Worm worries: Gastrointestinal nematodes, anthelmintic resistance, and worm management options

by

Kathleen E. Hipwell

A Thesis Submitted to

Saint Mary's University, Halifax, Nova Scotia

in Partial Fulfillment of the Requirements for

the Degree of Bachelor of Science (Honours) in Biology.

April 2015, Halifax, Nova Scotia, Canada

Approved: Dr. Gwyneth Jones

Supervisor

Approved: Dr. David Cone

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Abstract

Gastrointestinal nematodes cause significant morbidity and mortality in sheep. Anthelmintics are essential for worm management, but the sole reliance on these drugs to manage infections has led to widespread anthelmintic resistance (AR). Ivermectin is the only anthelmintic licenced for use in sheep in Canada, although off-label use of benzimidazoles and levamisole is common. In Nova Scotia during 2012, many farmers reported sheep deaths due to worms, despite anthelmintic treatment, and there is no information about AR in Eastern Canada. *Haemonchus contortus* has become the most problematic species in Nova Scotia. Closantel, a narrow spectrum drug that kills only *H. contortus*, was accessed through the Emergency Drug Release process. AR was investigated through Fecal Egg Count Reduction Tests on 13 farms over the grazing seasons of 2013 and 2014. Resistance to ivermectin, moxidectin, benzimidazoles, and levamisole was found on 5/6, 0/1, 6/7, and 0/8 farms, respectively**.** Post-treatment larval cultures showed that *H. contortus* was resistant to ivermectin and benzimidazoles, whereas closantel was effective against *H. contortus* and provided adequate worm control for several weeks. Levamisole was effective, but its short duration of activity necessitated more frequent treatments. Albendazole in combination with closantel was highly effective against all species, and drug combinations offer a means to extend the useful lifetime of both drugs. Seasonal monitoring showed a high peak in lamb infection levels in July-August of 2013 and 2014. Grazing lambs with and without ewes was investigated in 2014. Lambs grazed without ewes did not require treatment, whereas lambs grazed with ewes required treatment at the end of August. This grazing strategy may be an appealing and cost effective option to limit infections with *H. contortus*. To ensure the sustainability of sheep production in Nova Scotia, a transition to using anthelmintic combinations and incorporating alternative non-drug management strategies will be essential.

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List of Abbreviations

GIN	Gastrointestinal nematode(s)
L3	Third stage larvae
FEC	Fecal egg count(s)
epg	Eggs per gram of feces
AR	Anthelmintic resistance
FECRT	Fecal egg count reduction test
W.W.A.V.P.	World Association for the Advancement of Veterinary Parasitology

Introduction

Gastrointestinal nematodes (GIN) of sheep belong to the superfamilies Strongyloidea and Trichostrongyloidea (Roeber et al. 2013). GIN cause significant morbidity and mortality in sheep, and these health effects along with the costs of pharmacologic treatment reduce the profitability of sheep farming. Anthelmintics are essential for GIN management, but the sole reliance on these drugs to manage infections has led to widespread anthelmintic resistance (AR) in Europe (Papadopoulos et al. 2012), Australia (Leathwick and Besier 2014), New Zealand (Waghorn et al. 2006; Leathwick 2014), and North and South America (Falzon et al. 2013b; Torres-Acosta et al. 2012). The development of AR is influenced by many factors, including: the life cycle and epidemiology of GIN, the availability of refugia populations, the timing and frequency of treatment, and the quarantine of incoming animals (Abbott et al. 2012).

1.1 Species and life cycle

In Canada, as in other countries, there are several common species of GIN that parasitize sheep. The highly pathogenic species are *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus spp.*, and *Nematodirus battus.* Other less pathogenic species include: *Chabertia ovina*, *Strongyloides papillosus*, *Nematodirus filicollis*, *Cooperia spp.*, *Bunostomum spp*., *Trichuris ovis*, and *Oesophagostomum spp.* (Mederos et al. 2010; University of Guelph 2012). The location of GIN in the digestive tract of sheep is species specific. *H. contortus*, *T. circumcincta*, and *Trichostrongylus axei* live in the abomasum; *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, and *N. battus* live in the small intestine (University of Guelph 2012). The fecundity of these species

varies greatly. *H. contortus* is the most prolific, and a single female is capable of producing up to 10 000 eggs per day. Female *N. battus* produce around 50 eggs per day, and *Trichostrongylus spp*. and *T. circumcincta* produce a few hundred egg daily (Abbott et al. 2012; Roeber et al. 2013).

Almost all GIN have a similar direct life cycle, with both free living and parasitic stages. Adult GIN live in the gastrointestinal tract, and females lay eggs which are passed in the feces (Fig. 1). A first stage larva (L1) develops within the egg, hatches, and feeds on bacteria within the feces (Abbott et al. 2012). The L1 moults to the second stage larva (L2), which also feeds on bacteria. The L2 moults to the infective third stage larva (L3), but retains its L2 cuticle as a sheath, which protects the L3 from desiccation, but prevents feeding. L3s migrate out of the feces and onto forage where they are eaten by grazing sheep. Within the gastrointestinal tract, the L3 casts off its sheath and moults to a fourth stage larva (L4), which undergoes a final moult to an immature adult (L5). L4s and adults are parasitic and feed on the sheep. After maturing, adults reproduce, and females begin laying eggs. The prepatent period is approximately two to three weeks (University of Guelph 2012).

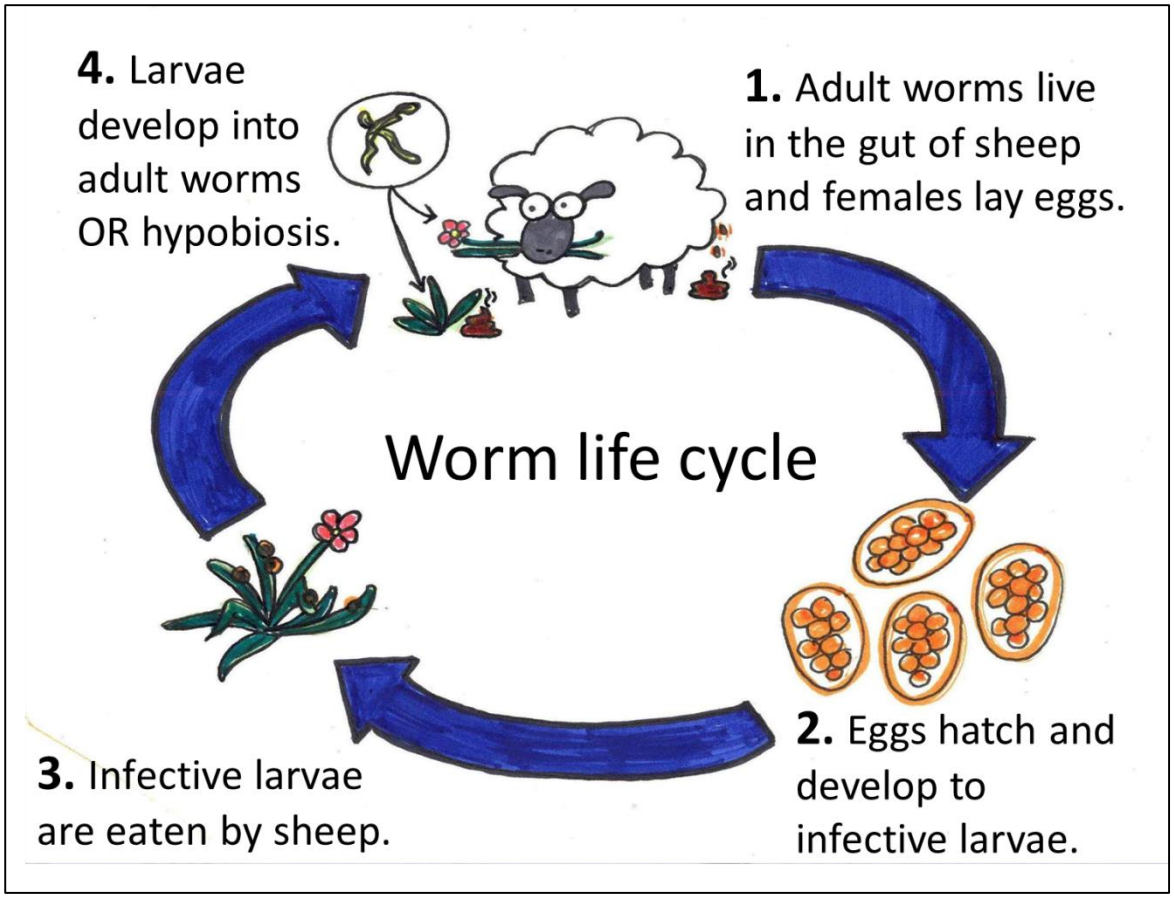

Figure 1. Life cycle of gastrointestinal nematode parasites of sheep. Image modified from: R. Betts 2012.

H. contortus is a sub-tropical species that has expanded its range to temperate climates (Waller and Chandrawathani 2005). This species is a relatively new problem in Nova Scotia. It was not identified in a previous study of GIN infections in 1997 (Maal-Bared 1998), but it is now ubiquitous on sheep farms (Jones 2013). Likewise, *H. contortus* has become prevalent in Central Canada (Mederos et al. 2010). *H. contortus* is the most pathogenic species of GIN in sheep, and unlike other species, even healthy adult sheep are at risk of illness from *H. contortus* in the summer because enormous numbers of L3s on pasture can overwhelm the sheep's acquired immunity (Abbott et al. 2012). *H. contortus* has a tooth to puncture the abomasal mucosa, and it feeds on blood (Angulo-

Cubillán et al. 2007). Each worm can consume 0.05mL of blood per day, and a burden of 500 worms can cause clinical illness. As a prolific breeder, each generation of *H. contortus* creates a huge effective population size and vast genetic diversity on which anthelmintic selection can act. Based on the mutation rate of *Caenorhabditis elegans*, 500 adult female *H. contortus* could produce enough offspring, per day, for a new mutation to occur at every nucleotide in the nuclear genome (Gilleard 2013). As a consequence of the high egg output, and the fact that larvae overwinter only in sheep and not on pasture, *H. contortus* can rapidly develop resistance to anthelmintics (Waller et al. 2004; Groz et al. 2013).

N. battus has a slightly different life cycle and epidemiology from most GIN. *N. battus* is an Arctic species (Hoberg 2005) that develops to a L3 inside the egg before hatching in the following spring, and disease transmission is from one year's batch of lambs to the next (Winter 2002). Typically, hatching is triggered by an increase in soil temperatures over 11°C in the spring, after eggs have overwintered on pasture (van Dijk and Morgan 2010). In the United Kingdom, a rapid increase in temperatures leads to mass hatches (National Animal Disease Information Service 2014). Lambs become infected, but develop immunity within a few weeks and expel adult worms (Israf et al. 1997). Immunity is age related, with older sheep maintaining their immunity and preventing the establishment of *N. battus* larvae. In contrast to other species, egg-laying adults do not cause significant disease. The emergence of immature adult worms from the intestinal mucosa causes damage to this tissue, resulting in lack of appetite and diarrhea, prior to the beginning of egg laying (Abbott et al. 2012). Hence, severe nematodirosis can occur before eggs are detected in the feces. *N. battus* continues to be a severe problem in

the United Kingdom (Morgan & van Dijk 2012), but this parasite is not considered prevalent in central Canada (University of Guelph 2012), and although it is prevalent in Nova Scotia, *N. battus* does not seem to cause significant health problems (Jones 2013). The long generation time and low egg output of *N. battus* are thought to be the reasons why AR has rarely been documented in this species, despite decades of exposure to anthelmintics (Mitchell et al. 2011).

1.2 Seasonal patterns

Pastures are considered contaminated when GIN eggs and/or free living larvae are present. Pastures are considered infective when L3s are present and environmental conditions are suitable for disease transmission (Abbott et al. 2012). In the spring, peri-parturient ewes are the main source of pasture contamination. Ewe immunity wanes around lambing, and hypobiotic larvae resume development, which leads to an increase in egg shedding known as the peri-parturient egg rise (Blitz and Gibbs 1972; Beasly et al. 2010; Falzon et al. 2013a). In the summer and fall, lambs are the main source of pasture contamination. Larval development on pasture is temperature (Reynecke et al. 2012) and moisture dependent, with higher temperatures and moist conditions accelerating development (Morgan and van Dijk 2012). Eggs laid early in the spring, when temperatures are lower, can take 10-12 weeks to develop to L3s. Eggs laid later in the year, when temperatures are higher, can develop to L3s in one to two weeks (Abbott et al. 2012). Under optimal conditions, *H. contortus* can become infective in just five days (University of Guelph 2012). Warm and dry periods reduce the survival of eggs and early stage larvae, but not L3s (Eysker et al. 2005), and for dry periods of about a week, fecal

pellets can act as a moist reservoir for larvae (Wang et al. 2014). Pastures can go from highly contaminated to highly infective over a few weeks, and pasture contamination and infectivity peak in late summer (Mederos et al. 2010). Lambs grazing on contaminated pasture can be overwhelmed by high larval challenge, and they can become severely affected quickly.

In temperate climates such as in Canada, there are two possible ways by which infective larvae can survive the winter when environmental conditions are not favourable for the completion of the GIN life cycle: they can survive freezing temperatures on pasture, or inside the sheep as hypobiotic larvae. Hypobiosis is an environmentally induced state of arrested development, similar to diapause in insects (Sommerville and Davey 2002). Overwinter survival of *H. contortus* L3s on pasture is minimal in cold winter climates (Waller et al. 2004; Troell et al. 2005; Groz et al. 2013), so that survival from one grazing season to the next occurs primarily as hypobiotic larvae inside sheep. By contrast, *T. circumcincta*, *Trichostrongylus spp.*, and *N. battus* all overwinter as both hypobiotic larvae and on pasture (Smith & Fulton 1989; Thomas 1991; Waller et al. 2004). Thus, for these species, a large pool of L3s remains on pasture at the beginning of the next grazing season and can infect sheep at turn-out.

GIN larvae that undergo hypobiosis halt development as early-stage L4s, inside the gastrointestinal mucosa (Sommerville and Davey 2002). The mechanisms for how hypobiosis is initiated, sustained, and terminated are not well understood. Environmental conditions such as temperature and photoperiod, as well as host immunity and magnitude of worm burden have all been implicated as factors involved. A Canadian study on out-

of-season lambing concluded that environmental and host immunological factors contribute to the entry and exit of *H. contortus* from hypobiosis (Falzon et al. 2013a). Typically, hypobiosis begins in late autumn or early winter, although it is unknown when hypobiosis begins in Canada, and there is probably regional variation in the onset and extent of hypobiosis, as has been observed in other countries with temperate climates (Capitini et al. 1990; Miller et al. 1998).

Under field conditions, GIN infections involve more than one species, but the contribution of different species to infections in lambs varies over the grazing season. Early in the season, infections are from species that overwintered on pasture. Infection levels then rise over the summer, as large numbers of eggs shed by peri-parturient ewes develop to L3s. *H. contortus* becomes the biggest contributor to worm burdens in mid to late summer (Miller et al. 1998; Jones 2013). In late summer and early fall *H. contortus* still predominates, but *T. circumcincta* and *Trichongylus spp.* begin to contribute more to worm burdens. These two genera produce far fewer eggs, relative to *H. contortus*, so it takes longer for sheep to pick up significant numbers of L3s. In Canada, GIN infections in lambs peak from August to October, and pasture management strategies and weather patterns influence the timing of the peak from year to year (Maal-Bared 1997; Mederos et al. 2010; Betts 2014).

1.3 Monitoring infections

Pasture raised adult sheep have acquired immunity to GIN and are usually able to manage their worm burdens well, without treatment. However, when immunity wanes, around parturition and lactation, some ewes may need treatment (Falzon et al. 2014a).

Immunity is never 100%, and continued exposure to GIN is needed to maintain immunity. In their first grazing season, it takes lambs or sheep four to six months to develop a good immune response to GIN, and anthelmintic treatment is often necessary to restore health and to ensure the survival of these animals (Abbott et al. 2012). Subclinical infections of GIN cause reduced weight gains and clinical infections result in lack of appetite, weight loss, diarrhea, sub-mandibular oedema (bottle-jaw), anemia, and in severe cases, death. Diarrhea is a common sign of GIN infection, but *H. contortus* causes anemia and constipation, which can mask this sign even when other species are abundant (University of Guelph 2012).

Clinical symptoms can be subjectively assessed with Body Condition scores (fatness), Dag scores (fecal soiling of hindquarters), and FAMACHA© scores (Abbott et al. 2012). Named after its creator, Dr. Faffa Malan (**FA**ffa **Ma**lan **CHA**rt), the FAMACHA© system was designed to monitor the anemia caused by *H. contortus* (van Wyk and Bath 2002; Kaplan et al. 2004). With this system, anemia is ranked on a five point scale based on the pinkness of the lower eyelid mucosa by comparison with a simple color chart. A score of four or five indicates serious anemia, which requires treatment. Subjective monitoring methods can be conducted quickly, on the farm, and with minimal training farmers can use the FAMACHA© system to correctly identify sheep suffering from haemonchosis (Burke et al. 2007; Maia et al. 2014). Yet, on their own, these methods are not reliable predictors of total GIN burden because there are many causes for diarrhea and weight loss, and anemia is an indicator of infections with only *H. contortus* (Mederos et al. 2014; Williams and Palmer 2012). To make treatment decisions, subjective methods need to be used in conjunction with fecal egg count(s) (FEC).

FEC are expressed as eggs per gram of feces (epg), and they are the most common method by which to gauge GIN burdens. Briefly, to calculate FEC, feces are mixed with a flotation solution and a small volume of this mixture is transferred to the two chambers of a McMaster counting slide (Ballweber et al. 2014). Eggs float to the top of the chambers and debris sinks to the bottom. Both chambers are etched with a grid. Eggs within the grids are counted, and the sum is multiplied by the dilution factor to give the epg. Whether a FEC is considered high depends on the species contributing to the count. According to Abbott et al's. (2012) guide for interpreting FEC, pathogenic levels of infection may be indicated by 500epg of *N. battus* and 1500epg in mixed infections, but 5000epg or more if *H. contortus* is the predominant species (Table A1).

With an adequate number of samples, mean FEC reflect the overall pattern of infection within a flock. At least 10 samples are essential because GIN infections are over-dispersed, with a few sheep having high worm burdens and the majority of sheep having low worm burdens (Morgan et al. 2005). Due to this pattern, too few samples are likely to underestimate the FEC in highly parasitized sheep. These results could incorrectly indicate that sheep do not need treatment, when in fact they do. Based on FEC, decisions can be made to treat the whole flock, or a portion of the flock. Normally, lambs have higher mean FEC than those of older sheep, so only lambs may need treatment. In Nova Scotia, few farms use FEC to monitor GIN infections – cost is the main inhibiting factor. For a registered farm each FEC costs around $6, and for an unregistered farm each FEC costs about $13 through the Department of Agriculture or as much as $20 through a veterinarian (Jones, personal communication 2014).

1.4 Anthelmintic resistance

Anthelmintic resistance (AR) is defined as the heritable loss of sensitivity in a worm population to a drug that was previously effective (Köhler 2001). AR is inevitable with repeated use of a drug that fails to kill the entire population of GIN in treated animals. In every GIN population there are a few individuals that carry alleles for resistance to drug treatment (Gilleard and Beech 2007). After treatment, these resistant individuals have a reproductive advantage, as it takes two to three weeks for newly picked up L3s to develop into egg-laying adults. Resistance builds up insidiously, and during this period there is still a clinical response to anthelmintics. As resistance increases further, sheep weight gains may be reduced and farmers may need to treat sheep within shorter intervals (Sargison et al. 2007). By the time it becomes obvious that a drug is ineffective the majority of the GIN population are resistant. At this point, a new drug is needed to manage infections.

AR has been the impetus for the development of new drugs with different modes of action, but this is a slow process that cannot keep pace with the development of AR. In the United Kingdom and New Zealand, six drug groups are licenced for use in sheep, and two of these drug groups were introduced there in the past four years (Abbott et al. 2012) (Table A2). But the first cases of resistance to one of these new groups, the amino-acetonitrile derivatives, have already been reported in New Zealand (Scott et al. 2013), and in the Netherlands (Van den Brom et al. 2015). Only one anthelmintic, ivermectin, is licenced for use in sheep in Canada (University of Guelph 2012). It is unlikely that new anthelmintics will be introduced to Canada in the near future because the sheep industry

is small and therefore not highly profitable. Sheep are considered a minor species, with about 12 000 breeding ewes in Nova Scotia and about 1 000 000 sheep in all of Canada (Jones, personal communication 2014). In Atlantic Canada, off label use of benzimidazoles is common, and there is little information on AR (Jones 2013). AR is widespread in Ontario and Quebec, and a recent study found resistance to ivermectin, fenbendazole, and levamisole on 97%, 95%, and 6% of farms tested, respectively (Falzon et al. 2013b). In Ontario, fenbendazole resistance of *H. contortus* was found on all 11 farms tested (Barrere et al. 2013).

Fecal egg count reduction tests (FECRTs) are the most popular and practical method for identifying AR (Coles et al. 1992). Through FECRTs, resistance can be identified before it is obvious and while the drug is still effective enough to be useful. This test involves collecting fecal samples from sheep before and after treatment and calculating the percent reduction in mean FEC (Ministry of Agriculture, Fisheries and Food [MAFF] 1986). When a drug is highly effective, treatment reduces mean FEC by ≥95%, and the lower limit of the 95% confidence interval is ≥90%. Resistance is confirmed if both criteria are not met, and if one criterion is not met resistance is suspected (Falzon et al. 2014b). Larval cultures can be used in conjunction with FECRTs to identify the genera or species that are resistant, but this procedure is time consuming and technically demanding (Roeber and Kahn 2014). Abbott et al. (2012) recommend that FECRTs be conducted every two to three years, but, as with FEC monitoring, most farmers consider the cost of testing prohibitive (Jones, personal communication 2014). A FECRT potentially costs as much as $865 through a veterinarian (Barrere et al. 2013). The reluctance to invest in FECRTs is short-sighted and expensive, for AR has been estimated to cost 10-15% of the

carcass value, which far exceeds the cost of routine testing (Sutherland et al. 2010; Miller et al. 2012).

As long as anthelmintics are in use AR cannot be prevented, but, through implementing sustainable integrated parasite management practices, the development of AR can be slowed and the useful lifetime of a drug can be maximized. With regard to anthelmintic treatment these practices include: using anthelmintics appropriately, monitoring and treating animals selectively, following quarantine protocols for animals that are new to the farm, and investigating treatment failure (University of Guelph 2012). Non-drug management practices are an important component of sustainable integrated parasite management. These practices reduce the need for anthelmintic treatment and include: pasture rotation, grazing sheep grouped by age, nutritional supplementation, and providing bioactive forages (Abbott et al. 2012).

Pasture rotation involves grazing sheep on a pasture for a short period of time followed by a move to another pasture. In this way, pasture contamination can be kept to low levels. Thus, sheep face a slower rate of larval challenge and have lower infection levels. Ideally, sheep do not return to previously grazed pasture in the same season because L3s can survive for several months on pasture in warm and moist conditions (University of Guelph 2012). *H. contortus* comes only from ewes, so grazing lambs separately from ewes can limit infections with this species (Abbott et al. 2012). Nutritional supplementation with by-pass protein such as soy meal can enhance the immunity of adult sheep (Houdijk 2012), and bioactive forages grown on pasture such as chicory and sulla have anthelmintic properties (Ramírez-Restrepo and Barry 2005).

Maintaining adequate refugia has been recognized as one of the most important factors in slowing the development of AR (Barnes et al. 1995; Besier 2012; Leathwick et al. 2012; Leathwick and Besier 2014). Refugium refers to the portion of the GIN population that has not recently been exposed to a drug. This includes GIN in untreated sheep as well as those on pasture (Kenyon et al. 2009). Susceptible genotypes in refugia act to dilute resistant genotypes that survive treatment. Refugia can be maintained by not treating whole flocks, especially in the fall and in the winter at lambing. Treatment of sheep in the fall after they have been taken off pasture selects for AR because susceptible GIN are killed, but sheep can no longer pick up susceptible L3s (Leathwick and Besier 2014; Falzon et al. 2013c). Treatment of ewes around lambing further selects for AR, for only resistant GIN remain to contaminate pastures at turn-out (Leathwick et al. 2006). Since *H. contortus* overwinters only in sheep in cold winter climates, fall and winter treatments can rapidly eliminate the susceptible population. Thus, with regard to *H. contortus*, whole flock treatments in the fall and at lambing are probably the biggest contributors to the development of AR (Le Jambre et al. 1999).

