<td>Median</td>
<td>100</td>
<td>200</td>
<td>225</td>
</tr>
<tr>
<td>N(^2)</td>
<td>89</td>
<td>63</td>
<td>88</td>
</tr>
<tr>
<td><strong>Late Gestation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>508</td>
<td>355</td>
<td>560</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(1749)</td>
<td>(404)</td>
<td>(634)</td>
</tr>
<tr>
<td>Median</td>
<td>25</td>
<td>200</td>
<td>275</td>
</tr>
<tr>
<td>N(^2)</td>
<td>20</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td><strong>Early Lactation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>944</td>
<td>879</td>
<td>1294</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(824)</td>
<td>(783)</td>
<td>(1120)</td>
</tr>
<tr>
<td>Median</td>
<td>650</td>
<td>650</td>
<td>1100</td>
</tr>
<tr>
<td>N(^2)</td>
<td>40</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td><strong>Late Lactation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2174</td>
<td>2047</td>
<td>2069</td>
</tr>
<tr>
<td>SD(^1)</td>
<td>(2706)</td>
<td>(1976)</td>
<td>(1757)</td>
</tr>
<tr>
<td>Median</td>
<td>725</td>
<td>1450</td>
<td>1525</td>
</tr>
<tr>
<td>N(^2)</td>
<td>66</td>
<td>23</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^1\)SD = standard deviation; \(^2\)N = number of animal observations with a fecal egg count

- = no data available
Table 2.2. Descriptive statistics, as well as coefficients and p-values for the continuous variables included in the univariable models of the natural logarithm transformation of fecal egg count in ewes from six farms in south-western Ontario, Canada (December 2009 to June 2011).

<table>
<thead>
<tr>
<th>Variable</th>
<th>n¹</th>
<th>Arithmetic Mean</th>
<th>Standard deviation</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed cell volume (%)</td>
<td>1662</td>
<td>30.5</td>
<td>4.08</td>
<td>14.0</td>
<td>45.0</td>
<td>-0.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total plasma protein (g/dl)</td>
<td>1670</td>
<td>7.1</td>
<td>0.67</td>
<td>4.8</td>
<td>10.4</td>
<td>-0.05</td>
<td>0.352</td>
</tr>
<tr>
<td>Crude protein ingested daily (%)</td>
<td>2673</td>
<td>13.8</td>
<td>1.75</td>
<td>10.2</td>
<td>17.4</td>
<td>-0.10</td>
<td>0.001</td>
</tr>
<tr>
<td>Litter size</td>
<td>992</td>
<td>1.8</td>
<td>0.75</td>
<td>1</td>
<td>4</td>
<td>-0.12</td>
<td>0.359</td>
</tr>
<tr>
<td>Number Weaned</td>
<td>537</td>
<td>1.5</td>
<td>0.70</td>
<td>0</td>
<td>4</td>
<td>0.22</td>
<td>0.239</td>
</tr>
<tr>
<td>Total birth weight (kg)</td>
<td>227</td>
<td>10.2</td>
<td>4.81</td>
<td>3.3</td>
<td>23.6</td>
<td>0.01</td>
<td>0.786</td>
</tr>
<tr>
<td>Total 50d weight (kg)</td>
<td>251</td>
<td>32.2</td>
<td>16.80</td>
<td>9.5</td>
<td>96.8</td>
<td>-0.02</td>
<td>0.142</td>
</tr>
</tbody>
</table>

¹n = number of animal observations
Table 2.3. Final general linear mixed model for the natural logarithm of fecal egg counts (eggs per gram) in 2581 fecal samples from ewes in different productions stages, sampled from six farms in south-western Ontario, Canada (December 2009 to June 2011).

<table>
<thead>
<tr>
<th>Fixed Effects</th>
<th>Estimate</th>
<th>95% CI²</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>7.41</td>
<td>(4.45 – 10.38)</td>
<td>6.43</td>
<td>0.0014</td>
</tr>
<tr>
<td>Production stage (PS)¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Gestation</td>
<td>-1.19</td>
<td>(-3.55 – 1.18)</td>
<td>5.25</td>
<td>0.0003</td>
</tr>
<tr>
<td>Late Gestation</td>
<td>0.22</td>
<td>(-2.51 – 2.95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Lactation</td>
<td>-0.35</td>
<td>(-2.83 – 2.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late Lactation</td>
<td>1.42</td>
<td>(-1.00 – 3.84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambing season (LS)²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>0.11</td>
<td>(-1.05 – 1.27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>2.53</td>
<td>(1.00 – 4.07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCV¹</td>
<td>-0.09</td>
<td>(-0.16 – -0.02)</td>
<td>69.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PS<em>LS</em>PCV²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effects</th>
<th>Estimate</th>
<th>95% CI²</th>
<th>Z-Value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm</td>
<td>0.27</td>
<td>(0.10 – 1.88)</td>
<td>1.50</td>
<td>0.0674</td>
</tr>
<tr>
<td>Sheep</td>
<td>2.00</td>
<td>(1.85 – 2.17)</td>
<td>24.46</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

¹When interpreting these variables, there is not just one coefficient to consider because these variables are involved in an interaction. The total effect for each variable is the combination of the relevant coefficients for the main effects and the interacting categories. Coefficients for the many levels of the interaction variable are not provided because they lack meaning in isolation from the main effect categories. The combined main and interaction effects are best represented in Figure 2.1.

²CI = Confidence Interval

³PCV = Packed Cell Volume
Table 2.4. Final general linear mixed model for the total plasma protein (g/dl) in 1670 blood
samples from ewes in different production stages, sampled from six farms in south-
western Ontario, Canada (December 2009 to June 2011).

<table>
<thead>
<tr>
<th>Fixed Effects</th>
<th>Estimate</th>
<th>95% CI(^2)</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>5.93</td>
<td>(5.20 – 6.67)</td>
<td>20.81</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Production stage (PS(^1))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Gestation</td>
<td>0.52</td>
<td>(0.04 – 0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late Gestation</td>
<td>0.54</td>
<td>(0.01 – 1.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Lactation</td>
<td>0.44</td>
<td>(-0.10 – 0.98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late Lactation</td>
<td>1.19</td>
<td>(0.67 – 1.72)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambing season (LS(^1))</td>
<td></td>
<td></td>
<td>8.06</td>
<td>0.0003</td>
</tr>
<tr>
<td>Winter</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>-0.56</td>
<td>(-0.87 – -0.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>0.31</td>
<td>(0.04 – 0.58)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCV(^3)</td>
<td>0.02</td>
<td>(0.01 – 0.03)</td>
<td>19.32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LnFEC(^1,4)</td>
<td>0.16</td>
<td>(0.05 – 0.26)</td>
<td>0.56</td>
<td>0.4555</td>
</tr>
<tr>
<td>PS*LS</td>
<td></td>
<td></td>
<td>4.03</td>
<td>0.0002</td>
</tr>
<tr>
<td>LnFEC(^4)*PS(^1)</td>
<td></td>
<td></td>
<td>10.30</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effects</th>
<th>Estimate</th>
<th>95% CI(^2)</th>
<th>Z-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm</td>
<td>0.04</td>
<td>(0.01 – 0.28)</td>
<td>1.49</td>
<td>0.0678</td>
</tr>
<tr>
<td>Toeph (1)(^5)</td>
<td>0.34</td>
<td>(0.28 – 0.40)</td>
<td>10.92</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Toeph (2)(^5)</td>
<td>0.18</td>
<td>(0.08 – 0.27)</td>
<td>3.58</td>
<td>0.0003</td>
</tr>
<tr>
<td>Toeph (3)(^5)</td>
<td>0.30</td>
<td>(0.09 – 0.51)</td>
<td>2.75</td>
<td>0.0059</td>
</tr>
</tbody>
</table>

\(^1\)When interpreting these variables, there is not just one coefficient to consider because these
variables are involved in interactions and are categorical. The total effect for each variable is the
combination of the relevant coefficients for the main effects and the interacting categories.
Coefficients for the many levels of the interaction variables are not provided because they lack
meaning in isolation from the main effect categories. The combined main and interaction effects
are best represented in Figures 2.2 and 2.3.

\(^2\)CI = Confidence Interval

\(^3\)PCV = Packed Cell Volume

\(^4\)LnFEC = natural logarithm of fecal egg counts (eggs per gram)

\(^5\)Toeph (1) (2) (3) = Correlation in total plasma protein between different sampling time-points
Table 2.5. Final general linear mixed model for the packed cell volume (%) in 1662 blood samples from ewes in different production stages, sampled from six farms in south-western Ontario, Canada (December 2009 to June 2011).

<table>
<thead>
<tr>
<th>Fixed Effects</th>
<th>Estimate</th>
<th>95% CI</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>22.59</td>
<td>(18.34 – 26.85)</td>
<td>13.65</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Production Stage (PS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Gestation</td>
<td>0.66</td>
<td>(-0.49 – 1.82)</td>
<td>2.56</td>
<td>0.0372</td>
</tr>
<tr>
<td>Late Gestation</td>
<td>1.26</td>
<td>(-0.14 – 2.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Lactation</td>
<td>0.31</td>
<td>(-0.99 – 1.61)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late Lactation</td>
<td>-0.67</td>
<td>(-1.96 – 0.58)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambing Season (LS)</td>
<td></td>
<td>3.40</td>
<td>0.0337</td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>-0.03</td>
<td>(-1.82 – 1.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>-0.66</td>
<td>(-2.17 – 0.85)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Plasma Protein</td>
<td>0.80</td>
<td>(0.51 – 1.10)</td>
<td>27.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LnFEC</td>
<td>1.62</td>
<td>(0.81 – 2.43)</td>
<td>15.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LnFEC*LnFEC</td>
<td>-0.20</td>
<td>(-0.27 – -0.13)</td>
<td>29.28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PS*LS</td>
<td>5.89</td>
<td></td>
<td>9.89</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effects</th>
<th>Estimate</th>
<th>95% CI</th>
<th>Z-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm</td>
<td>0.93</td>
<td>(0.34 – 7.00)</td>
<td>1.46</td>
<td>0.0727</td>
</tr>
<tr>
<td>Toeph (1)</td>
<td>0.30</td>
<td>(0.23 – 0.36)</td>
<td>9.15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Toeph (2)</td>
<td>0.28</td>
<td>(0.19 – 0.37)</td>
<td>6.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Toeph (3)</td>
<td>0.55</td>
<td>(0.40 – 0.70)</td>
<td>7.20</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(^1\)When interpreting these variables, there is not just one coefficient to consider because these variables are involved in interactions and are categorical. The total effect for each variable is the combination of the relevant coefficients for the main effects and the interacting categories. Coefficients for the many levels of the interaction variables are not provided because they lack meaning in isolation from the main effect categories. The combined main and interaction effects are best represented in Figure 5.

\(^2\)CI = Confidence Interval

\(^3\)LnFEC = natural logarithm of fecal egg counts (eggs per gram)

\(^4\)Toeph (1) (2) (3) = Correlation in packed cell volume between different sampling time-points.
Figure 2.1. Median Fecal Egg Counts (gastrointestinal nematode eggs per gram of feces) (± standard error) predicted from a general linear mixed model, plotted against the Production Stages for the minimum (24), mean (30.5) and maximum (37) PCV values, for the (a) winter, (b) spring and (c) autumn lambing seasons, on 6 farms in south-western Ontario, Canada (December 2009 to June 2011).

Note: None of the ewes sampled during the autumn season were in maintenance at time of sampling.
Figure 2.2. Total plasma protein (g/dl) (± standard error) predicted from a general linear mixed model, plotted against the different production stages for all three lambing seasons (autumn, spring and winter), for 1670 blood samples from ewes on six farms in south-western Ontario, Canada (December 2009 to June 2011).
Figure 2.3. The Total Plasma Protein (g/dl) (± standard error) predicted from a general linear mixed model, plotted against the different production stages, for the quartiles of gastrointestinal nematode fecal egg counts (0 eggs per gram, 200 eggs per gram, 800 eggs per gram and 13,000 eggs per gram) for 1670 observations from ewes on six farms in south-western Ontario, Canada (December 2009 to June 2011).

Note: None of the ewes that were sampled for blood were in maintenance
Figure 2.4. The curvilinear relationship between Packed Cell Volume (％) and natural log transformation of Fecal Egg Counts (eggs per gram) for ewes on six farms in southwestern Ontario, Canada (December 2009 to June 2011), as determined from a general linear mixed model of packed cell volume.
Figure 2.5. The packed cell volumes (%) (± standard error) predicted from a general linear mixed model, plotted against the different production stages for three lambing seasons (autumn, spring and winter), for 1662 blood samples from ewes on six farms in south-western Ontario, Canada (December 2009 to June 2011).

Note: None of the ewes sampled during the autumn season were in maintenance at time of sampling.
CHAPTER 3

Pilot project to investigate over-wintering of free-living gastrointestinal nematode larvae of sheep, in Ontario, Canada

In preparation for Small Ruminant Research

Abstract

Gastro-intestinal nematodes (GINs) have a direct life-cycle, with both free-living and parasitic stages. This pilot study was carried out to describe pasture-level environmental conditions that may be associated with overwintering survival and infectivity of free-living GIN stages on pasture on sheep farms in Ontario, Canada. One acre sections of pasture grazed in the previous season by GIN-infected sheep were assessed on three farms, starting in the winter after animal removal, until the following spring before turnout (January - April). Environmental data were collected using electronic data-loggers, which measured air and soil temperature, soil moisture and air relative humidity. The presence and type of GIN larvae on pasture were assessed by collecting monthly herbage and soil samples over the winter months. The infectivity of larvae in the spring was assessed by grazing 16 naïve lambs for 28 days, after which the lambs were slaughtered and the contents of their gastro-intestinal tracts analyzed for the presence of GINs. The lowest air and soil temperatures (-25.7°C and -19.8°C, respectively), and the lowest air relative humidity (15.0%), were recorded in January, while the lowest daily soil water content (-0.20 m³/m³) was recorded in March. Free-living stages of *Trichostrongylus* spp. (84.7 L3/kg dry matter [DM]) and *Nematodirus* spp. (42.4 L3/kg DM) were isolated from herbage samples collected in March on one farm; no larvae were isolated from the other
herbage and soil samples collected. GINs were recovered from the tracer lambs on all three farms; however, the mean GIN burdens for lambs on one farm were significantly less (1023 vs. >6600 for the other 2 farms), and were associated with lower mean daily soil temperatures and water content, possibly a consequence of limited snow cover observed on its pasture. Overall, Teladorsagia sp. was the predominant species recovered, followed by Nematodirus spp. and Trichostrongylus spp. Haemonchus contortus was recovered from one animal on one of the farms, but at very low levels. Since these farms were selected on the basis of a reported history of clinical haemonchosis, the results suggest that Haemonchus larvae do not survive well on pasture during a central Canadian winter. In contrast, Teladorsagia sp., Trichostrongylus spp. and Nematodirus spp. are able to overwinter on pasture, even with limited snow cover, and are still infective for sheep in the spring. These observations need to be taken into consideration when making recommendations on pasture management and treatment strategies for sheep flocks in Ontario, Canada.

3.1 Introduction

Gastro-intestinal nematodes (GINs) are ubiquitous on many sheep farms, and in addition to compromising animal welfare can cause important economic losses (van Dijk et al., 2010). All these helminth parasites have a direct life-cycle, with both free-living and parasitic stages (Taylor et al., 2007). The free-living stage involves the development of GIN eggs to third-stage infective GIN larvae (L3) in feces, and the subsequent migration onto pasture grasses which are then consumed by definitive hosts (O’Connor et al., 2006).
The survival and development of free-living stages is largely influenced by environmental factors, such as air and soil temperature, soil moisture and air relative humidity (Veglia, 1915; Stromberg, 1997; O’Connor et al., 2007; van Dijk et al., 2010; Reynecke et al., 2011), and several studies have reported a seasonality in larval availability on pasture (Gordon, 1948; Ayalew and Gibbs, 1973; Uriarte et al., 2003; Waller et al., 2004; Waghorn et al., 2011). This seasonality has often under-pinned sheep nematodosis management practices, such as timing of anthelmintic treatment and grazing strategies (Reynecke et al., 2011). With the emergence of anthelmintic resistance (Papadopoulos, 2008), regional knowledge of the factors that influence the dynamics of the free-living stages has assumed an even more important role, as more producers are turning to targeted anthelmintic treatment (Leathwick et al., 2011) and pasture management strategies to control GIN infections on sheep farms (O’ Connor et al., 2006). Anthelmintic resistance has recently been reported in sheep flocks in Ontario, Canada (Falzon et al., in press), highlighting the need to improve our knowledge of environmental factors that might influence the presence of GIN on pasture under central Canadian climate conditions.

A recent study on the epidemiology of GIN infections on sheep farms in Ontario and Quebec, Canada, showed that the most predominant nematode genera during the summer months were Teladorsagia sp., Haemonchus sp. and Trichostrongylus spp. (Mederos et al., 2010). While no recent studies have been conducted to determine the epidemiology of GINs on pasture during the winter months in central Canada, previous studies in the Maritime provinces (Smith and Archibald, 1965) and Quebec (Ayalew and Gibbs, 1973) indicated that both Teladorsagia sp. and Trichostrongylus spp. survived on
pasture in the winter, while *Haemonchus* sp. were not found. Nonetheless, in recent work conducted in Ontario and Quebec, *Haemonchus* sp. L3s were isolated from pasture samples collected in the spring months, before sheep were put out to graze (Mederos, 2010). This finding suggests that *Haemonchus* sp. parasites were able to over-winter on pasture and might have adapted to central Canadian climate conditions. However, the infectivity of these L3s was not determined.

*Haemonchus contortus* has been described as the single most important parasite affecting sheep, given its high pathogenicity and high biotic potential (Waller *et al.*, 2004). It is therefore essential to determine whether this parasite has adapted to, and is able to survive, winter weather conditions found in central Canada, as this will inform future recommendations on pasture management strategies for the control of GIN parasitism. Thus, the objectives of this study were twofold: (a) to describe the environmental factors that may affect the over-wintering survival of GIN on three commercial sheep farms in south-western Ontario; and (b) to determine if *H. contortus* larvae are (i) able to over-winter on pasture and/or soil under central Canadian winter conditions, and (ii) capable of establishing a patent infection in naïve tracer lambs the following spring.

### 3.2 Materials and methods

#### 3.2.1 Farm selection

A longitudinal study was conducted between December 2009 and June 2010 in which three commercial sheep farms were purposively selected in south-western Ontario (farm A= 42°19’N latitude and 71°20’W longitude; farm B=44°05’N latitude and
79°45’W longitude; farm C=43°55’N latitude and 80°25’W longitude). The sample size was dictated by logistical and financial constraints. The farms were selected based on their willingness to participate in the study, distance from the University of Guelph (within a 200 km radius) due to a requirement for frequent sampling and animal monitoring, and a known history of *H. contortus* parasitism on the farm. Specifically, both farms A and B reported a veterinary diagnosis of lambs dying of haemonchosis in the summer of 2009, while farm C participated in a study on the epidemiology of GIN parasites in Ontario sheep flocks between 2006-2009 (Mederos *et al.*, 2010) and larval cultures indicated that *H. contortus* was present on that farm. Within each farm, a one-acre representative section of pasture, where GIN-infected ewes and lambs had grazed the previous summer, was selected.

### 3.2.2 Environmental data

A HOBOware® Pro Data-Logger (Onset Computer Corporation, Bourne, MA, USA) was set up on the one-acre section of each of the three farms in December, after the sheep were taken off pasture. The data-logger had four probes which measured, at hourly intervals, the air temperature (°C) and relative humidity (%) at 1.5 m above ground level, and the soil temperature (°C) and moisture (m³/m³) just below ground level. These data were down-loaded at monthly intervals, from December 2009 to May 2010. It was not possible to collect snow coverage data at the probe level; however, reports from the nearest weather stations indicated that, during the period December 2009 to April 2010, the least snowfall was recorded for all the province of Ontario since observations began in 1843, with a mean of 46.2 cm recorded in Toronto (Environment Canada – Ontario
Weather Review, 2010). Anecdotally, less snow cover was observed on farm C, compared to farms A and B.

3.2.3 Sampling of herbage and soil

Herbage and soil samples were collected on a monthly basis during the winter months (January to April 2010) from the one-acre sections on each of the three farms. Both herbage and soil samples were collected by walking two “W” routes in the one-acre section and stopping every 20-30 paces. The herbage was clipped as close as possible to the ground, avoiding fecal and soil contamination; the total amount of herbage collected in each paddock section did not exceed 500 g wet weight. Soil samples were collected using a 30 cm soil auger; core samples were divided into ‘upper 15 cm soil’ (i.e. 0-15cm) and ‘lower 15 cm soil’ (i.e. >15-30cm) segments (Ministry of Agriculture, Fisheries and Food, 1986).

3.2.4 Tracer lambs

In April-May 2010, 16 Rideau-Arcott X Dorset weaned lambs of 3-4 months of age, were selected from the Ponsonby Sheep Research Centre at the University of Guelph, weighed, and treated with 10 mg/kg bodyweight fenbendazole (Safe-Guard™ Suspension 10%, Intervet Canada Ltd.). The flock at the Ponsonby Sheep Research Centre has been closed for the past 22 years, and from data acquired from repeated monitoring of fecal egg counts (FECs) and necropsy examinations, is considered to be free of infection from all GIN species, except for Nematodirus filicollis. Therefore, the lambs could be considered naïve. Moreover, these animals had never been on pasture,
and fecal samples collected from the lambs on day 0 (i.e. when lambs were put on pasture) showed zero GIN FECs.

The lambs were put out to graze for 28 days, starting at the same time the rest of the flock was put on pasture (11th May on farm A, 14th April on farm B, and 13th May on farm C): five animals on each of farms A and B, and six animals on farm C. Six lambs were used on farm C instead of five because an additional lamb was available at the Ponsonby Sheep Research Centre. On two of the farms (farm A and C), the same one-acre sections of the paddocks that were sampled in the winter months were fenced off, and the tracer lambs were left to graze on these sections without comingling with other sheep. On farm B, the owner wanted the tracer lambs rotationally grazed with the rest of the flock, which was moved onto different fenced-off paddocks, including the paddock with the one-acre section sampled in the winter, every 3-4 days. The sheep only grazed the pastures that had been grazed in the previous season but not yet that spring, and the tracer lambs did not return to the same paddock during their 28-day grazing period. Since this was done in April when maximum daily temperatures were still cool, it was improbable that any GIN eggs shed by the rest of the flock would develop into infective L3s within 3-4 days, as this normally takes a minimum of at least 5 days under optimal environmental conditions (Taylor et al., 2007). After 28 days of grazing, the lambs were weighed and slaughtered at the abattoir of the Food Science Department, University of Guelph. At necropsy, the abomasum, small intestine and large intestine from each lamb were tied, isolated, and transported in buckets over a period of 1h to the Ontario Veterinary College (OVC) necropsy room for further processing.
3.2.5 Laboratory methods

Herbage and soil samples were processed at the Parasitology laboratory, Department of Pathobiology, OVC, University of Guelph. Herbage samples were placed in a 9” diameter, 2.5 L capacity, funnel fitted with a scientific cleaning wipe (Kimwipes®, Kimberly-Clark Company, Texas, USA) which was laid over a removable wire mesh. A small length of hard rubber tubing was attached to the stem of the funnel, and the end of the tubing was fitted with a 50 ml plastic centrifuge tube. The funnel was filled with lukewarm water until the grass sample was submerged, and the sample was left overnight. The next day, the water was removed with a suction apparatus, and the centrifuge tube was detached. The tube was centrifuged at 1800 g for 2 minutes, and the supernatant discarded. The sediment (approximately 1 ml) was collected; two drops of the mixed sediment were transferred to a microscope slide and one drop of Lugol’s iodine was added to kill and stain the larvae. A 24x50mm cover slip was placed over the mixture and the slide was examined at a magnification of 100-400x (as required). This was repeated until all the sediment collected had been examined. All L3s recovered were identified to the genus level and counted. The grass sample was then transferred onto a tray, dried in an incubator at 37°C until brittle, and its weight recorded. The number of larvae in the herbage samples was expressed as larvae per kg dry matter (DM).

The same Baermann procedure described above was used for examination of soil samples, except that samples were submerged in lukewarm water for 48 hours. Free living soil nematodes were differentiated from infective L3s based on morphological features and Lugol-staining properties. Larval recovery was presented as presence or absence of larvae at a depth of 0-15 cm or >15-30 cm below the surface.
The lamb necropsies to determine GIN parasite infection were performed according to the method described by the Ministry of Agriculture, Fisheries and Food (1986), with minor modifications. Briefly, at necropsy, the abomasum, small intestine and caecum of each lamb were separated, opened, and the contents collected in separate buckets. The organs were then each washed in 5 L of lukewarm water. While mixing vigorously, a 1 L sample was collected in a pre-labeled plastic bottle and left to stand for 5-6 hours, after which the top 100 ml was removed and replaced with 100 ml 40% formaldehyde solution (i.e. 100% formalin).

After the aforementioned washing, the organs were placed in 5 L warm water and left overnight, to recover any larvae remaining in the mucosa (Gasbarre, 1987). The following day, the organs were scraped using a scalpel, and 1 L aliquots were collected and kept with a final concentration of 10% formalin until further analysis. All worms recovered were counted and identified microscopically to the species level using identification keys provided by Ministry of Agriculture, Fisheries and Food (1986).

3.2.6 Statistical analysis

The environmental data were first exported into a Microsoft Excel (Microsoft Office Excel®, 2007) spreadsheet, and then into SAS® 9.3 (SAS Institute Inc., Cary, NC, USA) for data cleaning. The data for each month from the three farms were merged together, then separated into four datasets for each of the four environmental variables measured (air and soil temperature, soil volumetric water content and air relative humidity). Only data from 26\textsuperscript{th} December 2009 to 31\textsuperscript{st} May 2010 were kept in the dataset as this corresponded to the relevant study period. Descriptive statistics were carried out to
calculate the daily mean, minimum and maximum measurements on the three farms, for each environmental variable – these measurements were chosen to be consistent with the measurements used by meteorological services, as suggested by O’Connor et al. (2006).

Herbage, soil and tracer lamb data were manually entered into a Microsoft Excel spreadsheet, and a student’s t-test used to compare the mean tracer lamb worm counts between the three farms. An alpha value $\leq 0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Farm description

All three study farms kept Rideau or Rideau X Polled Dorset breed sheep for meat purposes, practiced out-of-season lambing, and used ultrasound for pregnancy diagnosis. However, the farms had different flock sizes and represented 3 of the 11 Ontario Sheep Marketing Agency districts (Ontario Sheep Marketing Agency, 2012): Farm A was in District 7 and had 2000 breeding ewes; Farm B was in District 6 and had 400 breeding ewes; Farm C was in District 5 and had 130 breeding ewes.

3.3.2 Environmental data

The overall daily mean air temperature in January, the coldest month for the study period December 2009 to May 2010, was -7.0°C. The daily mean air temperatures showed a similar level and trend on all three farms (Fig. 3.1). Temperatures remained below freezing point for most of January and February, then increased above freezing point in March, though a dip in the temperature was observed in the second part of
March. On all three farms, the lowest temperature was recorded in January (-23.3°C, -25.7°C and -23.7°C, on farms A, B and C, respectively), while the highest temperature was recorded in May (31.8°C, 30.8°C and 31.3°C, on farms A, B and C, respectively).

The overall daily mean soil temperature in January was -3.1°C. The daily mean soil temperatures showed marked differences between farms (Fig. 3.2). While both farms A and B had a fairly constant soil temperature between December and February, the soil temperature on farm A declined at the end of January, reaching a minimum of -14.4°C, the soil temperature on farm B never declined below -5.0°C. In contrast, on farm C, the soil temperature fluctuated substantially between December and February; a minimum temperature of -19.8°C was recorded in January, while the maximum temperature between December and February was often above freezing point. On all three farms, the soil temperature increased considerably during May; a maximum temperature of 42.1°C, 37.4°C and 41.8°C was recorded on farms A, B and C, respectively.

The overall daily mean soil water content in January was -0.07 m³/m³. On all three farms the daily mean soil volumetric water content measurements showed a similar trend (Fig. 3.3); negative data were recorded in January and February, and positive data were recorded between March and May. On farms A and B, the minimum measurements were recorded in January (-0.07 m³/m³) and in February (-0.09 m³/m³), respectively. On both farms, the soil water content peaked in March, with maximum recordings of 0.40 m³/m³ and 0.47 m³/m³ for farms A and B, respectively. The soil moisture then decreased, though on farm B it remained at a higher level than farm A. In contrast to farms A and B, the soil moisture on farm C was lower (i.e. more negative values) during the winter months, with a minimum of -0.20 m³/m³ recorded in early March. It then increased
rapidly at the end of March, and fluctuated between 0.25 m$^3$/m$^3$ and 0.39 m$^3$/m$^3$ throughout April.

