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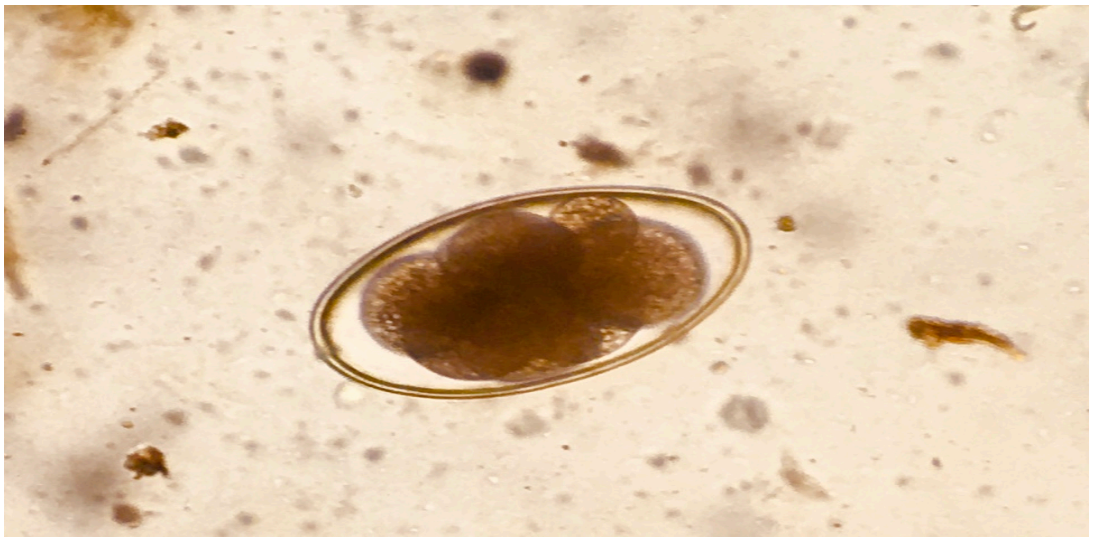

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Urea's ability to break the life cycle of *Nematodirus* spp. *in situ*

A field study on sheep in New Zealand

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SUMMARY

Nematodirus spp., along with other gastrointestinal nematodes, are a common cause of disease among sheep and remains a major animal health concern, and cause of reduced productivity for sheep farmers around the world. As the resistance against anthelmintics is substantial among gastrointestinal nematodes, there is an urgent need of significant alternatives to anthelmintic treatment. The life cycle of *Nematodirus* comprises stages both within and outside the host, with the pre-parasitic development to the infective third stage, occurring on pasture. With evidence suggesting that nitrogenous fertilisers could have the ability to inhibit egg hatching and larval survival in numerous gastrointestinal nematodes, the aim of this study was to investigate urea's ability, *in situ,* to break the life cycle of *Nematodirus* spp. outside of the host. The method used was through targeted application of liquid urea to experimental pastures at times when *Nematodirus* eggs are present on pasture. The study included seven experimental paddocks previously grazed by sheep with a known infestation of *Nematodirus* spp. and other *Strongyles.* The sheep were of different genetic lines, bred for either resistance or resilience against gastrointestinal nematodes. The application of liquid urea involved five repeated sprayings of 40kg N/ha every three weeks before the start of grazing season, or one single spraying of 200kg N/ha three months before start of grazing, with control paddocks left untreated. The effect of treatments was evaluated utilising three methods, i.e. observing pasture larval concentration, faecal egg counts and concentration of eggs and larvae in soil. The study had a main focus on *Nematodirus* spp., although "other" *Strongyles* were included in the results to give a better understanding of the overall effects of the treatments. Overall the results were equivocal, with absence of consistently statistically significant differences between treatments and control. However not significant, although a subject for discussion, was that urea could have a stimulating effect on egg hatching and larval development. In conclusion, the results from this field trial have demonstrated insufficient evidence that nitrogenous fertilisers provide epidemiological benefits in reducing larval contamination on pasture. It has been demonstrated that it is challenging to translate the *in vitro* results into the field, with the parasite having complex hatching requirements arising of the environment, thus difficult to influence.

CONTENT

List of abbreviations

FEC	Faecal egg count
FECRT	Faecal egg count reduction test
RPM	Rising plate meter
EPG	Eggs per gram
PLC	Pasture larval count
Ha	Hectare
ML	Milliliter
DM	Dry matter
Kg	Kilogram
°C	Degree Celcius

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INTRODUCTION

Nematodirus spp., along with other gastrointestinal nematodes, are a common cause of disease and remains a major animal health concern, and cause of reduced productivity for sheep farmers in New Zealand and around the world (Lawrence *et al.*, 2007). A lamb suffering from subclinical parasitism commonly results in a significant reduction in body condition scores, fleece weights and carcass value (Miller *et al.*, 2012). Today, the control of gastrointestinal parasites depends mainly on anthelmintic treatment. Nevertheless because of the widespread and emerging anthelmintic resistance among gastrointestinal nematodes of sheep in New Zealand and globally, there is an urgent necessity for alternatives (Waghorn *et al.*, 2006).