To date, there is limited data on management practices on sheep farms in Canada (Falzon et al. 2013c), and only anecdotal evidence for the Atlantic Provinces. Falzon et al. (2013c) determined that in Ontario, mean treatment frequency was 2.6 times per year, and treatment was usually administered to the whole flock, on a set schedule, in addition to being based on symptoms. Contrary to current recommendations to treat incoming sheep with two anthelmintic groups (Abbott et al. 2012; University of Guelph), no drug, or only one drug group, was used on quarantined sheep. Furthermore, almost half of the farms treated all sheep around the time they were taken off pasture in the fall, and around

lambing. Thus, not only is AR widespread in Ontario, current farming practices favor the development and acceleration of AR.

Anecdotal evidence from Atlantic Canada is consistent with the findings in Ontario. In Nova Scotia in 2012 and 2013, many farms reported lamb and sheep deaths due to GIN, and some farms have resorted to raising animals indoors to circumvent unmanageable GIN infections (Jones 2013). Treating whole flocks in the fall, and all ewes at lambing in the winter, is common (Jones, personal communication 2014). Over the past three summers (2012-2014), our research group has identified *H. contortus* as the most problematic GIN species in Nova Scotia. On the basis of these findings, closantel, a narrow spectrum drug that targets *H. contortus*, has been approved for veterinary access through the Emergency Drug Release process. AR of GIN, in particular *H. contortus*, poses a serious threat to the sustainability of sheep production in Nova Scotia. Hence, it is essential to determine the efficacy of available anthelmintics in order to extend the use of these drugs for as long as possible and to emphasise the need for alternative non-drug management options (sustainable integrated parasite management). The main objectives of this study are to document AR and to identify the genera of infective larvae that survive anthelmintic treatment. Additionally, the utility of administering closantel alone or in combination with a drug to which *H. contortus* is resistant, and a non-drug management strategy to limit infections of *H. contortus* will be examined.

Materials and Methods

This study was part of a larger project, involving collaboration between Saint Mary's University and the Dalhousie Agricultural College, which examined the epidemiology of *H. contortus* (Jones 2013), and options for integrated parasite management on sheep farms in Nova Scotia. The research team at Saint Mary's University: Kathleen Hipwell, Rebecca Betts, Danielle Thibault, Natasha Osborne, Tanya Lawlor, and Julie Poirier, analyzed approximately 2450 fecal samples in 2013, and 2540 samples in 2014. Overall, analysis of about half of the samples, and approximately 75% of the FECRT samples, as well as all larval identification, was performed by Kathleen Hipwell. All researchers were trained to perform FEC. Their technique was verified through the acceptable variation in counts among multiple (~15) subsamples from a single fecal solution (Torgerson et al. 2012), and consistency of FEC of the same McMaster slide was confirmed among researchers.

2.1 a. Sentinel farm characteristics and FEC monitoring

This study was conducted in Nova Scotia, Canada, with one farm contribution from Prince Edward Island, over two consecutive grazing seasons (May to November 2013 and 2014). Nant y Mor farm (Farm 1), in Noel Shore, Nova Scotia (45°18'47" N, 63°37'55" W) acted as the sentinel farm for the duration of the study (Figure 1). Nant y Mor has well-kept records for the past 20 years, and pasture management and anthelmintic treatment of sheep has remained consistent since 1997. The Nant y Mor flock consists of approximately 100 purebred Clun Forest sheep from five genetic lines, as well as a few crossbreds. New sheep are rarely added to the flock, and incoming

animals are quarantined according to the protocol of Abbott et al. (2012), to prevent the introduction of AR to the farm. From May to November 2013-2014, Nant-y-Mor provided fecal samples from at least 15 lambs, on approximately a bi-weekly basis, and the arithmetic mean FEC±SE was used to monitor GIN infection levels in the lamb flock. Prevalence (percentage of lambs infected), intensity (arithmetic mean FEC of infected lambs), and range (minimum and maximum FEC) were also calculated for each group of fecal samples. *N. battus* FEC were recorded for interest.

2.1 b.FEC monitoring of peri-parturient ewes

From late April to early July in 2013, and from late April to early June in 2014, Nant y Mor provided fecal samples from peri-parturient ewes to give an indication of peri-parturient egg shedding. In 2013 and 2014, four and five other farms, respectively, submitted ewe samples at or around lambing (April-June). Most farms provided one batch of samples. Arithemetic mean FEC±SE, prevalence (percentage of ewes infected), intensity (arithmetic mean FEC of infected ewes), and range (minimum and maximum FEC) were calculated for each group of fecal samples.

2.2 Farm selection and anthelmintics used in FECRTs

Based on their willingness to participate, sheep producers for FECRTs were recruited by the project leader, Dr. Gwyneth Jones. All farms were located around 45-46° N, 63° W (Fig. 2). To be included in the study, farms had to have at least 20 sheep (lambs or sheep up to three years old) that were raised on pasture. FECRTs were conducted on

nine farms in 2013 (Farms 1-9, Table 1) and eight farms in 2014 (Farms 1, 3, 5, 6, and 10-13, Table 2).

Figure 2. FECRT sites in Nova Scotia and Prince Edward Island. = location of Nat-y-Mor Farm, = two farms, = one farm. (Image modified from: http://www.wtcchalifax.com/site-tcl/media/wtcchalifax/Nova%20Scotia%20Map.jpg)

Anthelmintic dosages were as per the manufacturer's instructions or the recommended dose of levamisole (University of Guelph 2012), and were as follows: 0.2mg/kg ivermectin (Ivomec® Drench for Sheep, Merial Canada Incorporated); 0.2mg/kg moxidectin (Cydectin®, Zoetis); 5mg/kg fenbendazole (Safe-Guard®

Suspension 10%, Intervet Canada Limited; Panacur®, Intervet Canada Limited); 5mg/kg albendazole (Valbazen®, Pfizer Animal Health); 10mg/kg closantel (Flukiver®, Jansen Animal Health); and 7.5mg/kg levamisole (Chiron Compounding Pharmacy Incorporated). Dosages were calculated based on individual sheep weight on Farms 1, 2, 4, 5, and 8-13, and by estimating the weight of the heaviest sheep on Farms 3, 6, and 7.

All treatments were administered by drench (deposited into the rumen), with a recently calibrated drench gun. On Farms 1, 2, 4-6, and 10-13, anthelmintics were administered by Dr. E. Semple, Dr. G. Jones, or D. Thibault. Farms 3 and 7-9 treated their own sheep. Five anthelmintics were tested in the Nant y Mor flock. Most producers tested one or two anthelmintic(s), using their choice of treatment (Tables 1 and 2). Due to flock size constraints or concerns about severe parasitism, Farms 5-7, 9-11, and 13 did not use a control group.

Table 1. Farm identification and anthelmintic(s) tested with FECRT in 2013.

Farm	Drug(s) tested	Dosage calculated by	Control group
1	Closantel, levamisole, albendazole, ivermectin	Individual weight	+
2	Closantel, levamisole, ivermectin	Individual weight	+
3	Levamisole, ivermectin	Estimating heaviest weight	+
4	Levamisole	Individual weight	+
5	Levamisole, albendazole	Individual weight	-
6	Albendazole, pyrantel	Estimating heaviest weight	-
7	Ivermectin	Estimating heaviest weight	-
8	Albendazole, ivermectin	Individual weight	+
9	Ivermectin	Individual weight	-

Table 2. Farm identification and anthelmintic(s) tested with FECRT in 2014.

Farm	Drug(s) tested	Dosage calculated by	Control group
1	1st: Closantel, levamisole, ivermectin 2nd: Closantel, albendazole, moxidectin 3rd: Closantel, closantel+albendazole	Individual weight	+
3	Closantel, levamisole, albendazole, ivermectin	Estimating heaviest weight	+
5	Closantel, levamisole	Individual weight	-
6	Levamisole, albendazole	Estimating heaviest weight	+
10	Fenbendazole	Individual weight	-
11	Levamisole	Individual weight	-
12	Levamisole, albendazole	Individual weight	+
13	Closantel	Individual weight	-

2.3 FECRTs and FEC

With the exception of Farms 2, 3, and 11, lambs were used in FECRTs, as recommended by Coles et al. (1992). Sheep were randomly assigned to treatment or control groups, and where possible at least n=10 per group were used. To identify which group that sheep belonged to they were marked with livestock marker on their head, neck, back, or rump. Fecal samples were collected at the time of treatment, 7-10 days post-treatment for levamisole, and 14 days post-treatment for all other anthelmintics (Coles et al. 2006). One month post-treatment samples were collected for the closantel, levamisole, and control groups on Farm 1. The majority of samples were collected by Dr. G. Jones and D. Thibault. Farms 4 and 6-8 collected their own samples. Feces were collected rectally or off the ground in disposable gloves, and the gloves were labeled with ear tag numbers to identify the sheep. Following collection, samples were kept at ~4°C in

coolers with ice packs and transported to Saint Mary's University, in Halifax, Nova Scotia, where they were refrigerated at 4°C until analysis. As recommended by McKenna (1998), samples for culture were refrigerated for less than 24 hours.

FEC were conducted at the Department of Biology, Saint Mary's University. Most samples were analyzed within two weeks of collection, but due to a backlog of samples in 2013, some were stored for up to three weeks. Individual FEC were determined using a modified McMaster technique, with a detection limit of 50epg (MAFF 1986; Coles et al. 1992). A saturated sodium chloride floatation solution (400g/L), with a specific gravity of 1.18-1.20 (Gibbons et al. 2012) was used. Three grams of feces (±0.1g) were weighed (Ohaus Scout® *Pro* SP202, USA) and suspended in 45mL of floatation solution. Smaller samples, weighing at least 1.0g, were suspended in an appropriate volume of solution (volume = weight x 15). The suspension was passed through a tea-strainer to remove large pieces of debris.

After thoroughly stirring the filtered suspension, two sub-samples were quickly transferred by pipette to the two chambers of a McMaster counting slide (Chalex Corporation, USA) (Fig. B1). Slides were left for at least five minutes before counting, to allow eggs to float to the top of the chambers, and GIN eggs within the grids etched on the McMaster slide were observed with a compound microscope (Nikon Eclipse E200, Japan), at 100x magnification (Fig. B2). Eggs within both grids were counted, and the sum was multiplied by 50 to give the epg. The eggs of most species of strongyloidea and trichostrongyloidea look very similar, so they were collectively counted as GIN eggs (Fig. B3). *N. battus* eggs were counted separately, as they could be differentiated. Since

there is no AR in *N. battus*, their eggs acted as a control because if *N. battus* eggs were present after treatment, treatment failure may not have been due to AR. *N. fillicolis*, *S. papillosus*, and *T. ovis* eggs were not counted. Photographs of GIN eggs commonly found in sheep feces, and the table used to record FEC, are presented in Appendix B (Fig. B4-9, Table B1).

2.4 L3 culture and identification

Post-treatment fecal samples were cultured to identify the genera or species of L3s that survived anthelmintic treatment. In 2013, larval cultures were performed for Farms 1, 2, and 6. In 2014, larval cultures were performed for Farm 1. Individual fecal samples were cultured in their collection gloves, at room temperature, for at least 14 days. Larval identification was performed on most fecal cultures within 4 weeks, but one culture was seven weeks old. Cultured samples were pooled by treatment group, and L3s were collected using the Baermann technique (Gibbons et al. 2012). With an inverted microscope (Olympus Tokyo CK, Japan), the presence of L3s was determined by scanning a Petri dish containing a few mL of water drained from the Baermann funnel. A drop of this liquid was pipetted onto a microscope slide, and L3s were killed and stained with a drop of Lugol's iodine. The first 100 L3s were identified, and if there were few larvae at least 50 slides were examined to find as many L3s as possible.

L3s were identified at 400x magnification, with a compound microscope set on Ph2 (Nikon Eclipse E200, Japan). Morphological identification of L3s was based primarily on head-shape and tail-sheath-length by comparison with two keys (van Wyk et al. 2004; Gibbons et al. 2012). Gut cell shape and number were recorded when they were

visible. Using a calibrated ocular micrometer, sheath-length was measured to one decimal place using an 'X'-system, where each large division of the micrometer equalled 1X (25μm) (van Wyk et al. 2004). Due to the overlapping sheath-lengths of several species, L3s were categorized based on lengths of: <2.0X, 2.0-4.0X, 4.1-7.5X, >7.5X , and for the last two categories, filament length (solid section of sheath) was also measured (Table 3). For each treatment and/or control group, data were expressed as the percent and number of L3s in each category. To document the genera and species identified, photographs of L3s were taken with an Infinity*lite* camera (Lumen*era*® Corporation, Canada) mounted on an Olympus CX41 compound microscope (Olympus Corporation, Japan). Photographs of L3 morphological characteristics, and the table used to record L3 data, are presented in Appendix B (Fig. B10-13, Table B2).

Table 3. Classification system used to identify genera or species of GIN, based on L3 characteristics (van Wyk et al. 2004; Gibbons et al. 2012).

Genera/species group	**Sheath length (X-value)**	**Filament length (X-value)**	**Head shape**	**Gut cell number**	**Gut cell shape**
T. circumcincta, *Trichostrongylus spp.*	<2.0	No filament	Square	16	Triangular
H. contortus *Cooperia spp*.	2.0-4.0	No filament	Round	16	Triangular
Bunostomum spp. *Oesophagostomum spp.* *C.ovina*	4.1-7.5	1.0-2.5	Broad round or square	16 18-22 28-32	Triangular Rectangular
N. battus	>7.5	>2.5	Broad square	8	Triangular

2.5 Statistical analysis

Data were entered into Excel spreadsheets, and Microsoft Office Excel© 2010 was used to calculate FECRs. As yet, there is no standardized method for calculating FECRs, and the various methods give inconsistent results (Calvete and Uriarte 2013; Falzon et al. 2014b). For this reason, several common formulae were used and the results were compared. The criterion for resistance was a <95% reduction in FEC. Percent efficacies were rounded to the nearest percent, but in the case of efficacies falling between 99.5% and 99.9%, efficacies were rounded down. Anthelmintic efficacy based on arithmetic group mean FEC was calculated with four formulae:

- FECRT1 = 100 x (1 – T2/C2) (Coles et al. 1992)
- FECRT2 = 100 x (1 – T2/C1) (McKenna 2006)
- FECRT3 = 100 x (1 – T2/T1) (Kochapakee et al. 1995)
- FECRT4 = 100 x (1 - T2/T1 x C1/C2) (Dash et al. 1988)

Where:

- T1 = pre-treatment mean of the treatment group
- T2 = post-treatment mean of the treatment group
- C1 = pre-treatment mean of the control group
- C2 = post-treatment mean of the control group

Anthelmintic efficacy based on individual FEC was calculated with two formulae:

- iFECRT3 = (1/n) Σ (100 x (1 – Ti2/Ti1)) (Cabaret and Berrag 2004) – individually based equivalent of FECR3

- iFECRT4 = (1/n) Σ (100 x (1 – Ti2/Ti1 x Cj1/Cj2)) (Caberet and Berrag 2004) - individually based equivalent of FECR4

Where:

- Ti1 = pre-treatment FEC of individual i from n hosts in the treatment group
- Ti2= post-treatment FEC of individual i from n hosts in the treatment group
- Cj1 = pre-treatment FEC of individual j from n hosts in the control group
- Cj2 = post-treatment FEC of individual j from n hosts in the control group

2.6 Grazing management to reduce treatment frequency

At the Dalhousie Agricultural College in Bible Hill, Nova Scotia, a grazing management strategy to limit infections with *H. contortus* was investigated with the campus sheep flock (Farm 8). Over the grazing season of 2014, 10 lambs were grazed with 10 ewes, and 25 lambs were grazed without ewes. Ewes had lambed in mid-March and lambs were weaned before turnout on June 1 (Hines, personal communication 2014). On an approximately bi-weekly basis, the Dalhousie Agricultural College provided lamb and ewe samples from the lambs with ewes group, and lamb samples from the only lambs group. Arithmetic mean FEC±SE was used to monitor GIN infection levels in the ewes and each group of lambs. Prevelance (percent of and lambs or ewes infected), intensity (arithmetic mean FEC of infected lambs or ewes), and range (minimum and maximum FEC of lambs or ewes) were also calculated for each group of fecal samples. To identify the genera or species of L3s contributing to GIN infections in the only lambs group, a larval culture was conducted near the end of the grazing season.

Results

Data are expressed as arithmetic mean FEC in eggs per gram feces (epg). Range of FEC and the number of sheep sampled are given in parenthesis (range, n), and prevalence is given as a percentage. FEC from treated and untreated sheep were combined for FEC monitoring *(3.1a-d*).

3.1 a. Sentinel farm FEC monitoring

Figure 3 shows a comparison of the peak of infections to the end of the grazing seasons in 2012-2014 with previous work conducted in 1997 (Maal-Bared 1998). There has been a drastic increase in mean FEC of lambs, and this increase has been consistent for the last three years 2012-2014. Also the peaks in infections came earlier and lasted longer than in 1997.

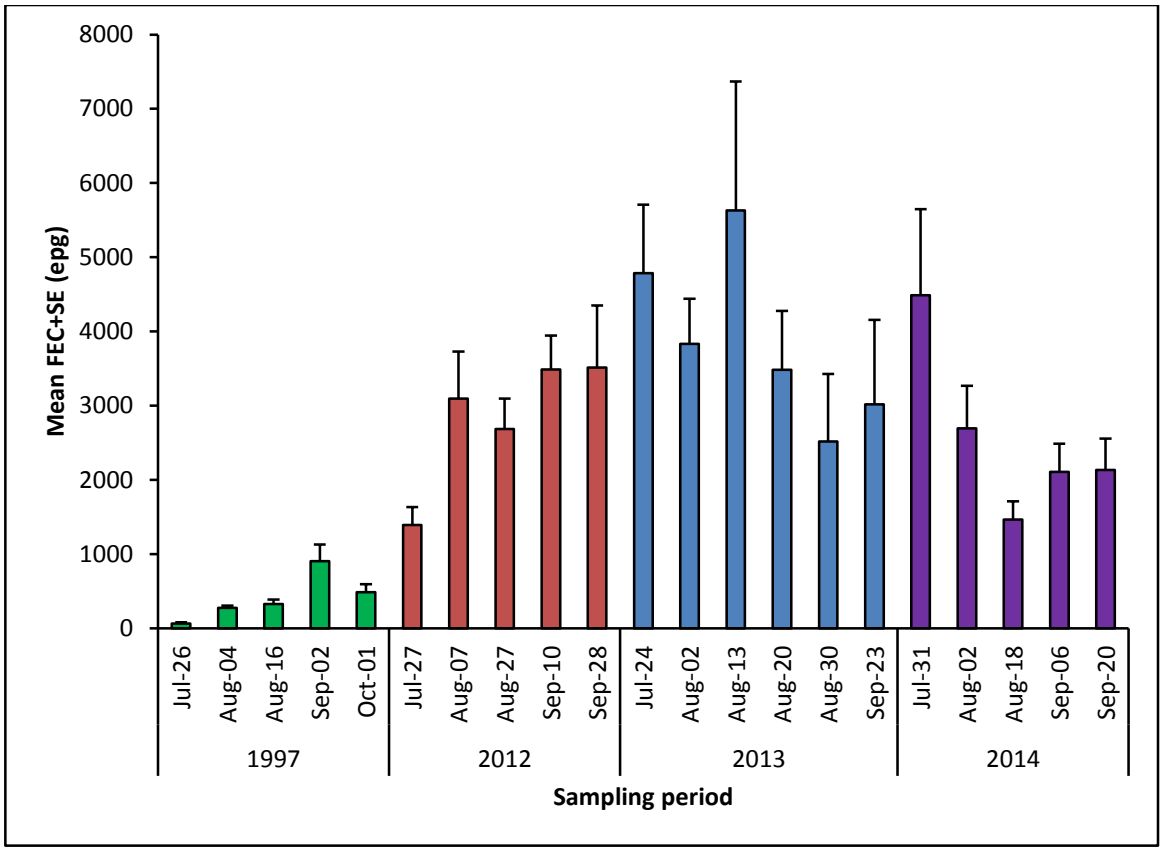

Figure 3. Seasonal patterns of GIN infections in the lamb flock on Farm 1, over the grazing seasons of 1997 and 2012-2014.

The seasonal patterns of GIN FEC in lambs throughout the grazing seasons, over the course of the study, are presented in Figure 4. Due to lack of feed, lambs were turned out on April 30 in 2013 and GIN eggs first appeared in the feces of one lamb on May 20 (0-100, n=9) (Fig. 4). FEC remained low in June, but prevalence increased to 56%. Mean FEC increased rapidly in July, exceeding 1000epg (0-7000, n=26) by July 4, and reaching almost 5000epg (0-31 700, n=31) by July 24. At that time, nine lambs had FEC over 5000epg, and another nine lambs had FEC over 10 000epg. Prevalence reached a seasonal maximum of 98% on July 10. Part of the flock was treated with anthelmintics on July 10, July 24, September 15, and October 15 as part of FECRTs. All lambs received

treatment at least once over the grazing season. Other than FECRT treatments, only lambs with high FEC were given additional treatment. Mean FEC declined from mid to late August (0-14 750, n=20) and rose again in September (0-17 350, n=18). Mean FEC decreased in October (0-16 600, n=32) in response to treatment with closantel, but remained high until the end of the sampling period. From July until the end of the sampling period, prevalence ranged from 75% to 97%, with short term decreases following anthelmintic treatment. Intensity of infections never exceeded mean FEC by more than 2000epg.

Mean FEC followed a similar pattern in 2014, but FEC were lower than in 2013 (Fig. 4). Lambs were turned out on May 12, and GIN eggs first appeared in the feces of seven lambs on June 5 (0-50, n=37). FEC remained low in June, but prevalence increased to 46%. Mean FEC increased rapidly in July, reaching almost 1900epg (25-26 500, n=53) by July 21, and almost 4500epg (700-12 050, n=9) by July 31. Prevalence reached 100% on July 21, and at this time three lambs had FEC over 5000epg. Part of the flock was treated with anthelmintics on July 21, August 2, August 18, and September 20, as part of FECRTs. Other than FECRT treatmens, only lambs with high FEC were given additional treatment, and four lambs were never treated. Mean FEC decreased from July 31 (700-12 050, n=9) to August 18 (0-9150, n=60), but increased again until September 20 (0-8300, n=22). Mean FEC decreased in October (0-5200, 25), but remained high until the end of the sampling period**.** From July 31 until the end of the sampling period, prevalence ranged from 82% to 100%. Intensity of infections never exceeded mean FEC by more than 600epg. In both years of the study (2013-2014) FEC from the whole lamb flock

were included, not just lambs that acted as controls in FECRTs. Had only control lambs been used, none of the FEC would have been 0epg past mid-July.