The overall daily mean air relative humidity was 83.5% in January, the coldest month, and 70.3% in May, the warmest month. The mean air relative humidity fluctuated daily on all three farms, and the differences between the minimum (day-time) and maximum (night-time) relative humidity reported became more marked between March and May when precipitation came in the form of rain, compared to snow being the predominant precipitation in December to February (Fig. 3.4). The minimum relative humidity between December 2009 and May 2010 was 15.0%, 21.6% and 21.9%, while the maximum relative humidity was 98.3%, 100% and 100%, on farms A, B and C, respectively.

3.3.3 Herbage and soil samples

Herbage and soil samples were collected from January to March 2010 on farm A, and from January to April 2010 on farms B and C. On farm A, 127.1 L3/kg DM were isolated in the pasture samples collected in March and, of the L3s isolated, 84.7 L3/kg DM (67%) were identified as Trichostrongylus spp., and 42.4 L3/kg DM (33%) were identified as Nematodirus spp.. No L3s were isolated in the herbage samples collected in January and February on farm A. Similarly, no L3s were isolated from any of the herbage samples collected on farms B and C. Furthermore, no L3s were isolated in either the ‘upper 15cm soil’ or ‘lower 15cm soil’ segments collected from all three farms.
3.3.4 Tracer lambs

The mean count of parasites (adults + immature stages) isolated in the tracer lambs was 6678, 6945 and 1023, on farms A, B and C, respectively (Table 3.1). There was a significant difference between the mean parasite count for tracer lambs on farm A and farm C (p<0.0001), and on farm B and farm C (p=0.0005); the mean worm count for tracer lambs on farm A and farm B was not significantly different (p=0.85).

On both farms A and B, *Teladorsagia circumcincta* was the predominant parasite species isolated (79.2% – 89.1%), followed by *Ostertagia trifurcata* (4.1%) on farm A, and *Trichostrongylus colubriformis* (9.7%) and *Nematodirus spathiger/filicollis* (7.7%) on farm B. On farm C, *N. spathiger/filicollis* was the most commonly isolated GIN species (35.8%), followed by *T. circumcincta* (28.9%), *Nematodirus battus* (19.4%) and *T. colubriformis* (14.2%). An arithmetic count of 8.3 adult *Haemonchus contortus* worms (0.5%) was isolated in the six tracer lambs on Farm C (i.e. two adult *H. contortus* parasites in one tracer lamb).

3.4 Discussion

3.4.1 Environmental factors

In this study, the air temperature on the three farms showed a similar level and trend (Fig. 3.1), despite differences in the geographical location of the farms. On all farms, the temperatures varied greatly between the winter (December - February) and spring (March - May) months, ranging from just under -20°C in the winter months to just over 30°C in the spring months. This range confirms that the weather experienced during the winter/spring of 2010 was representative of the continental climate experienced by
farms in south-western Ontario, with cold winters and warm to hot springs/summers (World Maps of Köppen-Geiger Climate Classification, 2012).

The soil temperatures recorded in our study were higher compared to the air temperatures recorded during the same time period. Similar observations were made by Andersen et al. (1970), who suggested that soil temperatures are affected by factors other than direct sunlight, such as soil type, topography, and type and amount of vegetative cover. Since environmental conditions at ground level may differ considerably from air measurements, it is important to measure micro-climate data, defined as “the immediate environment of an organism and the conditions that prevail there” (Andersen et al., 1970), as these provide a better reflection of the conditions to which larvae are exposed (Veglia, 1915; Gordon, 1948; Krecek et al., 1992).

There was considerable variation in the soil temperatures measured on the different farms (Fig. 3.2). On both farms A and B, the soil temperatures recorded during the winter months were fairly constant, whereas on farm C, greater fluctuations in the soil temperature, and associated freeze-thaw cycles, were recorded. This difference observed could be the result of a difference of level and/or persistency of snow cover on the pastures on the three farms. During the field visits, the researchers observed that on farm C, the snow coverage was less, and it was often harder to collect soil samples as the ground was deeply frozen, compared to the other two farms where the snow coverage was more constant throughout the winter months. Other studies have also suggested that snow can act as a buffer, as it prevents large fluctuations in soil temperature, keeping it at a constant freezing point (Smith and Archibald, 1965; Andersen et al., 1970; Stromberg, 1997; Troell et al., 2005).
In our study, the minimum soil temperature ranged between -20.0 and -5°C (Fig. 3.2). van Dijk et al. (2010) reported that -3.0°C was the minimum temperature at which *H. contortus* L3 larvae could survive, while both *T. circumcincta* and, less successfully, *Trichostrongylus* spp. could survive at -10°C (O’Connor et al., 2006). This suggests that, on all three farms, the soil temperature in the winter months was not amenable for the survival of *H. contortus*, at least in the one-acre sections of pasture tested. However, it should be noted that on farm C a few *H. contortus* larvae did survive on the one acre plot indicating that, in at least one location, the microclimate allowed for some survival, but at a very low level.

The soil volumetric water content is a good indicator of the moisture availability within soil (O’Connor et al., 2007). In this study, the soil water content was measured using a capacitive sensor, which measures the dielectric permittivity of soil. This permittivity is influenced by the soil’s water content, and can therefore be used as a surrogate measurement for water content (International Atomic Energy Agency, 2008). In the work described here, negative soil water content was recorded in the winter months. The dielectric permittivity of ice (5 F/m) is lower than that of water (80 F/m), and may have resulted in the negative recordings observed during the winter months when the ground was frozen. Frost events decrease the survival of L3s on pasture, as they are associated with lower temperatures and reduced water availability (van Dijk et al., 2010). Therefore, frozen ground and consequent negative soil water content may have acted as a limiting factor for the overwintering of L3s in our study.

On all three farms, the relative humidity fluctuated considerably throughout the study period, and these fluctuations became more evident between March and May (Fig.
3.4. However, the relative humidity was rarely less than 20%, which has been described by van Dijk et al. (2010) as the minimum threshold for the development of GIN free-living stages. Therefore, it is unlikely that relative humidity was a limiting factor for the survival of GIN free-living larvae during the winter months on the sampled farms.

3.4.2 Herbage and soil samples

Free-living parasite larvae were recovered from herbage samples collected on farm A in March, and the species identified were *Trichostrongylus* spp. and *Nematodirus* spp., suggesting that these parasite genera had overwintered on pasture. These parasites have been described as more cold-tolerant than *H. contortus*, and other studies have also shown that they can survive on pasture throughout the winter in temperate areas such as the Maritime provinces and Quebec, Canada (Smith and Archibald, 1965; Ayalew and Gibbs, 1973), Spain (Uriarte et al., 2003), and New Zealand (Waghorn et al., 2011).

No larvae were isolated in the herbage samples collected in January and February on farm A, or in any of the months on farms B and C. These results are in agreement with previous work conducted on sheep flocks in Ontario and Quebec, where no L3s were recovered from pasture samples collected in January and March (Mederos et al., 2010). In both our study and the study by Mederos et al. (2010), the herbage samples were collected using the standard “W” method, which assumes that infective larvae are distributed evenly on pasture, and that forage availability and use are homogeneous (Couvillion, 1993). However, larvae distribution and forage availability also depend on other factors such as stocking density and rate of pasture growth (Familton and McAnulty, 1994). Moreover, parasites are often not randomly distributed on pasture, but
are often concentrated around sheep fecal droppings (Couvillion, 1993). In December 2010 and April 2011, pasture samples were collected from the same three farms, but this time the samples were collected purposively within 10 cm of fecal samples. Free-living larval stages were isolated in the samples collected in December 2010 as follows: *Trichostrongylus* spp. on farm A, *Teladorsagia* sp., *Trichostrongylus* spp., *Haemonchus* sp. and *Oesophagostomum/Chabertia* spp. on farm B and *Trichostrongylus* spp. and *Oesophagostomum/Chabertia* spp. on farm C. In April 2011, free-living larval stages were isolated in pasture samples as follows: *Trichostrongylus* spp. and *Nematodirus* sp. on farm A, *Teladorsagia* sp., *Trichostrongylus* spp., *Nematodirus* spp. and *Oesophagostomum/Chabertia* spp. on farm B, and *Trichostrongylus* spp., *Teladorsagia* sp. and *Oesophagostomum/Chabertia* spp. on farm C (data not presented). These results suggest that our negative findings in winter 2010 may be due to limitations of the sampling method used.

Soil samples were collected in our study because previous work has suggested that parasites may migrate deep in the soil, especially when exposed to unfavourable weather conditions (Callinan and Westcott, 1986; Holasová et al., 1989). More recently, both Leathwick et al. (2011) and Waghorn et al. (2011) have described soil as a reservoir for free-living larval stages. However, in our study, no larvae were isolated in the soil samples collected during the winter months. The discordant results between our study and other studies (Callinan and Westcott, 1986; Holasová et al., 1989; Leathwick et al., 2011) may be a consequence of different environmental parameters, such as rainfall or temperatures which might influence the migration of larvae into the soil. Also, studies that reported the recovery of free-living larvae in soil samples were either conducted in
controlled laboratory settings (Callinan and Westcott, 1986) or during the summer (Leathwick et al., 2011), whereas our study was conducted on commercial farms during the winter months. These factors might have influenced the results observed in our study. Additionally, Waghorn et al. (2011) estimated the larval extraction efficacies from herbage and soil samples using the Baermann technique to be 24% and 17%, respectively. These low extraction efficacies lower the sensitivity of the Baermann technique, and may have influenced the negative observations in our study.

3.4.3 Tracer lambs

The use of tracer lambs is a more sensitive test to assess pasture contamination, compared to pasture larval recovery methods, as it represents the GIN infection an animal acquires over a period of time (Stromberg, 1997; O’Connor et al., 2006). In this study, several steps were taken to ensure that any parasites recovered from the tracer lambs were as a consequence of ingestion of free-living larvae that had overwintered on pasture from the previous grazing season: the pasture used had not been grazed since the previous November; the lambs were GIN-naïve, which was confirmed on FECs the day they were put on pasture; the lambs were additionally treated with a short-acting anthelmintic on the day they were moved to the farms; and, on farm B, the lambs were moved every 3-4 days to ensure that co-grazing would not lead to false-positive results.

Parasites (adults + immature stages) were isolated from all sixteen tracer lambs placed on pasture in April (farm A) and May (farms B and C); however, the mean worm count recovered from tracer lambs on farm C was significantly lower (six times lower), compared to the mean worm counts recovered from lambs on farms A or B (Table 3.1).
Moreover, on both farms A and B, *Teladorsagia* sp. was the predominant parasite recovered, while on farm C, *N. spathiger/filicollis* was the predominant parasite recovered.

O’Connor *et al.* (2006) described short-term fluctuations in temperature as being more harmful to GIN, compared to gradual changes in temperature. Other authors have described freeze-thaw cycles as detrimental to GIN free-living stages, and snow as a protective factor for the survival of GIN L3s, as it buffers freeze-thaw cycles and maintains the soil at a constant temperature (Smith and Archibald, 1965; Stromberg, 1997; Troell *et al.*, 2005). More freeze-thaw cycles were observed on farm C between December and February, and the volumetric soil water content during the winter months was also lower, compared to the other two farms. These observations may explain the lower number of nematodes found in tracer lambs on farm C. We recognize that the differences observed between parasite counts in tracer lambs on different farms could also be attributed to different parasitism levels in the flocks from the previous year. However, all three farms reported clinical signs of gastrointestinal parasitism in their flocks the previous grazing season, suggesting that the level of GIN pasture contamination was high at that time.

Despite historical evidence of serious clinical haemonchosis problems on the three study farms, *H. contortus* parasites were only isolated in one tracer lamb in low numbers from one farm in our study. These results are in accordance with recent studies by Uriarte *et al.* (2003), Waller *et al.* (2004) and Troell *et al.* (2005), which also found that *H. contortus* did not overwinter successfully on pasture in temperate winter conditions. In contrast, *Teladorsagia* sp., *Nematodirus* spp. and *Trichostrongylus* spp.
were all recovered from the tracer lambs in our study, which suggests that these parasite genera were more tolerant than *H. contortus* to cold temperatures. These results are in agreement with the recovery of *Trichostrongylus* spp. and *Nematodirus* spp. from the herbage samples collected in our study, and also with findings reported in other studies (Smith and Archibald, 1965; Ayalew and Gibbs, 1973). It is unclear why *Teladorsagia* sp. parasites were found in tracer lambs but not from herbage samples on farm A.

### 3.4.4 Study limitations and future research

This descriptive pilot study was conducted on three commercial sheep farms over one winter season, leading to limited external validity. However, the farms were purposively selected to be representative of the industry and geographic distribution of sheep farms in Ontario, and important differences in both environmental factors and GIN populations were noted among the farms. Enrolment of more farms over a longer sampling period would provide more regional and season-to-season information. With more farms and more time, geographical information systems could be used to describe trends and monitor changes over a wider geographical distribution, while mathematical modelling could be employed to predict future changes in the temporal dynamics of the parasites on pasture as a result of future climate change. Additionally, precise information on snow cover and soil type could be collected to determine whether these factors affect the development, migration and survival of L3s on pasture. However, our budget was for a pilot project on three farms for one winter only.

The small probability of *H. contortus* being able to over-winter on pasture could be exploited to eradicate this parasite from Ontario sheep flocks, a goal that could be
tested in future research (Barger et al., 1991; Waller et al., 2006; Sargison et al., 2007). *Haemonchus contortus* does however overwinter as hypobiotic larvae within the host (Blitz and Gibbs, 1972; Waller et al., 2004), and periparturient ewes have been identified as the primary source of pasture contamination with *H. contortus* the following spring, when arrested larvae resume development (Waller et al., 2006). An effective anthelmintic drug could therefore be used to treat ewes before turn-out on pasture, to kill the over-wintering *H. contortus* nematodes in ewes, and prevent pasture contamination (Waller et al., 2004; Sargison et al., 2007). Unfortunately, recent research in Ontario sheep flocks has shown that resistance to ivermectin and fenbendazole, the two most commonly used anthelmintics in Canada, is widespread, and most of the anthelmintic resistance reported is associated with *H. contortus* (Falzon et al., in press). Since these two anthelmintics have been shown to be ineffective against *H. contortus* on many of the sheep farms surveyed, more research is required to determine whether the use of other anthelmintics could be used as alternative drugs in Ontario sheep flocks. In particular, anthelmintics which have been shown to be effective against ivermectin- and fenbendazole-resistant strains of *H. contortus*, such as the new anthelmintic monepantel (Mason et al., 2009), or the narrow-spectrum anthelmintic closantel (Uppal et al., 1993; Waruiru, 1997), might be a promising alternative.

### 3.5 Conclusion

Results from this pilot study on the over-wintering and infectivity of parasite larvae on pasture in the spring suggest that very few *Haemonchus* larvae were able to over-winter on pasture in the temperate climate of Ontario. In contrast to *Haemonchus*, other important parasite genera such as *Teladorsagia, Nematodirus* and *Trichostrongylus*,
did survive on pasture during the Ontario winter, and were infective in the spring. These observations need to be taken into consideration when making recommendations on pasture management and timing of anthelmintic treatment for parasite control strategies.

3.6 Acknowledgements

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3.7 References


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with the overwintering of hypobiotic fourth stage larvae. Vet. Parasitol. 147, 326-331.


Table 3.1. Arithmetic means of gastrointestinal nematode counts (and percentage distribution) of 16 tracer lambs put out to graze, and slaughtered after 28 days, in south-western Ontario, between April and May 2010.

<table>
<thead>
<tr>
<th></th>
<th>Farm A</th>
<th>Farm B</th>
<th>Farm C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total mean worm count</strong></td>
<td>6678</td>
<td>6945</td>
<td>1023</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>5225-8050</td>
<td>3200-10360</td>
<td>385-1750</td>
</tr>
<tr>
<td><strong>Abomasum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Teladorsagia circumcincta</em></td>
<td>5925 (89.1%)</td>
<td>5350 (79.2%)</td>
<td>254 (28.9%)</td>
</tr>
<tr>
<td><em>Ostertagia trifurcata</em></td>
<td>275 (4.1%)</td>
<td>50 (0.6%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td><em>Haemonchus contortus</em></td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>8.3 (0.5%)</td>
</tr>
<tr>
<td><strong>Small intestine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trichostrongylus colubriformis</em></td>
<td>106 (1.5%)</td>
<td>690 (9.7%)</td>
<td>142 (14.2%)</td>
</tr>
<tr>
<td><em>Nematodirus battus</em></td>
<td>130 (2.0%)</td>
<td>185 (2.6%)</td>
<td>141.7 (19.4%)</td>
</tr>
<tr>
<td><em>Nematodirus spathiger and filicolis</em></td>
<td>240 (3.2%)</td>
<td>650 (7.7%)</td>
<td>475 (35.8%)</td>
</tr>
<tr>
<td><strong>Large intestine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Oesophagostomum columbianum</em></td>
<td>2 (0.04%)</td>
<td>20 (0.3%)</td>
<td>1.7 (0.4%)</td>
</tr>
</tbody>
</table>
Figure 3.1. Daily maximum, mean and minimum air temperatures, recorded by HOBOware® Pro Data-Loggers placed on each of three commercial sheep farms in south-western Ontario, between December 2009 and May 2010.
Figure 3.2. Daily maximum, mean and minimum soil temperatures, recorded by HOBOware® Pro Data-Loggers placed on each of three commercial sheep farms in south-western Ontario, between December 2009 and May 2010.
Figure 3.3. Daily mean soil volumetric water content, recorded by HOBOware® Pro Data-Loggers placed on each of three commercial sheep farms in south-western Ontario, between January and May 2010.

Note: Data from May 2010 are not presented for farm C as an error was observed in the recordings.
Figure 3.4. Daily maximum, mean and minimum air relative humidity, recorded by HOBOware® Pro Data-Loggers placed on each of three commercial sheep farms in south-western Ontario, between December 2009 and May 2010.
CHAPTER 4

Anthelmintic resistance in sheep flocks in Ontario, Canada

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Abstract

Gastrointestinal nematodes (GIN) are a significant constraint to pasture-based sheep production worldwide. Anthelmintic resistance (AR) has been reported in most sheep-raising areas in the world, yet little is known about the AR status in Canada. This study was conducted to determine the frequency of AR in GIN in sheep flocks in Ontario, Canada. Forty-seven sheep flocks were enrolled in the study, and their level of parasitism was monitored monthly throughout a grazing season by analyzing owner-acquired fecal samples from 15 grazing lambs per flock. When the mean GIN fecal egg count (FEC) reached a threshold of 200 eggs per gram (epg), oral ivermectin was supplied to producers to check ivermectin efficacy; the reduction in mean FEC 14 days after ivermectin treatment was calculated. ‘Drench failure’ was defined as a reduction in mean FEC of <95%. In those flocks with apparent drench failure, researchers performed a Fecal Egg Count Reduction Test (FECRT), dividing sheep into 4 treatment groups (n=10-15): control (i.e. untreated), ivermectin, and, if sufficient numbers of animals - fenbendazole and levamisole. AR was defined as a reduction in mean FEC <95% and a lower 95% confidence interval <90%. Larval cultures were performed on pooled post-treatment FECRT samples. Larval Development Assays (LDAs) to detect the presence of resistance to thiabendazole and levamisole were performed prior to the ivermectin drench check on pooled owner-acquired fecal samples that reached the 200 epg threshold.
Approximately 89% (42/47) of the farms reached the FEC threshold of 200 epg; 93% (39/42) of these farms performed an ivermectin drench check, and 88% (34/39) of these farms had drench failure. The FECRT was performed on 29 of the 34 farms. Resistance to ivermectin, fenbendazole and levamisole was demonstrated on 97% (28/29), 95% (19/20) and 6% (1/17) of the farms tested, respectively, with considerable variability in resistance levels among farms. *Haemonchus* sp. was the most commonly cultured parasite from post-treatment fecal samples. LDA results were available for 21 farms; of these, 14% (3/21) and 62% (13/21) had low and high levels of thiabendazole resistance, respectively, while none of the farms exhibited resistance to levamisole. Amongst these tested farms, resistance to both ivermectin and benzimidazoles was very common. These findings strongly suggest that AR, particularly in *Haemonchus* sp., is a serious problem in these sheep flocks. Thus, marked changes in GIN management need to be instituted immediately to mitigate a worsening situation.

**Keywords:** Gastro-intestinal nematodes; ivermectin drench failure; fecal egg count reduction test; anthelmintic resistance; larval development assays; post-treatment larval cultures

**4.1 Introduction**

Parasitic gastroenteritis caused by gastrointestinal nematodes (GINs) is widely considered the most important disease of grazing sheep worldwide, causing weight loss, diarrhea and death (Sutherland and Scott, 2010). Gastrointestinal nematode infections are typically controlled with anthelmintic drugs, and sheep producers worldwide have
customarily relied heavily on such drugs to maintain sheep health and productivity, while improving the overall profitability of the sheep industry (Sargison, 2008).

In North America, three broad-spectrum anthelmintic drug classes are most commonly used in sheep: macrocyclic lactones (e.g. ivermectin and moxidectin), benzimidazoles (e.g. thiabendazole, fenbendazole and albendazole) and imidazothiazoles (e.g. levamisole) (Adams, 2001). In Canada, only ivermectin is licensed for use in sheep (Compendium of Veterinary Products, Canada, 2012). Thiabendazole was the first benzimidazole to be marketed in Canada in the early 1960s (Adams, 2001), but was subsequently replaced with other structurally similar, but improved drugs, such as fenbendazole and albendazole. Fenbendazole and albendazole are licensed for use in Canada in cattle (Compendium of Veterinary Products, Canada, 2012), but are often used in sheep in an extra-label manner. Levamisole has not been licensed for use in sheep in Canada for the past 10 years (Health Canada – Drug Product Database Online Query, 2012).

Anthelmintic resistance (AR) is defined as the ‘heritable ability of the parasite to tolerate a normally effective dose of the anthelmintic’ (Abbott et al., 2009), and if sufficiently prevalent in a parasite population, results in treatment failure. However, treatment failure may also be caused by other confounding factors (McKenna, 1990), such as under-dosing or incorrect administration of anthelmintic drugs (El-Abdellati et al., 2010).

Anthelmintic resistance is an escalating problem in most sheep-rearing countries worldwide (Papadopoulos, 2008), and is a threat to both agricultural income and sheep welfare (Wolstenholme et al., 2004). It is widespread in New Zealand (Waghorn et al.,
In recent years, AR has also been described in the United States (Kaplan and Vidyashankar, 2012) and in several European countries including Greece (Gallidis et al., 2009), Italy (Cringoli et al., 2009) and the United Kingdom (Jackson and Coop, 2000). In 2007, the first case of AR in Canada was described in a sheep flock in Ontario (Glauser et al., 2007). Ontario is considered to have a humid continental climate, with cold snowy winters and warm-to-hot summers (World Maps of Köppen-Geiger Climate Classification, 2012). While recent studies have investigated how this climate affects the epidemiology of GIN infections in sheep (Mederos et al., 2010), no surveys have been published on how widespread the problem of treatment failure and AR is in sheep flocks in Canada and, in particular, Ontario.

The Fecal Egg Count Reduction Test (FECRT) is the standard test for determining AR under field conditions (Coles et al., 1992), and provides an indirect measurement of anthelmintic efficacy by determining the reduction in fecal egg counts (FECs) after treatment (McKenna, 2006). Several authors have suggested different threshold values for defining the presence of AR (McKenna, 1990; Wood et al., 1995; Smart, 2009), but the most commonly accepted threshold is that endorsed by the World Association for the Advancement of Veterinary Parasitology (WAAVP), which defines AR as a Fecal Egg Count Reduction (FECR) of <95% and a lower 95% confidence interval (CI) of <90%; if only one of these two factors is present, the farm is defined as being “suspected” of having resistance (Coles et al., 1992).
Despite being the standard test for AR determination, the FECRT is laborious, expensive and time-consuming (Craven et al., 1999; El-Abdellati et al., 2010). As a result, various alternative diagnostic tests have been suggested for the determination of anthelmintic susceptibility (Coles et al., 2006). The Larval Development Assay (LDA) described by Taylor (1990) is based on culturing a known number of GIN eggs in the presence of different anthelmintics. It is reported to be relatively easy to perform, more sensitive than the FECRT, and allows for the identification of parasite larvae to the genus level (Taylor, 1990). However, this methodology cannot reliably detect resistance to avermectins (Grimshaw et al., 1994), and is considered by some to require a high level of technical expertise, thus limiting its use outside of research laboratories (Kaplan and Vidyashankar, 2012).

The objectives of this study in Ontario sheep flocks were: (i) to determine the frequency of ivermectin treatment failure; (ii) to determine the frequency of resistance to ivermectin, fenbendazole and levamisole using a FECRT; and (iii) to assess the frequency of resistance to thiabendazole and levamisole using the LDA.

4.2 Materials and Methods

4.2.1. Number and selection of sheep farms

The study was conducted in Ontario, Canada, for two consecutive grazing seasons (May to November 2010 and May to November 2011). The target population was sheep farms in Ontario, while the study population was eligible and willing sheep producers (as defined below) that were members of the Ontario Sheep Marketing Agency (OSMA) – a producer-operated agency formed under the Ontario Farm Products Marketing Act that
represents all producers that raise and sell market lambs. All registered producers (n=3600) receive the magazine ‘Ontario Sheep News’, with over 70% of these producers also receiving emails from the OSMA email list-serve (OSMA office, personal communication).

Forty-seven sheep farms were recruited during the summers of 2010 or 2011. This sample size is associated with a precision of 14%, using a 95% level of confidence and an estimated ivermectin drench failure prevalence of 60% (latter estimate based on an unpublished pilot study conducted on Ontario sheep farms in 2009, in which 8 of 13 farms tested had ivermectin drench failure).

Recruitment required volunteer participation and was carried out through talks given at various OSMA sheep producer meetings held across the province and letters posted in the ‘Ontario Sheep News’ and distributed via the email list-serve. To be included in the study, farms had to: (i) have a minimum of 30 animals (lambs or yearling ewes) in their first grazing season; and (ii) keep the animals on pasture for at least 3 months during the grazing season. The first criterion was set to include both lambs and yearling ewes since a number of producers in Ontario opt to keep lambs indoors during the summer months to reduce risk of predator attacks, thereby minimizing their GIN exposure and immunity development during their first year of life. This study was approved by the Animal Care Committee (Protocol Number: 09R056) and the Research Ethics Board (Protocol Number: 09DC005) at the University of Guelph.
4.2.2. Farm monitoring

Starting in May each year, all producers enrolled in the study received a kit for fecal sample collection. This kit consisted of a Styrofoam cooler with 2 ice-packs, transparent plastic sealable bags, elastic bands, disposable gloves, cellophane packing tape, a bag with 5 g of coffee beans (to provide producers with a visual estimate of approximately 5 g of sheep feces), a courier (Purolator Inc., Canada) shipping label, and a consent form. Producers were asked to collect 5 g of fresh fecal pellets from the ground or per rectum, using the gloves provided, from each of 15 lambs or yearling ewes in their first grazing season, and to package these fecal samples individually, using the plastic sealable bags and elastic bands. The producers were then asked to place the samples in the Styrofoam cooler with the chilled ice-packs, and to courier the taped box, along with the signed consent form, to the Parasitology Laboratory, Department of Pathobiology, Ontario Veterinary College, University of Guelph.

The fecal samples were analyzed individually and results used to determine the flock mean FEC, as an indicator of flock-level GIN parasitism. A mean FEC of 200 eggs per gram (epg) was the threshold used for conducting the ivermectin drench check, since a previous study has indicated that this is a sufficient level for detecting changes in FEC following treatment (Miller et al., 2006). If the mean FEC on the farm was <200 epg, another kit for fecal sample collection was sent to the producer, and they were asked to repeat the process in 3-4 weeks; kits were thus sent approximately monthly until either the mean FEC attained was ≥200 epg or until early September. If the mean FEC on the farm reached ≥200 epg, an ivermectin drench check was conducted.
4.2.3. Ivermectin drench check

For the ivermectin drench check, a package containing a sample-collection kit (see section 4.2.2) and a 1 litre bottle of ivermectin (0.8 mg/mL, Ivomec® Drench for Sheep, Merial Canada Inc.) was couriered to the producers. The producers were asked to treat the sampled group with the ivermectin drench as they normally would; no recommendations on the dosage or methodology of treatment were provided. This was done to assess what producers normally do on their farms.