Effective parasite control relies on understanding the life cycle and the epidemiology of the present parasite (Familton & McAnulty, 1997). The life cycle of *Nematodirus* comprises of stages both within and outside the host, with the preparasitic development to the infective third stage, occurring on pasture. Moreover, *Nematodirus* has a unique life cycle amongst the Strongylid nematodes, in that the development to the third infective larval stage occurs within the egg, which makes the parasite particularly resistant to the environment (Kates & Turner, 1955). *Nematodirus spathiger* and *N. filicollis* are the species commonly found infecting young lambs in New Zealand (Oliver *et al.*, 2014) and cause clinical disease of different levels depending on magnitude of infestation (Kates & Turner, 1955).

Since it has been estimated that more than 90% of the total burden of gastro-intestinal nematodes is present in the phase outside of the host, a suitable strategy may be targeting this stage to depress the parasitic burden (Familton & McAnulty, 1997). This is especially the case for *Nematodirus* spp. which may spend many months as an egg on pasture (Vlassoff *et al.*, 2001).

Evidence suggests that nitrogenous fertilisers have the ability to inhibit egg hatching and larval survival in numerous gastrointestinal nematodes (Rodriguez-Kabana, 1986). In particular, *in vitro* studies have demonstrated urea's potential ability to break the life cycle of *Trichostrongylus colubriformis,* another member of the 'superfamily' that *Nematodirus* belongs to, through inhibiting egg hatching (Cairns *et al.*, 2017). Further *in vitro* studies have suggested this effect may extend to *Nematodirus* spp., though validation in the field is still required (J Bennett, 2017, unpublished honours dissertation, Lincoln University).

The aim of this study was to investigate urea's ability, *in situ,* to break the life cycle of *Nematodirus* spp. outside the host, through targeted application of liquid urea to pastures at times when *Nematodirus* eggs are present on pasture. This may provide significant alternatives to anthelmintic treatment, and the resistance among gastrointestinal nematodes can possibly be opposed.

LITERATURE REVIEW

Nematodirus

Gastrointestinal nematode parasites belonging to the genus *Nematodirus* (figure 1) cause disease in domesticated and wild ruminants throughout the world. An individual sheep usually co-harbour several species at various sites in the gastrointestinal tract. The most numerous and pathogenic of these are *Haemonchus contortus, Ostertagia* spp., and *Trichostrongylus axei,* which are found in the abomasum. Important parasites of the small intestines includes *Trichostrongylus* spp., *Nematodirus* spp., and *Cooperia* spp. (Vlassoff & McKenna, 1994).

Kingdom:	Animalia
Phylum:	Nematoda
Class/subclass:	Secernentea
Order:	Strongylida
Superfamily:	Trichostrongyloidea
Family:	Molineidae
Subfamily:	Nematodirinae
Genus:	*Nematodirus*
Species:	*filicollis, spathiger, abnormalis, battus* (and other)

Figure 1. *Taxonomy (Taylor, 2016).*

Distribution

Worldwide

The geographical distribution of *Nematodirus* spp. is generally global, although depending on species and environment, the parasite is generally more prevalent in temperate zones. *Nematodirus battus* is of more clinical importance in the British Isles but does occur in a number of European countries, parts of USA and Canada, and Australia, though it has never been reported in New Zealand (Taylor, 2016).

New Zealand

Nematodirus filicollis, N. spathiger and *N. abnormalis* are the species that frequently occur among sheep in New Zealand. The two former are the important species responsible of outbreaks of disease. Comparing the North and South Islands of New Zealand, between November 2010 and April 2011, Oliver *et al*. (2014) showed 100% prevalence for *N. spathiger* at all the tested farms, 22 farms from the North Island and 28 farms from the South Island, and 76% prevalence for *N. filicollis,* indicating that *Nematodirus* has a widespread distribution over the country and that the two species frequently occur as coexisting infections (Oliver *et al.*, 2014; Brunsdon, 1961). Outbreaks of nematodirosis during the 1950s seemed to

occur mostly in Canterbury, Otago and Southland, which could indicate that, at the time, nematodirosis was a larger problem in the South Island. Subsequent, preventative drenching reduced the incidence of the outbreaks (Brunsdon, 1967). Other studies have also showed *Nematodirus* to be of more importance in the South Island than the North Island (Vlassoff & McKenna, 1994).

Sweden

Nematodirus filicollis and *N. spathiger* are the most common species infecting sheep in Sweden. However, in 1999, *N. battus* was recorded for the first time (Lindqvist *et al.*, 2001).