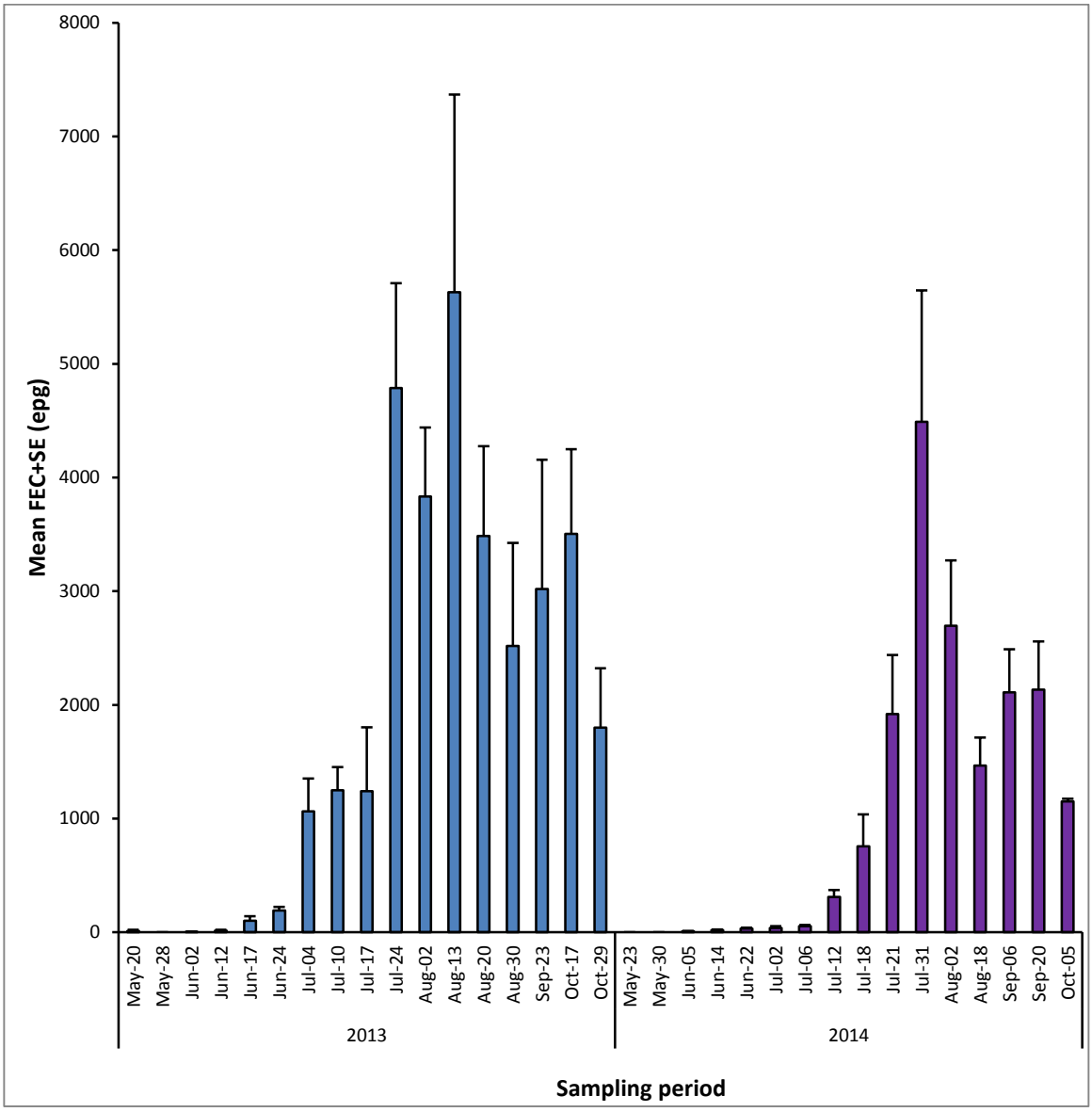

Figure 4. Patterns of GIN infection in the lamb flock on Farm 1, over the grazing seasons of 2013 and 2014.

3.1b. Sentinel farm FEC monitoring of N. battus

The seasonal patterns of *N.battus* FEC are presented in Figure 5. In 2013, *N. battus* eggs first appeared in the feces of lambs on May 20. By May 28, 38% of sampled lambs were infected (0-100, n=13). Mean FEC increased until July 17 (0-250, n=31), decreased steadily to a low of 5 on August 7 (0-200, n=47), and then rose again until the end of the sampling period. From June 2 to July 10, prevalence ranged from 62% to 88%. Following anthelmintic treatment on July 10, as part of a FECRT, prevalence fell sharply to 29% on July 17 (0-250, n=37). Prevalence remained below 22% until the end of the sampling period. Intensity of infections never exceeded mean FEC by more than 209epg

More variability was observed in mean FEC in 2014, but counts were generally higher than in 2013 (Fig. 5). *N. battus* eggs first appeared in the feces of lambs on May 30. Mean FEC rose until June 22 (0-550, n=26), when prevalence reached a maximum of 96%. Following anthelmintic treatment on July 21, as part of a FECRT, prevalence decreased to 40% on August 18 (0-550, n=60). Prevalence remained below 45% until the end of the sampling period. Intensity of infections never exceeded mean FEC by more than 162epg. Throughout 2013 and 2014 no lambs required treatment to manage *N. battus* infections. The prevalence of *N. battus* two weeks post-treatment was due to the controls, with the exception of one lamb (200epg) and two lambs (50epg) in two of the treatment groups.

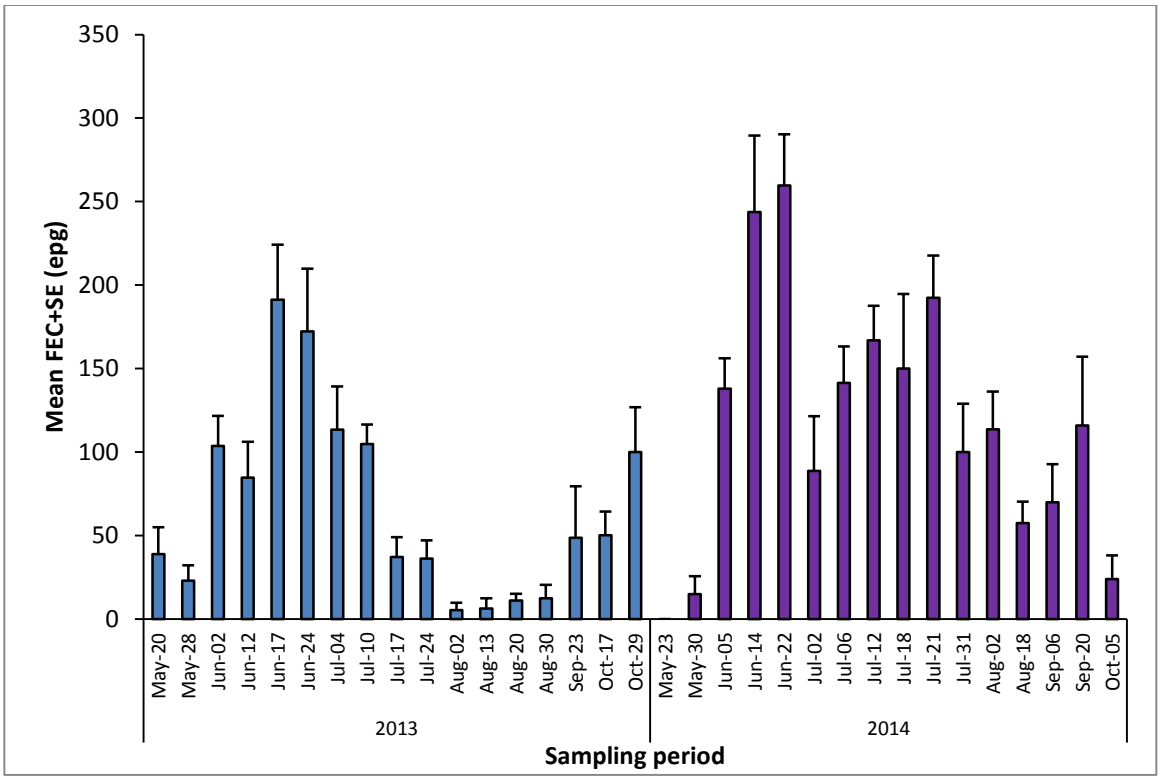

Figure 5. Patterns of *N. battus* infection in the lamb flock on Farm 1, over the grazing seasons of 2013 and 2014.

3.1c. Sentinel farm FEC monitoring of peri-parturient ewes

Figure 6 represents two groups of peri-parturient ewes. The first three samplings were from the late lambing group (late April to early May) before and around lambing (pattern filled columns). All subsequent samplings were from the early lambing group (late February to early March) during late lactation. On Farm 1 in 2013, mean FEC of ewes was low in late April (0-2700, n=8) and early May (0-2650, n=14) (Fig. 6). Mean FEC rose rapidly in mid-May (50-34 250, n=14) and remained high until mid-June (400-11 550, n=8). Prevalence was 100% during this period. By early July prevalence had decreased to 70%, and with the exception of three ewes, with FEC of 12 950, 17 875, and 20 100epg, FEC were well below pathogenic levels.

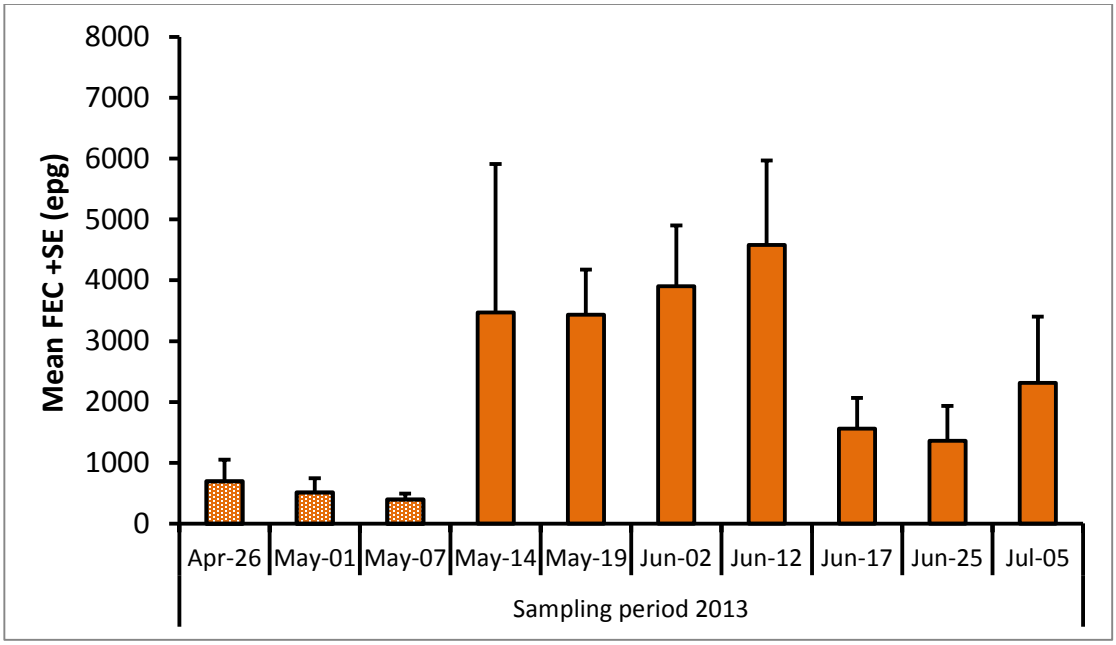

Figure 6. Pattern of GIN infection in ewes on Farm 1, around lambing and lactation in 2013.

Figure 7 represents two groups of peri-parturient ewes. The first sampling was from the early lambing group (late February to early March) during late lactation (pattern filled column). All subsequent samplings were from the late lambing group (late April to early May) before and around lambing. On Farm 1 in 2014, mean FEC of ewes was high when sampling began in late April (100-9700, n=16), and remained high until June 5 (100-11 400, n=7) (Fig. 7). At each sampling, at least three ewes had counts above 5000epg, and several ewes had counts exceeding 8000epg. Prevalence was 100% during this period.

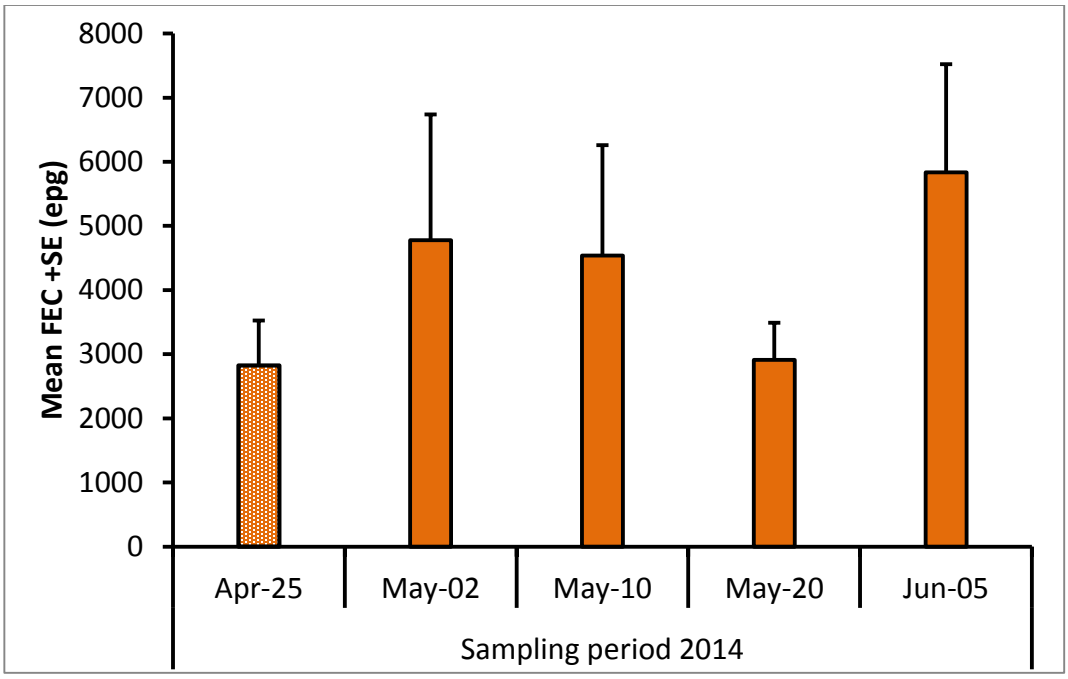

Figure 7. Pattern of GIN infection in ewes on Farm 1, around lambing and lactation in 2014.

3.1d. FEC monitoring of peri-parturient ewes

In May 2013, mean FEC of ewes on Farms 2 and 3 were 1021epg (150-2650, n=12) and 1682epg (0-6000, n=14), respectively. On Farm 3, two ewes had counts over 5000epg. In June, ewes from Farms 3, 5, and 7 had mean FEC of 954epg (0-4950, n=14), 4396epg (100-22 000, n=12), and 972epg (0-2550, n=9), respectively. Three ewes on Farm 5 had FEC over 5000epg.

On Farm 3 in 2014, from May 20 to May 29, mean FEC in ewes increased from 1169epg (0-5000, n=76) to 1588epg (0-14 050, n=64). Most ewes in this flock were treated with anthelmintics on May 14, and mean FEC was 627epg (200-2250, n=11) on

June 3. Around mid-June, mean FEC on Farms 2, 3, 8, 10, and 12 ranged from 1509epg to 5441epg. On each farm at least one ewe had a FEC over 11 000epg.

3.2 FECRTs

The results of the FECRTs in 2013 are shown in Table 4. Resistance to albendazole was found on 3/4 farms. Depending on the formulae used, resistance to ivermectin was found on 6/7 or 7/7 farms. On Farm 1, a double dose of ivermectin improved efficacy, but the status remained resistant. Depending on the formulae used, resistance to levamisole was found on 3/5 or 1/5 farms. Typically, the resistant status resulted from one levamisole treated lamb with a high FEC post-treatment, which may reflect poor dosing technique. Pyrantel also showed resistance or susceptibility. Apparent resistance to closantel was found on 2/2 farms tested, but the resistant status is misleading because closantel kills only *H. contortus*.

The results of the FECRTs in 2014 are shown in Table 5. Resistance to albendazole was found on 4/4 farms tested. A re-test of ivermectin on Farm 3 showed resistance, and all of the percentage reductions were negative. Moxidectin, which is in the same drug group as ivermectin, was effective on Farm 1. Depending on the formulae used, resistance to levamisole was found on 1/6 or 0/6 farms, and resistance to closantel was found on 3/4 or 4/4 farms. As in 2013, the apparent resistant status of closantel is misleading because closantel kills only *H. contortus*. For each of the six formulae, percent reductions for closantel on Farm 1 were higher in the first trial, in July, compared to the second trial, in October. GIN were susceptible to the closantel and albendazole combination on Farm 1.

Table 4. Fecal egg count reduction percent and AR status for nine farms in 2013, calculated using six formulae. GIN were classified as resistant (R) when treatment reduced mean FEC by <95% and susceptible (S) when treatment reduced mean FEC by ≥95%.

Anthelmintic	Farm	FECRT1		FECRT2		FECRT3		iFECRT3		FECRT4		i FECRT4	
albendazole	1	71	R	-311	R	-73	R	-177	R	12	R	43	R
	5	-	-	-	-	-15	R	-	-	-	-	-	-
	6	-	-	-	-	63	R	65	R	-	-	-	-
	8	100	S	100	S	100	S	100	S	100	S	100	S
ivermectin	1	5	R	-1222	R	-488	R	-391	R	-57	R	-1	R
	2	31	R	-15	R	-49	R	11	R	-56	R	38	R
	3	48	R	-20	R	-39	R	-149	R	60	R	-105	R
	7	-	-	-	-	77	R	75	R	-	-	-	-
	8	-46	R	11	R	97	S	95	S	99	S	98	S
	9	-	-	-	-	37	R	26	R	-	-	-	-
2X ivermectin	1	10	R	29	R	77	R	77	R	71	R	81	R
levamisole	1	99	S	99	S	99	S	98	S	99	S	99	S
	2	95	S	89	R	92	R	96	S	97	S	97	S
	3	88	R	81	R	84	R	99	S	91	R	98	S
	4	99	S	99	S	99	S	99	S	99	S	98	S
	5	-	-	-	-	79	R	-	-	-	-	-	-
pyrantel	6	-	-	-	-	97	S	90	R	-	-	-	-
closantel	1	68	R	70	R	72	R	69	R	64	R	22	R
	2	86	R	90	R	92	R	90	R	89	R	89	R

A dash indicates that FECRT calculations were not possible due to the lack of a control group, or an insufficient number of pre- and post-treatment samples from the same animals for iFECRT3.

Table 5. Fecal egg count reduction percent and AR status for eight farms in 2014, calculated using six formulae. GIN were classified as resistant (R) when treatment reduced mean FEC by <95% and susceptible (S) when treatment reduced mean FEC by ≥95%.

Anthelmintic	Farm	FECRT1		FECRT2		FECRT3		iFECRT3		FECRT4		iFECRT4	
albendazole	1	66	R	43	R	73	R	78	R	84	R	76	R
	1	-	-	-	-	71	R	97	S	-	-	-	-
	3	47	R	34	R	12	R	-98	R	29	R	-48	R
	6	-160	R	-941	R	-95	R	-374	R	52	R	-353	R
	12	-8	R	68	R	78	R	80	R	82	R	82	R
fenbendazole	10	-	-	-	-	55	R	25	R	-	-	-	-
ivermectin	3	-196	R	-266	R	-438	R	-388	R	-334	R	-599	R
moxidectin	1	98	S	92	R	98	S	98	S	99	S	98	S
levamisole	1	99	S	98	S	97	S	99	S	99	S	99	S
	3	-	-	-	-	99	S	97	S	-	-	-	-
	5	-	-	-	-	99	S	99	S	-	-	-	-
	6	100	S	100	S	100	S	100	S	100	S	100	S
	11	-	-	-	-	97	S	73	R	-	-	-	-
	12	95	S	99	S	99	S	-	-	98	S	-	-
closantel	1	90	R	68	R	92	R	82	R	97	S	94	R
	1	66	R	42	R	87	R	71	R	84	R	68	R
	3	88	R	85	R	62	R	16	R	69	R	-3	R
	5	-	-	-	-	75	R	65	R	-	-	-	-
	13	-	-	-	-	64	R	-	-	-	-	-	-
closantel + albendazole	* 1	-	-	-	-	98	S	95	S	-	-	-	-

A dash indicates that FECRT calculations were not possible due to the lack of a control group, or an insufficient number of pre- and post-treatment samples from the same

animals for iFECRT3 and iFECRT4. *Efficacy was 100% except in one lamb for the closantel + albendazole group.

There was overlap in the farms tested in 2013 and 2014. Some farms tested a different anthelmintic(s) in 2014, and others retested an anthelmintic in addition to another drug(s). In total, FECRTs were performed on 13 farms and most tested one or two anthelmintics. A summary of the FECRT results over the two years of the study is presented in Figure 8. For farms that used a control group, the AR status was defined according to iFECRT4, except for Farm 12 where FECRT4 was used due to an insufficient number of pre- and post-treatment samples from the same animals. For farms that did not use a control group the AR status was defined according to iFECRT3, or FECRT3 if there was an insufficient number of pre- and post-treatment samples from the same animals. The susceptibility of GIN to albendazole and ivermectin occurred on only Farm 8. All other farms that tested these anthelmintics had resistance to either or both drug(s). Retesting of levamisole showed that GIN were susceptible on all eight farms. For anthelmintics other than closantel it was noted that *N. battus* FEC were 0epg in post-treatment groups, except for a single lamb in three groups and two lambs in one group. In the five lambs, *N. battus* FEC ranged from 50epg to 200epg.

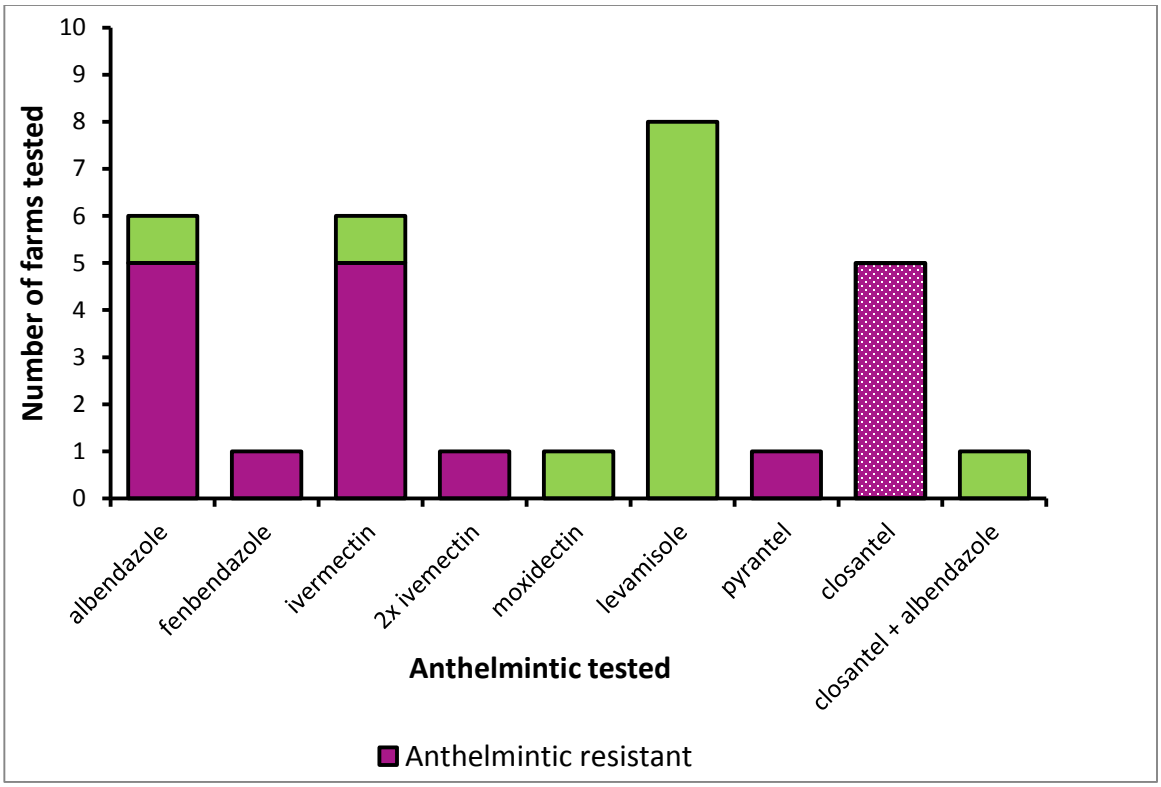

Figure 8. Summary of the resistance status of GIN on the 13 farms tested with FECRTs over the course of the study (2013-2014). Apparent resistant status of closantel is misleading, as it kills only *H. contortus*.