Fourteen days after treatment, the producers were asked to collect fecal samples from 15 of the treated animals, which were not necessarily the same animals sampled before treatment, and then package the samples individually and send for analysis as described above. The post-treatment fecal samples were analyzed individually, and the GIN FECR following ivermectin treatment was calculated for the group sampled as:

\[ 100 \times \left( \frac{\text{mean FEC before treatment} - \text{mean FEC after treatment}}{\text{mean FEC before treatment}} \right) \]

In the published literature, drench failure is ascribed when the post-treatment FECs are positive (Sargison, 2008); however, specific cut-points to indicate drench failure when post-treatment FECs were positive could not be found. A review by Campbell and Benz (1984) describes ivermectin efficacy in the absence of resistance as varying between 95-100% for different stages and species of ovine GINs. As such, in the work described here, ivermectin drench success was defined as a mean FECR \( \geq 95\% \), and ivermectin drench failure as a mean GIN FECR <95%. If a farm had ivermectin drench
success, no further sampling was performed on that farm. If a farm had ivermectin drench failure, a FECRT was initiated.

4.2.4 Fecal Egg Count Reduction Test

The FECRT was conducted a minimum of three weeks after treatment with ivermectin. Each farm was visited twice by the research team, 14 days apart (day 0 and day 14), and lambs or yearling ewes in their first grazing season (as described previously) were included.

On the first visit, eligible animals were divided into one of a maximum of 4 treatment groups, each with 10-15 animals: control (i.e. untreated), or treated with ivermectin, fenbendazole or levamisole. In the first year of the study, the animals were divided into groups sequentially (i.e. the first 15 animals passing through a chute were put in one group; the following 15 animals were put in a second group, etc.), except for 3 farms (farms 1, 3 and 7) where the animals were divided into different pens prior to the researchers’ visit, based on different age groups or breeds. In the second year, the animals were systematically assigned into groups to allow for better randomization; this was carried out by running the animals through a chute and the first animal allocated to the first group, the second animal allocated to the second group, etc. The age and sex of lambs was mixed, and all animals used were <2 years of age. The number of animals in each group was based on recommendations that 10 animals per group are sufficient to detect differences in FEC between groups (Coles et al., 1992); up to 15 animals per group were sampled to help account for any losses or non-suppliers of fecal samples that might occur. On some farms, less than 40 animals were eligible for inclusion in the study; fewer treatment groups were therefore used on these farms.
All animals involved in the FECRT were identified using ear-tag numbers, weighed, and then either left untreated or treated by the researchers according to the treatment group assigned. Treatments were as follows: 0.2 mg/kg ivermectin (Ivomec Drench for Sheep, Merial Canada Inc.); 5 mg/kg fenbendazole (Safe-guard™ Suspension 10%, Intervet Canada Ltd.); or 10.5 mg/kg levamisole (levamisole hydrochloride suspension, Chiron Compounding Pharmacy Inc., Canada). Doses were calculated based on each animal’s individual weight. Both the ivermectin and levamisole drenches were administered using a drench-gun that was calibrated before use, and after half of the animals per treatment group had been treated. The fenbendazole drench was administered using a 5 mL syringe since small doses were required, and the dose was administered orally over the back of the tongue.

Fecal samples were collected by the researchers directly from the rectum of each selected animal on day 0 and day 14, and these samples were analyzed individually and used to determine the arithmetic mean FEC pre- and post-treatment for each of the treatment groups. Due to logistic constraints related to the distant locations of farms, on two farms in the first year (farms 4 and 9), the producers were asked to collect fecal samples from the rectums of selected animals 14 days after the first visit and to courier the samples to the researchers for analysis.

4.2.5 Laboratory analysis

All fecal samples were examined at the Parasitology Laboratory, Department of Pathobiology, Ontario Veterinary College, University of Guelph. During transportation to the laboratory, all fecal samples were shipped with ice-packs. Thereafter, they were kept refrigerated at 4°C before being tested. Time from fecal sample collection on farm to
receipt by the laboratory took, on average, two business days, and most fecal samples were examined within seven days of collection. In the first year of the study, some fecal samples were stored for up to three weeks before being processed, due to a backlog of samples in the laboratory. All researchers processing the fecal samples were blinded to the treatment status of individual sheep. Fecal egg counts were performed on individual fecal samples using a modified McMaster concentration method (Ministry of Agriculture, Fisheries and Food [MAFF], 1986), with a lower detection limit of 50 epg.

4.2.6 Larval culture of post-treatment fecal samples

Larval cultures were performed on post-treatment fecal samples (FECRT day 14), to identify the resistant GIN genera. In the first year, larval cultures were not carried out due to a backlog of samples in the laboratory; in the second year, the larval cultures were performed on post-treatment fecal samples (FECRT day 14) for all farms on which a FECRT was conducted to identify the resistant GIN genera.

For each farm, fecal samples were pooled together using 2 g of feces per animal, for the control and each treated group, and the pooled feces were broken up finely using a pestle and mortar. If the fecal mixture was too dry, just enough water was added to moisten the feces, whereas, if the mixture was too wet, vermiculite was added to bring it to the necessary consistency (MAFF, 1986). The culture wells were then filled with the mixture, and separate culture wells were used for the different groups. The wells were incubated at 27°C for 7 days, after which third-stage GIN larvae (L3s) were harvested by Baermannization (Coles et al., 1992). The first 100 L3s, or all L3s when <100 developed, were identified to the genus level, following identification keys (MAFF, 1986).
4.2.7 Larval Development Assay

Larval Development Assays (Taylor, 1990) were performed to assess the in vitro anthelmintic susceptibility of GIN on sheep farms. In the first year, a member of the research group learned the technique at the Central Science Laboratory, York, United Kingdom and then established it at the Parasitology Laboratory, Department of Pathobiology, Ontario Veterinary College, University of Guelph. In the second year, LDAs were performed for all farms that reached the mean FEC threshold of ≥200 epg; this included farms enrolled in the second year, and some farms from the first year that re-submitted fecal samples in the second year. The samples used for the LDAs were from animals that had not been treated with an anthelmintic for at least 28 days.

The LDA used was a modification of the method originally described by Taylor (1990). In the modified method, the culture medium comprised lyophilised *Escherichia coli* and fecal extract, the final volume of fecal solution was adjusted to give 300 eggs/mL, and 2 concentrations of thiabendazole (0.1 and 0.3 µg/mL) and levamisole (1.0 and 3.0 µg/mL) were tested. The anthelmintics used were in the pure form: 99% thiabendazole (Sigma Life Science, T8904-100G, batch number: 079K1429) and 99% levamisole (ACROS Organics, CAS: 16595-80-5, Lot: A0287589), and therefore were diluted to the appropriate concentrations. All tests were run in duplicate, with a total of 10 culture wells per farm (2 control wells and 8 test wells) that were incubated at 26 ± 3°C for seven days. Recovered L3s in each well were counted and the first 100 L3s were identified microscopically to the genus level (MAFF, 1986).
4.2.8 Descriptive statistics

4.2.8.1 Fecal Egg Count Reduction

All data were entered manually into an Excel spreadsheet (Microsoft Office Excel®, 2007). The FECR was calculated in SAS® 9.3 (SAS Institute Inc., Cary, NC, USA), using the method endorsed by the WAAVP (Coles et al., 1992):

\[
\text{FECR} = 100 \times (1 - \frac{T2}{C2}),
\]

where \(T2\) and \(C2\) are the arithmetic means of epg, 14 days after treatment, for the treated and control groups, respectively;

The 95% CI was estimated as: \(100 \times (1 - \frac{T2}{C2} \exp \pm 1.96 \sqrt{Y^2})\), where \(Y^2\) is the variance of the reduction percentage.

Farms were classified as resistant when the FECR was <95% and the lower 95% CI limit was <90%; if only one of these two criteria was met, the farm was classified as being suspected of resistance (Coles et al., 1992).

4.2.8.2 Genera-specific Reduction

The percentage reduction for specific genera for each treatment was calculated for each farm in which the FECRT was performed using an equation described by Waghorn et al. (2006). Briefly, the pre-treatment FEC by genera was obtained by multiplying the proportion of larvae for each genus in the control well with the average pre-treatment FEC; any genus failing to achieve the equivalent of >50 epg in the pre-treatment counts was excluded from further calculations as their number was considered too low to give a reliable result (Coles et al., 2006). The efficacy by genus, for each treatment, was calculated by dividing the genus-specific post-treatment FECs by the genus-specific FECs from the pre-treatment samples, and then multiplying this value with the reduction
in the untreated group, to control for any changes occurring in the latter group. When the reduction was <95%, the genus was considered to be resistant to that anthelmintic treatment. If the overall FECR was ≥95% (i.e. anthelmintic was effective) and/or <50 L3s were found in an individual treatment culture (culture results deemed inconclusive for that anthelmintic), the genus-specific reduction was not calculated. A one-way analysis of variance using proc GLM for unbalanced design (SAS 9.3) was performed to compare genus-specific FECRs within treatment groups.

4.2.8.3 Larval Development Assay

The LDA was considered reliable when a mean of >100 larvae was isolated in the 2 control wells. Farms that met this criterion were defined as having a low level of drug resistance when the total number of L3s that developed in wells containing anthelmintics at the discriminatory drug concentration of 0.1 µg/mL thiabendazole or 1.0 µg/mL levamisole (Mitchell et al., 2010) was >5% of the number that developed in the control wells, while there was ≤5% of the number of larvae than in the control wells in the higher concentration wells. Farms were defined as having a high level of drug resistance when the total number of L3s that developed in wells containing anthelmintics at the higher concentration of 0.3 µg/mL thiabendazole or 3.0 µg/mL levamisole was >5% of the number that developed in the control wells. The 5% cut-offs were selected to be consistent with the definition of resistance used for the FECRT, whereby resistance is said to be present if the reduction in GIN eggs following treatment is <95%, compared to the number of GIN eggs in the control or pre-treatment group.
When the proportion of *Haemonchus* sp., *Teladorsagia* spp. and/or *Trichostrongylus* spp. in the control well was \(\geq 5\%\) of the total larvae isolated in the control well, the percentage reductions were separately calculated, for both drugs at both concentrations, for each genus as:

\[ 100 \times \left( \frac{\text{Mean number of genus-specific larvae in control wells} - \text{Mean number of genus-specific larvae in treatment wells}}{\text{Mean number of genus-specific larvae in control wells}} \right) \]

When the percentage reduction was <95%, the genus was considered to be resistant.

### 4.3. Results

#### 4.3.1 Study population

Forty-seven farms participated in the study from across southern and central Ontario (latitude from 42.6°N to 47.3°N; longitude from 75.4° to 82.3°W); 25 farms in Year 1, and 22 farms in Year 2. Flock sizes ranged between 50-2000 animals (mean flock size=300 animals).

#### 4.3.2 Farm monitoring

Forty-two of 47 farms (89%) reached the fecal GIN threshold of \(\geq 200\) epg; 22 farms in Year 1 and 20 farms in Year 2. Of these 42 farms, 10 farms reached the threshold in June, 26 farms in July, 5 farms in August, and 1 farm in September. Four farms never reached the threshold, while another farm stopped submitting samples. On average, the producers sent 1.7 and 1.4 sets of samples in the first and second year, respectively, before reaching the threshold.
4.3.3 Ivermectin drench check

Thirty-nine of 42 farms (93%) performed the ivermectin drench check; 21 farms in Year 1 and 18 farms in Year 2. The other three farms did not submit post-ivermectin treatment samples. Of the 39 farms, 5 (13%) farms had ivermectin drench success, while 34 (87%) farms had ivermectin drench failure. All cases of drench success occurred in the first year. Of the 34 farms with ivermectin drench failure, 16 cases were reported in the first year and 18 cases were reported in the second year. For farms that performed an ivermectin drench check, the mean and median pre-ivermectin FECs were 1663 epg and 696 epg, respectively (range: 207 – 8302 epg), while the mean and median post-ivermectin FECs were 1455 epg and 804 epg, respectively (range: 0 – 5956 epg). The mean and median overall reductions were -103% and -19%, respectively (range: -1667% – 100%). Negative reduction values indicate that the FEC increased after treatment with ivermectin.

4.3.4 Fecal Egg Count Reduction Test

A FECRT was conducted on 29 farms with ivermectin drench failure. In the first year, tests were conducted on 11 farms and in the second year, the FECRT was conducted on 16 farms. In addition, a FECRT was performed on two farms from the first year for which drench failure was reported too late in the season. In the first year, fecal samples from 5/11 (45%) farms were stored in the refrigerator for up to 3 weeks before being processed due to a backlog of samples in the laboratory; in the second year, all samples were analyzed within a week of collection on the farm.
Due to a limited number of animals on some farms, fenbendazole and levamisole were evaluated for efficacy on fewer farms than ivermectin. Overall, ivermectin was evaluated on 29 farms (11 farms in Year 1 and 18 farms in Year 2); fenbendazole on 20 farms (7 farms in Year 1 and 13 farms in Year 2); and levamisole on 17 farms (6 farms in Year 1 and 11 farms in Year 2). All treatment groups contained 10-15 animals.

Ivermectin resistance was reported on 28/29 (97%) farms, with another farm (farm 16) suspected of resistance (Table 4.1). Fenbendazole resistance was reported on 19/20 (95%) farms, with another farm (farm 1) suspected of resistance. Levamisole resistance was reported on 1/17 (6%) farms, with another 2 farms (farms 10 and 27) suspected of resistance.

4.3.5 Larval culture results

Larval cultures were performed using FECRT post-treatment fecal samples from the 18 farms that had FECRT results in Year 2. Figure 4.1 illustrates the number of *Trichostrongylus* spp., *Teladorsagia* spp. and *Haemonchus* sp. larvae identified in the first 100 larvae harvested from pooled fecal samples collected from the control and treated sheep. Less than 100 larvae were harvested from the feces of animals treated with levamisole on 10/11 farms. The most predominant parasite genus in both untreated and treated animals was *Haemonchus* sp., followed by *Teladorsagia* spp., and *Trichostrongylus* spp.

The genus-specific reduction was calculated when the FECR was <95%, when >50 larvae were isolated in each individual treatment culture well, and when the genus pre-treatment FEC was >50 epg (Table 4.2). Ivermectin was tested on 18 farms, but 1
farm (farm 16) was excluded since the FECR was ≥95%. Of the remaining 17 farms:

*Haemonchus* sp. was present (i.e. >50 epg) and was resistant on all farms; *Teladorsagia* spp. was present on 8 farms, and was resistant on 2/8 (25%) farms; and *Trichostrongylus* spp. was present on 6 farms, and was resistant on 1/6 (17%) farms. Fenbendazole was tested on 13 farms, and of these: *Haemonchus* sp. was present on 13 farms, and was resistant on 12/13 (92%) farms; *Teladorsagia* spp. was present on 6 farms, and was resistant on 3/6 (50%) farms; and *Trichostrongylus* spp. was present on 5 farms, and was resistant on 2/5 (40%) farms. Levamisole was tested on 11 farms; however, all farms were excluded from the genus-specific analysis either because the FECR was ≥95% or because the number of larvae in the treatment well was <50.

The FECRs for ivermectin were significantly different among parasite genera (p<0.001), with FECRs in *Haemonchus* sp. being significantly lower than those in *Teladorsagia* spp. FECRs (p=0.004) and *Trichostrongylus* spp. (p=0.003); there was no difference in FECRs among *Teladorsagia* spp. and *Trichostrongylus* spp. (p=0.893).

Similarly, the genus-specific FECRs for fenbendazole were significantly different overall (p=0.01), with *Haemonchus* sp. FECRs being significantly lower than those with *Teladorsagia* spp. FECRs (p=0.03) and *Trichostrongylus* spp. (p=0.04).

### 4.3.6 Larval Development Assay

The LDA was performed on 24 farms (Table 4.3) that reached the set FEC threshold in the second year of the study; these included 20 farms which were enrolled in the second year and 4 farms that were initially enrolled in the first year and resubmitted samples. Of these 24 farms, 21 had >100 larvae develop in the control wells. In a
comparison between 13 farms for which both the LDA and FECRT were performed in the same year, *Haemonchus* sp. was the most commonly represented genus in the LDA control wells (10/13; 77%), followed by *Trichostrongylus* spp. (2/13; 15%) and *Teladorsagia* spp. (1/13; 8%). *Haemonchus* sp. was the most commonly represented parasite on all farms at the time the FECRT was performed. It should be noted that the LDA and FECRT were conducted at different times during the grazing season.

With resistance being defined as a reduction <95%, 5/21 (24%) farms had no evidence of thiabendazole resistance, 3/21 (14%) farms had low thiabendazole resistance, and 13/21 (62%) farms had high thiabendazole resistance; 3 farms were excluded since <100 larvae developed in the control wells. None of the farms tested were considered to have levamisole resistance.

Figures 4.2, 4.3 and 4.4 present the mean number of *Trichostrongylus* spp., *Teladorsagia* spp., and *Haemonchus* sp. isolated from the LDAs for each farm, in the control wells, and wells containing 0.1 µg and 0.3 µg thiabendazole/mL, respectively. While the mean number of L3s isolated varied per farm, *Haemonchus* sp. was the most commonly isolated parasite. Only averages of 1 and 0.5 larvae were isolated from 2 farms in the 1.0 µg levamisole/mL wells, and none were isolated in the 3.0 µg levamisole/mL wells.

The percentage reduction for *Haemonchus* sp., *Teladorsagia* spp., and *Trichostrongylus* spp. were calculated for both 0.1 and 0.3 µg thiabendazole/mL when the genus constituted >5% of the control population (Table 4.3). *Haemonchus* sp. was present on 17 farms (i.e. >5% of the total parasite population) and was classified as
resistant on 14/17 (82%) farms for 0.1 μg thiabendazole/mL, and on 13/17 (76%) for the 0.3 μg thiabendazole/mL wells. Teladorsagia spp. was present on 13 farms and was classified as resistant on 5/13 (39%) farms for 0.1 μg thiabendazole/mL and on 4/13 (31%) farms for 0.3 μg thiabendazole/mL. Trichostongylus spp. was present on 7 farms and was classified as resistant on 2/7 (29%) farms for 0.1 μg thiabendazole/mL, and on none of the farms for 0.3 μg thiabendazole/mL.

4.4. Discussion

4.4.1 Fecal monitoring and ivermectin drench check

On almost 90% of the farms enrolled in the study, the FEC reached the set threshold of ≥200 epg for the ivermectin drench check at some point over the summer months. While clinical practitioners may use a threshold of >800 epg to indicate a high FEC (Sargison, 2008), a lower threshold of 200 epg was selected based on previous work on the distribution and abundance of GIN on farms in Ontario (Mederos et al., 2010). This work showed that pasture larval counts peak during the summer months, and because the time interval between sampling for this study was 3-4 weeks, it was planned that animals were treated before GIN infections became too severe and compromised their health.

An ivermectin drench check was conducted to assess what normally happens on sheep farms, when producers are not provided any information on correct anthelmintic dosages. Of the 39 farms that performed an ivermectin drench check, 87% had drench failure. This suggests that ivermectin treatment failure is a common occurrence on Ontario sheep farms. While AR is often incriminated as the main cause for anthelmintic
treatment failure, several other factors can be responsible. These include under-dosing or incorrect administration of the anthelmintic (McKenna, 1990; Taylor et al., 2002; El-Abdellati et al., 2010).

The reduction in egg count in the ivermectin drench check was estimated using the first mean FEC that reached or exceeded the 200 epg threshold as the pre-treatment FEC. However, since approximately two weeks elapsed between submission and analysis of the pre-treatment fecal samples, delivery of ivermectin to the farms, and producers treating the animals, it is likely that the actual FEC on the day of treatment by the producers was higher. Therefore, approximately four weeks passed between the pre- and post-treatment FECs, which might have led to an under-estimation of the ivermectin efficacy and, consequently, an over-estimation of the frequency of drench failure.

4.4.2 Fecal Egg Count Reduction Test

Using the FECRT, ivermectin susceptibility was assessed on 29 farms, fenbendazole susceptibility on 20 farms, and levamisole susceptibility on 17 farms. Most of the farms that participated in the study were classified as having parasites resistant to ivermectin (97%) and fenbendazole (95%), the two anthelmintic drugs that are most frequently used by Canadian producers, while only 6% of the farms tested had parasites resistant to levamisole (Table 4.1). Ivermectin is the only anthelmintic licensed for use in sheep in Canada (Compendium of Veterinary Products, Canada, 2012), and many sheep producers have relied exclusively on this drug for the past two decades. This might partially explain the widespread resistance to ivermectin observed in this study. Similarly, while fenbendazole and albendazole are not licensed for use in sheep in
Canada, many sheep producers have used products formulated for use in cattle in an extra-label manner for many decades. Side-resistance between drugs within the same drug class has been described (Martin et al., 1989; Sangster and Gill, 1999). Furthermore, it has been hypothesized that cross-resistance could also occur between different drug classes, whereby one genetic mutation might facilitate further mutations, or enhance the activity of particular enzymes, allowing the parasite to develop multiple-drug resistance (Sargison et al., 2010).

Most of the farms in our study had parasites that were susceptible to levamisole. Since levamisole has not been commercially available in Canada for the last 10 years (Health Canada – Drug Product Database Online Query, 2012), very few producers in our study had routine access to the drug, reducing the selective pressure for resistance. Levamisole resistance has been reported in other countries where the drug is readily available (Waghorn et al., 2006; Cezar et al., 2010; Sargison et al., 2010). On the few farms where resistance, or suspected resistance, to levamisole was shown, the mean reduction percentage was likely affected by a few outlier sheep; the reduced efficacy was due to a few sheep having low, as opposed to zero, post-treatment FECs (farm 10) or one sheep with a high post-treatment FEC out of a group with negative post-treatment FECs (farms 27 and 28). Cabaret and Berrag (2004) have suggested that, in situations like these, when the overall efficacy is high, low efficacies in one or a few of the individuals are often not indicators of resistance in those animals, but rather may be due to other factors such as poor metabolism or poor bioavailability of the drug in those animals.

Coles et al. (2006) suggest that when assessing the efficacy of levamisole, post-treatment fecal samples should be collected 7-10 days after treatment. However, due to
logistic constraints, fecal samples were collected from all animals in this study on day 14 following treatment for the FECRT. The aforementioned recommendations are based on studies by Grimshaw et al. (1994 and 1996), where a number of larvae were found at necropsy, despite treatment of animals with levamisole 11 days earlier. The authors suggested that levamisole was ineffective against immature stages, which could lead to misinterpretation of positive FECs 14 days after treating animals with levamisole, since this could be a result of the immature larvae developing into adult worms, rather than the presence of resistant parasites. However, our study did not support these findings, since the majority of the farms had 0 post-treatment FECs 14 days after treatment with levamisole, and the LDA results confirmed these findings. While one may argue that the FECRT results could be due to the higher dosage (10.5 mg/kg) used in this study (Pugh, 2001), other studies (McKenna, 1974; Andrews, 2000) have shown that levamisole was effective against arrested larvae in sheep; both studies treated sheep at a dosage of 8.0 mg/kg. It is also possible that during the summer months when this study was conducted, there were few arrested larvae.

4.4.3 Larval cultures of post-treatment fecal samples

Larval cultures provide insight into the parasite genera present in animals, and therefore allow for the determination of the efficacy at the genus level (McKenna, 1990). In the work described here, the fecal samples for larval culture were obtained from animals that had previously been treated with ivermectin (i.e. drench check prior to the FECRT). However, a minimum of five weeks elapsed between the ivermectin treatment for the drench check and the second FECRT visit, when larval cultures were conducted on the control (i.e. untreated) animals, providing sufficient time for the animals to
become re-infected with GIN. As such, the results from the larval cultures from the control group samples are likely true representations of the unselected parasite genera present on that farm, and the post-treatment results represent the resistant genera.

Results from the larval cultures showed that the most predominant parasite genera in both untreated and treated animals was *Haemonchus* sp., followed by *Teladorsagia* spp., and *Trichostrongylus* spp. (Fig. 4.1). Table 4.2 indicates that most of the resistance to ivermectin and fenbendazole observed in this study was associated with *Haemonchus* sp.. This is of concern since *Haemonchus* sp. is the most pathogenic GIN and has high biotic potential, which allows for rapid spread of AR (Sutherland and Scott, 2010) (Vidyashankar *et al.*, 2012).

4.4.4 Larval development assay

In our study, LDAs on fecal samples obtained prior to the ivermectin drench check were performed for both thiabendazole and levamisole in the second year of the study. More than 100 L3s (from 300 eggs) developed in the control wells of 21/24 of the LDAs performed, indicating good hatchability (Taylor, personal communication). Of these 21 farms, 3 farms (14%) showed low levels of resistance to thiabendazole, while 15 farms (62%) showed high levels of resistance to thiabendazole (Table 4.3). In contrast, there was no resistance to levamisole observed on any of the study farms.

In both the control and thiabendazole wells (Figs. 4.2 and 4.3, respectively), the predominant genus isolated was *Haemonchus*, followed by *Teladorsagia* and *Trichostrongylus*. *Haemonchus* sp. was the most common GIN on most of the Ontario sheep farms studied, and was also the most commonly resistant parasite (Table 4.3). A
possible explanation for this observation is that few *Haemonchus* sp. larvae appear to
successfully overwinter on pasture in countries with cold winters (Waller *et al.*, 2006),
like Canada, resulting in a scarce number of *Haemonchus* sp. in *refugia* (parasites that are
not exposed to an anthelmintic drug) in the spring. However, *Haemonchus* sp. can
survive the winter as hypobiotic larvae within sheep, maturing into egg-producing adults
in the spring (Blitz and Gibbs, 1972). A lack of susceptible parasites in *refugia* on spring
pasture may be an important contributing factor in the development of AR (van Wyk,
2001); it is a common practice for Ontario sheep farmers to treat all ewes with an
anthelmintic immediately before lambing time in the spring (unpublished results). Since
there are few *Haemonchus* sp. in *refugia* on pasture at this time, any resistant eggs that
are shed into the environment may accelerate the development of AR in *Haemonchus* sp.
if they contribute to future generations of resistant parasites.

Both farms 14 and 21 had a lower percentage reduction in *Teladorsagia* sp. larvae
in the 0.3 µg thiabendazole/mL well (i.e. higher concentration), compared to the 0.1 µg
thiabendazole/mL well (Table 4.3). However, these percentage reductions are based on
small numbers of larvae (e.g. on farm 21, 6 *Teladorsagia* sp. larvae were isolated in the
control well, while 2 *Teladorsagia* sp. larvae were isolated in the 0.3 µg/mL
thiabendazole well), and should therefore be interpreted cautiously.

4.4.5 Study limitations

The target population of this study was sheep farms in Ontario, and farm
enrolment for the study was derived from solicitation of volunteers via a variety of
communications from OSMA. Our original objective was to have a sufficient number of
volunteer producers to allow for random selection from these producers. However, presumably due to the longitudinal nature of the study, and the inherent owner-provided labor required for sampling, too few producers volunteered to allow us this opportunity; we therefore had to include all producers that met the inclusion criteria. It is recognized that this might introduce a bias to the study since the self-selected sample might not be representative of all Ontario sheep farms. However, based on the wide distribution of the farms across Ontario and the diversity in size and management practices reported on the farms (Falzon et al., unpublished), the study is likely representative of Ontario sheep farms that practice grazing. Moreover, performing the ivermectin drench check prior to the FECRT might have also resulted in a selection bias, since a FECRT was only performed on farms where an indication of ivermectin resistance was detected with the former test. However, given the high frequency of ivermectin drench failure (88% of the farms tested), few farms were not subjected to a FECRT, and so we believe the high levels of AR observed in this study with the FECRT are applicable to other Ontario sheep farms.