3.3 L3 culture and identification

Larvae were allocated to a species group based on tail-sheath-length and head shape, and classified as: short with square head (*Teladorsagia circumcincta/Trichostrongylus spp.*), medium with round head (*Haemonchus contortus/Cooperia spp.*), or long with a broad square or broad round head (*Chabertia ovina/Oesophagostomum spp./Bunostomum spp*.). Gut cell shape and number were used to identify some GIN to species (Table B2).

In 2013, a pre-treatment larval culture from Farm 1 contained over 90% *Haemonchus contortus/Cooperia spp.*. Two weeks post-treatment larval cultures for ivermectin on Farms 1 and 2, and for albendazole on Farm 6 contained only *Haemonchus contortus/Cooperia spp.*. In 2014, two weeks post-treatment larval cultures and FEC from Farm 1 showed that only *Haemonchus contortus/Cooperia spp.* survived treatment with moxidectin, but moxidectin was effective at reducing FEC to well below pathogenic levels (Fig. 9). *Haemonchus contortus/Cooperia spp.* was the dominant species in the control culture. Few larvae were recovered from the albendazole culture. The culture of closantel had a smaller proportion of *Haemonchus contortus/Cooperia spp.*, relative to the control, moxidectin, and albendazole cultures. Only one FEC was not 0epg in lambs treated with the closantel and albendazole combination. Thus, no larvae could be recovered from the other lambs in the treatment group. Levamisole was so effective in reducing FEC that no larvae were recovered from the culture. When anthelmintics are highly effective few larvae are recovered, which makes the intrepretation of results difficult.

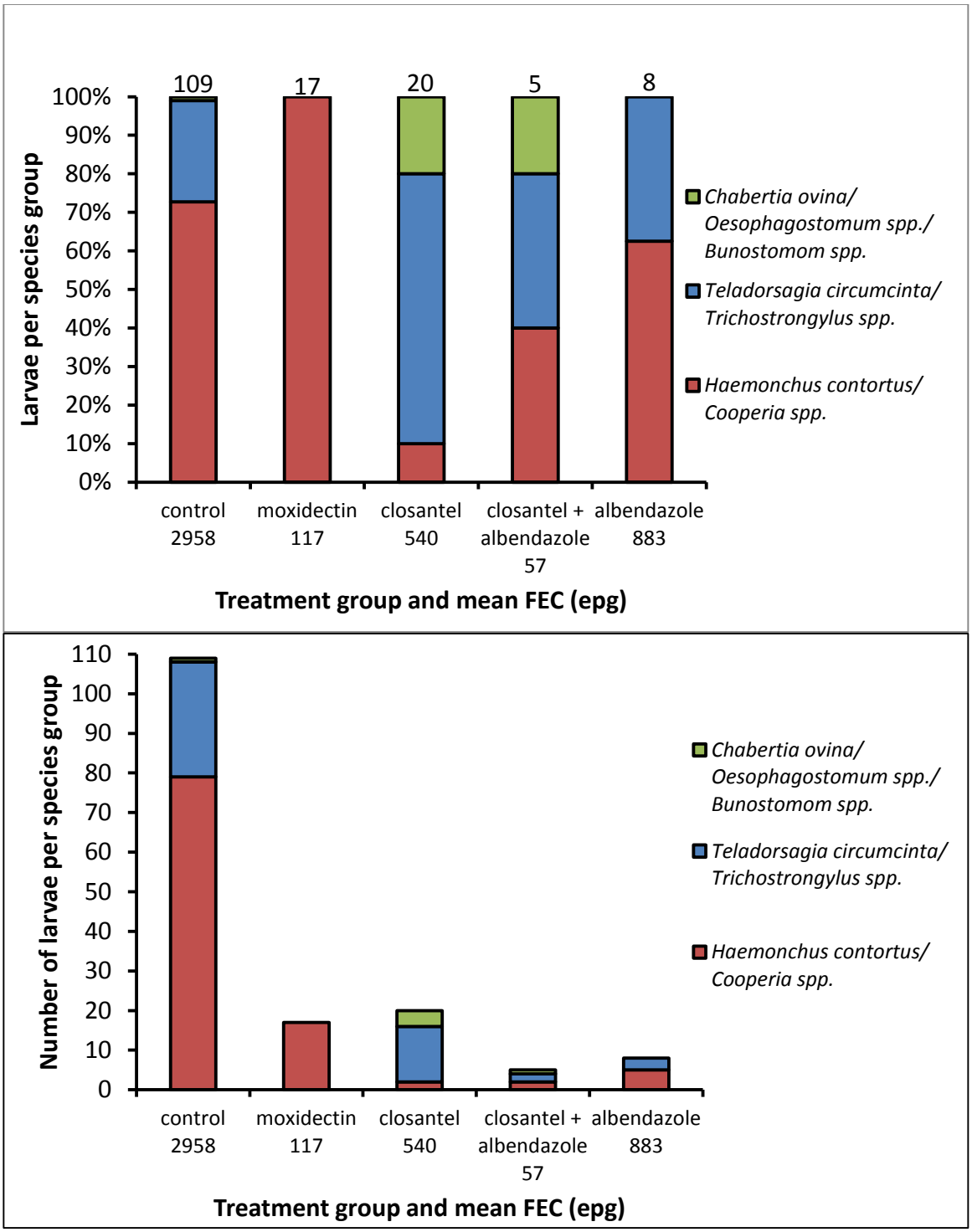

Figure 9. Percent (top) and number (bottom) of infective larvae per species group recovered from fecal cultures from Farm 1 in 2014, two weeks post-treatment. Numbers above the columns in the top graph indicate the total number of larvae.

In 2014, one month post-treatment larval cultures from Farm 1 showed that levamisole treated lambs had a similar proportion of *Haemonchus contortus/Cooperia spp.* as the control group, but a greater proportion of *Teladorsagia circumcincta/Trichostrongylus spp.*, and a lower proportion of *Chabertia ovina/Bunostomum spp./Oesophagostomum spp.* (Fig. 10). Also, levamisole treated lambs had a higher mean FEC than the control group. The proportion of *Haemonchus contortus/Cooperia spp.* was much lower in the closantel group, relative to the control and levamisole cultures. FEC of the closantel group were lower one month post-treatment than two weeks post-treatment.

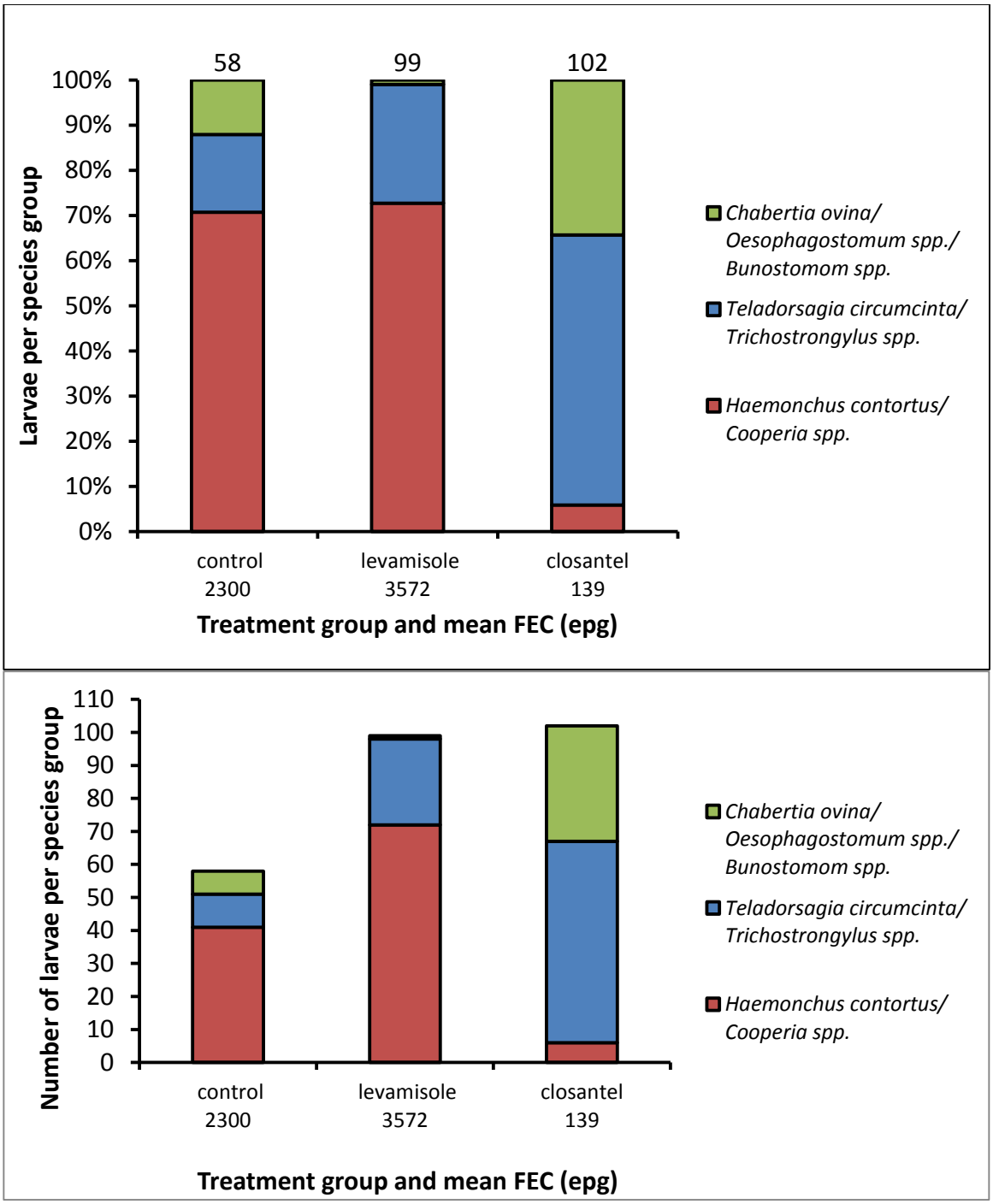

Figure 10. Percent (top) and number (bottom) of infective larvae per species group recovered from fecal cultures from Farm 1 in 2014, one month post-treatment. Numbers above the columns in the top graph indicate the total number of larvae.

Pre- and two weeks post-treatment larval cultures and FEC from albendazole treated lambs on Farm 1 in 2014 showed that *Haemonchus contortus/Cooperia spp.*, *Teladorsagia circumcincta* and *Trichostrongylus spp.* survived treatment (Fig. 11).

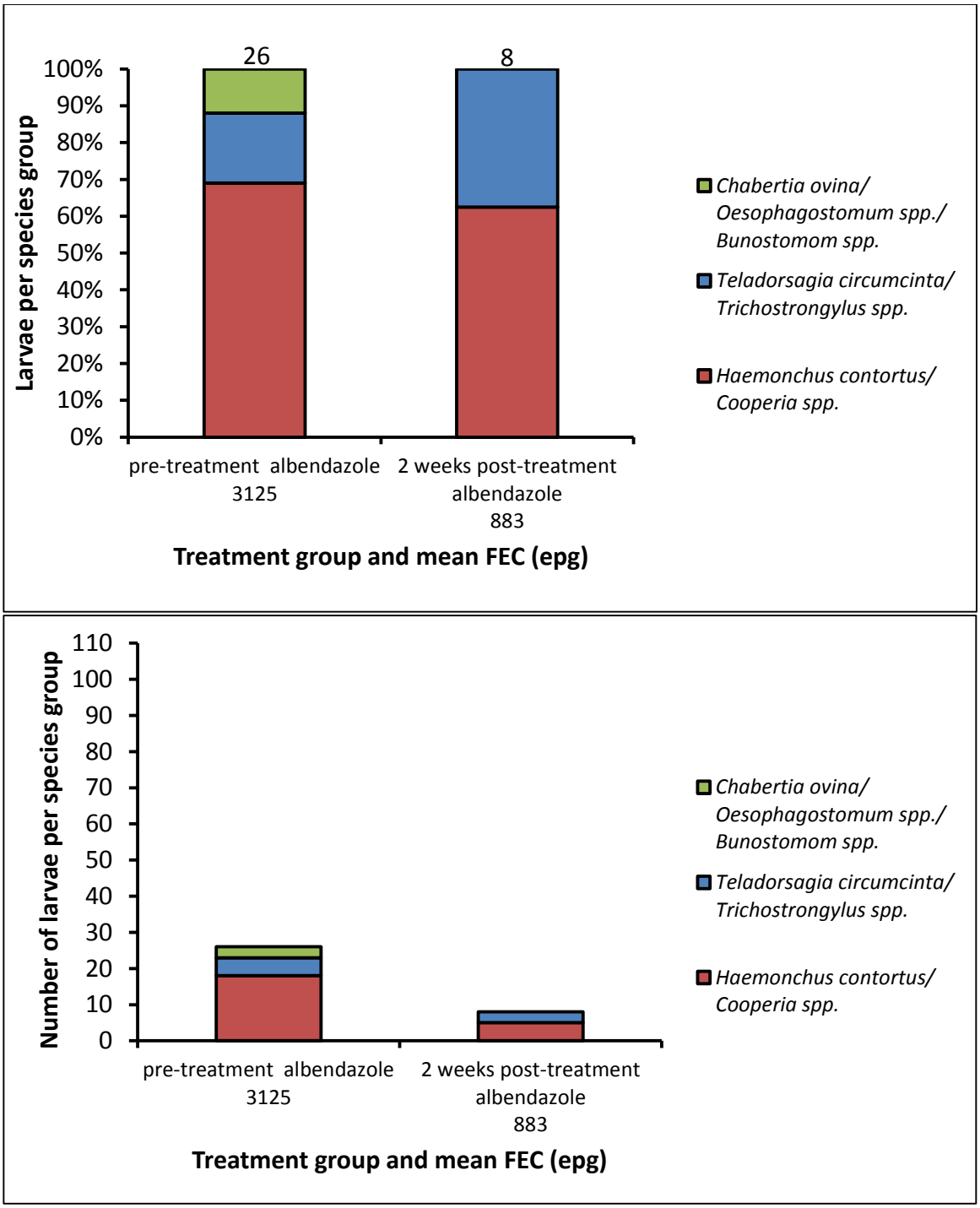

Figure 11. Percent (top) and number (bottom) of infective larvae per species group recovered from fecal cultures from Farm 1 in 2014, pre- and two weeks post-treatment with albendazole. Numbers above the columns in the top graph indicate the total number of larvae.

Although only *Haemonchus contortus/Cooperia spp.* survived treatment with moxidectin, by six weeks post-treatment, lambs had become re-infected with *Teladorsagia circumcincta* and *Trichostrongylus spp.* (Fig. 12).

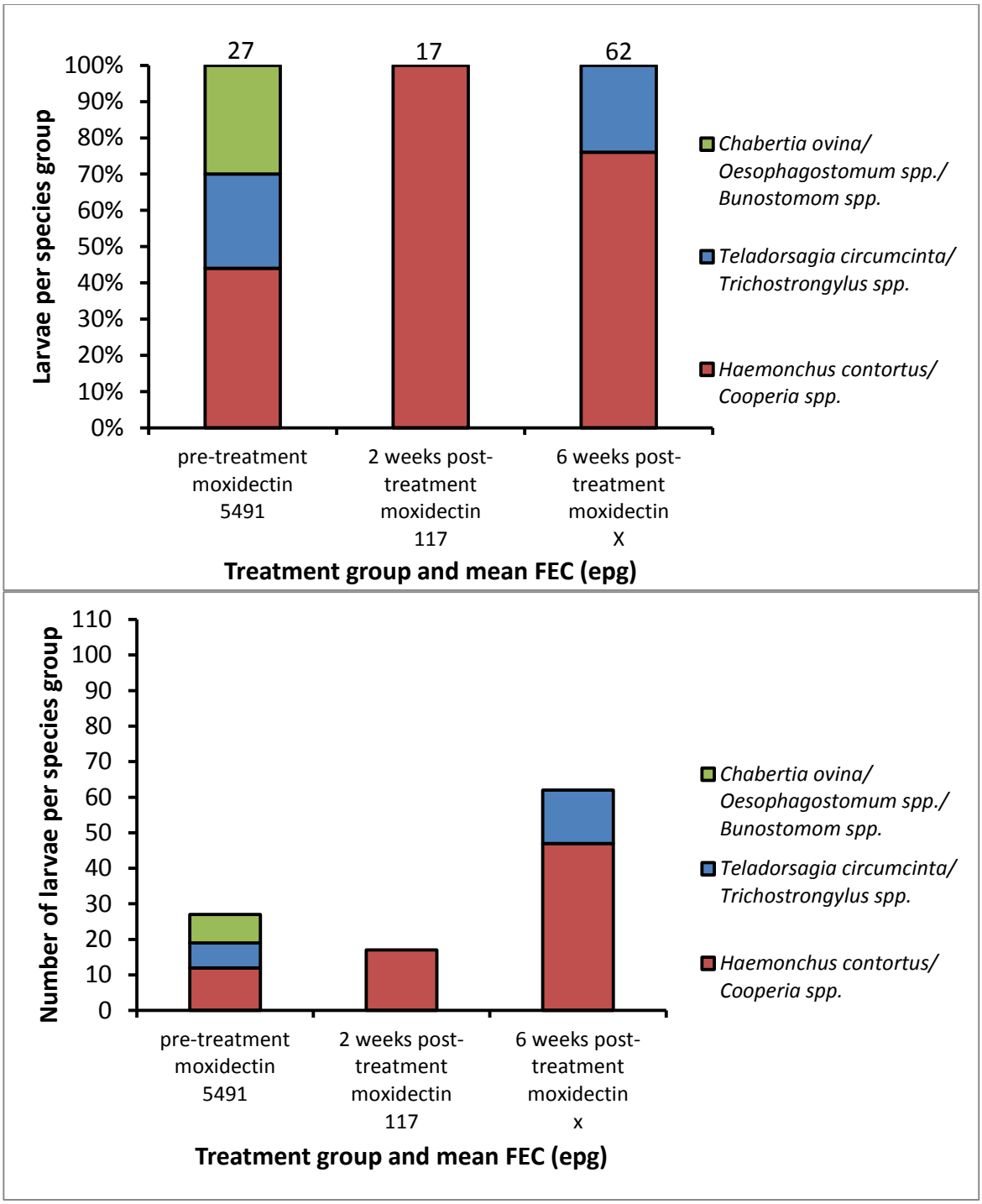

Figure 12. Percent (top) and number (bottom) of infective larvae per species group recovered from fecal cultures from Farm 1 in 2014, pre-, two weeks, and six weeks post-treatment with moxidectin. Numbers above the columns in the top graph indicate the total

number of larvae. X indicates that mean FEC was not available for the six weeks post-treatment samples.

3.4 Grazing management to reduce treatment frequency

On Farm 8 in 2014, the mean FEC of ewes was high when sampling began and two ewes had FEC over 7000epg (0-11 500, n=10) (Fig. 13). FEC dropped rapidly in July. Prevalence in ewes decreased over the grazing season, from 100% on July 8 to 20% on August 26 (0-600, n=10). In the group of only lambs, mean FEC remained low and lambs went untreated. By August 26, only two lambs had FEC over 1500epg (0-1800, n=16). Lambs grazed with ewes required anthelmintic treatment on August 12. On this date four lambs had FEC ≥2400epg, and another lamb had a FEC of 19 150epg (0-19 150, n=10). On August 3 and August 12, the mean FEC of lambs grazed with ewes was influenced by the one lamb with a very high FEC. In the only lambs group, two lambs consistently had higher counts than the rest of the group, from August 3 to August 26. With the exception of July 8, prevalence in the group of only lambs was lower than in the group of lambs with ewes. From July 21 to August 26, prevalence in the only lambs group ranged from 64% to 75%. Over the same period, prevalence in the lambs with ewes group ranged from 88% to 100%. At the end of the grazing season, a larval culture from the only lambs group showed that over 30% of larvae were *Haemonchus contortus/Cooperia spp.* (Fig. 14). This composite culture contained feces from four of the lambs with higher counts.

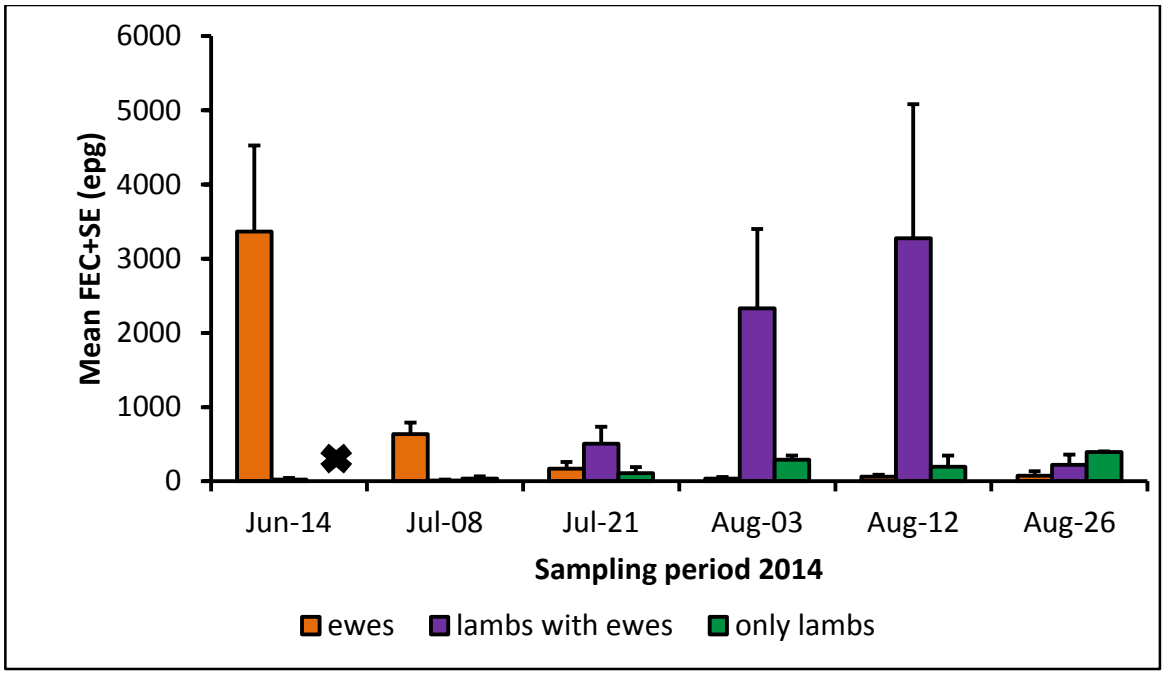

Figure 13. Seasonal patterns of GIN infection in ewes, a group of lambs grazed with ewes, and a group of only lambs on Farm 8 in 2014.✖ No data was available for the only lambs group on June 14.

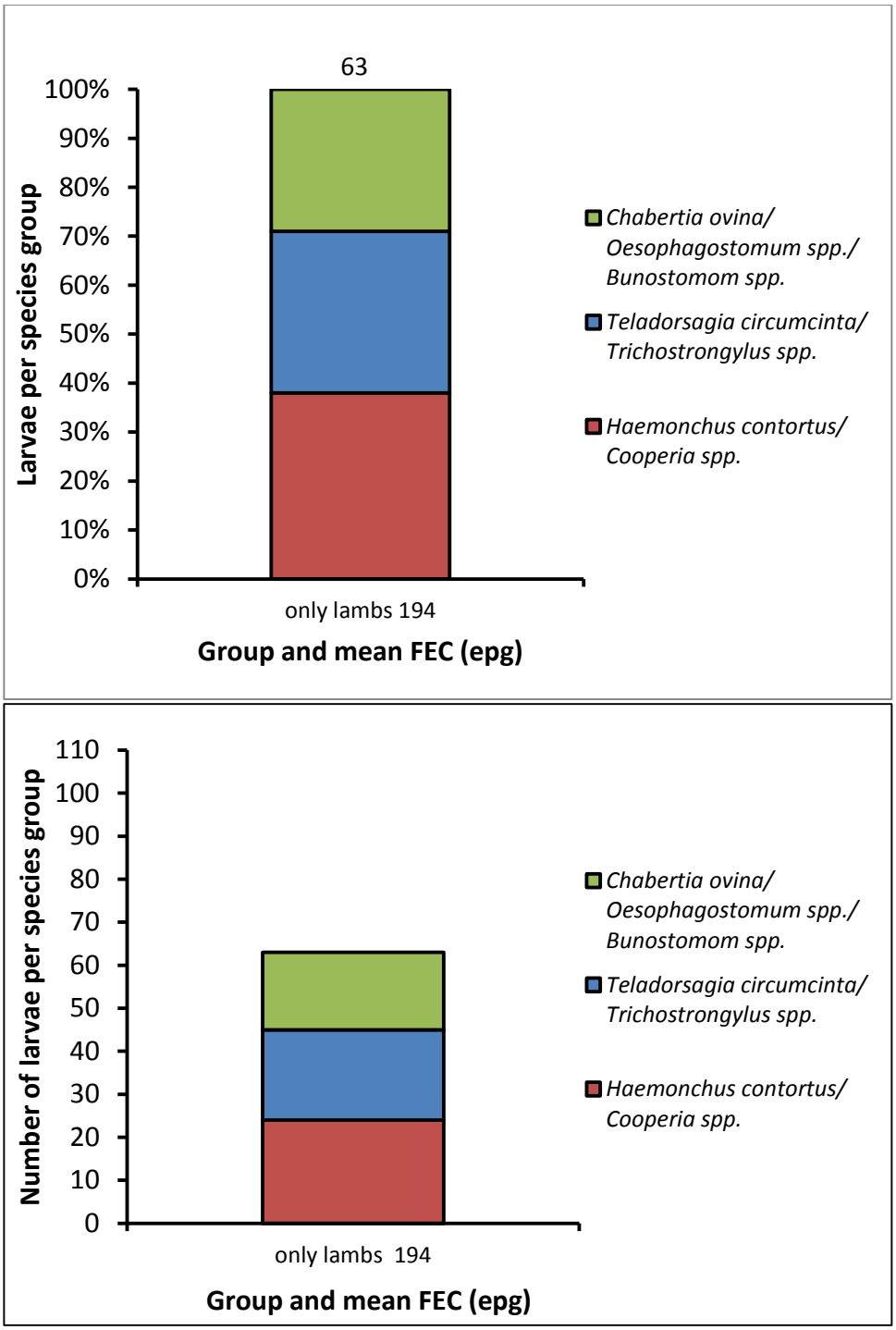

Figure 14. Percent (top) and number (bottom) of infective larvae per species group recovered from a fecal culture from the only lambs group on Farm 8, near the end of the

grazing season in 2014. The number above the column in the top graph indicates the total number of larvae.

Discussion

4.1 a. Sentinel farm FEC monitoring

Over the grazing season, GIN infections in lambs come from three sources: overwintered larvae on pasture at turnout (Smith and Fulton 1989), larvae from eggs shed by peri-parturient ewes in early summer, and larvae from eggs shed by lambs as they re-infect themselves in mid-summer to fall (Abbott et al. 2012). Sheep went out to pasture 12 days later in 2014, which explains the later appearance of eggs in the feces. In both 2013 and 2014, GIN eggs first appeared in the feces of lambs around three weeks after turnout. *N. battus* eggs first appeared in the feces of lambs 18 days after turnout on Farm 2 in 2013, and 18 days after turnout on Farm 1 in 2014. This timing coincides with the pre-patent periods of *T. circumcincta* and *Trichostrongylus spp.* (2-3 weeks) and *N. battus* (14-16 days). Since *H. contortus* does not survive overwinter, this species would not have contributed to initial lamb infections (Troell et al. 2009; Waller et al. 2004; Groz et al. 2013).

Most GIN eggs cannot be differentiated by microscopy. Yet, for species other than *H. contortus*, FEC over 1500epg indicate infections severe enough to cause excessive diarrhea and even death (Abbott et al. 2012). *H. contortus* causes anemia and masks diarrhea (University of Guelph 2012). Thus, in sheep with high FEC (≥2000epg) that were not scouring, *H. contortus* was the major contributor to infections. Ewe FEC were high at turnout in 2013 and 2014 and several ewes had pale conjunctiva (a sign of anemia), and no diarrhea (Jones, personal communication 2014). Consequently, these ewes were contaminating the pasture with huge numbers of *H. contortu*s eggs from May

through June. For example, a ewe with 500 adult female *H. contortus* (a pathogenic but manageable worm burden) (Abbott et al. 2012) could deposit up to 5 000 000 *H. contortus* eggs per day onto pasture.

As eggs shed by peri-parturient ewes developed to infective larvae over the grazing seasons, FEC rose rapidly in lambs in July as they became infected with *H. contortus. H. contortus* can take up to two months to develop to the infective stage at lower temperatures (<20°C), but as little as 5 days in hotter conditions (University of Guelph 2012). So, pastures can go from being highly contaminated to highly infective within a few weeks, as eggs laid earlier and later in the season develop to infective L3s at the same time. In 2013 and 2014, it took 46 and 47 days after turnout, respectively, for mean FEC to exceed 1000epg. Based on high FEC, pale conjunctiva indicating anemia, the absence of scouring, (Jones, personal communication 2014), and larval cultures, *H. contortus* was identified as the predominant species contributing to lamb infections from July through October. These results are consistent with those of Mederos et al. (2010), who identified *H. contortus* as the predominant species in Ontario and Quebec, from April through September of 2007. The inclusion of ewes in their study explains the dominance of *H. contortus* throughout the sampling period.

T. circumcincta and *Trichostrongylus spp.* lay far fewer eggs per day and develop more slowly than *H. contortus* (Abbott et al. 2012), so, although *T. circumcincta* and *Trichostrongylus spp.* continued to contribute to infections throughout the season, it took longer for lambs to pick up sub-clinical or pathogenic burdens of these species. On farm 1 in 2013 and 2014, very few lambs (<5) were scouring in August, a symptom of

infection with *T. circumcincta* and *Trichostrongylus spp.* (Jones, personal communication 2014). The decreases in FEC observed in late September of 2013 and 2014 were consistent with incoming L3s becoming hypobiotic rather than developing into adults (Waller et al. 2004; Groz et al. 2013), as well as the lambs' development of acquired immunity to GIN (Abbott et al. 2012).

In lambs, the lower infection levels observed in 2014 compared to 2013 were influenced by different weather patterns. Based on monthly soil temperature and precipitation averages over the grazing seasons, 2014 was hotter and drier in June than in 2013 (Jones and Semple, personal communications 2015). Eggs, L1s, and L2s are particularly susceptible to desiccation (Morgan and van Dijk 2012). L3s are protected from desiccation by their cuticle, but they cannot feed. In tropical regions, higher temperatures cause L3s to use up their limited energy reserves quickly, resulting in larval mortality and reducing pasture infectivity. However, in temperate climates such as in Nova Scotia, temperatures are not high enough to kill L3s in this manner (University of Guelph 2012). Although pastures became highly contaminated in both years, the dry conditions in 2014 would have reduced pasture infectivity by killing off eggs, L1s, and L2s, thus leading to lower infection levels. GIN that survived to the L3 stage would have been little affected (Eysker et al. 2005; Reynecke et al. 2011). The use of moxidectin and closantel in July of 2014 were additional factors in lowering the infection levels, since both drugs were effective and had persistent activity. Mean FEC for closantel treated lambs was lower (139epg) (50-1100, n=11) one month post-treatment than two weeks post-treatment (397epg) (0-1300, n=11). Hence, moxidectin and closantel treated sheep reduced the mean FEC of the flock.

4.1b Sentinel farm FEC monitoring of N. battus

N. battus eggs are easily differentiated from GIN eggs, and infections arise from lamb to lamb transmission, from one year's lambs to the next year's lambs. The typical pattern of infection is a rapid rise in FEC early in the grazing season, followed by a rapid decrease in FEC a few weeks later, as lambs develop immunity to this species (Winter 2002). A pronounced peak in mean FEC was observed from June through July in 2013, and a peak followed by a more gradual decline was observed from mid-June through August in 2014. Minimal exposure is needed for lambs to develop a good immune response to *N. battus* (Taylor and Thomas 1986), and the declines observed in mean FEC support the quick development of immunity. In both years (2013-2014), mean *N. battus* FEC never exceeded levels indicating moderate infections (150-300epg), and even lambs with FEC over 700epg (indicating severe infections) were not scouring (Abbott et al. 2012). These results are consistent with anecdotal evidence from other farmers who have not encountered problems with this species (Jones 2013). Unlike other parts of Canada where *N. battus* is not thought to be prevalent (University of Guelph 2012), 24/25 farms in Nova Scotia that submitted random samples in 2013 and 2014 had *N. battus* (Jones 2013). Farm 7 was the only farm without *N. battus*, and this farm lambed in June. Overwintered *N. battus* would not have survived on pasture long enough to infect the lambs on Farm 7.

In contrast to the United Kingdom where gradual and consistent increases in temperature in the spring result in mass hatches of *N. battus*, high rates of larval challenge, and consequently lamb mortality (National Animal Disease Information

Service 2014), the erratic pattern of temperature increases in the spring in Nova Scotia does not favor mass hatches. Accordingly, lambs in this region face a slower rate of larval challenge, thus reducing clinical illness. Moreover, FEC do not necessarily correlate with disease severity, for it is the immature stages and egg laying adults that are the source of illness (Abbott et al. 2012). Mean *N. battus* FEC were higher in 2014 than in 2013. This may be explained by the development of *N. battus* to the infective stage inside the egg. The dry conditions of 2014 that lowered pasture infectivity of GIN would have had a lesser impact on the development and survival of *N. battus* since its thick shelled, hardy egg is more desiccation resistant than those of GIN, and L1s and L2s are not exposed to the environment (Abbott et al. 2012).

Throughout the grazing seasons of 2013 and 2014, *N. battus* counts trickled on, followed by increases in mean FEC at the end of the grazing seasons. This suggests that *N. battus* is completing its life cycle in the same season. In response to climate change, *N. battus* has adapted to produce a second generation in the autumn in England and Scotland (van Dijk and Morgan 2010). Given that L3s hatched from eggs that overwintered on pasture survive for only a few weeks (Morgan and van Dijk 2012), it is unlikely that late season infections arose from overwintered eggs. Autumn hatching is further supported by *N. battus* L3s recovered from a fecal culture from Farm 8, which was collected on August 13 and analyzed on October 1 (Fig. B14-15). According to van Dijk and Morgan (2010), a second generation of *N. battus* can develop to the L3 stage in about seven weeks. This timing, plus the 14-16 day pre-patent period, coincides with the periods between the first appearance of *N. battus* eggs in the feces and the beginning of the second increase in both

years, as well as the age of the fecal culture from Farm 8. Larval cultures and/or herbage samples could confirm autumn hatching on Farm 1.

4.1c FEC monitoring of peri-parturient ewes

The high FEC of peri-parturient ewes on other farms (Farm 2, 3, 5, 7, 8, 10, and 12) corroborate the results from Farm 1. In Nova Scotia, peri-parturient egg shedding is a major source of pasture contamination with *H. contortus*. Most farms provided samples during late-lactation. In Ontario, this stage is associated with the highest FEC (Falzon et al. 2013a). It would be useful to determine the patterns of FEC over the entire lambing period. A survey of peri-parturient ewes from before and around lambing on, with and without supplementation with soy meal, is underway on Farm 1. Soy meal provides additional protein, which can improve immunity (Houdijk 2012, Houdijk et al. 2012). Preliminary results indicate low FEC before lambing.

4.2 FECRTs

Typically, FECRT calculations include two criteria to classify GIN as anthelmintic resistant: a <95% reduction in mean FEC, and a lower limit of a 95% confidence interval <90%. If both criteria are met GIN are classified as resistant, but if one criterion is not met resistance is suspected. Otherwise, GIN are classified as susceptible (Coles et al. 1992). In this study, one criterion for resistance was used: a <95% reduction in mean FEC. Many methods for calculating percent reductions can be found in the literature. Options include using: group mean FEC (Cole et al. 1992; McKenna 2006), group composite FEC (Rinaldi et al.2014), individual FEC (Caberet and

Berrag 2004), a general linear mixed model (Mejía et al. 2003), a negative binomial maximum likelihood model (Torgerson et al. 2005), and Bayesian analysis (Torgerson et al. 2014). Several of the above methods also have corresponding versions using geometric means (Presidente 1985), log transformed values and/or bias correction terms (Falzon et al. 2014; Torgerson et al. 2005; Dobson et al. 2012). The lack of consensus regarding which statistical method is best stems from the many factors that affect the results of FECRTs such as: FEC techniques, baseline FEC, sample size, aggregation of FEC, FECRT formulae, and the inclusion of a control group (Vidyashankar et al. 2012). Also, for field use, trade-offs among accuracy, affordability, and animal welfare are necessary so that farmers are willing and able to adopt routine testing.

Efficacies in this study were either so high or so low that confidence intervals were unnecessary. Furthermore, methods for calculating confidence intervals were not user friendly, and for the six formulae used, confidence intervals are based on the erroneous assumption of normality of FEC (Coles et al. 1992). Log transformation of FEC is often employed to make data conform to a normal distribution and to eliminate FEC of 0epg. However, the constant added to FEC in log transformations is inconsistent, with some researchers adding 1 and others adding half the detection limit of the FEC technique (Dash 1988; da Sliva et al. 2011). This hampers the comparison of results across studies. Indeed, the method advocated by the W.W.A.V.P. (for FECRT1) does not specify the constant to be added to FEC (Coles et al. 1992), and many studies that use this formula do not include this information.

A preliminary assessment of this study's data did not identify any groups as normally distributed based on the Anderson-Darling normality test, and log transforming FEC did not always produce normally distributed data. Confidence intervals are considered to be useful when resistance is emerging, because FECRTs suffer from poor sensitivity when efficacies fall between 90% and 95%. Additionally, for any sample size or level of aggregation, FECRTs cannot accurately identify resistance or susceptibility when true drug efficacy ranges between 87.5% and 92.5% (Levecke et al. 2012). For research purposes, where conditions are rigorously controlled and accuracy is important, confidence interval methods that incorporate the over dispersion of FEC may be appropriate (Cabaret and Berag 2004; Torgerson et al. 2014). However, for field use the biologically relevant information lies in the individual FEC and percent efficacies. These provide sufficient information to make treatment decisions and to quantify AR. As well, percent efficacies are easily understood and not intimidating to farmers.

The detection limit of FEC techniques ranges from 1epg for the FLOTAC method (Cringoli et al. 2010) to 50epg for the modified McMaster method used in this study (MAFF 1986). Regardless of the technique, sampling errors are then exaggerated by the multiplication of raw egg counts by the dilution factor (Torgerson et al. 2012). Accuracy and precision of FEC are improved with lower detection limit techniques (Godber et al. 2015; Bosco et al. 2014), but this improvement is marginal if baseline FEC are high (Levecke et al. 2011). Furthermore, FLOTAC is labour intensive, requires two centrifugation steps, and the counting apparatus is not commercially available (Cringoli et al. 2010). By contrast, the modified McMaster technique is widely used in FECRTs and flock monitoring for both research purposes and in the field (University of Guelph

2012; Abbott et al. 2012). This method requires limited training and equipment, and due to its practicality and affordability the McMaster technique could be taught to farmers in Nova Scotia. This would enable them to monitor their own sheep and avoid the high cost of testing by a laboratory ($6 - $20 per sample) (Jones, personal communication 2014). To date, our research team has trained two farmers to perform FEC. The author and other members of our research team have demonstrated the technique at workshops and at the PSBANS Annual Fall Sheep Sale for the last three years.

FECRTs produce unreliable data when baseline FEC are low (Vidyshankar et al. 2012). According to Falzon et al. (2013b) FECRTs require a minimum group mean FEC>300epg to provide reliable results, but current recommendations of the W.W.A.V.P. require a minimum group mean of only FEC>150epg (Coles et al. 2006). These low pre-treatment thresholds can result in some sheep having FEC of 0epg, creating zero inflated outcomes. Zero counts are uninformative for they may reflect a true lack of infection, making such sheep unsuitable for drug testing, or result from variation due to random sampling of the fecal filtrate (Torgerson et al. 2012).

Yet, in this study low baseline FEC and zero inflation were not an issue. In 2013, with the exception of three FECRT groups with mean FEC ranging from 192-513epg, all groups had mean FEC over 1000epg. Similarly in 2014, with the exception of four groups with mean FEC ranging from 275-685epg, all groups had mean FEC near or over 1000epg. In fact, FEC monitoring of just the sentinel farm was sufficient to determine when FEC on other farms would be high enough for FECRTs. Concerning FEC of 0epg, in 2013, 8/26 groups of lambs had a single FEC of 0epg, and 3/26 groups had two lambs

with FEC of 0epg. In 2014, 1/22 groups involved in FECRTs had a single FEC of 0epg. A bias correction can be added to eliminate FEC of 0epg, but this can have a great effect on the percentage efficacy of the anthelmintic. When Falzon et al. (2014b) reanalyzed FECRT data using a bias correction term of 25epg (half the detection limit of the FEC technique) the resistant status of levamisole for one farm remained the same, but the percent reduction increased from 6% to 92%.

In this study, inclusion or exclusion of lambs with FEC of 0epg did not greatly affect percent efficacies or change the resistance status, and these lambs could have been excluded from the analysis (data not shown). Lambs with pre-treatment FEC of 0epg were retained for consistency with current protocols and because post-treatment counts >0epg were obtained for some treated lambs. For some of these lambs, FEC of 0epg likely reflected variation due to random sampling and/or the high detection limit of the FEC technique, rather than a lack of infection at the beginning of the FECRT.

The impacts of over-dispersion and small sample sizes can be seen in the percent efficacies, and the disagreement in resistance status, for ivermectin on Farm 8. One lamb in the treatment group had an extremely high pre-treatment FEC of 24 500epg, and thus mean FEC were dissimilar between the treatment group 6010epg (0-24 500, n=5) and the control group 192epg (0-450, n=6). When this lamb was excluded from the analysis, efficacies were 100% for all six formulae. But, exclusion of lambs with high FEC is inappropriate, as they are not outliers in the traditional sense but are part of the typical pattern of parasite distribution within a population (Barger 1985). Treatment of this lamb reduced its FEC from 24 500epg to 850epg, a 97% reduction. Due to the single high

count at the beginning of the test, FECRT1 and FECRT2 failed to capture the efficacy of the drug, and erroneously indicated high levels of resistance (-46% and 11% efficacy, respectively). The other four formulae, which included pre- and post-treatment FEC from treated lambs, provided a more reliable assessment of drug performance and gave susceptible statuses with efficacies ranging from 95% to 99%. Sample sizes on Farm 8 were small (n=5-6), and given the over-dispersion of parasitic infections, a larger sample size may have resulted in a similarity of counts between the treatment and control group, more consistency among percentage efficacies, and agreement in resistance status across formulae. The recommendation of Coles et al. (1992) to include at least 10 sheep per group may be problematic on farms with few lambs.

AR to the only two available drug groups in Canada, the benzimidazoles (albendazole and fenbendazole) and the macrocyclic lactones (ivermectin and moxidectin), was present and in many cases, efficacies were too low to provide adequate worm control. Sheep disease and even death are likely when efficacy is below 60% (Abbott et al. 2012). Albendazole is no longer useable on 3/6 farms tested (Farms 3, 5, and 6). Similarly, on 4/6 farms that tested ivermectin, (Farms 1, 2, 3, and 9) efficacies were so low that this drug needs to be abandoned. On the aforementioned farms, slight decreases in mean FEC and increases as high as 4000epg were observed post-treatment. AR to both albendazole and ivermectin was documented on 2/3 farms (Farms 1 and 3) that tested both drugs.

Farm 8 was the only farm where GIN were susceptible to both albendazole and ivermectin. However, lambs on Farm 8 were grazed with fall lambed ewes (October)

(Hines, personal communication 2014). These ewes were several months past parturition and would have had immunity to GIN, and thereby shed low numbers of eggs. So, there would have been less contamination of pastures with *H. contortus.* This is supported by the relatively low pre-treatment mean FEC of lambs from Farm 8 compared to the other farms, all of which lambed in the winter and/or the spring.

On Farm 1 the efficacy of albendazole was approximately 70% in 2014. Post-treatment larval cultures from albendazole treated lambs yielded just eight larvae, even though the mean FEC was over 800epg. Such low numbers were due to the ovicidal properties of albendazole. Resistant survivors produce eggs, but many do not hatch (Abbott et al. 2012). On Farms 1, 8, and 12, albendazole may be effective enough (>70%) to prevent production losses with frequent use. Fenbendazole is in the same drug group as albendazole, and high levels of resistance were identified on the one farm tested.

Ivermectin, the only anthelmintic licenced for use in sheep in Canada, showed the highest levels of resistance. This is unsurprising, as it has been used extensively for years, and many farmers have stopped using it due to its obvious ineffectiveness (author's observation). Moxidectin is a stronger but similar drug to ivermectin, and it was highly effective on Farm 1, where GIN were resistant to a double dose of ivermectin. Reportedly, moxidectin works on other farms too, and it has become a popular alternative to ivermectin (Jones, personal communication 2015). But, as was observed in a post-treatment larval culture, *H. contortus/Cooperia spp.* survived treatment, albeit in low numbers. Since moxidectin and ivermectin work by a similar mechanism (Bygarski et al. 2014; Kotze et al. 2014), and *H. contortus* can develop resistance rapidly, it is unlikely

that switching to moxidectin will provide adequate worm control for more than a few years. This phenomenon is called side resistance, whereby resistance to one drug confers resistance to other drugs in the same class. According to Bygarski et al. (2014), GIN resistant to ivermectin are at least partially resistant to moxidectin. Also, moxidectin is an injectable drug, which is licenced for use in cattle, but it is given to sheep as a drench. This route of administration results in a high drug concentration in the abomasum - where *H. contortus*, *T. circumcincta*, and one species of *Trichostrongylus* live - and reduces the period over which GIN are exposed to a sub-therapeutic concentration of the anthelmintic (Lanusse et al. 2014). Oral administration of injectable anthelmintics is not advised in Canada (University of Guelph 2012)

GIN were susceptible to levamisole on 8/8 farms tested, and efficacies ranged from 96% to 100%. Where the resistance status depended on the FECRT formulae, such as for Farms 2 and 3 in 2013, efficacies were typically higher for the individually based and more complete formulae (96% to 99%). Additionally, a retest of levamisole on Farms 3 and 5 in 2014 showed efficacies of 97% and 99%, confirming susceptibility. However, levamisole is not licenced in Canada and is available only from a veterinary compounding pharmacy. Since the vehicle for drug delivery may differ among pharmacies there is no withdrawal time (to enter the food chain) available for this anthelmintic. The high efficacies of levamisole were expected because it has not been commercially available for the last 10 years (Lexchin 2005), which predates the recent problems with *H. contortus*. Despite its efficacy, levamisole's short duration of activity made frequent treatment necessary. On Farm 1 in 2014, one month post-treatment FEC of 6/11 lambs were high enough to warrant re-treatment.

Pyrantel is similar to levamisole and is for use in horses. Very little of the drug is absorbed, and most is passed unchanged in the feces. Efficacy depends on the period over which GIN are exposed to the anthelmintic in the lumen of the horse's digestive tract (Gokbulut et al. 2001). There are no dosage guidelines for its use in sheep. It is unknown if there are differences in metabolism between horses and sheep, as there are for other animals, that could affect efficacy. For example, goats require double the dose of anthelmintics given to sheep because goats metabolize anthelmintics faster (University of Guelph 2012). As for levamisole, there is no withdrawal time for pyrantel. On Farm 6 in 2013, the farmer used pyrantel out of desperation, and efficacies were 90% or 97%, depending on the formulae used (Jones, personal communication 2014). In 2014, levamisole was 100% effective. This is promising since the pre-treatment FEC were very high (4500-26 950, n=10). Levamisole is commonly used in sheep in other countries and dosage guidelines are available. Levamisole presents a better option to pyrantel.