The inclusion criteria were designed to ensure that the animals enrolled in the study had a sufficiently high FEC, since studies have shown that low FECs hinder the interpretation of a FECRT (Miller et al., 2006). Younger animals usually have higher FECs, and since most sheep develop some immunity to most of the GIN species by 4-7 months of age (Sutherland and Scott, 2010), adults tend to have lower GIN burdens. However, as described earlier, in our study we had to expand one of our initial inclusion criteria (only lambs would be enrolled in the study) to include yearling ewes in their first grazing season, since many producers in Ontario keep their lambs indoors.
In the first year of the study, the animals included in the FECRT were divided into treatment groups sequentially, as opposed to the systematic method used in the second year. In additional work, analysis of the FECRT data showed that there was no statistically significant difference (p >0.10) in the FECs and in the FECR between the two years (Falzon et al., unpublished). This indicates that the non-random group allocation of animals that might have occurred in the first year should not have influenced our results. Also, in the first year, some fecal samples were stored for up to three weeks before being processed. While it is general practice to process fecal samples within the first week after collection, Foreyt (1986) indicated that refrigeration of fecal samples was the best method of preservation, and 87% of strongyle eggs were detected after 50 days of storage at 4°C. Moreover, a study conducted by Falzon et al. (results not published) indicated that when pooled fecal samples were refrigerated and analyzed on a weekly basis over 13 weeks, the FECs did not change over time. Therefore, we do not believe that the storage of fecal samples for more than a week should be a cause of concern in the present study. However, we recognize that refrigeration of fecal samples negatively influences the hatchability of the GIN eggs, with Haemonchus sp. being more affected than others (McKenna, 1998). For this reason, we chose not to report any larval culture or LDA results from the first year of the study.

While some LDA methodologies allow for the in vitro assessment of ivermectin resistance (Howell et al., 2008), we were unable to assess the in vitro ivermectin susceptibility in this study, since the LDA methodology used here has not been shown to be effective in detecting resistance to ivermectin (Grimshaw et al., 1994). Finally, the modified McMaster method used in this study has a detection limit of 50 epg. While we
recognize that this may introduce a bias when calculating FECR values (El-Abdellati et al., 2010), we believe that, given the high post-treatment FECs on many of the farms, this would have had minimal influence on the high frequency of ivermectin and fenbendazole resistance detected on the farms in this study. While recent studies have suggested including pre-treatment egg counts in calculations for FECRTs to account for this bias (Levecke et al., 2012), we have elected to utilize the calculation endorsed by the WAAVP (Coles et al., 1992).

4.5 Conclusion

Drench failure to ivermectin occurred on most of the volunteer farms tested. FECRT results indicated that resistance to ivermectin and fenbendazole, the two drugs most frequently used by Canadian sheep producers, was common on the Ontario sheep farms tested, while levamisole was mostly effective. Results from the LDA on fecal samples obtained prior to the ivermectin drench check also indicated widespread resistance to benzimidazoles, while confirming susceptibility to levamisole on all farms tested. *Haemonchus* sp. was the most commonly isolated parasite in both the LDA and post-treatment FECRT cultures. It would therefore appear that most of the ivermectin and benzimidazole resistance detected on the Ontario farms was associated with *Haemonchus* sp., which is a concern as this is typically the most pathogenic of the GIN that infect sheep in Ontario. Overall, these findings strongly suggest that anthelmintic resistance, particularly in *Haemonchus* sp., appears to be a serious problem in Ontario sheep flocks. Thus, veterinarians and sheep producers should exercise more judicious use of anthelmintics and incorporate sustainable integrated parasite management strategies to
mitigate a worsening situation, and to maintain the sustainability of the Ontario sheep industry.

4.6 Acknowledgements

This research was supported by the Animal Health Strategic Initiative, with additional support from the University of Guelph for summer student positions and in-kind assistance from Merial, Canada. The authors are very grateful to William Sears for statistical advice and to Brad De Wolf, Steve Roche, Grazyna Adamska-Jarecka, Katie Sippel, Kirstie Puskas, Lee Siertsema, Jacqueline Sinclair, Benjamin Schlegel and David Baker for laboratory and field assistance. We especially acknowledge the sheep producers that participated in the study.

Conflict of interest

The authors declare no conflict of interest.
4.7 References


World Maps of Köppen-Geiger Climate Classification, 2012. Available at:

Table 4.1. The fecal egg count reduction percentages (and 95% confidence intervals) following treatment with ivermectin, fenbendazole, and levamisole on sheep farms in Ontario (2010 and 2011).

<table>
<thead>
<tr>
<th>Farm</th>
<th>Ivermectin (n = 29 farms)</th>
<th>Fenbendazole (n = 20 farms)</th>
<th>Levamisole (n = 17 farms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage reduction</td>
<td>95% CI</td>
<td>Percentage reduction</td>
</tr>
<tr>
<td>1</td>
<td>76 (-13 – 95)</td>
<td>97 (72 – 100)</td>
<td>100 $(†)$</td>
</tr>
<tr>
<td>2</td>
<td>28 (-98 – 74)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>3</td>
<td>-93 (-1138 – 70)</td>
<td>46 (-39 – 79)</td>
<td>n/a</td>
</tr>
<tr>
<td>4</td>
<td>29 (-42 – 65)</td>
<td>79 (59 – 89)</td>
<td>100 $(†)$</td>
</tr>
<tr>
<td>5</td>
<td>-8 (-88 – 38)</td>
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<td>n/a</td>
</tr>
<tr>
<td>6</td>
<td>59 (26 – 77)</td>
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<td>n/a</td>
</tr>
<tr>
<td>7</td>
<td>-157 (-579 – 2)</td>
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<td>n/a</td>
</tr>
<tr>
<td>8</td>
<td>-23 (-175 – 45)</td>
<td>0 (-116 – 54)</td>
<td>100 $(†)$</td>
</tr>
<tr>
<td>9</td>
<td>54 (-3 – 80)</td>
<td>34 (-25 – 65)</td>
<td>100 (99 – 100.0)</td>
</tr>
<tr>
<td>10</td>
<td>93 (60 – 99)</td>
<td>74 (57 – 96)</td>
<td>96 (70 – 99)</td>
</tr>
<tr>
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<td>-14 (-120 – 41)</td>
<td>100 (99 – 100.0)</td>
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<td>100 $(†)$</td>
</tr>
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</tr>
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<td>60 (34 – 76)</td>
<td>93 (52 – 99)</td>
</tr>
<tr>
<td>29</td>
<td>55 (0 – 80)</td>
<td>24 (-64 – 64)</td>
<td>100 $(†)$</td>
</tr>
</tbody>
</table>

Note: the minus sign "-" in front of a number indicates that the FEC increased after treatment.
CI=Confidence Intervals
n/a=Fenbendazole and levamisole were not tested due to insufficient numbers of animals.
†No 95% confidence intervals were computed since the reduction was 100%.
Table 4.2. The fecal egg count reduction, and percentage reductions (%) in *Haemonchus* sp., *Teladorsagia* spp., and *Trichostrongylus* spp., for ivermectin (n=18 farms), fenbendazole (n=13 farms) and levamisole (n=11 farms) from post-treatment larval cultures from sheep farms in Ontario (2011).

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<th></th>
<th>Fenbendazole</th>
<th></th>
<th></th>
<th></th>
<th>Levamisole</th>
<th></th>
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<td>Tel.³</td>
<td>Tri.⁴</td>
<td>FECR %¹</td>
<td>Hae.²</td>
<td>Tel.³</td>
<td>Tri.⁴</td>
<td>FECR %¹</td>
<td>Hae.²</td>
<td>Tel.³</td>
<td>Tri.⁴</td>
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<td>100</td>
<td>100</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
</tbody>
</table>
n/a=Fenbendazole and levamisole were not tested on all farms due to insufficient numbers of animals.

FECR=Fecal Egg Count Reduction; Hae=Haemonchus; Tel=Teladorsagia; Tri=Trichostrongylus;

† the genus specific fecal egg count reduction for these farms was not calculated since the fecal egg count reduction was ≥95%
‡ the percentage reduction for these species was not calculated since there were <50 epg in the pre-treatment samples
§ the percentage reduction for this farm was not calculated since <50 larvae developed in the levamisole culture well
Table 4.3. The mean number of larvae isolated from the two control wells, the farm thiabendazole resistance status, and the percentage reduction of *Haemonchus* sp., *Teladorsagia* spp., and *Trichostrongylus* spp., in the TBZ 0.1 and TBZ 0.3 wells, in the Larval Development Assay for 24 sheep farms in Ontario (2011).

<table>
<thead>
<tr>
<th>Farm</th>
<th>Number of larvae</th>
<th>TBZ R</th>
<th>Percentage reduction in larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TBZ 0.1</td>
</tr>
<tr>
<td>12</td>
<td>201</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>142</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>157</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>88</td>
<td>n/a</td>
<td></td>
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<tr>
<td>16</td>
<td>183.5</td>
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<td></td>
</tr>
<tr>
<td>17</td>
<td>155.5</td>
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<td></td>
</tr>
<tr>
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<td>164</td>
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<td></td>
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<tr>
<td>19</td>
<td>115</td>
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<tr>
<td>40</td>
<td>93</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

n/a=<100 larvae were isolated in the control well, and therefore was excluded.

†=number of larvae of a particular species in the control well was <5% of the total number of larvae in the control well, and therefore the reduction percentage was not calculated.

1^TBZ R=thiabendazole farm resistance status;
2^TBZ 0.1=0.1 µg/mL thiabendazole
3^TBZ 0.3=0.3 µg/mL thiabendazole
a) Control (i.e. no treatment)

b) Ivermectin
c) Fenbendazole

![Bar chart showing the number of Trichostrongylus spp., Teladorsagia spp., and Haemonchus sp. in the first 100 (±5) larvae isolated from the culture of pooled fecal samples obtained from (a) control sheep (i.e. no treatment) and sheep treated with (b) ivermectin (n=18 farms) (c) fenbendazole (n=13 farms) or (d) levamisole (n=11 farms), 14 days after treatment, on farms in Ontario (2011).

Note: Fenbendazole and levamisole were not tested on all farms due to insufficient animals; less than 100 larvae were isolated from the feces of many of the sheep treated with levamisole on 10/11 farms.

d) Levamisole

![Bar chart showing the number of Trichostrongylus spp., Teladorsagia spp., and Haemonchus sp. in the first 100 (±5) larvae isolated from the culture of pooled fecal samples obtained from (a) control sheep (i.e. no treatment) and sheep treated with (b) ivermectin (n=18 farms) (c) fenbendazole (n=13 farms) or (d) levamisole (n=11 farms), 14 days after treatment, on farms in Ontario (2011).
Figure 4.2. The mean number of *Trichostrongylus* spp., *Teladorsagia* spp. and *Haemonchus* sp. larvae identified in the control wells (i.e. no anthelmintics) of the larval development assays performed on gastrointestinal nematode eggs from 24 farms in Ontario (2011).
Figure 4.3. The mean number of *Trichostrongylus* spp., *Teladorsagia* spp. and *Haemonchus* sp. larvae identified in wells containing 0.1 µg/mL thiabendazole in larval development assays performed on gastrointestinal nematode eggs from 24 farms in Ontario (2011).
Figure 4.4. The mean number of *Trichostrongylus* spp., *Teladorsagia* spp. and *Haemonchus* sp. larvae identified in wells containing 0.3 µg/mL thiabendazole in larval development assays performed on gastrointestinal nematode eggs from 24 farms in Ontario (2011).
CHAPTER 5

Comparison of tests and methods used for the determination of anthelmintic resistance in sheep

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Abstract

Anthelmintic resistance (AR) in parasites of sheep has been reported worldwide. The Fecal Egg Count Reduction Test (FECRT) is the standard field test for diagnosing AR, but there are different methods for calculating the Fecal Egg Count Reduction (FECR) percentages. The Larval Development Assay (LDA) also tests for anthelmintic susceptibility, yet few studies have been carried out to correlate results between the FECRT and the LDA. This study was undertaken to: i) compare results obtained with different FECR calculation methods for defining resistance to ivermectin, fenbendazole, and levamisole; and ii) compare categorized results obtained with the FECRT and LDA for resistance to benzimidazoles and levamisole. Four different methods were used to calculate FECR percentages for the treatment groups: FECR₁ and FECR₂ used pre- and post-treatment Fecal Egg Counts (FECs) from both treated and control animals; FECR₁ used arithmetic means while FECR₂ used geometric means. FECR₃ was calculated using arithmetic means for post-treatment FECs from treated and control animals, while FECR₄ was calculated using mean FEC estimates from a General Linear Mixed Model. For all treatment groups, FECR₁ and FECR₃ showed fair to almost perfect correlation, as did FECR₂ and FECR₄, while the other FECR pair-wise combinations showed poor to fair correlation. For the second objective, Kappa values between the LDA and the four
different FECR methods were computed; FECR values ≥95% were considered as indicative of no resistance while five different threshold percentages (90%, 80%, 70%, 60% and 50%) were used to indicate low to high resistance. Results evaluating benzimidazole resistance indicated a poor to moderate agreement; however, the Kappa value was statistically significant when the percentage reduction was calculated using FECR₁ with an 80% threshold, and using FECR₂ with a 70% threshold. In conclusion, the classification of farm resistance status varied depending on whether arithmetic or geometric means were used, especially when low levels of resistance were present. In contrast, inclusion of both pre- and post-treatment, or only post-treatment, groups in the FECR formula was less influential. The LDA and FECRT showed an overall poor to moderate Kappa agreement in this study; however, the applicability of these tests depends on the goal of the monitoring program and the levels of resistance present. Since the classification of resistance varied with the method used, there is a need for consensus standardization on classification of AR

5.1 Introduction

Gastro-intestinal nematodes (GINs) are of concern on sheep farms worldwide as they impair milk, meat and wool production in sheep, and are an important cause of morbidity and mortality (Knox et al., 2012). For many years, producers have relied primarily on the use of anthelmintics for the control of GINs in sheep (Sargison, 2008). However, this reliance has led to the development of anthelmintic resistance (AR), and many countries are now reporting both multi-drug and multi-nematode species resistance (Jackson and Coop, 2000; Kaplan, 2004; Kaplan and Vidyashankar, 2012).
In every parasite population, a number of genotypically resistant parasites are typically present (Prichard et al., 1980). When these parasite populations reach a certain frequency threshold, they become phenotypically resistant in animals (Kaplan and Vidyashankar, 2012), and are associated with treatment failure and, inevitably, losses in sheep health and productivity (Kaplan, 2004). It is therefore important to diagnose AR before it reaches this ‘critical frequency’, where resistance becomes a clinical and economic problem (Kaplan and Vidyashankar, 2012).

The Fecal Egg Count Reduction Test (FECRT) is the standard field test for the diagnosis of AR in sheep (Coles et al., 2006). However, the results from a FECRT may be influenced by several factors, including the study design, the host-parasite interaction (Levecke et al., 2012), and the mathematical formulae used to calculate drug efficacy (Miller et al., 2006). In the literature, there are several methods to calculate the Fecal Egg Count Reduction (FECR); these methods differ based on whether the arithmetic or geometric mean is used, and whether pre-and post-treatment Fecal Egg Counts (FECs), or just post-treatment FECs, are used in the calculation (Cabaret and Berrag, 2004). Some authors have suggested the use of arithmetic means as they are unbiased estimators of the true mean (Fulford, 1994), and therefore provide better estimates of the parasite egg output compared to geometric means (Coles et al., 1992; Dobson et al., 2009).

Meanwhile, other authors have described geometric means as more appropriate estimators of the central tendency parameter for parasite populations which are usually overdispersed and, therefore, do not have a constant variance (Smothers et al., 1999). Further, while some FECR formulae take into account both pre- and post-treatment FECs in both treated and control animals (Presidente, 1985; Dash et al., 1988), other variations
of the FECR calculation only take into consideration the post-treatment FECs of both treated and untreated animals (Coles et al., 2006), thereby reducing the number of fecal samples required. Lastly, Mejia et al. (2003) described an alternate approach for calculating FECR, using a General Linear Mixed Model (GLMM) to provide FEC means corrected for other co-variable effects, such as animal weight and treatment. While McKenna (2006) reported that different FECR formulae provided similar estimates of anthelmintic efficacy, Miller et al. (2006) found that different methods may generate different FECR percentages, hence influencing the decision as to whether AR is present, and whether an anthelmintic should be used on a farm (Torgerson et al., 2005). It is therefore important to further assess the correlation between these different methods in order to improve and standardize the method of FECR calculation and limit misclassification of farm resistance status (Coles et al., 2006; Denwood et al., 2010).

Larval Development Assays (LDAs) are a laboratory-based test for the diagnosis of AR that have been described as a suitable alternative to the FECRT since they are rapid and comparatively inexpensive (Taylor et al., 2002). However, for a laboratory technique to be widely applicable, it should provide results that correlate closely with those obtained from a field test (Roush and Tabashnik, 1990). To date, studies that have compared AR data obtained from laboratory and field tests either used different in vitro techniques, such as an egg hatch assay (Maingi et al., 1998), a larval feeding inhibition assay (Díez-Baños et al., 2008) or different LDA methodologies (Hubert and Kerboeuf, 1992); or were carried out in animal species other than sheep (Craven et al., 1999; Königová et al., 2003). To determine the potential of the LDA methodology described by Taylor (1990) for field screening in sheep in Canada, it is important to calculate the
agreement between the results obtained with the LDA and the different methods of calculating the FECR, on sheep farms in Canada.

The objectives of this study were to: i) compare the FECR percentages obtained using different formulae, for resistance to ivermectin, fenbendazole, and levamisole; and ii) compare categorized results obtained with the FECRT and LDA for resistance to benzimidazoles and levamisole.

5.2 Materials and methods

5.2.1 Farm selection, Fecal Egg Count Reduction Test and Larval Development Assay

Full details of the farm selection, FECRT and LDA have been described in Falzon et al. (in press). In brief, 47 sheep flocks across Ontario, Canada, were enrolled over 2 years in a study to determine the frequency of AR in Ontario sheep flocks. A FECRT was performed on those farms that reported ivermectin drench failure (defined as a FECR < 95% following ivermectin treatment by producers); a mean of 28 days (range = 21 to 35 days) elapsed between ivermectin treatment and the FECRT. The FECRT for ivermectin (0.2 mg/kg), fenbendazole (5.0 mg/kg) and levamisole (10.5 mg/kg) was conducted on 29, 20 and 17 farms, respectively, over 2 years, and 10-15 animals were included in each treatment group. Fenbendazole and levamisole were tested on fewer farms due to a limited number of animals meeting the inclusion criteria on some farms. LDAs (Taylor, 1990) to detect the presence of resistance to thiabendazole and levamisole were performed for 24 farms in the second year of the study (20 farms that were enrolled in the second year and 4 farms from the first year that re-submitted fecal samples in the second year), using two drug concentrations for each drug. The LDAs were carried out on
composite fecal samples when the mean FEC was >200 epg and before any treatment was administered to the animals. All matching LDAs and FECRTs were conducted in the second year of the study; however, LDAs were performed at the beginning of the grazing season (June-July) while FECRTs were performed five to eight weeks later.

5.2.2 Fecal Egg Count Reduction calculations

FECR calculations were conducted using SAS® 9.3 (SAS Institute Inc., Cary, NC, USA) as follows:

(i) \( \text{FECR}_1 = 100 \times (1 - \frac{T2}{T1})\frac{C1}{C2} \)

where \(T1\) and \(T2\) were pre- and post-treatment arithmetic means of the GIN eggs per gram (epg) in treated groups, respectively, and \(C1\) and \(C2\) were pre- and post-treatment arithmetic means of the epg in the controls (i.e. untreated animals), respectively (Dash et al., 1988).

(ii) \( \text{FECR}_2 = 100 \times (1 - \frac{T2}{T1})\frac{C1}{C2} \)

where \(T1\) and \(T2\) were pre- and post-treatment geometric means of the epg in treated groups, respectively, and \(C1\) and \(C2\) were pre- and post-treatment geometric means of the epg in the controls, respectively (Presidente, 1985).

For both \(\text{FECR}_1\) and \(\text{FECR}_2\), the 95% Confidence Intervals (CIs) were estimated as:

\[
100 \times [1 - \exp \{ \log \left( \frac{T2}{T1} \right) \left( \frac{C1}{C2} \right) \}] \pm 1.96 \times \text{SE} \\{ \log \left( \frac{T2}{T1} \right) \left( \frac{C1}{C2} \right) \}
\]

where \(\text{SE}\) was the standard error of the reduction, and was estimated as: \(1/T1 + 1/T2 + 1/C1 + 1/C2\)^{1/2}.

(iii) \( \text{FECR}_3 = 100 \times (1 - [T2/C2]) \)

where \(T2\) and \(C2\) were the post-treatment arithmetic means of epg in the treated and control groups, respectively. Ninety-five percent CIs
were estimated as: 100 x [1 - [T2/C2] exp( ± 1.96 \sqrt{Y^2})] , where Y^2 was the variance of reduction (Coles et al., 1992).

(iv) FECR_4 was calculated by building a GLMM, as described by Mejia et al. (2003). The GLMM was fit using PROC MIXED (SAS 9.3®), with the natural logarithm of the post-treatment FEC as the response variable. The dependence of the data was modeled by a fixed effect for farm (to obtain a coefficient for each farm) and a random slope parameter at the treatment level (due to different variances between treatment groups) (Littell et al., 1996). While accounting for the treatment random effect, each fixed effect variable was examined on its own to screen for variables to start the modeling process. Variables screened included the natural logarithm of the pre-treatment FEC, animal weight, farm, treatment (control, ivermectin, fenbendazole and levamisole) and year of study (first and second). Due to the relatively small sample size, a liberal alpha value of ≤0.20 was used to indicate which terms to initially include in the model. The linearity of continuous variables was assessed graphically by plotting lowess smoother curves and by including a quadratic term in the model, as described by Dohoo et al. (2009).

A final GLMM was built using a manual backwards stepwise procedure, by first including all variables that were significant in the univariable analyses. After the main effects model was built, predictors of interest that were not significant in the univariable analysis were forced into the model to assess potential confounding and conditional effects. All possible two-way interactions between significant predictors were tested. The model assumptions were assessed by plotting residuals against the predicted outcomes and explanatory variables, to look for homoscedasticity, non-linearity and outliers. Normality was visually assessed with histograms of the residuals and normal quantile
plots, and assessed statistically using four different tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling). Observations that were identified as outliers or influential were cross-checked with the original data sheets for any abnormality in the data to explain their behavior. The model was repeated without those observations, and differences in model estimates were noted.

The predicted post-treatment FECs were back-transformed using the exponential function (equivalent to geometric means), and the FEC means were then used to calculate the FECR following the same method described for FECR\textsubscript{3} (Coles et al., 1992).

For all FECR methods, farms were classified as resistant when the reduction was <95% and the lower 95% CI was <90%; if only one of these two criteria was met, the farm was classified as being suspected of resistance (Coles et al., 1992).

5.2.3 Comparison of data from Fecal Egg Count Reduction Calculation methods

The FECR percentages obtained with the different FECR methods were compared by computing a Concordance Correlation Coefficient (CCC) using SAS\textsuperscript{®} 9.3, as described by Lin (1989). Bland-Altman plots were used to evaluate the distribution of the observations, and outliers were cross-checked with the original data sheets for any abnormality in the data that may have explained their influential behavior. The CCC was re-computed without the influential observations, and changes in the CCC were noted. The CCCs were interpreted using a scale described by Shoukri and Pause (1998), where values equal to 0.0 or between 0.01-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80 and 0.81-1.00, were considered indicative of poor, slight, fair, moderate, substantial and almost perfect agreement, respectively.
5.2.4 Comparison of Larval Development Assay and Fecal Egg Count Reduction Test results

The LDA was performed for thiabendazole and levamisole; LDAs and matching FECRTs were only conducted in the second year of the study. To estimate the Kappa agreement between the LDA and FECRT, results from both tests were first categorized into ordinal variables (0=no resistance; 1=low resistance; 2=high resistance) using the following rubric, and the results entered into 3X3 frequency tables.

The LDA results for thiabendazole and levamisole were categorized as having: “no resistance” if ≤5% of the GIN eggs (compared to the control well) developed in the 0.1 µg/mL thiabendazole well or 1.0 µg/mL levamisole well; “low resistance” if >5% of the GIN eggs (compared to the control well) developed in the 0.1 µg/mL thiabendazole well or 1.0 µg/mL levamisole well and ≤5% of the GIN eggs (compared to the control well) developed in the 0.3 µg/mL thiabendazole well or 3.0 µg/mL levamisole well; and “high resistance” if >5% of the GIN eggs (compared to the control well) developed in the 0.3 µg/mL thiabendazole well or 3.0 µg/mL levamisole well (Taylor, 1990). The discriminatory concentrations of 0.1 µg/mL thiabendazole and 1.0 µg/mL levamisole were used as described by Hong et al. (1992, 1996). The 5% cut-off was selected to be consistent with the definition of resistance used for the FECRT, whereby resistance is said to be present if the FEC following treatment is >5% compared to the FEC in the control or pre-treatment group (Coles et al., 1992).

Since no references for thresholds of high or low levels of resistance based on the FECRT could be found in the literature, the FECR percentages obtained with the four
different calculation methods discussed previously \( (\text{FECR}_{1,4}) \) were categorized using five different threshold levels:

i) No resistance present when the FECR was \( \geq 95\% \); low resistance present when the reduction was \( <95\% \) and \( \geq 90\% \); and high resistance when the reduction was \( <90\% \);

ii) No resistance present when the FECR was \( \geq 95\% \); low resistance present when the reduction was \( <95\% \) and \( \geq 80\% \); and high resistance when the reduction was \( <80\% \);

iii) No resistance present when the FECR was \( \geq 95\% \); low resistance present when the reduction was \( <95\% \) and \( \geq 70\% \); and high resistance when the reduction was \( <70\% \);

iv) No resistance present when the FECR was \( \geq 95\% \); low resistance present when the reduction was \( <95\% \) and \( \geq 60\% \); and high resistance when the reduction was \( <60\% \); and,

v) No resistance present when the FECR was \( \geq 95\% \); low resistance present when the reduction was \( <95\% \) and \( \geq 50\% \); and high resistance when the reduction was \( <50\% \).

Therefore, by way of example using a threshold of 70\%, farms with a FECR \( >95\% \) were classified as susceptible, farms with a FECR between 70 to 95\% were classified as having low resistance, and farms with a FECR \( <70\% \) were classified as having high resistance.
A weighted Kappa using squared values (Fleiss and Cohen, 1973) was calculated using an in-house Fortran program (Stat-Menu) that was developed based on subject matter covered in Fleiss et al. (2003) and Agresti (2002). The weighted Kappa better accounts for partial agreement between tests, compared to the non-weighted Kappa (e.g. “no resistance” is in closer agreement with “low resistance” than with “high resistance”) (Dohoo et al., 2009). The squared approach was used since this puts more weight on the observations that are more discrepant, and is therefore more consistent with the notion of intra-class correlation. A McNemar-Bowker test (Agresti, 2002) was also calculated to determine if the distribution of discordant cells was equal.

Since the LDA and FECRT were conducted at different times during the grazing season (Falzon et al., in press), the proportion of Haemonchus sp., Teladorsagia sp., and Trichostrongylus spp. present at each time-point was calculated to assess whether the parasite population on each farm changed during the grazing season, and to facilitate interpretation of the Kappa agreement results. This was carried out using information on parasite species obtained from the LDA, and from the FECRT post-treatment larval cultures (Falzon et al., in press).

5.3 Results

5.3.1 Descriptive results of different FECR calculation methods

Ivermectin, fenbendazole and levamisole FECR percentages and 95% CIs were calculated using the four different FECR methods (Tables 5.1, 5.2 and 5.3, respectively). The percentage of farms classified as resistant varied, depending on the FECR calculation method and anthelmintic used. The FECR$_4$ reduction could not be calculated for farms 9
and 28 since no animal weight data were available for the control animals and the weight variable was required in the GLMM estimate for FECR\textsubscript{4}. The final model of the natural logarithm of post-treatment FECs (Table 5.4) met the assumptions of both homoscedasticity and normality; however, residual analyses revealed two major outliers. The model was rerun without these observations, but this did not change the final fit of the model, and there was no reason to omit the observations; hence, the outliers were retained in the final model.

Ivermectin resistance was reported on: 28/29 (97\%) farms when the FECR\textsubscript{1} and FECR\textsubscript{2} methods were used; on 28/29 (97\%) farms, with an additional farm suspected of resistance, when the FECR\textsubscript{3} method was used; and on 25/27 (93\%) farms, with 2 additional farms suspected of resistance, when the FECR\textsubscript{4} method was used (Table 5.1). Fenbendazole resistance was reported: on 20/20 (100\%) farms when the FECR\textsubscript{1} and FECR\textsubscript{2} calculations were used; on 19/20 (95\%) farms, with one additional farm suspected of resistance, when the FECR\textsubscript{3} calculation was used; and on 17/18 (94\%) farms, with 1 additional farm suspected of resistance, when the FECR\textsubscript{4} method was used (Table 5.2). Levamisole resistance was reported: on 0/17 (0\%) farms, with 1 farm suspected of resistance, when the FECR\textsubscript{1} calculation was used; on 4/17 (24\%) farms, with an additional 3 farms suspected of resistance, when the FECR\textsubscript{2} calculation was used; on 1/17 (6\%) farms, with 1 additional farm suspected of resistance, when the FECR\textsubscript{3} calculation was used; and on 3/15 (20\%) farms when the FECR\textsubscript{4} method was used (Table 5.3). Farms 10, 25 and 26 had the lowest estimates of levamisole reduction for both FECR\textsubscript{2} and FECR\textsubscript{4}. 

5.3.2 Comparison of different FECR calculation methods

The CCCs and level of correlation between the four FECR calculation methods for the ivermectin, fenbendazole and levamisole treatment groups are shown in Table 5.5. The level of correlation varied depending on which FECR methods were compared, and which treatment group was being assessed. Overall, FECR1 and FECR3, and FECR2 and FECR4 showed better correlation, compared to the other pair-wise combinations.

In all treatment groups, farms 1, 3 and 7 were identified as influential observations. On these three farms, the animals were divided into the treatment groups by the producers, prior to the researchers’ arrival on the farm; it was suspected that the group allocation was based on the animals’ age, where older and younger animals were put in separate groups. These three farms were removed from the dataset since this non-randomization is likely to have influenced the results obtained for these three farms (Dohoo et al., 2009). In both the fenbendazole and levamisole treatment groups, farm 25 was identified as an influential observation and its removal improved the CCC between all FECR pair combinations (e.g. for the fenbendazole treatment group, the correlation between FECR1 and FECR3 increased from 0.35 to 0.77 when farm 25 was removed); however, no data errors or other issues were identified that justified its removal, and therefore it was retained in the dataset.
5.3.3 Comparison of the Larval Development Assay and Fecal Egg Count Reduction Test results

5.3.3.1. Categorization of the LDA and FECRT results

LDAs for thiabendazole and levamisole were performed for 24 farms; however, matching FECRT data were available for only 13 and 11 of these farms, for fenbendazole and levamisole respectively, as there were insufficient numbers of animals available on some farms. Of the 13 farms with matching benzimidazole results, 1 farm was classified by the LDA as having no resistance to thiabendazole (i.e. ≤5% GIN eggs in 0.1 µg/mL thiabendazole well), 2 farms were classified as having low resistance (i.e. >5% and ≤5% GIN eggs in 0.1 and 0.3 µg/mL thiabendazole wells, respectively) and 10 farms were classified as having high resistance (i.e. >5% GIN eggs in 1.0 µg/mL thiabendazole well). Of the 11 farms with matching FECRT and LDA results for levamisole, all farms were classified as having no resistance (i.e. ≤ 5% GIN eggs in 1.0 µg/mL levamisole well).

Regardless of the FECR calculation method used, none of the farms tested for fenbendazole resistance with the FECRT were classified as having “no resistance present” (i.e. all farms had FECR <95%) (Table 5.6). When 90% and 80% cut-offs were used as thresholds to differentiate between high and low resistance, the majority of the farms were classified as having high resistance; when 70%, 60% and 50% were used as cut-offs, the number of farms categorized as having low resistance increased. When the 50% threshold was used, all farms were classified similarly among the FECR calculation methods used.
The levamisole FECR percentages were classified similarly across the five thresholds used to differentiate between high and low resistance, when calculated using FECR₁ and FECR₃ (Table 5.7). With the FECR₂ and FECR₄ methods, the classification of farms as having high or low resistance changed when higher (90%, 80% and 70%) or lower (60% and 50%) thresholds were used.

5.3.3.2 Kappa agreement

Kappa agreement analyses were carried out on the ordinal results (i.e. no, low or high resistance) from both LDA and the four FECR calculation methods, for both benzimidazoles and levamisole. For the benzimidazole results, the agreement (beyond chance alone) between the two tests was highest when the 80% and 70% thresholds were used, especially for FECR₁ and FECR₂ (Table 5.8). In particular, the agreement between the LDA and the FECR₁ method using an 80% threshold, and with the FECR₂ method using a 70% threshold, was statistically significant (p≤0.05), indicating that these two thresholds could be used to differentiate between high and low levels of resistance. The results of the McNemar-Bowker test of symmetry were only statistically significant for the FECR₁ and FECR₂ methods at the 50% threshold. At all the other thresholds, the McNemar-Bowker test of symmetry was not statistically significant, indicating that the marginal cells (and therefore false-positives and false-negatives) were equally distributed between the two tests. This suggests that the FECRT and LDA had similar test sensitivities, and were therefore able to detect the same number of positive cases.
For the levamisole results, the weighted Kappa was zero between the LDA and the four FECR methods, at all the different threshold percentages. The McNemar-Bowker test was not statistically significant for any of the tests.

Table 5.9 presents the percentages of *Haemonchus* sp., *Teladorsagia* sp., and *Trichostrongylus* spp. isolated from the control wells of the LDA and from larval cultures of pooled fecal samples collected from the control animals on the second visit for the FECRT. *Haemonchus* sp. was the most common species on 10/13 (77%) farms at the time the LDA was performed, and on 13/13 (100%) farms at the time the larval culture was performed.

### 5.4 Discussion

#### 5.4.1 Different Fecal Egg Count Reduction calculation methods

In the work described here, for both the ivermectin and fenbendazole treatment groups, almost all farms (28/29 [97%] and 19/20 [95%], respectively) were classified as having AR, regardless of the FECR calculation used. For fenbendazole, farm 1 had very different reduction percentages, depending on which FECR method was used. This may be due to the non-random allocation of older and younger animals into separate treatment groups which was associated with a large number of zero pre-treatment FECs in the fenbendazole treatment group, despite a mean FEC>200epg for the group. This, in turn, may be responsible for the discrepancy between FECR\(_1\) and FECR\(_2\) that included pre-treatment FECs, and FECR\(_3\) and FECR\(_4\) that did not include pre-treatment FECs.

The levamisole reduction percentages obtained with the different FECR calculations showed greater heterogeneity (Table 5.3), compared to the other two
treatment groups. Farms 10, 25 and 26 had the lowest estimates for drug efficacy, indicating greater levels of AR, with both the \( \text{FECR}_2 \) and \( \text{FECR}_4 \) methods. The heterogeneity in FECR percentages observed for the levamisole treatment group could be explained by over-dispersion of the parasite population within the sheep, as suggested by Levecke et al. (2012). On farm 10, 12/15 of the animals treated with levamisole had post-treatment FECs of 0 epg, while the other three animals had post-treatment FECs of 100 epg. However, this farm was classified as having resistance to levamisole when using \( \text{FECR}_2 \) and \( \text{FECR}_4 \) methods. Work carried out by Dobson et al. (2009) showed that geometric means are subject to increased variability, and become highly unstable, when there is a high level of aggregation and most of the animals sampled have fecal egg counts of 0. This might explain for farm 10 why these methods, which used geometric means, provided lower estimates, compared to the other FECR methods using arithmetic means. On farms 25 and 26, ewe lambs in their first grazing season were used in addition to lambs, for the FECRT, since the lambs were kept indoors as young lambs due to predator concerns; these animals were heavier (50-80kg) than the lambs (10-45kg). Since the \( \text{FECR}_4 \) method also took into account the animals’ weight, this might have influenced the overall estimate provided by the GLMM. As indicated in Table 5.4, weight was negatively correlated with the percentage reduction; lower FECR percentages were estimated for the heavier animals. Weight was included in the model since it was considered a potential confounder based on anecdotal evidence. Although in our study all animals were weighed individually prior to treatment to remove the risk of under-dosing, it has been hypothesized that heavier animals may carry more resistant parasites as they
are more likely to be under-dosed, which in turn may accelerate the development of resistance (Sutherland and Scott, 2010).

Overall, the FECR results obtained for the three different treatment groups are in agreement with Miller et al. (2006), who suggested that the FECRT is effective at diagnosing resistance when AR is present at high levels, but is less reliable when the drug efficacy ranges around 90 - 95%. This was particularly evident when geometric means were used, and many FECs of 0 epg were present, leading to biased estimates of overall treatment efficacy (Dobson et al., 2009). In contrast, inclusion of both pre- and post-treatment or only post-treatment data was less influential on the FECR percentage, regardless of the levels of resistance. These results are in agreement with a similar study by McKenna (2006), which reported that different FECR formulae (using only arithmetic means) detected a similar number of anthelmintic resistance cases, and therefore suggested that the simpler formulae (i.e. those using only post-treatment FECs) could be a suitable alternative to the more complex formulae where both pre- and post-treatment FECs are required. This would reduce the number of fecal samples required, thereby reducing the labour and costs associated with the FECRT and making it more accessible to sheep producers.

5.4.2 Comparison of different Fecal Egg Count Reduction Calculation methods

The concordance correlation coefficient “evaluates the degree to which pairs [of observations] fall on the 45° line” in a scatterplot (Lin, 1989), and was used to measure the correlation between the different FECR methods. For all treatment groups, FECR$_1$ and FECR$_3$, as well as FECR$_2$ and FECR$_4$, showed a fair to almost perfect agreement,
while the other FECR pairs showed a slight to moderate correlation. This was particularly
evident for the levamisole treatment group, where both FECR<sub>1</sub> and FECR<sub>3</sub>, and FECR<sub>2</sub>
and FECR<sub>4</sub> were almost perfectly correlated (0.96 and 0.93, respectively).

These results suggest that the type of mean (arithmetic vs. geometric) used in the
FECR formulae was more influential than which data (pre- and post-treatment vs. only
post-treatment) were taken into account; the differences between formulae were most
marked when low levels of resistance were present (i.e. to levamisole), resulting in a
large number of post-treatment FECs that were zero. These results are in agreement with
the previous discussion, whereby the right-skewness of the post-treatment results
increases the variability of the estimates, making the reduction percentages more unstable
(Levecke et al., 2011; Dobson et al., 2012), especially when geometric means are used
and the level of resistance is low.

For the fenbendazole treatment group, the differences between the FECR pair-
wise correlations were less distinct. As noted in Section 5.3.2, farm 25 was identified as
an influential observation, and its removal from the dataset improved the correlation
between the different FECR pair-wise combinations. As mentioned earlier, on farm 25
ewe lambs in their first grazing season were used for the FECRT; many of these animals
had zero pre-treatment FECs (8/15 animals in the fenbendazole treatment group),
resulting in a right-skewed distribution of the GIN egg counts within the group of
animals.
5.4.3 Comparison of the Larval Development Assay and Fecal Egg Count Reduction Test

To check for the agreement between the FECRT and the LDA used in this study, a weighted Kappa agreement was calculated. Kappa is defined as “the proportion of agreement corrected for chance, and scaled to vary from -1 to +1 so that a negative value indicates poorer than chance agreement, 0 indicates exactly chance agreement, and a positive value indicates better than chance agreement” (Fleiss and Cohen, 1973).

To estimate the weighted Kappa, the LDA results and the FECR values were categorized into three ordinal categories: no resistance, low resistance and high resistance to benzimidazoles and levamisole. Since we could not find any reference in the literature for FECR threshold percentages indicative of the critical frequency at which resistance starts to have an economic and clinical impact, we examined five different threshold percentages to evaluate how these affect the level of agreement between the FECRT and LDA. While the FECRT threshold percentage for no resistance was kept constant (i.e. reduction value >95%), five threshold percentages were used to distinguish between low and high levels of resistance (see Section 5.2.4 for definitions).

Overall, there was a poor to moderate agreement beyond chance, between the FECRT and LDA for benzimidazoles (Table 5.8). However, the agreement was statistically significant when the FECR$_1$ method and an 80% threshold were used, and when the FECR$_2$ and a 70% threshold were used (Weighted Kappa = 0.58 and 0.48, respectively). This occurred because, at these thresholds, there were fewer observations in the perfectly discordant cells, and most observations were in either perfect or partial, agreement. These results suggest that 80% or 70% might be appropriate thresholds to
differentiate FECR percentages as indicative of high or low levels of resistance. For levamisole, despite most of the farms being classified as having no resistance by both tests, there was no agreement between the LDA and FECRT results.

The poor agreement between methods observed in our study could be explained by the small sample size (n=13 and 11 for benzimidazoles and levamisole, respectively), and the fact that some of the concordant cells in the contingency tables were empty, either because of the high frequency of benzimidazole resistance (i.e. there were no cases of susceptibility classified by the FECRT) or the low frequency of levamisole resistance (i.e. there were no cases of resistance classified by the FECRT). Other authors (Craven et al., 1999; Wolstenholme et al., 2004) have also described a poor correlation between in vivo and in vitro tests for AR, and suggested that this may be due to the fact that the two types of tests measure different attributes of the parasites’ response to anthelmintics. LDAs measure the effect of anthelmintics on the growth of first stage larvae, whereas the FECRT reflects the anthelmintics’ efficacy against adult parasites (Taylor, 1990). Further research investigating the agreement between the two tests, using populations with higher resistance to levamisole and lower resistance to fenbendazole, is warranted.

With respect to limitations, the results from this study need to be interpreted with caution, since the LDA and the FECRT were not conducted simultaneously (Falzon et al., in press). The LDAs were conducted earlier in the grazing season, when the FECs of the fecal samples submitted by producers reached a mean threshold of ≥200epg, while the FECRT and larval cultures were conducted approximately 5 to 8 weeks later in the season, after the animals had been treated with ivermectin and diagnosed with drench failure. Therefore, the parasite populations, both in terms of species and number, and
possibly the resistance level, may have changed over time. However, on most farms, the parasite populations were similar (Table 5.9), with *Haemonchus* sp. being the predominant species at both times. Exceptions included farms 12, 23 and 24, where the percentage of *Haemonchus* sp. present on the farm changed over time. Interestingly, both farms 12 and 23 had a lower percentage of *Haemonchus* sp. present at the time of the LDA, and were classified as having “no” or “low resistance” to thiabendazole by the LDA, whereas in the larval culture post-FECRT, the proportion of *Haemonchus* sp. was considerably higher, and the FECR percentages for fenbendazole reduction were indicative of high levels of resistance. A field study conducted by Falzon *et al.* (in press) indicated that most of the ivermectin and fenbendazole resistance detected on the same farms was associated with *Haemonchus* sp. Therefore, an increase in the *Haemonchus* sp. population, relative to other species, as has been reported in Ontario during the grazing season (Mederos *et al.*, 2010), might be associated with more detectable levels of AR on farms when resistant strains of *Haemonchus* sp. are present. Farm 24, in contrast, was classified as having low levels of resistance to benzimidazoles by both tests, despite having a similar observed increase in *Haemonchus* sp. between the two sampling points. This might have occurred because the *Haemonchus* sp. present on this farm were more susceptible, compared to other farms, and therefore did not influence the farm’s resistance status.

The McNemar-Bowker’s test of marginal homogeneity for almost all FECR calculation methods, at the different threshold percentages, was not statistically significant, indicating that the false-positive and false-negative results were equally distributed for both the FECRT and LDA. This result was not expected since the FECRT
is described as a less sensitive test for detecting AR, compared to the LDA (Martin et al., 1989), and should therefore have more false-negative results compared to the LDA. However, this result might be explained by the fact that the FECRT was conducted at a later time in the grazing season, when apparent resistance levels may have increased as a result of an overall increase in the proportion of Haemonchus sp. due to the warmer weather conditions (Mederos et al., 2010). The McNemar-Bowker test was only statistically significant when FECR\textsubscript{1} and FECR\textsubscript{2}, and the 50\% threshold, were used; in these cases, more farms were classified as having high resistance with the LDA, compared to the FECRT, indicating that the LDA was a more sensitive test when lower threshold percentages for FECR were used.

Although the LDA and FECRT showed a poor to moderate agreement, the use of both tests within a monitoring program could still be useful, as the tests measure different parasite attributes and, as such, can be used at different stages of the monitoring program (Roush and Tabashnik, 1990). The LDA is a more sensitive test as it can detect resistance when 10\% of the parasite population are phenotypically resistant, compared to 25\% of the parasite population for the FECRT (Papadopoulos, 2008). LDAs also provide results more quickly and at less cost (Howell et al., 2008), and can therefore be used to provide early warning signs of impending resistance problems on sheep farms. In contrast, the FECRT reflects the phenotypic expression of resistance (Sangster and Gill, 1999), and can therefore be carried out at a later stage in a parasite control program, to monitor changes in the severity of the resistance situation.
5.4.4 Overall study strengths and limitations, and future research

This study had several strengths: FECRT and matching LDA data obtained during the same grazing season were available for 13 and 11 farms for fenbendazole and levamisole, respectively, allowing us to make direct comparisons between the two tests. Moreover, we explored the use of several different FECR formulae, using both arithmetic and geometric means, to analyse real farm data. We also explored the use of different threshold percentages to distinguish between low and high levels of resistance with the FECRT, and found 80% and 70% to be potentially suitable thresholds. However, research is needed to investigate at which level resistance has an economic impact on sheep productivity and profitability.

One of the limitations in our study is the accuracy of the diagnostic test used for detection of FECs. The McMaster technique used in this study has a minimum detection limit of 50 epg; this limited sensitivity may fail to detect low FECs, introducing a misclassification bias and making the FECR calculation less reliable (El-Abdellati et al., 2010). A way to avert this problem is to use more sensitive tests, such as FECPAK (detection limit = 10 epg) or the FLOTAC technique (detection limit = 1-2 epg) (El-Abdellati et al., 2010). The accuracy of the diagnostic test could also be improved by increasing the test precision, either taking measurements in triplicate (Kaplan and Vidyashankar, 2012), or using alternative analytical methods. Denwood et al. (2010) recommended the use of Monte Carlo Markov Chain simulations to calculate the FECR, as these provide confidence intervals with better defined properties and more precise estimates for the true FECR. However, all these techniques are either more labour intensive, or require additional training or apparatus, which might hinder their
widespread uptake and application. Research is therefore required to determine an appropriate trade-off between improved sensitivity of diagnostic and analytical methods, and the willingness and ability of veterinarians and producers to use these tests.

5.5 Conclusion

The different FECR methods evaluated in this study did not provide consistent FECR percentages following treatment with ivermectin, fenbendazole or levamisole. The correlation between the methods was influenced by which means were used in the FECR formulae, especially when low levels of resistance were present, resulting in a right-skewness of the parasite data. This suggests that arithmetic means should be used as they do not require any correction factors and are less prone to bias, especially when data is right-skewed. In contrast, whether both pre- and post-treatment or only post-treatment sample data were used in the FECR formulae was less influential. Therefore, the simpler formula could be used, reducing the cost and labour associated with the FECRT. While both the FECRT and LDA were able to diagnose AR, the agreement, overall, for benzimidazole resistance between the two tests was slight to moderate. However, the agreement improved when 80% or 70% were used as threshold percentages in the FECRT for defining resistance, indicating that these percentages might be useful cut-offs to differentiate between high and low levels of resistance. While there was no agreement between the LDA and FECRT for levamisole resistance results, these findings need to be interpreted cautiously given the low levels of levamisole resistance present on the farms investigated. Moreover, since the LDA and FECRT measure different parasite properties, the applicability of these tests depends on both the primary objective of the monitoring program, and the expected levels of resistance present on the farm. In conclusion,
different methods of calculating FECR and use of different diagnostic tests may lead to
different classifications of a farm’s resistance status. Thus, there is a need for consensus
on the method(s) used to define anthelmintic resistance, to provide more uniform results
to producers and to allow for standardised comparisons between different studies.

5.6 Acknowledgements

This research was supported by the Ontario Ministry of Agriculture, Food and
Rural Affairs - University of Guelph agreement through the Animal Health Strategic
Investment fund, with additional in-kind assistance from Merial. The authors are very
grateful to William Sears for statistical advice. We especially acknowledge the sheep
producers that participated in the study.
5.7 References


Table 5.1. Fecal egg count reduction (FECR) percentages (and 95% confidence intervals) following ivermectin treatment on 29 sheep farms in Ontario, Canada (2010 and 2011), calculated using four different FECR formulae.

<table>
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<tr>
<th>Farm</th>
<th>FECR₁</th>
<th>AR status</th>
<th>FECR₂</th>
<th>AR status</th>
<th>FECR₃</th>
<th>AR status</th>
<th>FECR₄</th>
<th>AR status</th>
</tr>
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<td>-24.5 (-49.9 – -3.4)</td>
<td>R</td>
<td>76.4 (-12.7 – 95.1)</td>
<td>R</td>
<td>62.7 (62.5 – 62.9)</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td>22.4 (16.8 – 27.6)</td>
<td>R</td>
<td>77.1 (73.9 – 79.8)</td>
<td>R</td>
<td>28.1 (-97.6 – 73.8)</td>
<td>R</td>
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<td>SR</td>
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<tr>
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<td>66.0 (46.6 – 78.4)</td>
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<td>R</td>
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<td>-1034.2 (-1041.1 – -1027.4)</td>
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<td>93.4 (60.1 – 98.9)</td>
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<td>S</td>
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<td></td>
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<td>Resistance</td>
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<td>FEC (lower – upper)</td>
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<td>4.3 (-150.6 – 63.5)</td>
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<td>28</td>
<td>21.1 (15.0 – 26.8)</td>
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<tr>
<td>29</td>
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<td>R</td>
<td>55.3 (-0.2 – 80.1)</td>
<td>R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: FECR₁ and FECR₂ used pre- and post-treatment Fecal Egg Counts (FECs) from both treated and untreated animals, but FECR₁ used arithmetic means while FECR₂ used geometric means. FECR₃ was calculated using arithmetic means for post-treatment FECs from treated and untreated animals, while FECR₄ was calculated using FEC estimates from a General Linear Mixed Model.

n/a = it was not possible to compute the predicted fecal egg counts for this farm since weight information for the control animals was not collected.

AR = anthelmintic resistance; R = resistance; SR = suspected of resistance; S = susceptible.

Minus sign (“-”) in front of a number indicates that the FEC rose after treatment.
Table 5.2. Fecal egg count reduction (FECR) percentages (and 95% confidence intervals), following fenbendazole treatment on 20 sheep farms in Ontario, Canada (2010 and 2011), calculated using four different FECR formulae.*

<table>
<thead>
<tr>
<th>Farm</th>
<th>FECR\textsubscript{1}</th>
<th>AR status</th>
<th>FECR\textsubscript{2}</th>
<th>AR status</th>
<th>FECR\textsubscript{3}</th>
<th>AR status</th>
<th>FECR\textsubscript{4}</th>
<th>AR status</th>
</tr>
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<tbody>
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<td>1</td>
<td>-8.1 (-39.1 - 16.1)</td>
<td>R</td>
<td>-309.3 (-557.1 - -155.0)</td>
<td>R</td>
<td>97.3 (71.7 - 99.7)</td>
<td>SR</td>
<td>93.4 (93.3 - 93.5)</td>
<td>SR</td>
</tr>
<tr>
<td>3</td>
<td>39.8 (11.5 - 59.1)</td>
<td>R</td>
<td>23.9 (-23.0 - 52.9)</td>
<td>R</td>
<td>45.6 (-39.2 - 78.7)</td>
<td>R</td>
<td>-3.7 (-4.2 - -3.2)</td>
<td>R</td>
</tr>
<tr>
<td>4</td>
<td>84.0 (81.6 - 86.1)</td>
<td>R</td>
<td>85.9 (82.8 - 88.4)</td>
<td>R</td>
<td>78.5 (58.5 - 88.8)</td>
<td>R</td>
<td>82.0 (81.9 - 82.1)</td>
<td>R</td>
</tr>
<tr>
<td>8</td>
<td>27.3 (23.8 - 30.5)</td>
<td>R</td>
<td>68.2 (66.0 - 70.2)</td>
<td>R</td>
<td>0.1 (-115.6 - 53.8)</td>
<td>R</td>
<td>49.5 (49.4 - 49.6)</td>
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<td>-53.6 (-70.6 - -38.3)</td>
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<td>33.9 (-24.5 - 65.0)</td>
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<td>40.6 (8.6 - 61.4)</td>
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<td>-92.1 (-107.9 - -77.5)</td>
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<td>41.7 (41.6 - 41.8)</td>
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<td>25.4 (25.3 - 25.5)</td>
<td>R</td>
</tr>
<tr>
<td>13</td>
<td>84.0 (80.6 - 86.8)</td>
<td>R</td>
<td>92.0 (89.6 - 93.8)</td>
<td>R</td>
<td>92.5 (75.2 - 97.7)</td>
<td>R</td>
<td>91.0 (90.9 - 91.1)</td>
<td>SR</td>
</tr>
<tr>
<td>16</td>
<td>72.2 (64.3 - 78.3)</td>
<td>R</td>
<td>60.1 (45.5 - 70.8)</td>
<td>R</td>
<td>90.8 (79.4 - 95.9)</td>
<td>R</td>
<td>86.1 (86.0 - 86.2)</td>
<td>R</td>
</tr>
<tr>
<td>18</td>
<td>79.4 (77.9 - 80.8)</td>
<td>R</td>
<td>68.0 (64.0 - 71.6)</td>
<td>R</td>
<td>78.0 (49.7 - 90.4)</td>
<td>R</td>
<td>75.1 (75.0 - 75.2)</td>
<td>R</td>
</tr>
<tr>
<td>20</td>
<td>74.3 (72.1 - 76.4)</td>
<td>R</td>
<td>53.2 (46.1 - 59.3)</td>
<td>R</td>
<td>70.9 (-5.5 - 92.0)</td>
<td>R</td>
<td>61.0 (60.9 - 61.1)</td>
<td>R</td>
</tr>
<tr>
<td>21</td>
<td>18.9 (6.4 - 29.7)</td>
<td>R</td>
<td>48.6 (25.0 - 64.7)</td>
<td>R</td>
<td>55.3 (-137.9 - 91.6)</td>
<td>R</td>
<td>36/6 (35.7 - 37.5)</td>
<td>R</td>
</tr>
<tr>
<td>23</td>
<td>88.0 (87.0 - 89.0)</td>
<td>R</td>
<td>89.0 (87.4 - 90.4)</td>
<td>R</td>
<td>79.4 (52.6 - 91.1)</td>
<td>R</td>
<td>83.8 (83.7 - 83.9)</td>
<td>R</td>
</tr>
<tr>
<td>24</td>
<td>89.6 (84.6 - 92.9)</td>
<td>R</td>
<td>67.8 (47.7 - 80.2)</td>
<td>R</td>
<td>87.1 (12.7 - 98.1)</td>
<td>R</td>
<td>75.5 (75.1 - 75.8)</td>
<td>R</td>
</tr>
<tr>
<td>25</td>
<td>0.4 (-37.2 - 27.7)</td>
<td>R</td>
<td>30.0 (-17.4 - 58.2)</td>
<td>R</td>
<td>-366.4 (-2977.7 - 29.8)</td>
<td>R</td>
<td>-56.6 (-58.9 - -54.4)</td>
<td>R</td>
</tr>
<tr>
<td>26</td>
<td>4.3 (-33.4 - 31.4)</td>
<td>R</td>
<td>-61.6 (-159.6 - -0.7)</td>
<td>R</td>
<td>-20.9 (-357.1 - 68.0)</td>
<td>R</td>
<td>-1.8 (-3.1 - 40.1)</td>
<td>R</td>
</tr>
<tr>
<td>27</td>
<td>36.2 (32.7 - 39.5)</td>
<td>R</td>
<td>47.8 (44.2 - 51.2)</td>
<td>R</td>
<td>27.6 (-88.5 - 72.2)</td>
<td>R</td>
<td>40.0 (39.9 - 40.1)</td>
<td>R</td>
</tr>
<tr>
<td>28</td>
<td>66.7 (63.9 - 69.3)</td>
<td>R</td>
<td>71.2 (68.4 - 73.7)</td>
<td>R</td>
<td>60.3 (33.5 - 76.3)</td>
<td>R</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>-3.0 (-9.9 - 3.4)</td>
<td>R</td>
<td>-53.9 (-71.1 - -38.5)</td>
<td>R</td>
<td>23.5 (-64.0 - 64.3)</td>
<td>R</td>
<td>59.3 (59.2 - 59.4)</td>
<td>R</td>
</tr>
</tbody>
</table>

*See Table 5.1 for description of different FECR methods.

n/a = it was not possible to compute the predicted fecal egg counts for this farm since weight information for the control animals was not collected.