This study was the first to use and test closantel in Canada through an Emergency Drug Release application. Based on data collected by our research team in 2013, Dr. E. Semple and B. Densmore made an application through the Veterinary Drugs Directorate to the Federal Ministry of Health for an Emergency Drug Release to import closantel for specified clients. On the basis of our results from testing closantel in October 2013, closantel is now available to any veterinarian in Canada through the Emergency Drug Release process. One other veterinarian in Nova Scotia has since used this process to import closantel in 2014. However, closantel is still not licenced. According to FECRTs, GIN were resistant to closantel on all five farms tested. This status is misleading because closantel kills only *H. contortus*. Other species are unaffected and continue to lay eggs,

reducing the apparent percent efficacy. In October of 2013, the FECRTs on Farms 1 and 2 showed over 60% and over 86% efficacy, respectively. This provides further support for *H. contortus* being the dominant species in GIN infections in the fall. FEC in 4/14 lambs in the control group on Farm 1 decreased over the course of the test, which reflected GIN becoming hybiotic in the fall. Closantel was tested twice on Farm 1 in 2014. The first FECRT in July showed 80% to 90% efficacy depending on the formulae, and FEC (0-1300, n=11) were reduced well below pathogenic levels for *H. contortus* (5000epg) (Abbott et al. 2012). Even though closantel kills only one species, efficacies were higher, and FEC were lower than those of albendazole treated lambs.

Similarly, post-treatment larval cultures showed that approximately 10% of larvae recovered from the closantel group were *H. contortus/Cooperia spp.*. In the control group approximately 70% of larvae recovered were *H. contortus/Cooperia spp.*, FEC were high (150-22 300, n=28), and 17/28 lambs had FEC >2000epg. The second FECRT in August showed lower efficacies of 70% to 80%. This was expected and is indicative of the accumulation of *T. circumcincta* and *Trichostrongylus spp.* later in the grazing season, rather than AR. The persistent activity of closantel provided excellent worm control of *H. contortus* for several weeks on Farm 1. One month post-treatment, over 90% of larvae belonged to other species, and the mean FEC was <200epg. Closantel reduced FEC well below pathogenic levels for *H. contortus* on all five farms. Farms 1, 2, 3, and 5 tested closantel in addition to levamisole and ivermectin and/or albendazole. Levamisole was the only anthelmintic that outperformed closantel.

At the end of September in 2014, a combination of closantel and albendazole was tested on Farm 1. When used alone each drug was 70%-80% effective. The combination was 100% effective except in one lamb (94B). This lamb had the second lowest pre-treatment FEC in the treatment group, and given that post-treatment FEC of 0epg were obtained for lambs with pre-treatment FEC over 4000epg, it seem likely that this lamb missed the dose of one anthelmintic. Nevertheless, the effectiveness of the combination confirmed that *H. contortus* was susceptible to closantel and that other species were still susceptible to albendazole.

4.3 FECRT formulae

The six FECRT formulae used in this study are effective at identifying AR, when the level of resistance is high (Maingi et al. 1996a; Cabaret and Berag 2004). Generally, there was agreement in AR status across formulae, but there were great variations in the percent efficacies. For example, on Farms 1 and 3 in 2013, efficacies for ivermectin ranged from -1222% to 5%, and from -149% to 48%, respectively. When resistance was low and/or GIN were susceptible, more consistency was observed in efficacies among formulae. These results are similar to those of other studies that compared different methods for FECRT calculations (Torgerson et al. 2005; Miller et al. 2006; Falzon et al. 2014b).

Falzon et al. (2014b) recommended that in Canada a bias correction term be added to all FEC of 0epg and that FECRT4 should be used for research. They also recommended that FECRT1 should be used for field testing to reduce costs (fewer samples are required), and to allow clinicians to compare results among farms. I disagree

with these recommendations. Given the low number of FEC of 0epg observed in this study, a bias correction term is unnecessary and "correction" seems inappropriate as it is essentially making up data. If a correction term is to be used, it should be added to all FEC, not just those of 0epg. It would be better to eliminate lambs with FEC of 0epg from the test, or to wait an extra week so that all lambs would be infected. FECRT1 should be avoided as it performs poorly when there are large differences in mean FEC between control and treatment groups (Calvete and Uriarte 2013). For field use, this may be the case because it is unreasonable to leave lambs untreated if they have symptoms of severe parasitism. Additionally, in practice it is difficult to get farmers to randomly allocate sheep to treatment and/or control groups. According to Dr. G. Jones, without encouragement farmers would put healthy looking lambs in the control group, and put sickly lambs in the treatment group(s), preferably the levamisole group. Furthermore, FECRTs in Nova Scotia have been limited to those conducted by our research group, and farmers only pay for the anthelmintic(s). Our group plans to continue providing FECRTs as it is unlikely that farmers will absorb the costs of testing elsewhere in the near future. Thus, clinicians comparing results among farms and limiting costs are irrelevant. Our research group has performed FEC on 4990 samples in the last two years (2013-2014). Given that the cost of each FEC is $6.58 at the Veterinary Pathology Laboratory in Truro (Jones, personal communication 2015) or up to $20 through a veterinarian, we have saved the sheep industry in Nova Scotia between $32 843 and $99 800.

Of the six FECRT formulae used in this study, I think that iFECRT4 is the most appropriate for AR testing in Nova Scotia. A control group is needed to correct for rapid increases in FEC during the test, thus providing a better estimate of the true efficacy of

the anthelmintic. Also, individually based calculations account for variation in FEC among sheep in a group. This is especially important when sample sizes are small. iFECRT3, which uses pre- and post-treatment counts from the same treated sheep, is the next best option. Although it may not show the true efficacy of the drug, it is sufficient to determine if the anthelmintic reduces FEC to a manageable level. FECRT2 should not be used. It does not accommodate increases in FEC during the test, variation in FEC among sheep, nor variation in FEC among groups.

In future testing, the inclusion of a control group will have to be decided on a farm-by-farm basis. Many flocks in Nova Scotia have 10-20 sheep, so control groups may not be feasible (Jones, personal communication 2015). Given the patterns of infection on Farm 1 over the last three years, the window of opportunity between FEC reaching the threshold for FECRTs and exceeding pathogenic levels is just a few weeks. This is also the case in Ontario and Quebec (Mederos et al. 2010). Due to such rapid increases in FEC, leaving lambs untreated for one or two more weeks (the length of the test) may result in lowered body condition or production losses. For example, during a FECRT on Farm 1 in August of 2012, close monitoring of lambs with FAMACHA scores was necessary to ensure that they would survive being left untreated for two more weeks. FEC of these lambs increased by approximately five times, they suffered a loss of body condition, and were anemic by the end of the test (Jones, personal communication 2012). On Farm 2, during the second week of August in 2012, 10 lambs died suddenly due to haemonchosis, and several others were anemic (Densmore, personal communication 2013). These lambs were not part of a FECRT, but had gone untreated. Fears of reduced weight gain, illness, and deaths due to GIN were the primary reasons why seven of the 13

farms tested did not use a control group (Jones, personal communication 2015). These fears underscore the severity of the problems with GIN management in Nova Scotia.

Myriad factors, other than AR, can reduce the efficacy of an anthelmintic. Unfortunately, these factors also select for AR. Underdosing has perhaps the greatest impact and can result from: failure to calibrate drench guns, incorrect use of the drench gun, or using a syringe to administer drugs. An anthelmintic needs to be administered over the back of the tongue so that the drug ends up in the rumen, where the drug is absorbed and metabolized slowly. When D. Thibault was assisting on Farm 5 in 2013, the farmer was observed squirting levamisole onto the sheep's teeth using a syringe. If an anthelmintic is deposited in the front of the mouth sheep can spit out the dose, or it can be swallowed into the abomasum where the drug is rapidly absorbed and metabolized. This results in GIN not being exposed to an adequate concentration of the drug for the necessary period of time (Abbott et al. 2012). Underdosing can also occur when dosages are calculated by estimating sheep weights. Conversely, estimating weights can lead to overdosing and toxicity with levamisole because it has a low safety margin (University of Guelph 2012).

In this study, anthelmintics were administered correctly, with calibrated drench guns, but this is not common on Canadian farms (Falzon et al. 2013c). *N. battus* eggs effectively acted as a control for satisfactory dosing technique in FECRTs. *N. battus* was present in 29/38 pre-treatment groups. For anthelmintics other than closantel post-treatment FEC of *N. battus* were 0epg in 21/25 groups. Of the four post-treatment groups with *N. battus*, three had a single sheep, and one had two sheep, with *N. battus* FEC of

50-200epg. Two of these groups were from Farm 3, where dosages were calculated by estimating the weight of the heaviest sheep. Underdosing may have occurred on this farm. On Farm 1, two lambs in the moxidectin group had *N. battus* post-treatment. Treatment with moxidectin involves a 1mL/50kg dose, so it is very easy to underdose lambs and for sheep to fail to swallow the whole dose. The FEC of 0epg of *N. battus* in the majority of groups indicated that there was no resistance in this species and confirmed that the dosing technique was acceptable. In future FECRTs, where farmers may be treating sheep themselves, *N. battus* could be used as an informal method for confirming correct dosing.

4.4 Anthelmintic combinations and refugia

Combinations of anthelmintics, with different modes of action, are increasingly being used to control GIN where AR is present to one or more anthelmintic(s). Combination formulations are available in Australia, New Zealand, and the United Kingdom (Geary et al. 2012). The literature abounds with studies reporting the successful use of anthelmintic combinations against AR GIN, and they have been used for many years (Maingi et al. 2002; Baker et al. 2012; Geurden et al. 2012; Geary et al. 2012; Leathwick and Besier 2014). Mathematical modeling has shown that using a combination of drugs delays the development of AR to both constituents, and is superior to rotations of single anthelmintics. The higher the efficacies of both drugs, the longer it takes for AR to develop to a detectable level (Barnes et al. 1995; Learmount et al. 2012; Leathwick 2012; Dobson et al. 2011). Except for a few mutations that confer resistance to either benzimidazoles or ivermectin, which are thought to act dominantly (Kotze et al. 2014),

alleles for resistance are generally considered to be recessive. Each drug protects the efficacy of the other because initial frequencies of resistance alleles are low and most are hidden in heterozygotes. Hence, there are very few doubly resistant survivors. Resistant worms are likely to mate with worms that are susceptible to both drugs, thus preserving susceptibility in the next generation (Bartram 2013). A recent field trial has corroborated the results of modelling studies (Leathwick et al. 2012). More field trials are needed to gauge the long-term performance of anthelmintic combinations under typical farm conditions.

Derquantel belongs to the newest class of anthelmintics, the spiroindoles, and is available only as a dual active with abamectin called STARTECT®. According to a simulation study by Learmount et al. (2012) resistance to derquantel was negligible after 20 years of continuous use, even when the efficacy of abamectin was 50%, provided that refugia are high. High refugia involved low stocking density, grazing lambs separately from ewes, targeted treatment of lambs with high FEC, and no treatment of ewes. When the efficacy of abamectin was 50% and refugia was low, STARTECT® was highly effective for five years, after which AR developed rapidly to derquantel and the efficacy of abamectin dropped to 0%.

In the Learmount et al. (2014) simulation study, the maintenance of the low efficacy of abamectin and the high efficacy of derquantel in a high refugia environment is promising. Considering the AR identified in this study, the ideal situation where both drugs in a combination are highly effective is unlikely to be realized. In a combination, both anthelmintics should have a similar duration of activity, so that throughout exposure,

only doubly resistant GIN survive treatment. Moxidectin and closantel have persistent activity, whereas levamisole's duration of activity is short. Compromises will need to be made on which anthelmintics to use in combinations. Multidrug resistance needs to be investigated so that farmers can make informed decisions on the best anthelmintics to administer. For example, on Farms 1, 8, and 12, percent efficacies of albendazole are high enough for it to be used in combination with another drug. Importantly, when the efficacy of both anthelmintics in the combination is less than about 70%, the ability of each anthelmintic to protect the efficacy of the other is greatly diminished (Leathwick et al. 2012). Therefore, this study is timely because we have access to a few highly effective anthelmintics. Levamisole and moxidectin are effective against all GIN, and closantel is effective against *H. contortus*. One or more of these anthelmintics could be used in combination with lower efficacy drugs on farms with AR. It should be specified that since multi-active formulations are not available in Canada, using anthelmintics in combination involves administering the full dose of each drug sequentially, not mixing them together (Abbott et al. 2012).

Increasing transparency about the use of anthelmintics among farmers would be a great benefit to farmers and the sheep industry. Currently, such communication between farmers selling and buying sheep in Nova Scotia is lacking (author's observation). Knowing the efficacy of anthelmintics on source farms would help farmers buying sheep to decide which anthelmintics to use in quarantine treatments, or at least convince them that quarantine treatments are necessary. Most farmers do not quarantine incoming sheep and those who do typically use only one anthelmintic (Falzon et al. 2013c; Jones, personal communication 2014). Since almost all farms use either a macrocyclic lactone or

a benzimidazole, there is a 50/50 chance that new sheep will be treated in quarantine with a drug from the same group as was used on the source farm, to which GIN are likely resistant. Anthelmintic combinations would be highly beneficial to quarantine treatments, and are recommended (Abbott et al. 2012).

The preservation of a refugia population of susceptible GIN is fundamental to the successful and sustainable incorporation of anthelmintic combinations in worm management (Besier 2012). This can be achieved by leaving some sheep untreated. The proportion of sheep that need to be left untreated depends on the level of AR (Barnes et al. 1995). With a higher level of AR, a greater proportion of sheep need to be left untreated (Barnes et al. 1995). This leads to the difficult situation where farmers must balance reducing treatments without compromising sheep health. What's more, it necessitates that farmers accept living with parasitism rather than trying to eliminate it.

Leathwick et al. (2012) determined that leaving 10% of lambs untreated when an anthelmintic is 95% effective can slow the development of AR. However, farmers in Nova Scotia may be unable to get through an entire grazing season without dosing all lambs at least once. For example, on Farm 1 no lambs would have survived without treatment in 2013, and just four lambs went without treatment in 2014. Leaving a portion of ewes untreated is a better alternative (Dobson et al. 2011a, 2011b). The above mentioned studies attempted to quantify the parameters for adequate refugia, but each farm is unique so quantifications are not broadly applicable. The general message can be boiled down to: for best results, treat as few sheep as possible.

Preserving susceptible *H. contortus* from one season to the next can only be achieved thorough hypobiotic larvae within ewes that have not recently been exposed to anthelmintics. Hence, ewes are the key to refugia. Anthelmintic use can be reduced with targeted selective treatment, which involves identifying and treating only sheep that need it (Kenyon et al. 2009; Besier et al. 2010; Learmount et al. 2015). This can circumvent the risk of disease inherent in leaving a certain proportion of the flock untreated and has been shown to reduce anthelmintic treatment by up to 50% (Busin et al. 2013). The FAMACHA© system is an excellent tool for identifying anemic sheep that need treatment for infections with *H. contortus* (van Wyk and Bath 2002). The FAMACHA© system has been validated in the United States and thousands of farmers there have been trained to use this system at workshops (Terrill et al. 2012). FAMACHA© has been readily adopted because it is easy to learn and employ. Several farmers in Nova Scotia have been provided with FAMACHA© cards at workshops.

4.5 Grazing management to reduce treatment frequency

Non-drug worm management strategies can reduce the need for anthelmintic treatment, thereby reducing the selection pressure for resistant GIN (Abbott et al. 2012). Grazing lambs separately from ewes was an excelllent strategy to limit infections with *H. contortus* and lambs grazed without ewes in 2014 were never treated. The simplicity and cost effectivenss of this strategy may be acceptable to farmers, particularly to those who have resorted to raising lambs in the barn. In the face of unmanageable AR *H. contortus*, lambs could be raised on pasture where forage is freely available. Other AR GIN could be managed too, because infections would be limited to those arising from overwintered

L3s. Additionally, preventative treatments of ewes and lambs, to reduce pasture contamination and subsequently prevent high worm burdens in lambs, would not be needed. This would help to maintain a refugia population of susceptible GIN in ewes from year-to-year.

The results of the larval culture from the only lambs group were unexpected. Over 30% of larvae recovered were *H. contortus/Cooperia spp.*. Correct labeling of the samples was confirmed, and through consultation with the shepherd of the flock it was discovered that two of the lambs in the only lambs group repeatedly escaped under the fence and grazed in the pasture with lambs and ewes (Hines, personal communication 2014). These two lambs consistently had higher FEC than the rest of their group. Feces from one of these lambs was part of the composite larval culture. It is likely that the *H. contortus/Cooperia spp.* came from this lamb, or from lambs infected from eggs shed by the escapees. The two escapee lambs inadvertently supplied the rest of the lambs in their group with exposure to *H. contortus,* but low infection levels were maintained.

Lambs need continual exposure to GIN to develop and maintain acquired immunity (Abbott et al. 2012). While immunity may be unnecessary for market lambs, immunity is essential for lambs that remain for breeding. Seeding a pasture of only lambs with GIN eggs from other sheep could provide adequate exposure for immune development without lambs facing high rates of larval challenge that could lead to clinical illness. Due to *H. contortus's* high biotic potential, this strategy is not as easy as it seems. For example, in 2013 two ewe lambs went from the lambing barn to a clean ¼ acre paddock. The ewe lambs shared their paddock with one other lamb for 10 days in

July. By the end of the grazing season the ewe lambs were anemic and one had a FEC over 33 000epg (Jones 2013). In 10 days, the other lamb had heavily contaminated the pasture with *H. contortus*. An intentional and controlled seeding of GIN on pasture where only lambs are grazed has great potential.

Conclusions

In this study, AR was present and in some cases severe. A transition to using anthelmintics in combination is vital to preserve the efficacy of available drugs and to provide adequate worm control. Multidrug resistance needs to be investigated and routine FECRTs are essential to monitor anthelmintic efficacy. Closantel is an excellent option for controlling resistant *H. contortus* and levamisole or moxidectin are suitable options where several species are resistant. It has been standard practice for many years to treat whole flocks in winter and/or at turnout (Falzon et al. 2013c; Jones, personal communication 2014). This has undoubtedly contributed greatly to the rapid development of AR in *H. contortus*. A paradigm shift from preventative whole flock treatments in the fall and winter to targeted treatments and the incorporation of non-drug worm management strategies to preserve refugia is desperately needed. Without such changes, sheep farming is unsustainable.

It is likely that the use of unlicensed anthelmintics and closantel will become a mainstay in worm management in Nova Scotia. A major limitation to GIN management in Canada is that few drug groups are available. While effective anthelmintics should not be held in reserve until other drugs fail, we must dissuade farmers from switching to the use of a highly effective drug as the sole means of controlling GIN. Bringing in a new drug without implementing changes to farming practices would be a very short-term solution. Monepantel, the first anthelmintic in a new group called the amino-acetonitrile derivatives, was introduced in the Netherlands in 2011. After two years of use on one farm, *H. contortus* had developed AR to monepantel, and a FECRT showed that efficacy

was 0%. The farmer did not think that other anthelmintics were effective and switched to frequently using solely monepantel (Van den Brom et al. 2015). The Van den Brom (2015) study highlights exactly the type of situation that we do not want to occur in Canada. The limited availability of levamisole, through a veterinary compounding pharmacy, and to closantel, via the Emergency Drug Release process, provides an opportunity for farmers to be educated about the drugs' sustainable use. For a farmer to access closantel, a veterinarian with whom the farmer has a Veterinarian-Client Professional Relationship (VCPR) must submit an application for an Emergency Drug Release. Currently, farmer outreach is insufficient, and educating farmers about GIN is exceedingly difficult when farmers are unaware that they have a problem.

Based on the results of this study, the challenges ahead seem daunting, but this situation is in no way unique. Indeed, Nova Scotia is similar to everywhere else that has investigated AR, particularly regions with cold winters where *H. contortus* has become a major problem (Maingi et al. 1996b; Höglund et al. 2009; Domke et al. 2012a, 2012b; Groz et al 2013; Peña-Espinoza et al. 2014). Developing an organized and accessible sustainable management education program for farmers could help achieve the necessary changes that are crucial to making sheep farming sustainable in Nova Scotia.

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Appendix A

Table A1. Guideline for the interpretation of FEC. Modified from: Abbott et al. (2012).

Species contributing to infection	Infection Level (epg)		
	Low	**Medium**	**High**
Mixed (no *H. contortus*)	<250	250-750	>750
Mixed (with *H. contortus*)	<500	500-1500	>1500
H. contortus	<500	1000-5000	>5000
T. circumcinta	100–500	500-1500	>1500
Trichostrongylus spp.	100–500	500-1500	>1500
N. battus	<150	150-300	>300

Table A2. Anthelmintic drug groups used against GIN (Abbott et al. 2012; University of Guelph 2012).

Drug group	**Chemical name(s)**	**Spectrum**	**Accessible in Canada**	**In Canada, licenced for use in**
Macrocyclic Lactones	Ivermectin,	Broad	+	Sheep
	Doramectin,	Broad	+	Cattle
	Eprinomectin	Broad	+	Cattle
	Abamectin	Broad	+	Cattle
and Milibemycins	Moxidectin	Broad	+	Cattle
Benzimidazoles	Fenbendazole	Broad	+	Cattle
	Albendazole	Broad	+	Cattle
Imidazothiazoles	Levamisole	Broad	+*	Not licenced*
and	Pyrantel	Broad	-	
Tetrahydropyrimidines	Morantel	Broad	-	
Amino-acetonitrile Derivatives	Monepantel	Broad	-	
Spiroindoles	Dual-active of derquantel and abamectin	Broad	-	
Salicylanilides	Closantel	Narrow	+**	Sheep**
and Substituted Phenols	Nitroxynil	Narrow	-	

Access to all drugs requires a prescription from a veterinarian

*Levamisole is available only through a compounding veterinary pharmacy

** Closantel is available only through the Emergency Drug Release process by veterinary application to the Federal Ministry of Health

Appendix B

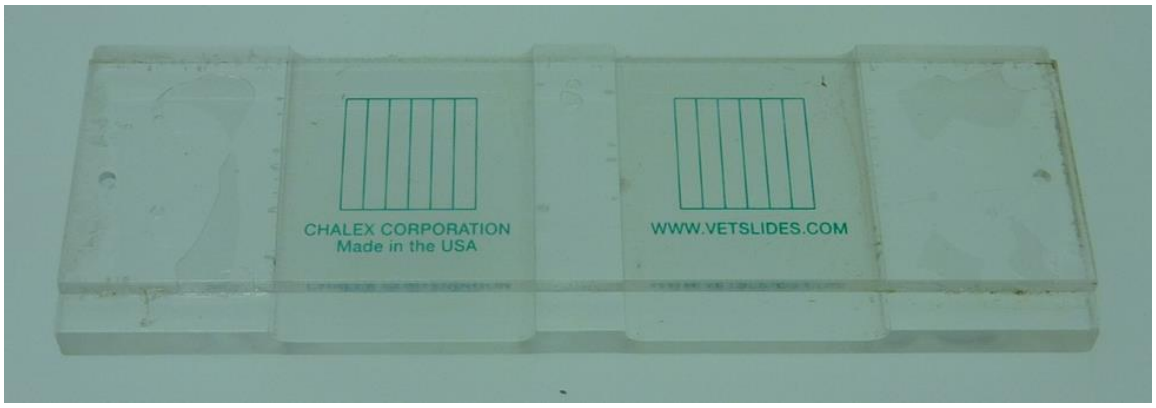

Figure B1. Two-chamber McMaster counting slide used for FEC floatation of ruminant feces (Chalex Corporation, USA).