AR = anthelmintic resistance; R = resistance; SR = suspected of resistance; S = susceptible.

Minus sign ("-".) in front of a number indicates that the FEC rose after treatment.
Table 5.3. Fecal egg count reduction (FECR) percentages (and 95% confidence intervals), following levamisole treatment on 17 sheep farms in Ontario, Canada (2010 and 2011), calculated using four different FECR formulae.*

<table>
<thead>
<tr>
<th>Farm</th>
<th>FECCR₁</th>
<th>FECCR₂</th>
<th>FECCR₃</th>
<th>FECCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FECR%</td>
<td>AR</td>
<td>FECR%</td>
<td>AR</td>
</tr>
<tr>
<td>1</td>
<td>100.0</td>
<td>S</td>
<td>-21.0 (-90.04 – 22.98)</td>
<td>R</td>
</tr>
<tr>
<td>4</td>
<td>100.0</td>
<td>S</td>
<td>98.2 (97.23 – 98.78)</td>
<td>S</td>
</tr>
<tr>
<td>8</td>
<td>100.0</td>
<td>S</td>
<td>99.6 (99.37 – 99.71)</td>
<td>S</td>
</tr>
<tr>
<td>9</td>
<td>99.7 (99.20 – 99.90)</td>
<td>S</td>
<td>94.7 (92.10 – 96.45)</td>
<td>SR</td>
</tr>
<tr>
<td>10</td>
<td>97.0 (95.23 – 98.10)</td>
<td>S</td>
<td>80.2 (67.95 – 87.80)</td>
<td>R</td>
</tr>
<tr>
<td>11</td>
<td>99.9 (99.80 – 99.97)</td>
<td>S</td>
<td>99.2 (98.82 – 99.46)</td>
<td>S</td>
</tr>
<tr>
<td>12</td>
<td>100.0</td>
<td>S</td>
<td>99.1 (98.72 – 99.43)</td>
<td>S</td>
</tr>
<tr>
<td>13</td>
<td>97.9 (97.02 – 98.50)</td>
<td>S</td>
<td>94.0 (91.62 – 95.77)</td>
<td>SR</td>
</tr>
<tr>
<td>16</td>
<td>100.0</td>
<td>S</td>
<td>94.0 (90.70 – 96.10)</td>
<td>SR</td>
</tr>
<tr>
<td>18</td>
<td>99.9 (99.93 – 99.98)</td>
<td>S</td>
<td>99.7 (99.62 – 99.82)</td>
<td>S</td>
</tr>
<tr>
<td>20</td>
<td>100.0</td>
<td>S</td>
<td>98.2 (97.32 – 98.81)</td>
<td>S</td>
</tr>
<tr>
<td>23</td>
<td>99.9 (99.86 – 99.97)</td>
<td>S</td>
<td>99.2 (99.85 – 99.46)</td>
<td>S</td>
</tr>
<tr>
<td>25</td>
<td>100.0</td>
<td>S</td>
<td>9.4 (-72.08 – 52.26)</td>
<td>R</td>
</tr>
<tr>
<td>26</td>
<td>100.0</td>
<td>S</td>
<td>66.9 (43.29 – 80.73)</td>
<td>R</td>
</tr>
<tr>
<td>27</td>
<td>98.2 (97.93 – 98.45)</td>
<td>S</td>
<td>99.3 (99.08 – 99.49)</td>
<td>S</td>
</tr>
<tr>
<td>28</td>
<td>92.3 (91.03 – 93.36)</td>
<td>SR</td>
<td>98.2 (97.49 – 98.70)</td>
<td>S</td>
</tr>
<tr>
<td>29</td>
<td>100.0</td>
<td>S</td>
<td>98.0 (97.04 – 98.67)</td>
<td>S</td>
</tr>
</tbody>
</table>

*See Table 5.1 for description of different FECR methods.

n/a = it was not possible to compute the predicted fecal egg counts for this farm since weight information for the control animals was not collected.

AR = anthelmintic resistance; R = resistance; SR = suspected of resistance; S = susceptible.

Minus sign (“-”) in front of a number indicates that the FEC rose after treatment.
Table 5.4. General linear mixed model for the natural-logarithm of the post-treatment fecal egg counts (eggs per gram) for 29 sheep farms in south-western Ontario, Canada (2010 and 2011).

<table>
<thead>
<tr>
<th>Fixed Effects</th>
<th>Estimate</th>
<th>95% C.I.</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>8.069</td>
<td>(5.989–10.148)</td>
<td>7.61</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lbefore</td>
<td>0.298</td>
<td>(0.113–0.482)</td>
<td>7.39</td>
<td>0.007</td>
</tr>
<tr>
<td>Weight</td>
<td>-0.165</td>
<td>(-0.232– -0.097)</td>
<td>17.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight*Weight</td>
<td>0.001</td>
<td>(0.0004–0.0015)</td>
<td>11.32</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ivermectin</td>
<td>-0.244</td>
<td>(-1.887–1.399)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>2.750</td>
<td>(0.956–4.544)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>-0.148</td>
<td>(-1.672–1.377)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm</td>
<td>†</td>
<td>†</td>
<td>4.90</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lbefore*weight</td>
<td>0.005</td>
<td>(0.001–0.010)</td>
<td>5.60</td>
<td>0.002</td>
</tr>
<tr>
<td>Lbefore*treatment</td>
<td>‡</td>
<td>‡</td>
<td>58.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lbefore*farm</td>
<td>‡</td>
<td>‡</td>
<td>4.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight*treatment</td>
<td>‡</td>
<td>‡</td>
<td>8.50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight*farm</td>
<td>‡</td>
<td>‡</td>
<td>1.79</td>
<td>0.007</td>
</tr>
<tr>
<td>Farm*treatment</td>
<td>‡</td>
<td>‡</td>
<td>4.23</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effects</th>
<th>Estimate</th>
<th>95% C.I. (^1)</th>
<th>Z-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment - control</td>
<td>1.459</td>
<td>(1.246–1.733)</td>
<td>11.90</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Treatment - ivermectin</td>
<td>1.444</td>
<td>(1.240–1.705)</td>
<td>12.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment - fenbendazole</td>
<td>1.453</td>
<td>(1.222–1.757)</td>
<td>10.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment - levamisole</td>
<td>0.187</td>
<td>(0.151–0.238)</td>
<td>8.59</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

C.I. = Confidence Interval
Lbefore = natural-logarithm of the pre-treatment fecal egg counts (eggs per gram)
\(^1\)These coefficients are not reported in the table since a separate coefficient was provided for each of the 29 farms included in the model.
\(^\ddagger\)When interpreting these variables, there is not just one coefficient to consider because these variables are involved in interactions and are categorical. The total effect for each variable is the combination of the relevant coefficients for the main effects and the interacting categories.
Table 5.5. The concordance correlation coefficients (and 95% confidence intervals) and level of agreement, between the different methods for calculating fecal egg count reduction (FECR) percentages following (a) ivermectin, (b) fenbendazole and (c) levamisole treatment for 26, 18 and 16 sheep farms, respectively, in Ontario, Canada (2010 and 2011).

a) Ivermectin

<table>
<thead>
<tr>
<th></th>
<th>FECR₁</th>
<th>FECR₂</th>
<th>FECR₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>FECR₂</td>
<td>0.33 (0.07 – 0.64)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Fair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FECR₃</td>
<td>0.65 (0.36 – 0.82)</td>
<td>0.63 (0.33 – 0.82)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Substantial</td>
<td>Substantial</td>
<td></td>
</tr>
<tr>
<td>FECR₄</td>
<td>0.10 (-0.31 – 0.48)</td>
<td>0.81 (0.61 – 0.91)</td>
<td>0.51 (0.13 – 0.77)</td>
</tr>
<tr>
<td></td>
<td>Slight</td>
<td>Almost perfect</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

b) Fenbendazole

<table>
<thead>
<tr>
<th></th>
<th>FECR₁</th>
<th>FECR₂</th>
<th>FECR₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>FECR₂</td>
<td>0.67 (0.38 – 0.84)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Substantial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FECR₃</td>
<td>0.35 (0.08 – 0.56)</td>
<td>0.22 (-0.21 – 0.58)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Fair</td>
<td>Fair</td>
<td></td>
</tr>
<tr>
<td>FECR₄</td>
<td>0.60 (0.12 – 0.85)</td>
<td>0.37 (-0.13 – 0.72)</td>
<td>0.50 (0.31 – 0.66)</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>Fair</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

c) Levamisole

<table>
<thead>
<tr>
<th></th>
<th>FECR₁</th>
<th>FECR₂</th>
<th>FECR₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>FECR₂</td>
<td>-0.02 (-0.10 – 0.07)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FECR₃</td>
<td>0.96 (0.89 – 0.99)</td>
<td>-0.01 (-0.10 – 0.07)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Almost perfect</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td>FECR₄</td>
<td>0.01 (-0.03 – 0.06)</td>
<td>0.93 (0.79 – 0.98)</td>
<td>0.02 (-0.05 – 0.08)</td>
</tr>
<tr>
<td></td>
<td>Slight</td>
<td>Almost perfect</td>
<td>Slight</td>
</tr>
</tbody>
</table>
Table 5.6. Number of farms that were classified as having high or low resistance to fenbendazole using five different threshold percentages (90%, 80%, 70%, 60% and 50%) to differentiate the fecal egg count reduction as indicative of low or high levels of resistance. Farms were classified as having no resistance when the fecal egg count reduction was ≥95%.

<table>
<thead>
<tr>
<th>FECR Method</th>
<th>90%</th>
<th>80%</th>
<th>70%</th>
<th>60%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>FECR1 (n=13)</td>
<td>13</td>
<td>0</td>
<td>10</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>FECR2 (n=13)</td>
<td>12</td>
<td>1</td>
<td>11</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>FECR3 (n=13)</td>
<td>11</td>
<td>2</td>
<td>10</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>FECR4 (n=12)</td>
<td>11</td>
<td>1</td>
<td>9</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

Threshold Percentages for the FECR to be Considered Highly Resistant

Note: None of the farms were classified as having no resistance (i.e. reduction value ≥95%).
FECR = Fecal Egg Count Reduction
Table 5.7. Number of farms that were classified as having high, low or no resistance to levamisole using five different threshold percentages (90%, 80%, 70%, 60% and 50%) to differentiate the fecal egg count reduction as indicative of low or high levels of resistance. Farms were classified as having no resistance when the fecal egg count reduction was ≥95%.

<table>
<thead>
<tr>
<th>FECR Method</th>
<th>90%</th>
<th>80%</th>
<th>70%</th>
<th>60%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>No</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>FECR₁ (n=11)</td>
<td>0 1 10</td>
<td>0 1 10</td>
<td>0 1 10</td>
<td>0 1 10</td>
<td>0 1 10</td>
</tr>
<tr>
<td>FECR₂ (n=11)</td>
<td>2 2 7</td>
<td>2 2 7</td>
<td>2 2 7</td>
<td>1 3 7</td>
<td>1 3 7</td>
</tr>
<tr>
<td>FECR₃ (n=11)</td>
<td>0 1 10</td>
<td>0 1 10</td>
<td>0 1 10</td>
<td>0 1 10</td>
<td>0 1 10</td>
</tr>
<tr>
<td>FECR₄ (n=10)</td>
<td>2 0 8</td>
<td>2 0 8</td>
<td>2 0 8</td>
<td>1 1 8</td>
<td>1 1 8</td>
</tr>
</tbody>
</table>

FECR = Fecal Egg Count Reduction
Table 5.8. Weighted Kappa values for the agreement, beyond that due to chance, between the farm resistance statuses based on the larval development assay and the fecal egg count reduction test for benzimidazoles using four different FECR calculations, for 13 sheep farms in Ontario, Canada (2011).

<table>
<thead>
<tr>
<th>Threshold value</th>
<th>Method of FECR calculation</th>
<th>Weighted Kappa</th>
<th>95% C.I.</th>
<th>Exact p-value</th>
<th>X²-test</th>
<th>Approximate p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>90%</td>
<td>FECR₁</td>
<td>0.32</td>
<td>(-0.17 – 0.82)</td>
<td>0.12</td>
<td>0.12</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>FECR₂</td>
<td>-0.15</td>
<td>(-0.31 – 0.01)</td>
<td>0.80</td>
<td>1.33</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>FECR₃</td>
<td>-0.18</td>
<td>(-0.39 – 0.02)</td>
<td>0.85</td>
<td>1.00</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>FECR₄</td>
<td>-0.11</td>
<td>(-0.29 – 0.08)</td>
<td>0.81</td>
<td>1.33</td>
<td>0.51</td>
</tr>
<tr>
<td>80%</td>
<td>FECR₁</td>
<td>0.58</td>
<td>(0.23 – 0.92)</td>
<td>0.02</td>
<td>1.00</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>FECR₂</td>
<td>0.41</td>
<td>(-0.07 – 0.89)</td>
<td>0.06</td>
<td>1.33</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>FECR₃</td>
<td>0.02</td>
<td>(-0.41 – 0.46)</td>
<td>0.56</td>
<td>1.33</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>FECR₄</td>
<td>0.29</td>
<td>(-0.25 – 0.83)</td>
<td>0.20</td>
<td>1.00</td>
<td>0.61</td>
</tr>
<tr>
<td>70%</td>
<td>FECR₁</td>
<td>0.28</td>
<td>(-0.15 – 0.70)</td>
<td>0.20</td>
<td>2.80</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>FECR₂</td>
<td>0.48</td>
<td>(0.09 – 0.87)</td>
<td>0.04</td>
<td>1.33</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>FECR₃</td>
<td>0.28</td>
<td>(-0.15 – 0.70)</td>
<td>0.20</td>
<td>2.80</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>FECR₄</td>
<td>0.35</td>
<td>(-0.09 – 0.78)</td>
<td>0.35</td>
<td>2.00</td>
<td>0.37</td>
</tr>
<tr>
<td>60%</td>
<td>FECR₁</td>
<td>0.19</td>
<td>(-0.21 – 0.60)</td>
<td>0.29</td>
<td>3.67</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>FECR₂</td>
<td>0.28</td>
<td>(-0.15 – 0.70)</td>
<td>0.20</td>
<td>2.80</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>FECR₃</td>
<td>0.19</td>
<td>(-0.21 – 0.60)</td>
<td>0.29</td>
<td>3.67</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>FECR₄</td>
<td>0.25</td>
<td>(-0.18 – 0.68)</td>
<td>0.24</td>
<td>2.80</td>
<td>0.25</td>
</tr>
<tr>
<td>50%</td>
<td>FECR₁</td>
<td>0.34</td>
<td>(0.03 – 0.65)</td>
<td>0.11</td>
<td>6.00</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>FECR₂</td>
<td>0.34</td>
<td>(0.03 – 0.65)</td>
<td>0.11</td>
<td>6.00</td>
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<td></td>
<td>FECR₃</td>
<td>0.12</td>
<td>(-0.26 – 0.50)</td>
<td>0.40</td>
<td>4.57</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>FECR₄</td>
<td>0.16</td>
<td>(-0.25 – 0.57)</td>
<td>0.35</td>
<td>3.67</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Different cut-off values (90%, 80%, 70%, 60% and 50%) were used to distinguish the FECR percentages obtained with the fecal egg count reduction test as indicative of low or high levels of resistance. Farms were classified as not resistant if the fecal egg count reduction was ≥ 95%.

FECR = Fecal Egg Count Reduction
C.I. = 2-tailed confidence intervals
Exact p-value = one-tailed p-value
*denotes statistically significant Kappa values (i.e. p≤0.05)
Table 5.9. Percentages (%) of *Haemonchus* sp., *Teladorsagia* sp., and *Trichostrongylus* spp. isolated from the control wells of the larval development assay, and from larval cultures of pooled fecal samples collected from the control (i.e. untreated) animals on the second visit for the fecal egg count reduction test, for 13 sheep farms in Ontario, Canada (2011).

<table>
<thead>
<tr>
<th>Farm</th>
<th>Larval Development Assay</th>
<th></th>
<th></th>
<th>Larval Culture</th>
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<tr>
<td></td>
<td>% Haemomonchus</td>
<td>% Teladorsagia</td>
<td>% Trichostrongylus</td>
<td>% Haemomonchus</td>
<td>% Teladorsagia</td>
<td>% Trichostrongylus</td>
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<tr>
<td>12</td>
<td>1</td>
<td>80</td>
<td>19</td>
<td>87</td>
<td>5</td>
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</tr>
<tr>
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<td>97†</td>
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<td>100</td>
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<td>95†</td>
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<td>29</td>
<td>96</td>
<td>2</td>
<td>2</td>
<td>92</td>
<td>6</td>
<td>2</td>
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</tbody>
</table>

†The total percentage does not add up to 100% because other gastrointestinal nematode species, such as *Oesophagostomum/Chabertia* sp., were present in small numbers.
CHAPTER 6

A survey of farm management practices and their associations with anthelmintic resistance in sheep flocks in Ontario, Canada

Prepared for submission to Small Ruminant Research

Abstract

Anthelmintic resistance (AR) has been reported on sheep farms worldwide. To better understand AR on Ontario sheep farms, this study was conducted to describe management practices, and their associations with AR, on sheep farms in Ontario, Canada. A questionnaire pertaining to farm practices considered risk factors for AR was administered on 38 farms that participated in a study to determine the frequency of ivermectin drench failure and resistance to ivermectin, fenbendazole and levamisole in Ontario sheep flocks. Most of the producers surveyed had used ivermectin and fenbendazole drenches (36/38 [95%] and 26/38 [68%), respectively), while only 4/38 (11%) had used levamisole drench, in the previous 5 years. Producers treated their animals a mean of 2.6 times per year. Routine treatment (defined as treatment of the whole flock at fixed times during the year) was practiced by 31/38 (82%) of the producers; most ewes were treated routinely either at lambing (17/31 [55%]) and/or at the beginning of winter housing (15/31 [48%]). The majority of the producers (31/38; 82%) also used targeted or targeted selective treatment (defined as treatment of the whole flock or individual animals, respectively, when gastro-intestinal nematode parasitism was suspected); however, it was often in addition to, rather than in lieu of, routine treatment. Twenty-five producers (66%) brought in new animals (including sheep, goats, llamas and/or alpacas, but not cattle or other livestock) in the previous year; of these 25 farms, 8
(32%) kept the animals off pasture for an average of 46 days, and treated the animals with an anthelmintic while in quarantine, 6 (24%) kept the animals off pasture for an average of 30 days, 4 (16%) treated the animals with an anthelmintic upon arrival but put them on pasture immediately, and 7 (28%) did not have any quarantine strategies. Many producers (17/38; 45%) did not calibrate the drench gun before use. Although univariable analyses identified several marginally significant risk factors (0.10<p>0.05), no predictor variables were significant in the final model for ivermectin resistance. The prior use of benzimidazoles was associated (p=0.01) with increased resistance (lower fecal egg count reduction percentages) to fenbendazole. Levamisole resistance could not be modeled due to the very low levels of resistance on the farms surveyed. This study has provided a picture of management practices currently employed by Ontario sheep producers on their farms, while allowing us to formulate hypotheses as to how these practices may be associated with AR.

6.1 Introduction

Gastrointestinal nematodes (GIN) cause significant disease in grazing sheep worldwide, and effective programs for GIN control are necessary to maintain sheep health, productivity and profitability (Scott, 2007). For many years, anthelmintic drugs have represented the cornerstone of GIN control, since they are relatively inexpensive and easy to use (Kenyon and Jackson, 2012; Taylor, 2012). However, reports of anthelmintic resistance (AR) have become increasingly more common over the past 20 years (Knox et al., 2012), and AR now represents the status quo in numerous sheep-rearing countries (Kaplan and Vidyashankar, 2012). A recent study in Canadian sheep flocks demonstrated that ivermectin drench failure was a common occurrence, and that
resistance to both ivermectin and fenbendazole was present on the majority of these farms (Falzon et al., in press).

To slow the development of AR in flocks, it is necessary to improve our understanding of the epidemiology of resistance, and to determine which management practices may be associated with the emergence and/or presence of AR (Coles, 2001). Several management practices, such as increased frequency of anthelmintic treatment (Coles, 2010; Calvete et al., 2012), and inadequate quarantine strategies for new animal introductions (Hughes et al., 2007; Sargison, 2011), have been described as risk factors for AR. However, the association of AR with these practices is based on complex theoretical principles (Sargison, 2011) or simulation studies (Leathwick et al., 1995). So far, few observational studies on risk factors associated with AR have been conducted on commercial sheep farms (Suter et al., 2004; Lawrence et al., 2006; Hughes et al., 2007; Calvete et al., 2012), and there is a lack of empirical evidence regarding which management practices should be recommended to sheep producers to lower the risk of development of AR on their farms. Moreover, a recent survey on sheep farms in the United Kingdom (Morgan and Coles, 2010) showed that, despite the widespread dissemination in 2005/2006 of theoretically plausible practical guidelines on how to counter AR (Abbott et al., 2009), very few changes in management practices ensued in the following two years. This reluctance to change could be because producers often do not perceive the economic impact of resistance until it reaches overt levels (Miller et al., 2012). Therefore, it is important to improve our knowledge of the management practices that are commonly used on farms, and to understand producers’ perceptions of AR risk on their farms, so that extension programs to stakeholders can be improved (Woodgate...
and Love, 2012). In Ontario, Canada, the ewe flock has been increasing steadily over the past few years, with 158,900 ewes reported in January 2006, and 189,000 ewes reported in July 2012 (Statistics Canada, 2012). However, information on farm management practices commonly practiced on Ontario sheep flocks, and producers’ awareness of AR, is currently lacking.

The objectives of this study were: (a) to describe parasite control and farm management practices commonly used on Ontario sheep farms; and (b) to determine whether any of these practices are associated with the presence of resistance to ivermectin, fenbendazole or levamisole.

6.2 Materials and methods

6.2.1 Farm selection

A description of the farm selection, ivermectin drench check, and the Fecal Egg Count Reduction Test (FECRT) can be found in Falzon et al. (Chapter 5). In brief, 47 sheep farms were enrolled in a study over two consecutive grazing seasons in 2010 and 2011, to determine the frequency of AR in Ontario sheep flocks. Among these 47 farms, animals on 39 farms (15 lambs or yearling ewes/farm) attained mean fecal egg counts (FECs) for GINs that reached the set threshold of 200 eggs per gram (epg) of feces. As a result, an ivermectin drench check was carried out by producers on these farms. On the basis of FECs before treatment and 14 days later, “drench failure” was defined as a reduction in mean FECs of <95%. FECRTs were then conducted in flocks with ivermectin drench failure; lambs or yearling ewes were divided into 4 drench treatment groups (n=10-15 lambs or yearling ewes per group): control (i.e. untreated), ivermectin
(dosage: 0.2 mg/kg), and, if sufficient numbers of animals, fenbendazole (dosage: 5.0 mg/kg) and levamisole (dosage: 10.5 mg/kg). The percentage reduction in mean FECs following treatment with ivermectin, fenbendazole and levamisole was calculated using the method endorsed by the World Association for the Advancement of Veterinary Parasitology (Coles et al., 1992). Farms were classified as resistant when the Fecal Egg Count Reduction (FECR) was <95% and the lower 95% confidence interval limit was <90%; if only one of these two criteria was met, the farm was classified as being suspected of resistance (Coles et al., 1992).

6.2.2 Farm-level questionnaire

A questionnaire on management practices and putative risk factors for AR was administered by one of the co-authors (LCF) in a face-to-face interview with the farm manager on the farms that performed the ivermectin drench check. Questionnaires took approximately 30-40 minutes each to complete and were carried out during the grazing seasons of 2010 and 2011.

The questionnaire contained 29 questions, and was a refinement of a questionnaire previously pretested on Ontario sheep farms. The questionnaire was divided into six main sections: (i) demographics of the farm; (ii) use of anthelmintics; (iii) quarantine strategies for new animal introductions (animals of interest included sheep, goats, llamas and/or alpacas, but not cattle or other livestock); (iv) pasture management and alternative strategies for parasite control; (v) manure disposal; and (vi) perceived anthelmintic resistance. The majority of the questions were closed-ended, with a few semi-open (i.e. a close-ended question with the addition of a category “other –
please specify”) and open-ended questions. Routine treatment was defined as treatment of the whole flock at fixed times during the year and not based on fecal egg count results or evidence of clinical parasitism. Targeted treatment and targeted selective treatment were defined as treatment of the whole flock or individual animals, respectively, when GIN parasitism was suspected. A copy of the questionnaire can be obtained from the authors upon request (Appendix III).

6.2.3 Data management and statistical analyses

The questionnaire data were entered into an Excel spreadsheet (Microsoft Office Excel©, 2007) and analysed using SAS® 9.3 (SAS Institute Inc., Cary, NC, USA).

Summary descriptive statistics were performed, and predictor variables were checked for missing values and variability. Due to the limited number of observations, the levels of outcomes for some of the categorical predictor variables were collapsed to two for modeling purposes. For example, flock purpose was reduced to ‘meat’ if they kept sheep for meat purposes, or ‘other’ if they kept sheep for breeding, to train herding dogs or for dairy purposes. Likewise, responses to a question on calibration of the drench gun were reduced to ‘no’ if they replied that they never calibrated the drench gun or only calibrated it once or twice a year, versus ‘yes’ if they calibrated the drench gun before each use. Similarly, when producers were asked how they determined the weight of the animal to calculate the dose of the anthelmintic to administer, the responses were reduced to ‘estimate’ if they estimated the weight, used the expected breed average, or weighed some animals and used the average weight, or ‘weigh’ if they weighed each animal or
weighed the larger animals in the group and used the heaviest weight to calculate the
dose of anthelmintic to administer.

The FECR percentages following ivermectin (n=29), fenbendazole (n=20) or
levamisole (n=17) treatment were used for three separate anthelmintic-based outcomes
and model-building processes. For each of the three models, univariable associations
between the predictor variables and the outcome were screened using linear regression
(for continuous predictor variables) and the Mann-Whitney-Wilcoxon Rank-Sum Test
(for categorical predictor variables), which computes all unique pair-wise differences
between the two groups and estimates the median difference. Predictor variables that
were significantly associated with outcomes at a liberal alpha value of ≤0.20 were
checked for collinearity (Pearson correlation coefficient >0.8); if pairs of highly
correlated variables were identified, one of them was retained in the model based on
biological plausibility or fewer missing observations (Dohoo et al., 2009). All remaining
variables at an alpha ≤0.20 were offered to a general linear model, with all predictor
variables considered as fixed effects. The model was built using a manual forward step-
wise procedure; predictor variables that were significant at an alpha value ≤0.05 were
retained in the final model. All possible two-way interactions between predictor variables
significant in the final main effects model were tested for significance (alpha ≤0.05). The
linearity of associations between continuous predictor variables and outcomes was
assessed graphically by plotting lowess smoothed curves, and by testing a quadratic term
in the model, as described by Dohoo et al. (2009).

The model assumptions were assessed by plotting residuals against the predicted
outcomes for the explanatory variables, to look for homoscedasticity, non-linearity,
outliers and potential influential observations. Normality was visually assessed with histograms of the residuals and normal quantile plots, and assessed statistically using four different tests offered by SAS (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling). If the assumptions of linearity or homoscedasticity were not met, different data transformations were performed and the residuals were re-assessed. Observations that were identified as outliers or influential were cross-checked with the original data sheets for any peculiarity in the data to explain the results. The model was repeated without the outlier or influential observation, and differences in the coefficients and goodness-of-fit tests were noted.

6.3 Results

6.3.1 Descriptive statistics

The questionnaire was administered on 38/39 farms that performed the ivermectin drench check (97% response rate); 20 questionnaires were administered in the first year of the study (2010), while 18 questionnaires were administered in the second year of the study (2011).

6.3.1.1 Farm demographics

Of the 38 farms surveyed, 29%, 26%, 34%, and 11% had flock sizes of <50 sheep, 50-99 sheep, 100-300 sheep, and >300 sheep, respectively. The sheep grazed a mean of 51 acres (range from 3 to 310 acres). Most of the producers kept sheep for meat purposes (31/38; 82%), while 5/38 (13%) of the producers kept sheep for breeding purposes; one farm kept sheep for training border collies, and another farm kept dairy sheep. Only 8/38 (21%) farms had purebred sheep of one breed, while the remaining
30/38 (79%) farms had crosses or several different breeds. The most common breeds were Rideau-Arcott (11 farms), Dorset (9 farms), Suffolk (7 farms) and North Country Cheviot (6 farms). None of the farms included in the study were organic or working towards organic status. Twenty-five (66%) flocks lambed once a year and, of these, 19 (76%) lambed in the spring, 5 (20%) lambed in the winter, and 1 (4%) lambed in the autumn. Another 10 flocks (26%) lambed in multiple seasons, while the remaining 3 flocks (8%) lambed year-round.