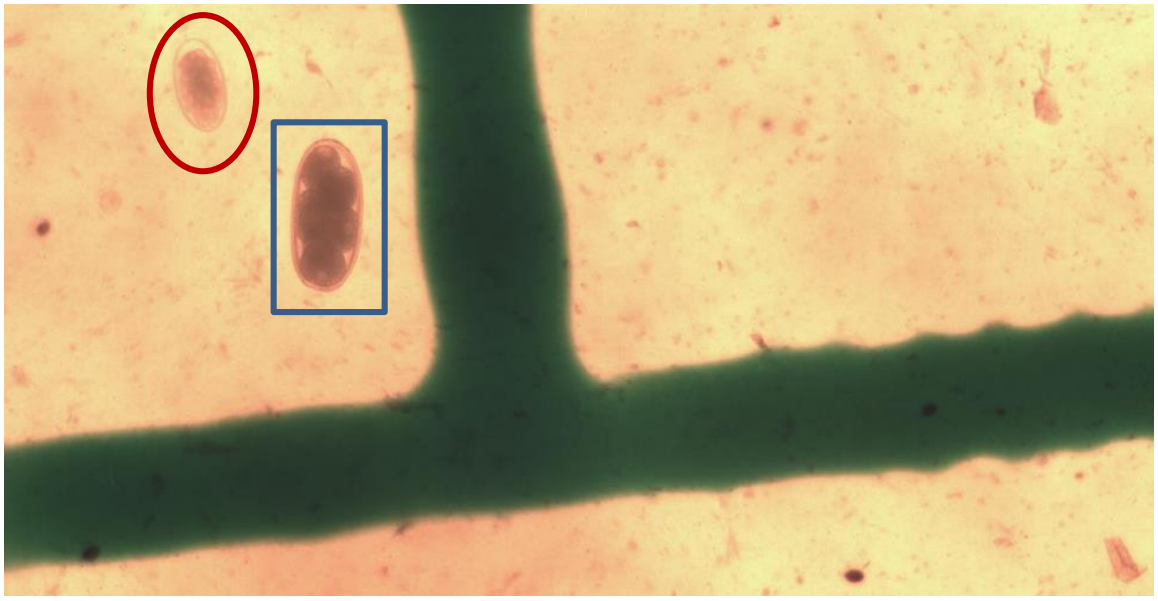

Figure B2. GIN eggs observed within the grid lines of a McMaster counting slide, at 100x magnification. *N. battus* egg (blue rectangle), and strongyle-type egg (red oval).

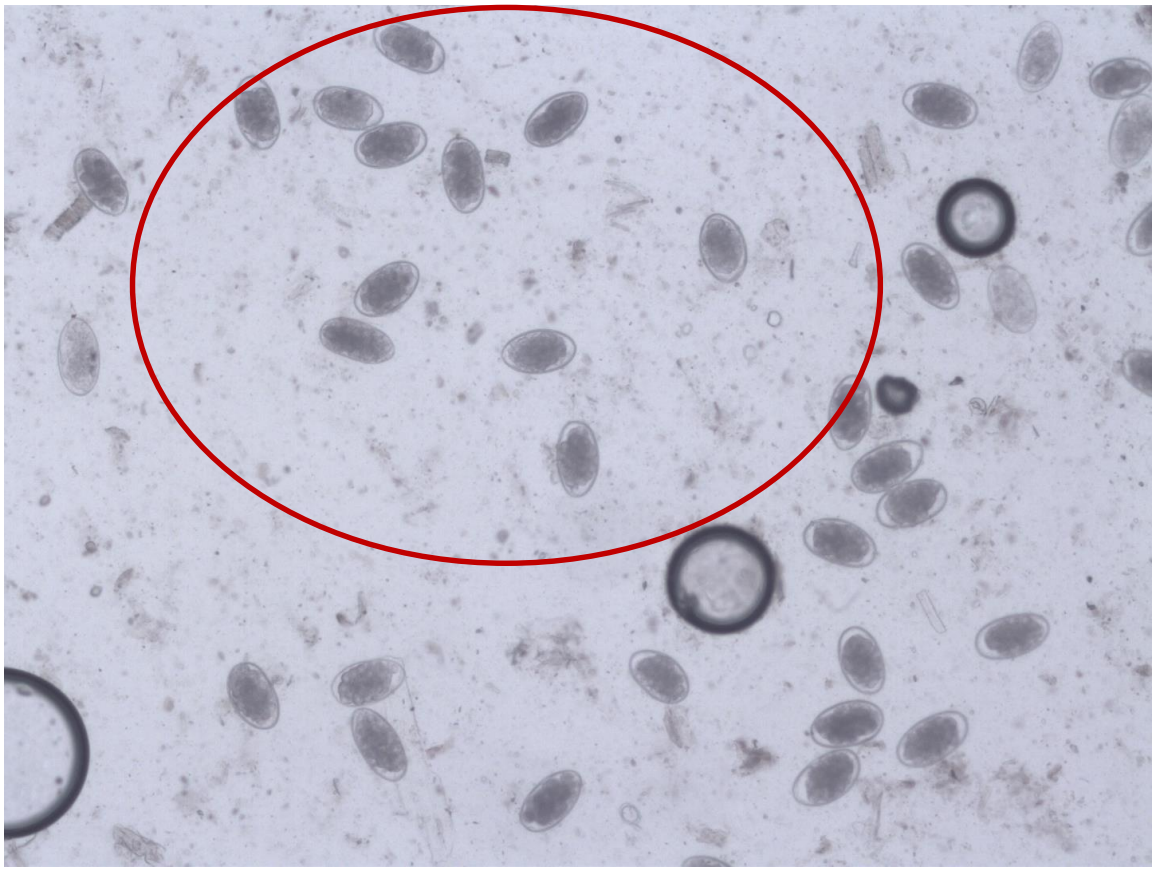

Figure B3. Strongyle-type eggs found in sheep feces, which cannot be differentiated by microscopy, at 100x magnification. These could be eggs of: *H. contortus*, *T. circumcincta*, *Trichostrongylus spp.*, *C. ovina*, *Cooperia spp.*, *Bunostomum spp*., and/or *Oesophagostomum spp.*. If this density of eggs was observed within the grids of the McMaster slide the FEC would be ~30 000epg.

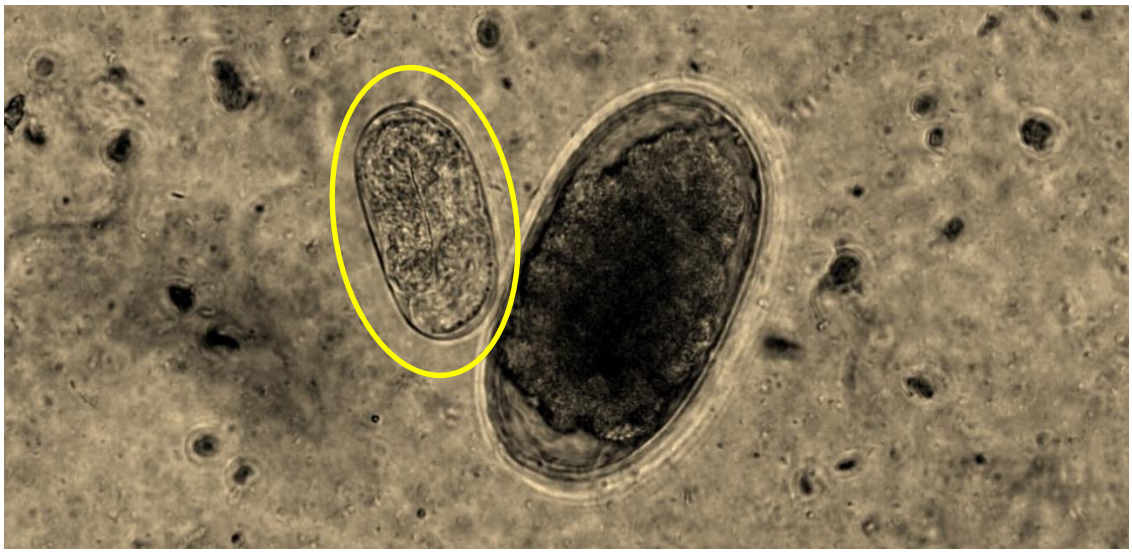

Figure B4. *S. papillosus* egg (yellow oval) next to a strongyle-type egg, at 100x magnification (expanded).

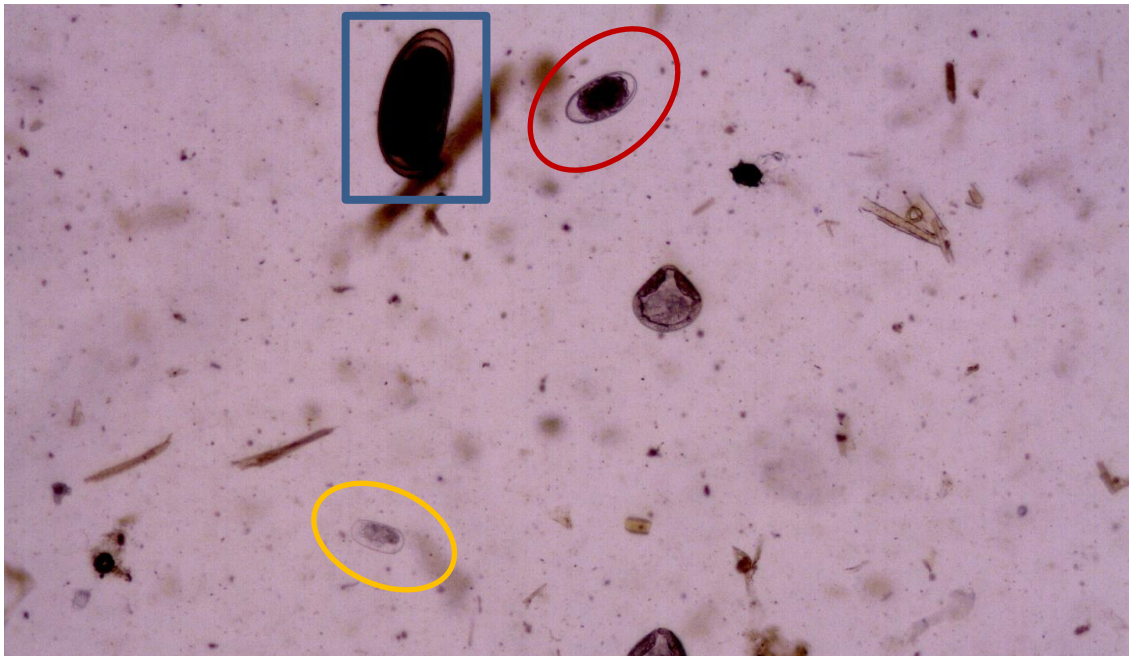

Figure B5. *N. battus* egg (blue rectangle), *S. papillosus* egg (yellow oval), and strongyle-type egg (red oval), at 100x magnification.

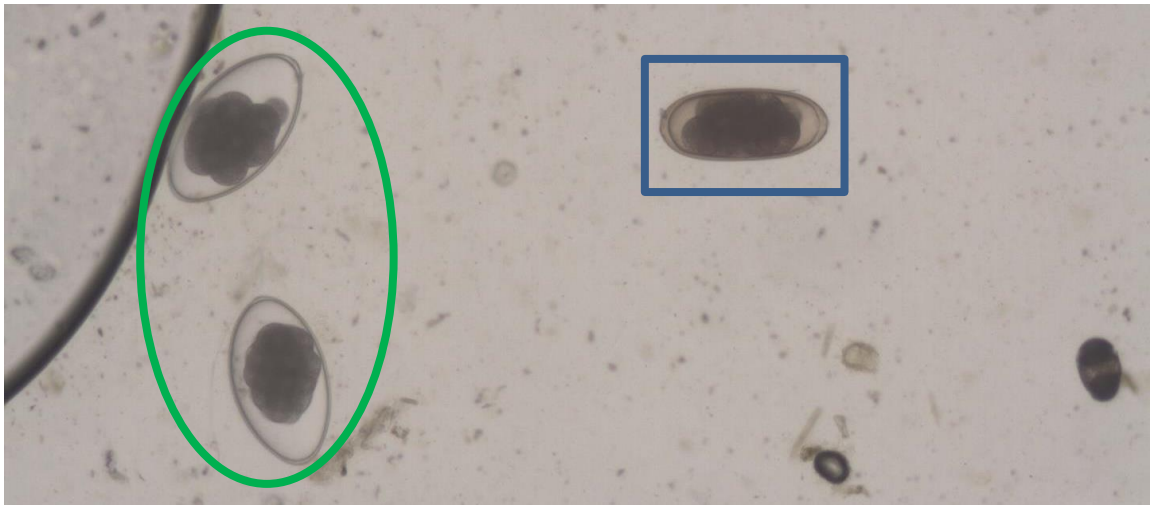

Figure B6. *N. battus* egg (blue rectangle) and *N. filicollis* eggs (green oval), at 100x magnification.

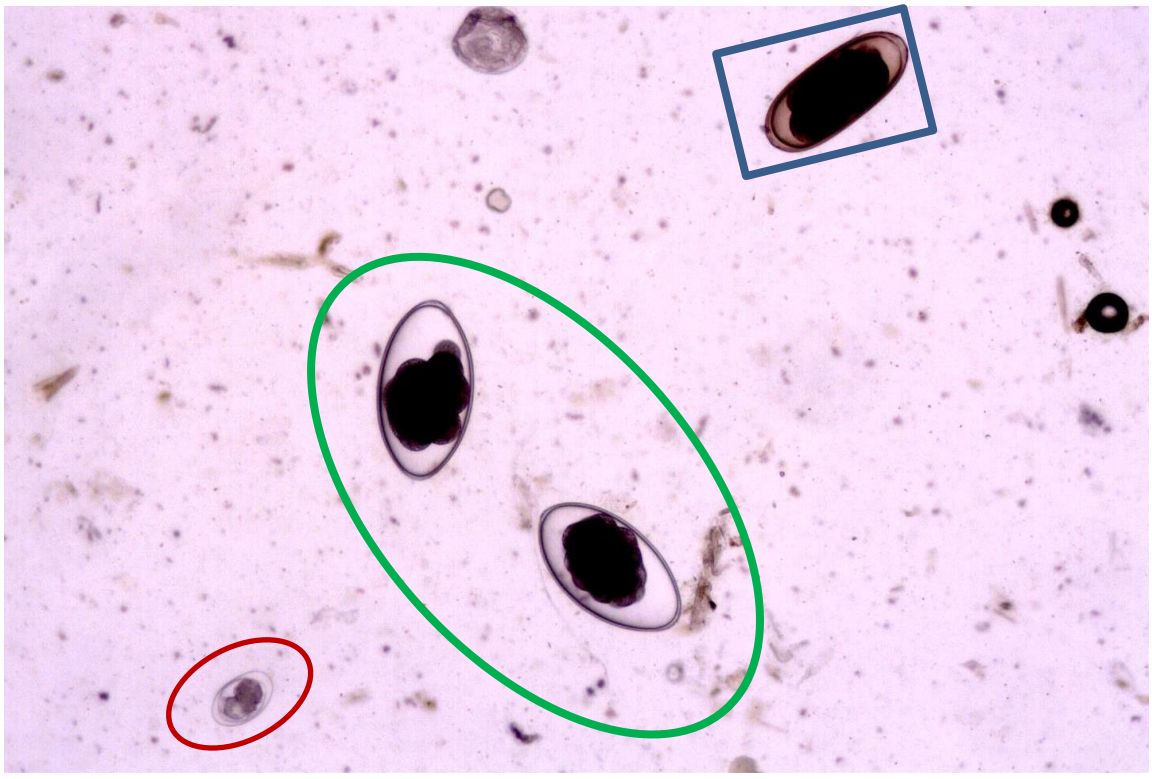

Figure B7. *N. battus* egg (blue rectangle), *N. filicollis* eggs (green oval), and strongyle-type egg (red oval), at 100x magnification.

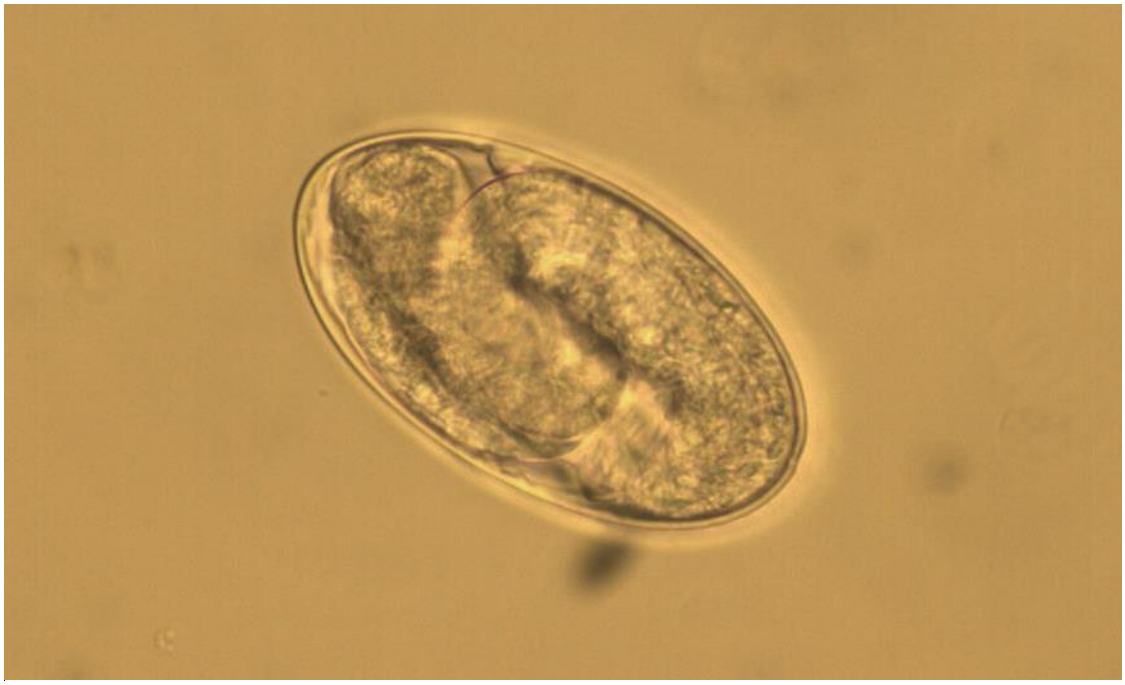

Figure B8. Developing larva inside a strongyle-type egg, at 400x magnification.

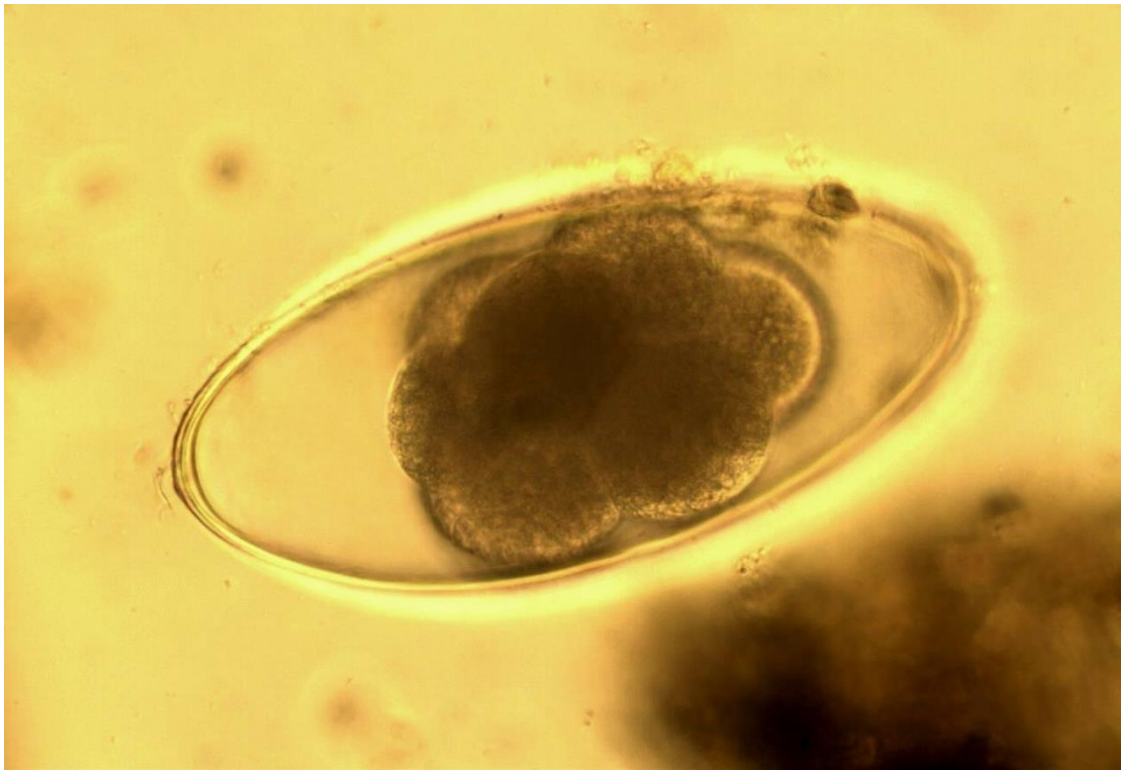

Figure B9. *N. filicollis* egg containing an early-stage embryo, at 400x magnification.

Table B1. Example of data sheet used to record FEC.

FARM ID: RECIEVED: PAGE # OF

DATE COLLECTED: ANALYZED:

FCS	Animal ID	Sex	Age	Category	FAMACHA	Raw GIN	Raw *N. b*	EPG GIN	EPG *N. b*

Age = L/Y/A lamb/yearling/adult Category = C control, TC treat Closantel, TI Ivermectin, TL levamisole, TV albendazole, TF fenbendazole, TM moxidectin

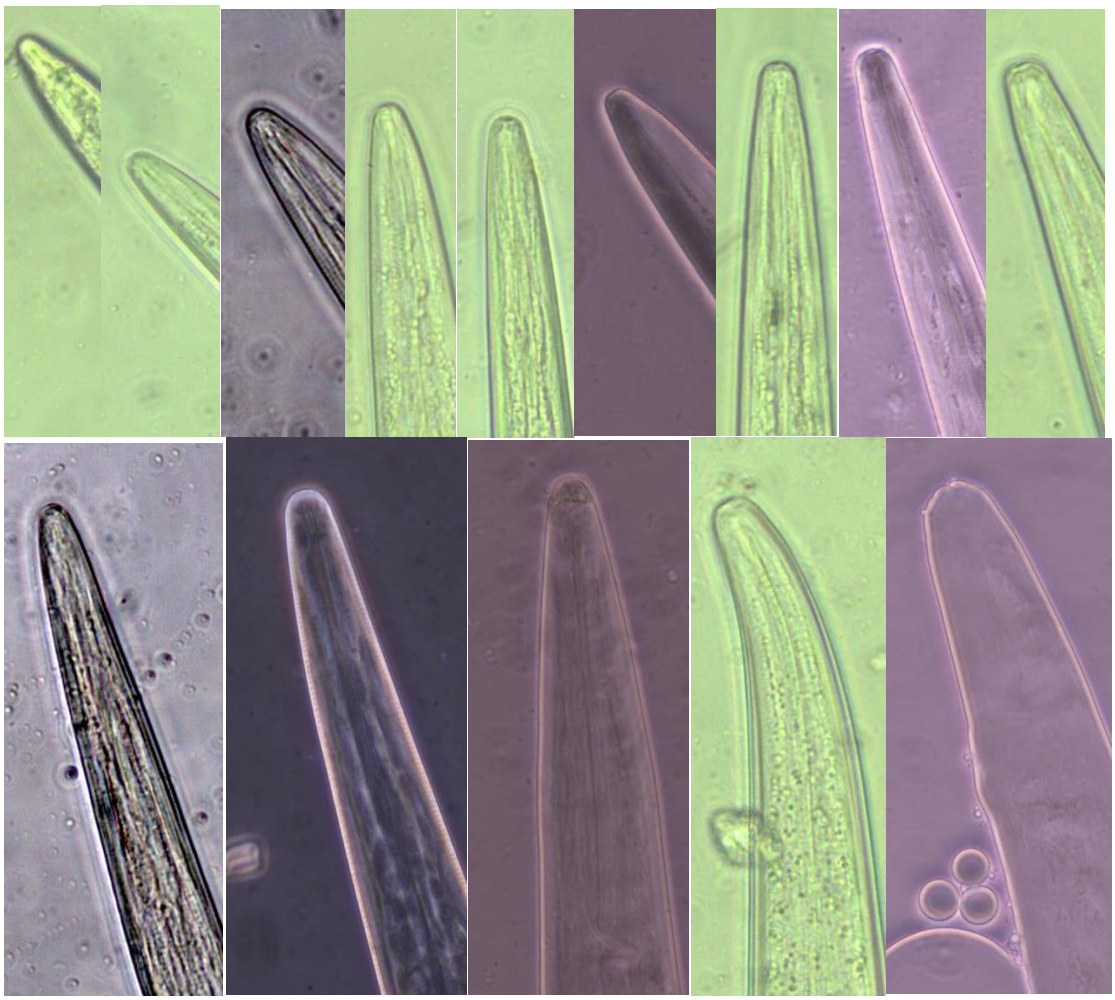

Figure B10. Variation in head shape of L3s, at 400x magnification. Shape can range from obviously round (top left) to obviously square (top right), and from narrow (bottom left) to broad (bottom right).