Producers were asked when they took the sheep off pasture the previous grazing season, and when they put the sheep out to graze that year. Animals were taken off pasture in September (1/38 [3%]), October (7/38 [18%]), November (19/38 [50%]) and December (4/38 [11%]) the previous grazing season; 1 producer did not have sheep the previous grazing season. During the year of the study, animals were put on pasture in April (4/38 [11%]), May (20/38 [53%]), and June (8/38 [21%]). Six other producers kept their sheep on pasture all year-round, including winter months with snow cover. Ewes and lambs were put out on pasture together on most of the farms (34/38; 89%), and co-grazed for a mean of 3.8 months (range from 1 to 7 months).

6.3.1.2 Use of anthelmintics

The producers used a mean of 2.6 (range from 1 to 6) different anthelmintic formulations in the preceding 5 years. Of the 38 producers surveyed, 36 (95%) reported using ivermectin drench. Other anthelmintic formulations used included: fenbendazole drench (26/38; 68%); albendazole drench (14/38; 37%); ivermectin injectable (10/38; 26%); moxidectin pour-on (cattle product) used as a drench (5/38; 13%); levamisole
drench (4/38; 11%); ivermectin pour-on (cattle product) used as a drench (3/38; 8%); and moxidectin injectable (1/38; 3%). In the preceding 12 months, producers had treated their flock or a portion of the flock with an anthelmintic drug a mean of 2.6 times (range from 0 to 5) - this did not include the ivermectin treatment the producers administered for the ivermectin drench check as part of this study.

The majority of producers (31/38; 82%) treated their sheep routinely, i.e. at fixed times of the year and not based on FEC results or evidence of clinical parasitism. Ewes were treated routinely a mean of 2.1 times/year (range from 1 to 4), rams were treated routinely a mean of 1.3 times/year (range from 0 to 4), while lambs were treated routinely a mean of 0.9 times/year (range from 0 to 4). Of those producers that treated their sheep routinely, 17/31 (55%) treated ewes at lambing regardless of season; 15/31 (48%) treated ewes at housing during the autumn, 13/31 (34%) treated the flock before turnout on pasture – separate from a lambing-time treatment, 12/31 (32%) treated the flock at some point during the grazing season, 3/31 (8%) treated adults at breeding, 2/31 (5%) treated lambs at weaning, and 2/31 (5%) treated the adults at shearing.

Thirty-two farms (85%) used targeted and/or targeted selective treatment. Specifically, targeted treatment (i.e. treatment of the whole group when GIN infection was suspected) was performed on 9/38 (24%) of the farms, while targeted selective treatment (i.e. treatment of individual sheep when GIN infection was suspected) was performed on 11/38 (29%) of the farms; 12/38 (32%) used both targeted and targeted selective treatment. To determine which animals to treat using either targeted or targeted selective treatment, 15/32 (47%) producers relied on clinical signs (such as bottle-jaw, poor body condition score, weight loss and diarrhea), 3/32 (9%) producers took fecal
samples for analysis to determine the FECs, while 1/32 (3%) producer used the FAMACHA® score; 7/32 (22%) producers used both clinical signs and FECs, 1/32 (3%) used both FAMACHA® and clinical signs, while 5/32 (16%) used clinical signs, FECs and FAMACHA®.

Thirty producers (79%) used a drench gun to administer the anthelmintic drench, while 8/38 (21%) producers never used a drench gun, preferring to administer the drench using a syringe. Of those producers that used a drench gun, 17/30 (57%) never calibrated the drench gun, while 1/30 (3%) and 2/30 (7%) checked it once or twice a year, respectively. In contrast, 10/30 (33%) producers calibrated the drench gun before each use.

When asked how they determined an animal’s weight before calculating the dose of anthelmintic, 17/38 (45%) producers estimated the weight by ‘eye-balling’ the animal, 4/38 (11%) used the expected breed average, 6/38 (16%) weighed some of the sheep and used the average weight, 6/38 (16%) weighed the larger animals, and dosed according to the heaviest weight, and 5/38 (13%) weighed each animal and calculated the dose accordingly.

6.3.1.3 Quarantine strategies for new animal introductions

More than half of the farms (25/38; 66%) had brought in new animals (i.e. sheep, goats, llamas and/or alpacas, but not cattle or other livestock) in the previous 12 months. Of these 25 farms, 8 farms (32%) kept the animals off pasture for an average of 46 days and treated the animals with an anthelmintic while in quarantine, 6 farms (24%) kept the animals off pasture for an average of 30 days, 4 farms (16%) treated the animals with an
anthelmintic upon arrival but put them on pasture or with the rest of the flock immediately, and 7 farms (28%) did not have any quarantine strategies. On those 14 farms that kept the animals in quarantine, the animals were kept off pasture for a mean of 34 days (range from 7 to 180 days). Of those 12 farms that treated the new animals with an anthelmintic, 7 (58%) used ivermectin drench, 2 (17%) used albendazole drench, 2 (17%) used fenbendazole drench, and 1 (8%) used moxidectin cattle pour-on as drench. Most of the producers that had brought in new animals (20/25; 80%) said that the newly acquired animals were eventually turned onto pastures that had been grazed by sheep in the previous 12 months.

6.3.1.4 Pasture management and alternative (non-anthelmintic) strategies for parasite control

A few farms practiced pasture management strategies for the control of parasites. Crop rotation (i.e. the field is used for hay or cultivation of another crop one year, and for grazing purposes the following year) was practiced on 9/38 (24%) farms, while 6/38 (16%) farms rested their pasture in fallow for a year or longer. Mixed-species co-grazing was practiced on 9/38 (24%) farms; sheep were grazed with cattle (5/9 farms) and horses (4/9 farms). Rotational grazing with other species was practiced on 8/38 (21%) farms; most of these farms had cattle (7/8 farms), while the other farm had horses.

Seven producers (18%) reported using alternative methods for the control of GIN; 5/7 (71%) used diatomaceous earth, 1/7 (14%) used diatomaceous earth and bentonite clay, while 1/7 (14%) culled the more susceptible animals based on repeated high FECs.
6.3.1.5 Manure disposal

Approximately one-third of the farms (15/38; 39%) reported spreading sheep manure in the previous 12 months on pastures currently grazed by sheep. On these 15 farms, the manure was stored for a mean of 7.2 months (range from 0 to 24 months) before being spread on pasture, and the manure was spread sometime between February and November. Potential for manure run-off spreading into grazed pastures was reported on 15/38 (39%) farms, and 23/38 (61%) farms reported that sheep had access to the manure pile.

6.3.1.6 Suspicion of anthelmintic resistance

More than half of the farms (21/38; 55%) reported that they suspected AR was present on their farm prior to the study, and this had been suspected for a mean of 25 months (range from 3 to 108 months). Of these 21 farms, 16 (76%) suspected AR because there was no improvement in clinical signs after anthelmintic treatment; 5 (24%) suspected AR because FECs were still high after treatment. When asked whether they suspected resistance to a specific drug, 14/21 (67%) producers suspected resistance to ivermectin, 2/21 (10%) producers suspected resistance to benzimidazoles, 3/21 (14%) suspected resistance to both ivermectin and benzimidazoles, and 2/21 (10%) did not suspect resistance to a specific drug.
6.3.2 Analytical statistics

6.3.2.1 Ivermectin Fecal Egg Count Reduction

The median FECR following treatment with ivermectin for the FECRT was 46.6%, with a range from -157.4% to 96.5% (negative FECR values indicate that the FEC increased after treatment) (Falzon et al., in press). Ten variables were significantly associated with the FECR following ivermectin treatment at a p-value ≤0.20. However, after testing for collinearity, “suspicion of anthelmintic resistance” showed multicollinearity (Pearson correlation coefficient >0.8) with both “suspicion of anthelmintic resistance based on high fecal egg counts after treatment” and “suspicion of anthelmintic resistance based on no improvement of clinical signs after treatment”. Therefore, only the variable “suspicion of anthelmintic resistance” was retained for multivariable modeling since it was based on the most observations, an important consideration for modeling.

Table 6.1 presents the explanatory variables that had associations with FECR with p-values ≤0.20. For the categorical variables, the difference in median FECR percentages expresses the median of all differences when subtracting the FECR of the second-mentioned category from the FECR of the first-mentioned category, for each explanatory variable. A positive median of differences indicates that the median FECR% was higher in farms within the first mentioned category, and therefore there was less resistance on farms in the first-mentioned category compared to farms within the second-mentioned category. For example, with a median difference of 54%, farmers who did not use levamisole had a 54% higher FECR%, on average, than farmers who did use...
levamisole, indicating less resistance on farms not using levamisole. Conversely, a 
**negative** median of differences indicates that the **FECR% was lower** in farms within the 
first mentioned category and therefore there was **more resistance** on farms in the first 
category compared to farms in the second category. For example, with a median of 
differences of -22%, flocks that were not treated with an anthelmintic during quarantine 
had a 22% lower FECR percentage, on average, than flocks that were treated during 
quarantine, indicating more resistance among flocks that were not treated with an 
anthelmintic during quarantine. None of the explanatory variables were statistically 
significant in the final model (i.e. \( p \leq 0.05 \)).

**6.3.2.2 Fenbendazole Fecal Egg Count Reduction**

The median FECR following treatment with fenbendazole was 57.8%, with a 
range from -366.4% to 97.3% (Falzon et al., in press). Three explanatory variables had p- 
values \( \leq 0.20 \) in univariable associations with FECR following fenbendazole treatment 
(Table 6.2) and none of these variables showed multicollinearity. In model-building, only 
‘previous use of benzimidazoles’ remained significant (\( p \leq 0.05 \)) in the final model, and 
there were no significant interactions, therefore no final model is presented. The median 
difference in FECR percentage after fenbendazole treatment was 45%, indicating that 
sheep flocks that had not used benzimidazoles prior to the study had 45% higher FECR 
percentages, on average, than flocks that had used benzimidazoles, therefore indicating 
less resistance among non-benzimidazole users.
6.3.3.3 Levamisole Fecal Egg Count Reduction

The median FECR following treatment with levamisole was 100%, with a range from 93.1% to 100% (Falzon et al., in press). We have not reported the univariable associations with levamisole FECR, since statistically significant differences were only associated with very small changes in the FECR, which were of limited biological significance because FECR percentages in both groups were still above the 95% cut-off for determining AR. For instance, whether the producer treated or did not treat sheep with an anthelmintic during quarantine was statistically significant (p=0.046), however, the difference in the medians of the FECRs was only 2.52, indicating that farms that did not treat animals with an anthelmintic during quarantine had a median FECR of 99.9% vs. a median FECR of 97.4% on farms that treated animals with an anthelmintic during quarantine.

6.4 Discussion

6.4.1 Descriptive statistics

The majority of the producers enrolled in this study had used ivermectin drench and fenbendazole drench in the previous 5 years (95% and 68%, respectively). In a complementary study to determine the frequency of anthelmintic resistance in the same sheep flocks, a high frequency of ivermectin and fenbendazole resistance (97% and 95% of the farms tested, respectively) was reported (Falzon et al., in press). Since most producers in Ontario have relied heavily on these two anthelmintics over the previous five years, this is likely to have been a major driver for the development of resistance to these two drugs.
While the frequency of treatment may increase the selection pressure for resistance, other epidemiological factors, such as timing of treatment, and which animals are treated, are also important contributors to the selection pressure for AR (Sutherland and Scott, 2010). Kettle et al. (1982) described treating mature sheep as an important contributor to the selection pressure for AR, and a study by Leathwick et al. (2006) showed that treating ewes at lambing may increase the risk of development of AR. Untreated ewes may harbour large parasite populations, and are therefore considered an important source of parasites in refugia (Sutherland and Scott, 2010). Refugia has been recognized as one of the most important concepts in selection for AR (Van Wyk, 2001), and relates to preserving susceptible worms on the farm, either within untreated hosts or as free-living parasites on pasture (Kenyon et al., 2009). In our study, the overall mean frequency of anthelmintic treatment was 2.6 times per year, which is relatively low compared to other countries such as New Zealand, where producers treat at least five to six times per year (Leathwick, personal communication). In our study, however, the ewes underwent routine treatment a mean of 2.1 times per year (range from 1 to 4 times per year), and more than half of the producers treated the ewes at lambing time (17/31 [55%]). The common practice of treating adult ewes at lambing at the beginning of the grazing season may therefore be an important contributor to the development of resistance in Ontario sheep flocks, as it removes the susceptible parasites in refugia within the ewe. Therefore, any resistant worms surviving treatment have a selective advantage, leading to an increased proportion of resistant parasite eggs shed by the ewe. Moreover, a study conducted recently by Falzon et al. (unpublished data) found that, while Teladorsagia sp. and Trichostrongylus spp. may survive on pasture over the winter,
few *Haemonchus* sp. larvae can overwinter in pasture on Ontario sheep farms, resulting in a scarce number of *Haemonchus* sp. in *refugia* on pasture at the beginning of the grazing season (Chapter 3). Therefore, any resistant *Haemonchus* sp. eggs that are shed into the environment may accelerate the development of resistance in that species, as they constitute the majority of parasites on pasture. However, neither “spring lambing” nor “treatment of ewes at lambing” were significantly associated with any of the outcomes investigated in this study. This may be a result of the limited variability in the outcome (i.e. ivermectin and fenbendazole resistance), and should be investigated further.

Targeted and targeted selective treatments are practical applications of the *refugia* theory. The latter is based on the notion that, while the majority of the parasite population is found on pasture, within a sheep flock, parasites are typically over-dispersed, with the majority of parasites harboured within a small proportion of the flock (Morgan *et al.*, 2005). Therefore, treating only those animals that require treatment should reduce the selection pressure at the farm level for resistant parasites, while maintaining adequate production levels (Cabaret *et al.*, 2009). A majority of the producers interviewed in our study reported using either targeted (whole flock treatment when GIN parasitism is suspected), or targeted selective (selected individual treatment when GIN parasitism is suspected) treatment (55% and 61%, respectively). However, these targeted treatment approaches were carried out in addition to, rather than in lieu of, producers’ routine deworming practices (i.e. whole-flock treatment at fixed times regardless of whether the animals showed signs of parasitism or not). Producers were not using targeted or selective treatment to reduce the number of treatments administered, but rather as a
means to avert possible treatment failure, when signs of parasitism emerged despite routine treatment.

Quarantine strategies need to be implemented to avoid introducing resistant parasites onto a farm (Dobson et al., 2001), and have been described in several practical guidelines for producers (Abbott et al., 2009; Love, 2010; Menzies, 2012). Nevertheless, in our survey, almost a third of the producers that brought in new livestock onto their farm (7/25; 28%) did not practice any quarantine strategies. Moreover, most producers that treated animals when they arrived on the farm only used one anthelmintic, which often was the same drug in current use for the rest of the flock (ivermectin [7/12; 58%] or benzimidazoles [4/12; 34%]). Coles (2010) suggests that using only one anthelmintic may be an inappropriate quarantine treatment, as this allows any parasites resistant to that anthelmintic to survive treatment. The general recommendation is to treat animals in quarantine sequentially with different anthelmintic drug classes (Sargison, 2011), thus avoiding the introduction of resistant parasites from these animals to the rest of the flock. This is especially relevant in light of the high frequency of ivermectin and fenbendazole resistance observed on the farms in this study. These results therefore highlight the importance of educating producers on correct quarantine strategies to prevent importation of resistant parasites onto their farms.

Different grazing management strategies, such as crop rotation, resting pastures and mixed-species grazing, have been described as alternative approaches to control GINs by reducing the numbers of infective larvae on pasture (Jackson and Miller, 2006). However, while many in our study anecdotally reported the benefits of these approaches, less than half of the producers employed them on their farm. During the interview, the
producers highlighted the difficulties associated with these strategies, such as the lack of land availability and added complexities in management (data not shown). Other studies have similarly described the practicality of implementation as an obstacle to the adoption of similar management approaches (Jackson and Miller, 2006; Besier, 2012).

6.4.2 Ivermectin and Fenbendazole reduction in the Fecal Egg Count Reduction Test

No predictor variables were statistically significant in the final model for ivermectin FECR which may be a result of the small sample size used in this study, limiting statistical power to detect differences between groups, or because of the limited variability in the outcome (i.e. all farms, except one, had ivermectin resistance [i.e. FECR <95% following ivermectin treatment]). Nonetheless, several variables had a p-value ≤0.20 (Table 6.1), and should be investigated further for their potential association with FECR following ivermectin treatment.

Producers that reported previous use of benzimidazoles in their sheep flock had higher levels of fenbendazole resistance, as the median FECR following fenbendazole treatment was 45% lower in sheep flocks that had used a benzimidazole prior to the study, compared to the median FECR in flocks that had not used benzimidazoles. Exposure to a certain drug class kills the susceptible parasites while conferring a selective advantage to the resistant parasites (Sargison, 2011). If the same drug is used repeatedly, the resistant parasites will accumulate on pasture, eventually reaching the critical threshold for a reduction in drug efficacy (Kaplan and Vidyashankar, 2012). It is therefore not surprising that more fenbendazole resistance was observed on those farms that had used the drug prior to the study. However, a similar finding was not associated
with ivermectin. This may be a result of the limited variability in the predictor variable “use of ivermectin drench”, since all producers that were surveyed in the study and performed the ivermectin FECRT reported using ivermectin drench on their farm prior to the study. In contrast, 16/20 (80%) producers that conducted a FECRT for benzimidazole resistance reported use of a benzimidazole product on their farm prior to the study.

6.4.3 Study limitations and future research

Since the majority of the farms surveyed in this study had AR, we used the FECR as our outcome, to determine whether certain management practices were associated with a lower FECR percentage, indicative of lower drug efficacy. However, the FECR percentages are subject to a wide variability (Vidyashankar et al., 2012), and therefore one must be cautious when interpreting the relevance of any changes in the FECR percentages.

Although the intent was to randomly sample flocks from those willing to participate in the study, volunteer numbers were so low that we enrolled all eligible flocks which volunteered, and this might have introduced a bias towards those flocks that thought AR was a problem on their farm. While we recognize that the non-random sample population might limit the external validity of this study, a comparison of the study flocks with flocks across Ontario (Ontario Sheep Industry Survey, 2009) found a similarity in flock sizes. Moreover, the majority of sheep flocks in Ontario are kept for meat purposes, with only approximately 50 dairy flocks, and the most commonly registered sheep breed is the Rideau, followed by Dorset and Suffolk (Menzies, personal
communication); this information suggests that the demographics of our study population is representative of the Ontario sheep flock.

Further research is required to identify evidence-based recommendations for protective management practices to reduce the incidence of AR. Current recommendations for managing AR are based on evidence regarding key processes involved in the selection for resistance (Dobson et al., 2001) or individual observational studies (Suter et al., 2004; Lawrence et al., 2006; Hughes et al., 2007; Calvete et al., 2012). While individual observational studies allow for the evaluation of multiple, and often complex, risk factors (Dohoo et al., 2009), they often require additional observational studies in other populations and locations, or randomized clinical trials, in order to produce enough evidence to warrant changes in recommendations (McGovern et al., 2001). In recent years, a number of clinical trials have been performed to investigate the effect of certain putative risk factors for AR (Leathwick et al., 2006; Leathwick et al., 2008; Waghorn et al., 2008; Waghorn et al., 2009). A systematic review and meta-analysis on risk factors for AR should be carried out to identify management practices associated with AR. This type of study provides the most substantive clinical evidence (Sargeant et al., 2006) and allows for the evaluation of several risk factors and synthesis of all current research. In turn, this information would enable development of better recommendations for the control of parasites.

6.5 Conclusion

In this study, both ivermectin and fenbendazole drench were the anthelmintics used most frequently on Ontario sheep farms, consistent with previous findings of high
levels of resistance to both these drugs on these farms. Quarantine strategies were poorly implemented on many of the farms surveyed. Targeted or targeted selective treatment was often used in conjunction with routine treatment. Few producers practiced pasture management strategies as a means to control parasites on their farms. Although univariable analyses identified several marginally significant risk factors for ivermectin resistance (0.10 < p > 0.05), no variables were significant in the final model. However, the prior use of benzimidazoles was associated with increased resistance (lower FECR percentages) to fenbendazole. Levamisole resistance could not be modeled due to the very low levels of resistance on the farms surveyed.

6.6 Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.
6.7 References


Love, S., 2010. Wormkill – the Basics. Available at:

[Accessed: 2nd July, 2012]


Table 6.1. Predictor variables that had a p-value ≤0.20 in univariable associations with the outcome ivermectin fecal egg count reduction (FECR) percentage on 29 Ontario sheep flocks (May to November 2010 and May to November 2011).

<table>
<thead>
<tr>
<th>Predictor variable (Categorical)</th>
<th>Median of the differences (95% confidence intervals)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Used levamisole (no vs. yes)</td>
<td>54.0 (0.00, 93.0)</td>
<td>0.138</td>
</tr>
<tr>
<td>Flock purpose (meat vs. other)</td>
<td>29.0 (0.0, 59.0)</td>
<td>0.094</td>
</tr>
<tr>
<td>Treated animals with an anthelmintic while in quarantine (no vs. yes)</td>
<td>-22.0 (-59.0, 7.0)</td>
<td>0.173</td>
</tr>
<tr>
<td>Weight determination (weigh vs. estimate)</td>
<td>-28.0 (-59.0, 0.0)</td>
<td>0.132</td>
</tr>
<tr>
<td>Practiced spring lambing (no vs. yes)</td>
<td>-35.0 (-59.0, 0.0)</td>
<td>0.083</td>
</tr>
<tr>
<td>Suspected anthelmintic resistance on their farm (no vs. yes)</td>
<td>-26.0 (-54.0, -1.0)</td>
<td>0.143</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Predictor variable (Continuous)</th>
<th>Coefficient (95% confidence intervals)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of times rams were routinely treated with anthelmintics</td>
<td>-8.6 (-18.4, 1.2)</td>
<td>0.081</td>
</tr>
</tbody>
</table>

Note: The estimate of the median of the differences expresses the median of all differences when subtracting the FECR of the second mentioned category from the FECR of the first mentioned category, for each predictor variable.
Table 6.2. Predictor variables that had a p-value ≤0.20 in univariable associations with the outcome fenbendazole fecal egg count reduction (FECR) percentage on 20 Ontario sheep flocks (May to November 2010 and May to November 2011).

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>Median of the differences (95% confidence intervals)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Used benzimidazole (no vs. yes)</td>
<td>45.0 (5.0, 79.0)</td>
<td>0.011*</td>
</tr>
<tr>
<td>Used targeted selective treatment based on FAMACHA score (no vs. yes)</td>
<td>38.5 (-28.0, 79.0)</td>
<td>0.126</td>
</tr>
<tr>
<td>Potential for manure run-off into pasture (no vs. yes)</td>
<td>-37.0 (-71.0, 5.0)</td>
<td>0.089</td>
</tr>
</tbody>
</table>

*denotes a variable that is statistically significant (p ≤0.05)

Note: The estimate of the median of the differences expresses the median of all differences when subtracting the FECR of the second mentioned category from the FECR of the first mentioned category, for each predictor variable.
CHAPTER 7

General discussion, study limitations and recommendations for future research

The sheep industry in Ontario is currently burgeoning, as the increased demand for both lamb and mutton meat has created a real opportunity for flock expansion. To ensure that sheep producers can meet this demand, it is important to identify and investigate the major causes of losses in their sheep flocks and address these issues appropriately.

Gastro-intestinal nematodes (GINs) have long been recognized as one of the most important production-limiting diseases for grazing sheep worldwide, including Canada (Pullin, 1961). While GIN parasitism has traditionally been controlled with the use of broad-spectrum anthelmintics, the emergence of anthelmintic resistance (AR) in many sheep-rearing countries has underscored the importance of providing and promoting more specific and sustainable recommendations for GIN control on Ontario sheep farms. This can be achieved by improving our understanding of the epidemiology of these parasites.

Recent research conducted in Ontario and Quebec sheep flocks has provided important baseline information on the epidemiology and distribution of GIN parasites in these areas (Mederos, 2010). However, it also highlighted important areas for further research, including the periparturient egg rise (PPER), overwintering of parasites on pasture, and the frequency of AR in Ontario sheep flocks. Consequently, the main goal of this thesis was to investigate these important epidemiological factors associated with GIN parasitism.
The PPER is described as one of the major sources of GIN pasture contamination, and the study described in Chapter 2 was conducted to improve our knowledge of the distribution and determinants of the PPER in Ontario sheep flocks that practice out-of-season lambing. This was carried out by following six Ontario sheep flocks that practiced out-of-season lambing, over three lambing seasons (winter-spring-autumn). Twenty pregnant ewes and 20 unbred/early gestation ewes were selected on each farm, for each lambing season, and both fecal and blood samples were collected from these 40 animals at fixed time-points before, mid-way, and after lambing. The samples were then processed to estimate GIN Fecal Egg Counts (FECs), blood Total Plasma Protein (TPP) and blood Packed Cell Volume (PCV); data were then analyzed to investigate differences between ewes in different production stages.

Overall, the FECs were significantly higher in the autumn, compared to the winter and spring lambing season. While a PPER was observed during all three lambing seasons evaluated in this study, the magnitude and distribution of maximum fecal egg shedding for each production stage varied between lambing seasons. In both the winter and spring, ewes that were unbred or in early gestation did not experience a rise in fecal egg shedding, in contrast to ewes that were in late gestation, in which the FEC increased and remained elevated during early lactation. By contrast, ewes lambing in the autumn experienced a rise in fecal egg shedding over the gestation period, which peaked at late gestation and then decreased. In the animals enrolled in this study, both TPP and PCV were associated with parasite FECs, though this association varied between production stages and lambing seasons. Collectively, the findings showed that both seasonal and animal physiological factors play an important role in determining fecal egg shedding,
and should be taken into consideration when making recommendations regarding targeted treatment of periparturient ewes.

As with most long-term field studies, we encountered several difficulties while conducting this study. Firstly, since it was a longitudinal study with repeated sampling on the same animals, it was important to maintain the producers’ compliance, which often proved difficult as time wore on. This was further complicated by the fact that the producers were asked to keep animals that were in different production stages in the same housing system (i.e. either indoors or put to graze on pasture), to allow for a fair comparison between the different groups. Moreover, randomization was sometimes difficult to execute, as producers could not understand why we had to run the whole flock through a chute when we were only sampling a small proportion of the flock. All these challenges were overcome by explaining the rationale of the study and the importance of proper epidemiological methods to ensure internal validity of the results, and by emphasizing the benefits to be gained by the sheep industry. Moreover, we tried to provide the producers with timely feedback by returning the FEC results promptly and addressing any concerns expressed.

Carrying out research on pregnant animals also had several difficulties. Firstly, not all producers used ultrasound to diagnose pregnancy, and therefore we sometimes had false-positive ewes, leading to a reduced sample size of the pregnant group. Secondly, producers were sometimes reluctant to allow the researchers to work on animals that were in late gestation/early lactation, as they feared this might cause injury, abortion or maternal neglect. Therefore, producers would often not contact us until all animals had lambed, and on one farm the producer did not allow us to sample the pregnant group,
leading to missing data for one lambing season. In another study which has not been included in this thesis (a clinical trial to evaluate the efficacy of targeted treatment of periparturient ewes to suppress the PPER), we tried to avert this problem by sampling the animals at the end of the lambing season (as opposed to midway through lambing), and by providing the producers with data collection forms with clear instructions on which data we required. This improved overall study compliance.

The study described in the second chapter was originally designed to compare the FECs between a group of ewes due to lamb that season, and a comparison group comprised of ewes that were unbred. However, since all the farms practiced out-of-season lambing, they bred their ewes approximately two months after lambing. Therefore, there were few unbred ewes, leading to the necessity of using animals that were bred, but were early in gestation, to act as a comparison group. Moreover, on different farms, animals in the comparison group were often at different stages in gestation. Therefore, to account for all this variability we decided to divide all the animals into different productivity stages, based on sampling dates relative to lambing dates which were provided by the producers. However, reliance on producer-collected data might have led to some misclassification. Moreover, it also reduced the study’s power to detect differences between certain groups, as not all producers collected the requested data on lamb birth weights, 50-day weights etc. All these challenges were an important eye-opener to the practical problems and limitations one encounters when working on commercial sheep farms.

The PPER in ewes is usually described as a rise in fecal egg shedding commencing 2-4 weeks before lambing and peaking at lambing, followed by a decrease
4-6 weeks later (Abbott et al., 2009). However, in our study, we did not observe the expected decrease in fecal egg shedding after lambing, even though we collected fecal samples up to eight weeks after lambing. The sustained high FECs eight weeks after lambing suggests that factors other than parturition, such as the duration of lactation, might have an important effect on the increase in fecal egg shedding, as has been shown in recent studies by Beasley et al. (2010 and 2012). Therefore, further studies should be conducted to determine the effect of lactation on the PPER, and when the PPER decreases, in Ontario sheep flocks.

In the same study, forage quality data were collected and incorporated into the data analysis. Studies have indicated that high levels of bypass protein (also referred to as rumen-undegradable protein) may reduce fecal egg shedding during the periparturient period (Beasley et al., 2012; Houdijk, 2012). However, since our study was observational, we had no control over the type and quantity of nutrition offered to the animals, which might have limited the variability of the predictor variable ‘Crude Protein Ingested’. A clinical trial could be conducted to determine whether the administration of different amounts of bypass protein (e.g. roasted soybeans) could have a protective effect on the PPER. Also related to nutrition, on several occasions during presentations of the research work, producers have asked about the potential use of bioactive forages such as birdsfoot trefoil and sulla, considered indirect sources of bypass protein (Niezen et al., 1998), as complementary strategies for parasite control. These plants contain an active metabolite (condensed tannins) which has been shown to reduce fecal egg output in sheep (Athanasiadou and Kyriazakis, 2004). However, studies need to be conducted to determine whether these plants could be integrated within Ontario grazing pastures, the
palatability of the plants, and whether they can be a cost-effective measure for parasite control.

Some studies have suggested that certain breeds of sheep are genetically more resistant to parasites (Burke and Miller, 2002; Good et al., 2006), and therefore experience a lower PPER. In Ontario, many producers have Rideau sheep, a breed developed through a long-term cross-breeding program using several different breeds (e.g. Suffolk, Finnish Landrace, East Friesen, Border Leicester) (Menzies, 2006), and heavily selected for maternal traits such as prolificacy (Shrestha and Heaney, 2003). However, no studies have been conducted to determine whether this breed is more resistant to parasites, compared to other common breeds in Ontario, such as the Suffolk or Dorset (Menzies, 2006).

Understanding the over-wintering survival of GIN free-living stages in central Canadian climates is important as it provides information on the level of parasites present on pasture in refugia at the beginning of the grazing season. Chapter 3 describes a study on the over-wintering survival of free-living GIN stages, especially Haemonchus contortus, on three Ontario sheep farms with a previous history of clinical haemonchosis. Monthly herbage and soil samples were collected from one-acre isolated paddocks, starting in December (after the sheep were taken off pasture), up to the following April (before the sheep were once again put out to graze). In the spring (April/May), naïve lambs were put out to graze on the one-acre isolated paddocks that had not yet been grazed that season, on each of the three farms. After 28 days, the lambs were slaughtered, and their gastro-intestinal contents were collected and analyzed for the presence of GINs.
Trichostrongylus spp. and Nematodirus spp. larvae were isolated from one herbage sample collected on one farm in March; no larvae were isolated from the other herbage samples and from any of the soil samples collected during the winter months on any of the three farms. Teladorsagia sp., Trichostrongylus spp. and Nematodirus spp. were isolated from the abomasal and small intestine contents of all tracer lambs, from all three farms. In contrast, Haemonchus sp. parasites (two) were only isolated in very low numbers in one tracer lamb, from one farm. These results suggest that very few Haemonchus sp. larvae were able to overwinter on pasture. However, in contrast to Haemonchus sp., other important parasite larvae such as Teladorsagia sp. and Trichostrongylus spp., did survive on pasture during the Ontario winter, and were still infective in the spring. The poor over-wintering survival of Haemonchus sp. documented in this study suggests that most, if not all, Haemonchus sp. parasites survive the winter within host sheep. This poor over-wintering survival, associated with the common practice of treating ewes before lambing in the spring (Chapter 6), could explain why AR has emerged as a significant problem in Haemonchus sp. (Chapter 4) on multiple Ontario sheep farms.

Since the overwintering of Haemonchus sp. larvae on pasture proved to be an extreme observation (i.e. only two adult worms in one tracer lamb, on one farm), and the study was very expensive and time-consuming, we decided not to repeat the study the following year as we agreed that an additional year would not likely change the study results significantly. While we recognize that one year is suggestive but not conclusive, we considered this study as a pilot project that could serve to inform future studies. Ideally, a long-term project involving a larger number of farms in Ontario should be
conducted. Environmental data-loggers could be set up on farms from all 11 Ontario Sheep Marketing Agency districts, and pasture and soil samples could be collected in November/December, when the sheep are taken off pasture, and once again in the spring before the animals are put on pasture again. These data, collected over several years, could be mapped out to indicate whether there are any observable trends in the number and type of parasites, and whether there is a correlation with the environmental data, in the different districts. However, one must also be aware of the limitations of the diagnostic methods (e.g. pasture sampling techniques) and confounding effects (e.g. the farm topography and stocking density) when conducting such long-term studies, as these will inevitably influence interpretation of the results.

Anthelmintic resistance has been reported in most sheep-rearing countries worldwide, and in some areas is now threatening the viability of the sheep industry. Thus, it is important to improve our understanding of the current AR status in Ontario sheep flocks and mitigate the risks associated with widespread AR. Chapter 4 describes a study that evaluated the frequency of ivermectin drench failure and AR in Ontario sheep flocks. Forty-seven sheep flocks were enrolled over two grazing seasons, and the FECs were monitored monthly starting in May of each year. Once the mean FEC reached a set threshold of 200 eggs per gram (epg), ivermectin was sent to each producer for an ivermectin drench check. If ivermectin drench failure was reported (i.e. Fecal Egg Count Reduction [FECR] <95% 14 days after ivermectin treatment), a Fecal Egg Count Reduction Test (FECRT) was conducted to determine if there was resistance to ivermectin and, if a sufficient number of animals was available, fenbendazole and levamisole. Larval cultures were conducted on pooled post-treatment FECRT samples to
identify resistant GIN genera. Larval Development Assays (LDAs) to assess thiabendazole and levamisole susceptibility were conducted on pooled owner-acquired fecal samples collected during the grazing season when the mean FECs reached the set threshold of 200 epg.

On the basis of the FECRT, the field study indicated that resistance to ivermectin and fenbendazole was common in GINs on Ontario sheep farms. In contrast, levamisole was effective on almost all of the farms tested. Results from the LDA indicated widespread resistance to thiabendazole, while levamisole was effective on all farms tested. *Haemonchus* sp. was the most commonly isolated parasite in both the thiabendazole LDAs and post-treatment FECRT larval cultures. It would therefore appear that most of the ivermectin and fenbendazole resistance detected on the Ontario farms was associated with *Haemonchus* sp., which is a concern as this is typically the most pathogenic of all the GINs that infect sheep in Ontario.

The greatest challenge of this study was its seasonality, since the ivermectin drench check could only be performed when the mean FECs reached 200 epg. Once this threshold was reached, we had to wait for the results of the ivermectin drench check to confirm whether a FECRT would be performed on that farm. Also, we had to allow for a minimum time period between the ivermectin drench check and FECRT, to ensure that the animals had a sufficient level of FECs that would allow us to detect changes in the FECs after treatment. However, on certain farms, the FECs reached dangerous levels despite treatment with ivermectin, and we had to conduct the FECRT as early as possible to ensure that the sheep’s well-being was not compromised. Therefore, it was very hard to balance the work-load, which sometimes led to a back-log of fecal samples. For this
reason, we were unable to perform LDAs and larval cultures in the first year of the study. In the second year, we decided to perform the LDAs when the FECs reached the 200 epg threshold; this helped balance the work-load distribution, but resulted in the LDAs and FECRTs being conducted at different time-points, making it harder to draw comparisons between the two tests.

Producers were sometimes reluctant to use ivermectin (as required by the study), since they had previously experienced ivermectin drench failure on their farm and were concerned regarding further losses when using this product; this led to some farms refusing to perform a FECRT after ivermectin drench failure was shown on their farm. Also, some producers were reluctant to keep a negative control (i.e. untreated) group when conducting a FECRT, as they were worried that this would compromise the health and productivity of the animals. We addressed this concern by reassuring the producers that they could treat any control animal that showed overt clinical signs, and asked them to keep a record of any treatments administered. Moreover, whenever possible, we tried to send them the pre-treatment FECs as quickly as possible so they could monitor, and treat if necessary, any high-shedding animals.

Since this research indicated that resistance to ivermectin and fenbendazole, the two most commonly used anthelmintics in Canada, is widespread, and most of the AR reported is associated with Haemonchus sp., research is required to determine whether the use of other anthelmintics could be used as alternatives in Ontario sheep flocks. In particular, monepantel (Mason et al., 2009) and closantel (Uppal et al., 1993; Waruiru, 1997) are promising alternative drugs as they have been shown to be effective against both ivermectin- and fenbendazole-resistant strains of H. contortus. However, more
research is required to ensure that the efficacy of any new anthelmintic that becomes commercially available for Ontario sheep producers is preserved. This includes investigation of the efficacy of targeted selective treatment of ewes at lambing time as a means to reduce treatment frequency and to slow down further development of resistance.

In the field study described in Chapter 4, we used the method endorsed by the World Association for the Advancement of Veterinary Parasitology to calculate the FECR percentages (Coles et al., 1992). However, the literature describes several FECR calculation methods, and no formal agreement has been reached as to which method is more appropriate. Moreover, few studies so far have compared results between the FECRT and LDAs. Therefore, the study described in Chapter 5 was conducted to compare different methods for calculating the FECR percentages, and to compare results obtained with the FECRT and LDA. Four different FECR methods were used (FECR\textsubscript{1} and FECR\textsubscript{2} used pre- and post-treatment FECs from both treated and control animals, but FECR\textsubscript{1} used arithmetic means while FECR\textsubscript{2} used geometric means; FECR\textsubscript{3} was calculated using arithmetic means for post-treatment FECs from treated and control animals; FECR\textsubscript{4} was calculated using mean FEC estimates from a General Linear Mixed Model), and the FECR percentages were then compared using a concordance correlation coefficient. Additionally, FECRT and LDA results were categorized into three classes defined as “no”, “low” or “high resistance”, and the results were compared using the Kappa agreement method.

The different FECR methods evaluated in this study did not provide consistent FECR percentages following treatment with ivermectin, fenbendazole or levamisole. The
correlation between the methods was influenced by which means (arithmetic vs. geometric) were used in the FECR formulae, especially when low levels of resistance were present, resulting in right-skewed parasite data. Therefore, the use of arithmetic means is recommended, since no correction factor is required, and they are less prone to bias, particularly when the date is right-skewed. In contrast, whether both pre- and post-treatment, or only post-treatment groups, were used in the FECR formulae was less influential. This suggests that the simpler formula (i.e. using only post-treatment data) could be used, reducing the cost and labour associated with the FECRT.

The LDA and FECRT showed an overall poor to moderate Kappa agreement in this study, which is in disagreement with a recent study by Taylor et al. (2009) which indicated a good agreement between the two tests. However, in the latter study, they only evaluated whether the tests detected the presence of resistance (i.e. yes/no), and no statistical test was performed to determine the agreement between tests. In our study, the Kappa agreement was statistically significant when an 80% threshold was used to differentiate FECR percentages as indicative of low or high levels of resistance, suggesting that this could be a useful threshold.

As mentioned earlier, the LDA and FECRT were conducted at different time-points during the grazing season, which limits the overall interpretation of test comparisons. Moreover, we were unable to perform LDAs for ivermectin, since the methodology used in this study has not yet been validated for ivermectin (Taylor, 1990). Lastly, the high prevalence of phenotypically benzimidazole-resistant parasites, and low prevalence of phenotypically levamisole-resistant parasites, were problematic when calculating the Kappa agreement, since “the prevalence of the condition being diagnosed
affects Kappa. Two tests will have a higher Kappa value if the prevalence of the underlying conditions is moderate than if it is very high or very low” (Dohoo et al., 2009).

The modified McMaster technique used in this study had a minimum detection level of 50 epg, which might have influenced the overall interpretation of the FECR, especially when the FEC levels were low. This test was used for practicality and cost issues, since we had to process many fecal samples in a short time-frame and had limited financial resources. While more sensitive diagnostic methods have been described, these would likely increase the overall cost of the FECRT, making it cost-prohibitive for sheep producers. Therefore, we need to evaluate the cost and benefits of increasing the test sensitivity, especially since one of our goals is to encourage producers to monitor for AR by performing FECRTs more regularly.

El-Abdellati et al. (2010) have suggested that the Bayesian approach could account for the limited sensitivity of the McMaster method used in this study. Moreover, these methods might be more suitable to calculate the FECR, as they allow for greater flexibility in the model specification, and account for different sources of variability, providing more accurate estimates (Denwood et al., 2010). Therefore, the FECRT data obtained in this study could be evaluated using Bayesian methods.

Chapter 6 describes an investigation of management practices currently employed by Ontario sheep producers on their farms, and their association with AR. A questionnaire was administered in a face-to-face interview on 38 of the 39 farms that conducted an ivermectin drench check. Questions were asked on farm demographics,
previous use of anthelmintics, quarantine strategies, pasture management and alternative control strategies, manure disposal, and perceived risk of AR. Ivermectin and fenbendazole drenches were the most commonly used anthelmintics in the previous five years, while levamisole drench was not commonly employed. Most producers treated their flock routinely; most ewes were treated at lambing and/or at the beginning of winter housing. The majority of producers also used targeted or targeted selective treatment, but this was often done in addition to, rather than in lieu of, routine treatment. Many producers (20/30; 67%) did not calibrate the drench gun before use, and more than half of the producers calculated the dose of anthelmintic to administer by estimating the weight of the animal or using the flock average weight. Almost a third of the producers that brought in new animals (i.e. sheep, goats, llamas or alpacas) did not perform any quarantine strategies. Just over half of the producers surveyed (21/38; 55%) used pasture management as an alternative parasite control strategy, and more than half of the farms surveyed indicated that they suspected the presence of AR on their farm prior to the study. This descriptive information has highlighted important aspects (e.g. proper quarantine strategies, risk of sub-optimal dosing) that should be addressed when disseminating information on parasite control strategies to mitigate the development of AR.

No management practices were significantly associated (i.e. p≤0.05) with the ivermectin fecal egg count reduction. In contrast, previous use of benzimidazoles was significantly associated with a lower fecal egg count reduction after treatment with fenbendazole, suggesting higher levels of resistance. We were unable to model the levamisole fecal egg count reduction, since there were low levels of phenotypically
levamisole-resistant parasites on the farms surveyed, and associations that were statistically significant were not of biological significance.

The study described in the sixth chapter had limited power due to the small sample size and limited variability of the outcome (i.e. high levels of ivermectin and fenbendazole resistance; low levels of levamisole resistance). We therefore opted to use the continuous FECR percentage as the outcome; however, there is currently no literature on how different FECR percentages correspond with more severe clinical signs of resistance, which hindered interpretation of some of the analytical results. Moreover, following analysis of the questionnaire data, we recognized that the questionnaire could be improved by modifying certain definitions to avoid overlap between the terms (e.g. the definition of routine and targeted treatment) and collecting additional data such as: how often (times/year) each anthelmintic formulation was used in the previous five years, and specific information on treatment of periparturient ewes in the spring before turn-out on pasture.

Other studies have suggested that treatment of ewes at lambing in the spring may lead to the development of AR (Leathwick et al., 2006; Waghorn et al., 2010), and our research indicated that treatment of ewes pre-lambing was a common practice on the sheep farms surveyed. As indicated earlier, we believe that this common practice, together with the poor over-wintering survival of Haemonchus sp. on pasture, is likely a major driver of the high levels of resistance observed in this species. Therefore, a clinical trial could be conducted to evaluate whether this management practice is causally associated with higher levels of AR in Ontario sheep flocks.
Furthermore, a systematic review and meta-analysis on risk factors for AR could be carried out to identify management practices associated with AR. This type of study provides the most substantive clinical evidence (Sargeant *et al.*, 2006), and allows for the evaluation of several risk factors and synthesis of all current research.

Lastly, as described by the theory of planned behaviour (Azjen, 1991) and the health belief model (Becker and Maiman, 1975), people’s perceptions (e.g. seriousness and threat of a certain disease), attitudes and motivation to change will influence their decision to adopt recommended preventive actions. Therefore, it is also necessary to conduct research on sheep producers’ perceptions of the risk of AR, and their motivations and barriers to making management changes, to inform our communication strategies on parasite control management practices and ensure that these translate into proactive changes.

Overall, this study has provided us with valuable information on several important epidemiological features of GIN parasitism, namely the distribution and determinants of the PPER, the frequency of *Haemonchus* sp. overwintering in the environment, and the frequency of AR in Ontario sheep flocks. Collectively, the results will be used to develop a strategic integrated parasite control program for commercial sheep flocks in Ontario, and to guide future research on AR.
7.1 References


APPENDIX I

Visit Schedule: Over-wintering in Ewes Study

Producer Name:

_________________________

Group 1:
Date of Ram Introduction (DRI): ______________________________
Estimated date 50% of lambing completed (L50%): (DRI + 148 days + __ days)________________

Group 2:
Date of Ram Introduction (DRI): ______________________________
Estimated date 50% of lambing completed (L50%): (DRI + 148 days + __ days):

---

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Sampling Dates</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks before L50%</td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>4 weeks before L50%</td>
<td></td>
<td>Sample as well</td>
</tr>
<tr>
<td>50% lambing completed</td>
<td></td>
<td>Sample as well</td>
</tr>
<tr>
<td>3 weeks after L50%</td>
<td></td>
<td>---</td>
</tr>
<tr>
<td>8 weeks after L50%</td>
<td></td>
<td>Sample as well</td>
</tr>
<tr>
<td>---</td>
<td></td>
<td>6 weeks before L50%</td>
</tr>
</tbody>
</table>

---

1 For flocks with natural exposure, 14 days from 1st lambing is predicted to be the L50%. For flocks with an induced estrus out-of-season, 5 days from 1st lambing is estimated to be the L50%. Can be modified based on previous experience.
<table>
<thead>
<tr>
<th>Sample as well</th>
<th>4 weeks before L50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample as well</td>
<td>50% lambing complete</td>
</tr>
<tr>
<td>---</td>
<td>3 weeks after L50%</td>
</tr>
<tr>
<td>Sample as well</td>
<td>8 weeks after L50%</td>
</tr>
</tbody>
</table>

**Procedures to be done on sampling day:**

**Producer:** Complete “Visit Information Form” prior to arrival of research team.

**Producer:** Gather ewes to be sampled into pens or handling facilities prior to arrival of research team, so animals can be sorted and sampled. If lambs at foot or you need labour, please notify team that you will need their help. This is to assist scheduling time for visit.

**Producer:** Make sure that lambing and lamb performance records are kept up-to-date.
APPENDIX II

Visit Information Form: Over-wintering in Ewes study

Producer Name: ________________________________ Date of Visit: ______________

Ewe ID’s to be sampled:

<table>
<thead>
<tr>
<th>From Lambing Group (15)</th>
<th>From Non-Lambing Group (15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Replacement Ewe ID’s: Replacement Ewe ID’s:

Since our last visit to your farm on ______________________________(date)

Have any ewes on this list left the flock? ☐ Yes ☐ No

If yes, please list Tag ID and reason left flock (including death)

________________________________________________________

Please record ewe diet since last visit (changes only):

Forage (type and estimated daily intake)

____________________________________________

Forage analysis?

____________________________________________

Mineral / Premix / Salt (brand, attach tags):

____________________________________________
Concentrate (type and estimated daily intake):
___________________________________________

Commercial supplement (Brand, attach tag)_________________________________________

Have ewes had access to pasture since last visit?  □ Yes  □ No
If yes, please indicate: Time on pasture _____________(days) Date left pasture: _____________

*Have the ewes received any treatments with a worming product* (anthelmintic) (e.g. Ivomec, Safeguard, natural product) *since the last visit?*  □ Yes  □ No
If yes, please indicate date: ___________________ and product used: _______________________ 

*Are there other issues or concerns you would like to make the research team aware of?*
__________________________________________________  __________________________
APPENDIX III

Administered questionnaire

Anthelmintic Use Practices on Ontario Sheep Operations

The purpose of this questionnaire is to understand better the usage of dewormers on Ontario sheep operations. For the purpose of this questionnaire, we shall be defining the following as:

- **Parasites** - internal parasites only (that is, not lice, keds or mange)
- **Drugs** - products used as de-wormers and sometimes also called anthelmintics
- **Quarantine** - to hold any new animals off pasture used for sheep grazing for 24-48 hours
- **Used pasture** - pasture that has been grazed by sheep in the last 12 months

Name of Producer: ___________________ Date: ______________

- **Background Information**

1. How many breeding ewes do you have in your flock?
   - □ <50
   - □ 50 – 99
   - □ 100 - 300
   - □ >300

2. What is the **primary** purpose of the flock?
   - □ Meat Production
   - □ Dairy Production
   - □ Wool Production
   - □ Breeding Stock for Replacement Sales
   - □ Other (please specify) _____________________
3. What breeds of sheep do you have on your operation? How many?
   
i. _______________________________ Number: _____________
   
ii. _______________________________ Number: _____________
   
iii. _______________________________ Number: _____________
   
iv. _______________________________ Number: _____________
   
v. _______________________________ Number: _____________

4. Are you a certified organic sheep producer?
   
   □ Yes       □ No
   
i. If yes, for how long have you been a certified organic sheep producer?

   ______________________________________________________________
   
   ii. If no, are you working towards organic status?

   □ Yes       □ No

- **Drench Failure**

5. Could you please name the dewormers you have used since Jan 1st 2006 for parasite control on your farm.

   ________________________________
   
   ________________________________
   
   ________________________________
   
   ________________________________

6. In addition to commercial dewormers, what other methods have you used for parasite control in your sheep since Jan 1st 2006.

   ______________________________________________________________
   
   ______________________________________________________________

7. In the last year, since ________ 2009, what products and procedures have you used for parasite control in your sheep flock? This could include both natural and/or commercial deworming products.
8. If you use a drench gun, how often do you test that it is delivering the indicated volume?

- Never
- Before each use
- Once a year
- Do not use a drench gun
- Twice a year

9. How do you determine the weight of the animal when calculating the dose of dewormer to be given? Please check the most frequently used method.

- Estimation of weight (eyeball)
- Weigh and use average weight of the group
- Expected breed average
- Other ____________________________
- Weigh group and use heaviest

**Quarantine**

10. In the last year, have you brought new animals into your flock or had animals return that had been on another farm? (Animals of interest include sheep, goats, llamas and/or alpacas, but not cattle or other livestock)

- Yes
- No (skip to question 11)

i) If yes, what did you do with them in regards to parasite control at arrival? Please check all applicable.

- Nothing
- Deworm upon arrival and release immediately into the flock
- Deworm while in quarantine
- Quarantine new arrivals off pasture for ____________ days
- Other ____________________________

---

Age: **PWL** = pre-weaned lambs; **WL** = weaned lambs; **A** = adults

Route: **SC** = subcutaneous; **OD** = oral using drench gun; **OS** = oral using syringe; **PO** = pour-on
If a deworming product is used in quarantine, please record.

<table>
<thead>
<tr>
<th>Age and Species type</th>
<th>Products Used (Dose and Frequency)</th>
<th>When was it administered (Day 0 = day of arrival)</th>
</tr>
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</table>

ii) If brought-in animals were turned onto pasture, had the pasture been grazed by sheep within the last 12 months, since ________ 2009?

☐ Yes  ☐ No  ☐ Not applicable (please explain) __________________________________________________________

_________________________________________________________________

• Pasture Management

11. How many acres of pasture do your sheep graze in a season? ___________________

12. What is your estimated current stocking density? __________________

13. In which months does your flock routinely lamb? Please check all applicable.

☐ Jan  ☐ May  ☐ Sep
☐ Feb  ☐ Jun  ☐ Oct
☐ Mar  ☐ Jul  ☐ Nov
☐ Apr  ☐ Aug  ☐ Dec

14. Are the ewes and nursing lambs ever out on pasture together?

---

4 Species type: PWL = preweaned lambs; WL = weaned lambs; A= adults; Others = goats, llamas, alpacas

5 1 adult sheep = 1 sheep-unit (SU). 1 ewe + nursing lambs = 1.5 SU. Estimate grazing by the number of months grazed. Please estimate SU per acre of sheep grazing pasture on your farm on an annual basis. E.g. 100 adult sheep grazing 40 acres over 5 months would be a stocking density of \[\frac{(100 \times 40) \times (5/12)}{40} = 1.04 \text{ SU per acre per year}\]
15. If yes, for how many months are they on pasture together? ________________

16. When were your sheep turned out to pasture to graze this spring or when are you planning to turn them out?

- 5 Apr – 11 Apr
- 12 Apr – 18 Apr
- 19 Apr – 25 Apr
- 26 Apr – 2 May
- 3 May – 9 May
- 10 May – 16 May
- 17 May – 23 May
- 24 May – 30 May
- 31 May – 6 Jun
- 7 Jun – 13 Jun
- 14 Jun – 20 Jun
- 21 Jun – 27 Jun
- Other __________________________

ii. What month were sheep permanently taken off pasture at the end of the previous grazing season (2009) and put into a barn, barnyard or dry lot?

17. Do you ever rest pastures that have been used for grazing sheep, goats, llamas and/or alpacas, for more than one year? (The pasture may be used for other things, but not grazing).

- Yes
- No (please skip to question 18)

ii. If yes, how often? __________________________________________

18. Do you practice crop rotation with the pastures used by the sheep? (That is, pasture ploughed every few years and seeded with grain, corn, cash crops, etc. or used for hay)

- Yes
- No

ii. If yes, how often? __________________________________________

19. Do you practice mixed species grazing, other than those animals used for guarding purposes? (that is, different species that are at pasture the same time as the sheep)

- Yes
- No (please go to question 20)

i. If yes, what species? Please check all applicable.

- Cattle
- Horses
- Goats
- Llamas and/or alpacas
- Other

20. Do you practice rotational grazing with other species? (that is, grazing one species first, removing, and then placing another species on the same pasture)
□ Yes  □ No (please go to question 21)

i. If yes, what species? Please check all applicable.
□ Cattle  □ Llamas and/or alpacas
□ Horses  □ Other
□ Goats

Dewormer Usage

21. Do you deworm your flock routinely (that is, without seeing signs of disease)?
□ Yes  □ No  (Please skip to question 24)

22. If yes, how many times a year do you routinely deworm your

Ewes

________ time(s)

Lambs (<12 months of age)

________ time(s)

Rams

________ time(s)

23. If yes, at which times do you treat?

□ Adults at breeding  □ Ewes at lambing
□ At housing in the fall  □ Other _________________
□ At a specific time after turn-out onto pasture (# days = ________________)
□ At a specific time during the grazing season (date = _________________)
□ Lambs at weaning

24. Do you deworm the flock because when you believe that it has parasites?
□ Yes  □ No

25. Do you deworm individual animals when you believe them to have parasites?
□ Yes  □ No (please skip to question 27)
26. How do you determine if the flock or individuals in the flock have parasites?

☐ High faecal egg counts  ☐ Animals showing clinical signs
☐ FAMACHA system  ☐ Other __________________________

Resistance

27. Do you believe you have ever had dewormer resistance in your flock with regards to internal parasites?

☐ Yes  ☐ No (please skip to question 28)

i. If yes, what made you believe dewormer resistance was present?

☐ Egg counts still high after deworming
☐ No improvement after deworming. Please explain.

________________________________________________________

☐ Other __________________________________________________

ii. If yes, when do you believe resistance first occurred?

________________________________________________________

iii. If yes, did you suspect resistance to a specific dewormer(s)?

☐ Yes  ☐ No

iv. If yes, to which dewormer(s)?

________________________________________________________

28. Within the last 12 months, since ________ 2009, did you spread sheep manure on pastures being grazed by sheep?

☐ Yes  ☐ No (please skip to question 29)

i. If yes, what was the minimum time from storage (i.e. moved out of the barn to a manure pile or storage area) until spread on pastures?
ii. If yes, which month was manure spread?

29. How is your sheep manure stored? Can you please show me? Please check all applicable.

☐ Potential for runoff into a grazing  ☐ Sheep have access to manure area

☐ None of the above