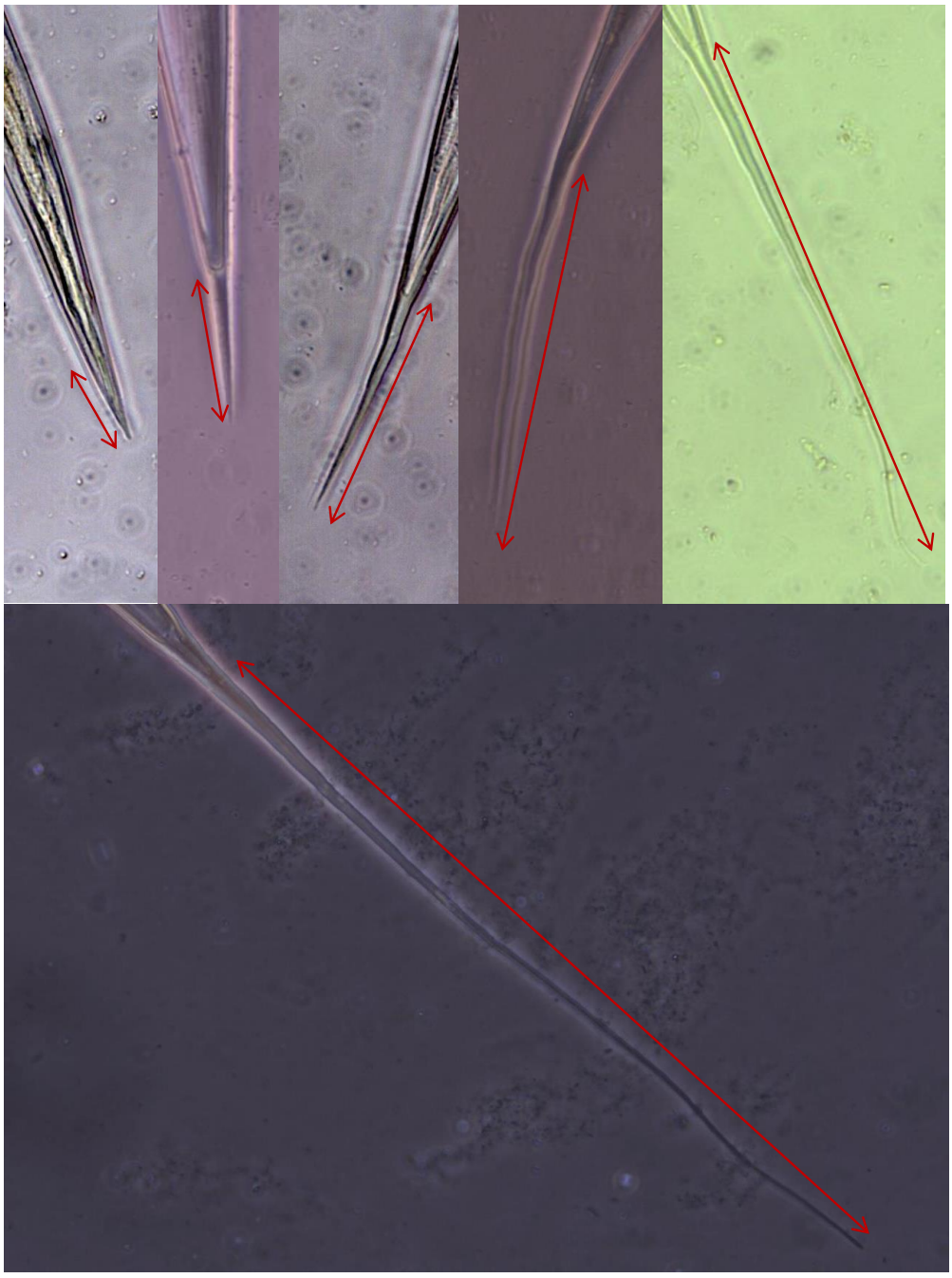

Figure B11. Variation in tail-sheath-length of L3s, at 400x magnification. Red arrows indicate the sheath of the larvae. From left to right (top): X=1.0, X=1.7, X=2.5, X=4.1, X=6.0; bottom X=9.3.

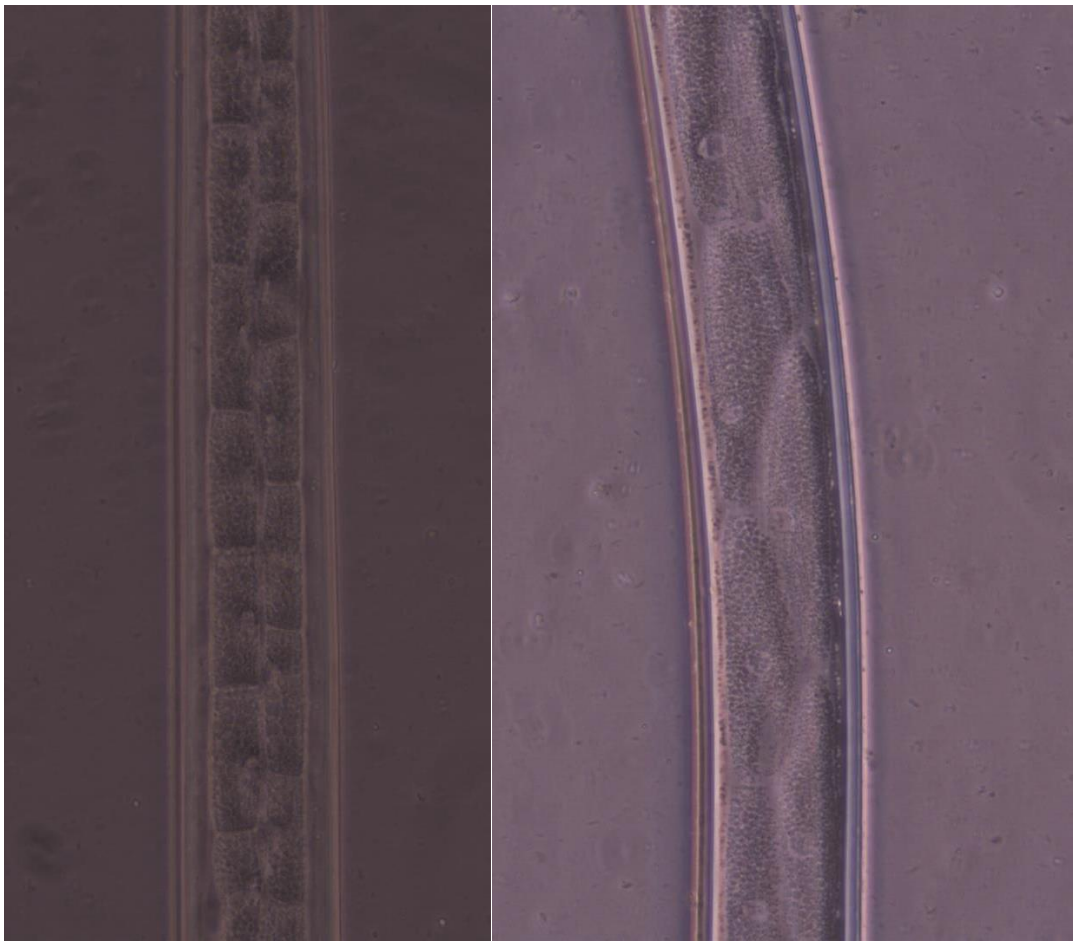

Figure B12. Gut cell shape of L3s. Rectangular (left) and triangular (right), at 400x magnification.

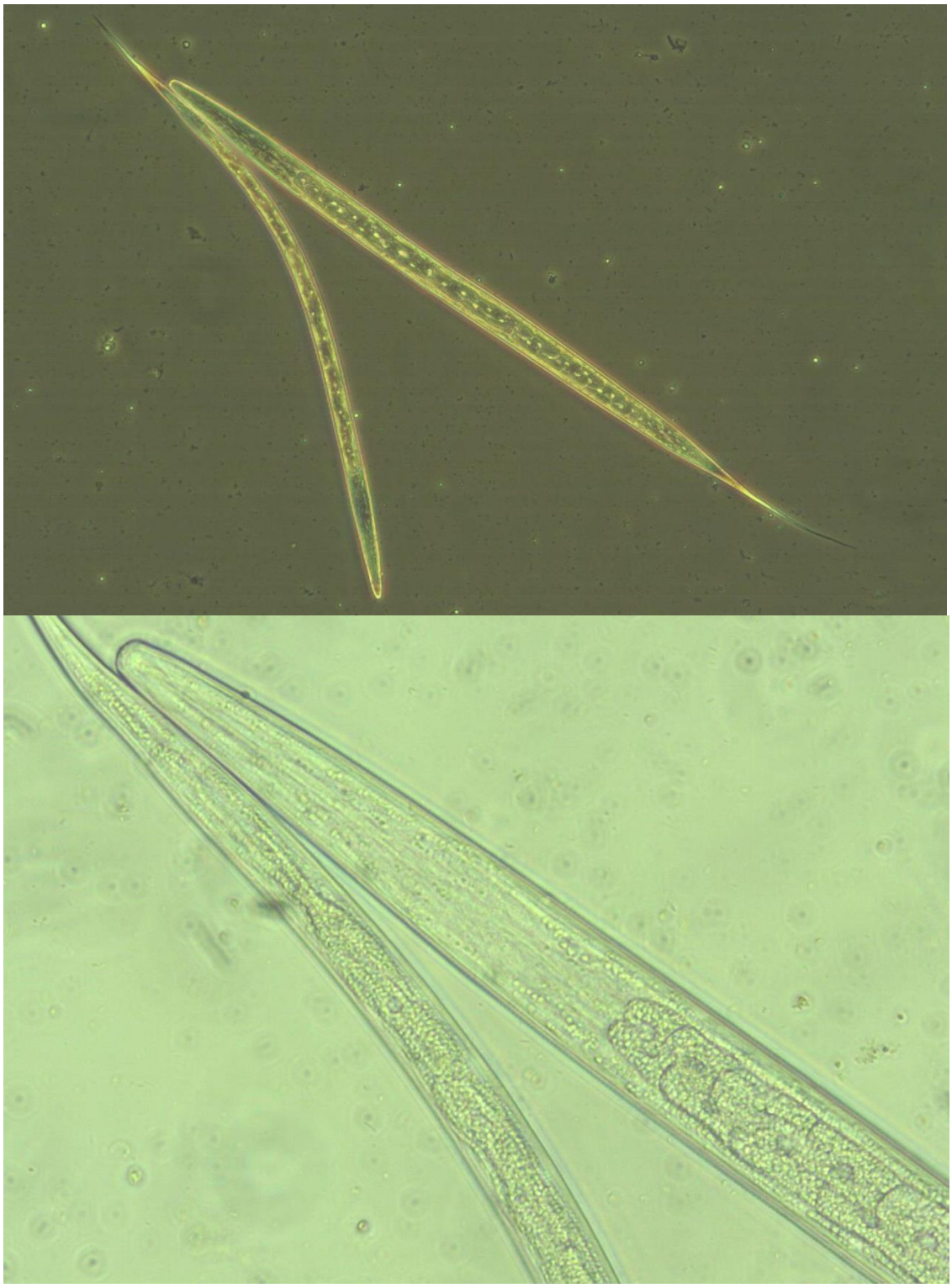

Figure B13. Variation in body width of L3s. Narrow (*H. contortus*) and wide (*C. ovina*) L3s at 100x magnification (top); same L3s at 400x magnification (bottom).

Figure B14. *N. battus* larva recovered from a seven week-old larval culture from Farm 8, at 100x magnification.

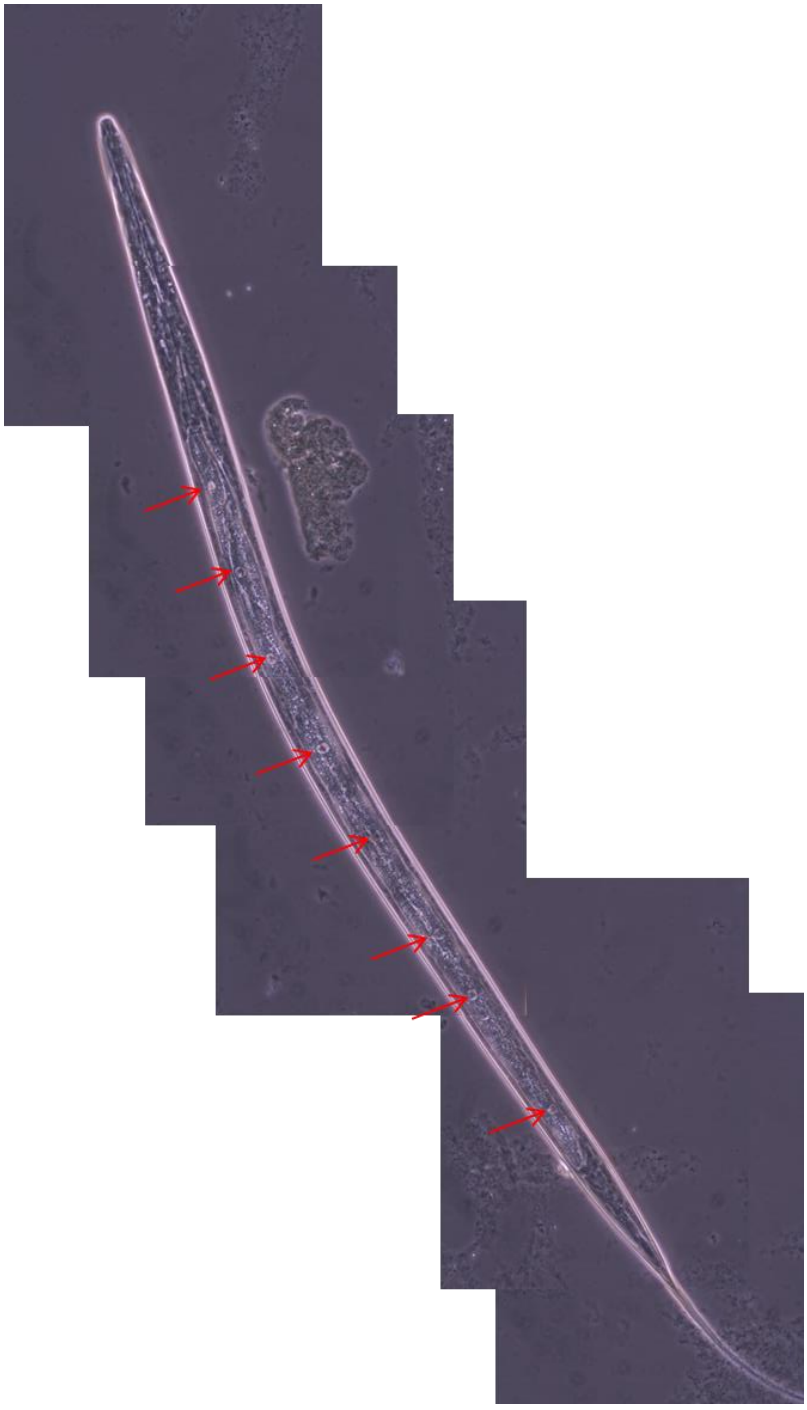

Figure B15. Composite photo of a *N. battus* larva recovered from a seven week-old larval culture from Farm 8. The nucleus inside each of the eight gut cells is visible as a bright circle (red arrows). Photographs taken at 400x magnification.

Figure B16. Head and tail of H. contortus, at 400x magnification.

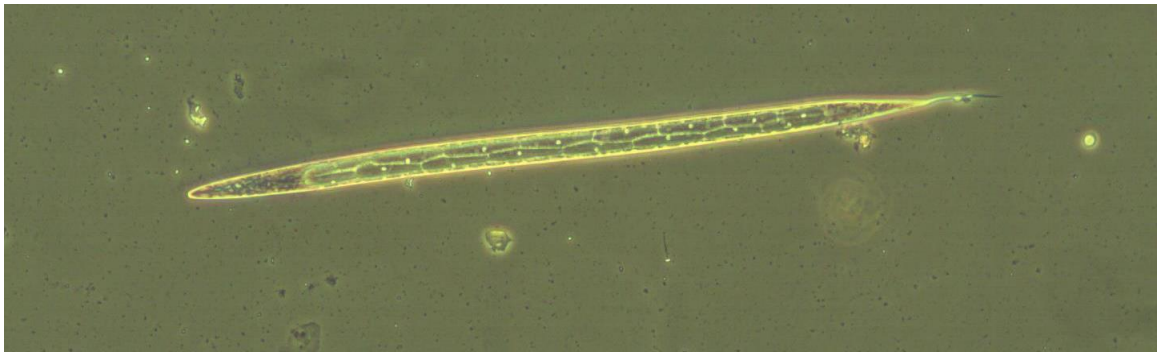

Figure B17. GIN with 16 triangular gut cells, at 100x magnification. The nucleus in each gut cell is visible a bright dot.

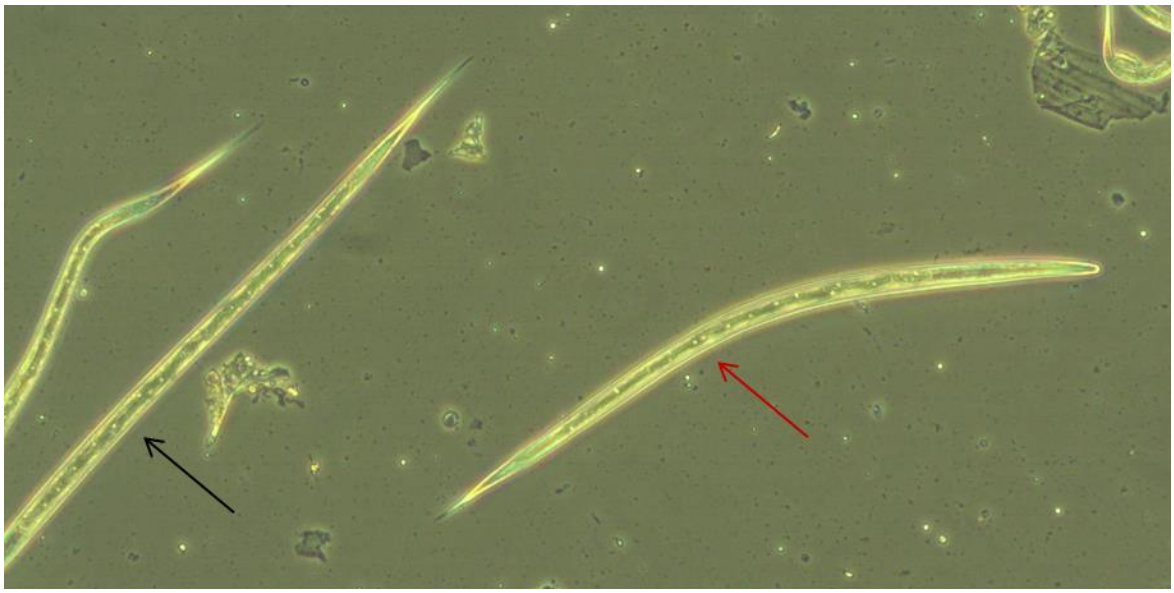

Figure B18. *H. contortus* (black arrow) and *T. circumcinta/Trichostrongylus spp.* (red arrow), at 100x magnification.

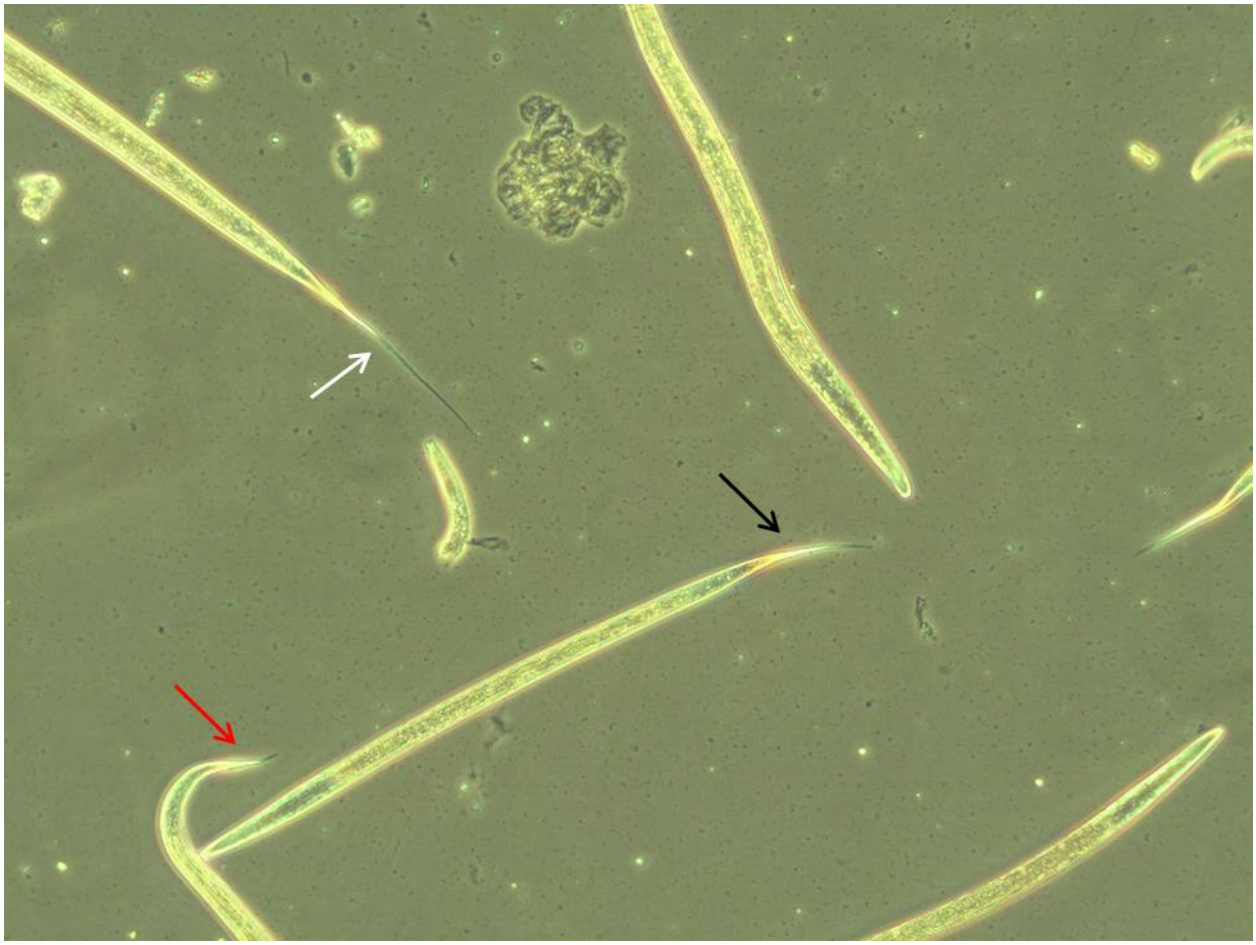

Figure B19. Short, medium, and long tailed GIN. Short: *T. circumcinta/Trichostrongylus spp.* (red arrow), medium: *H. contortus* (black arrow), and long: *C. ovina*, at 100x magnification.

Table B2. Example of data sheet used to record morphological characteristics of L3s.

FARM ID: ANALYZED: PAGE # OF

DATE OF CULTURE: DRUG GROUP:

Shape (length, width, straight/curved)	Sheath-length	Filament?	Head shape	Intestinal cells (#, shape)	Tail (straight, kinked)	Other