Life cycle

The fundamental life cycle for nematodes consists of eggs, followed by four larval stages and completing as an adult in the fifth stage. *Nematodirus* spp. has a direct life cycle, meaning there is no intermediate host, and the life cycle comprises a stage within the host and a stage outside the host. Reproduction is sexual and confined to the adult parasitic stage inside of the host. Each worm in the host has been ingested from larvae contaminated pasture. The females reside in the small intestines and lay eggs which are passed out in the faeces of the host (Familton & McAnulty, 1997). In the *Nematodirus* spp. life cycle there are four moults (shedding of the cuticle), the successive larval stages being termed L_1, L_2, L_3 (the infective stage), L_4, and finally L_5, which is the immature adult. The preparasitic phase for *Nematodirus* spp. is almost unique among the trichostrongyloids parasites in that the development to the L_3-stage takes place within the eggshell, which means that all the free-living larval stages are protected until the time of hatching. In contrast, most other trichostrongyloid species hatch as first stage larvae. Post hatching, the L_3 is infective to their hosts by ingestion (Taylor, 2016; Rickard *et al.*, 1989; Gibson & Everett, 1982). Within the small intestines the larvae develop to adults, following two further moults. The pre-patent period has been reported to be approximately 14 days, although even if sexual maturity is reached in 14 days, greater numbers of eggs are produced during the third week after infection (Thomas, 1959a; Kates & Turner, 1955).

Development rates outside the host are dependent of environmental components, especially temperature. *In vitro* studies have shown that eggs of *N. filicollis* incubated at 21° C. pass through the morula stage in 2-3 days and reach the first larval stage in 8-9 days. At this stage the larvae was undifferentiated and showed activity within the shell, but did not hatch. At day 12-16 from the start of development, the larvae became a second-stage larvae. At day 24-27, development was complete and the larvae had reached the infective larval stage, L_3. In contrast, the development took 40-45 days when the eggs were incubated at 15° C (Thomas, 1959a). The development to the infective larval stage proceeds mainly during summer. The larvae then remain dormant within the egg membranes and overwinter on pasture, to hatch the following spring. Signals for hatching appear to depend of a period of chilling, when temperatures are below 5° C, and this period works as sensitization of the eggs before the temperature rise in spring (Thomas & Stevens, 1960). However, if hatching of the egg occurs during autumn, the larvae have shown to have the capacity to survive on pasture during winter. Therefore, the potentially heavy source of infection for the spring lambs originates from larvae that have hatched in autumn and overwintered on pasture, or eggs that passed the

previous season and remained unhatched as overwintering eggs on pasture, only to simultaneously hatch when the temperature increases (Brunsdon, 1963).

Diapause, chilling and hatching requirements

Both *N. battus* and *N. filicollis* eggs can enter a state of diapause, an adaption where the hatching is postponed until spring, and gives the egg a greater opportunity of being ingested by a lamb, as that is also when the lambs are more numerous. In addition, it allows the larvae to also outlast unfavourable conditions, such as winter (Thomas & Stevens, 1960). Diapause is a form of arrested development which is irreversible, and development will not re-start, even in environments that are capable of supporting growth of the worm, until some intrinsic changes have been completed (Sommerville & Davey, 2002). This strategy has been seen to have a positive effect on hatching of *N. filicollis* and several studies have indicated that the eggs are stimulated to hatch first after a period of chilling that is followed by a rise in temperature, as seen in spring, suggesting that chilling is an important requirement in the diapause process and the ability to hatch (Van Dijk & Morgan, 2009). In contrast, Boag and Thomas (1975) described a spring peak and an autumn peak of larval occurrence which potentially could be explained by the hatching of non-chilled eggs (Boag & Thomas, 1975). Species differences occur regarding the critical hatching requirements and *N. spathiger* does not have the same strict chilling necessity to hatch. It seems that *N. spathiger* has the ability to hatch straight after reaching the L_3-stage, though studies have shown that a period of chilling before incubation did favour hatching (Viljoen, 1972). In general, a period of chilling will stimulate hatching of *N. filicollis* and *N. spathiger*, but it may not be obligate.

There is also evidence that *N. spathiger* eggs hatch more quickly than those of *N. battus* and *N. filicollis (Gibson & Everett, 1982)*. Important features that also are affecting the egg hatching, development and survival of nematode larvae are the level oxygen, moisture and pH. Absence of oxygen inhibits hatching and generally eggs and larvae need warmth and moisture to develop effectively (Familton & McAnulty, 1997). Additionally, pH appears to have the ability to affect the life cycle of nematodes. Reduced or no egg hatching of *Trichostrongylus colubriformis* was seen when pH was less than 5, although the hatching was unaffected at pH 6-13 (Cairns *et al.*, 2017). Additionally, there are factors from inside the egg that appears to stimulate egg hatching. These are increased levels of trehalose in the fluid surrounding the unhatched juvenile, increased cell permeability (caused by the trehalose), and biochemical action both inside and outside the egg. Subsequently, as the shell of the egg becomes more permeable, more biochemical agents can penetrate the biological membrane (Perry, 1989).

Seasonality

Nematodirus spp. are the first strongylid nematodes to be ingested in large numbers by the young lamb, with peak infestations occurring before, or at about the time of, weaning (Brunsdon, 1960). Infestation of *Nematodirus* is known as a "lamb to lamb" disease, where each crop of lambs are gaining the major percentage of its infestation from eggs laid down on pasture by the previous year's lambs (Vlassoff, 1973). The regular appearance of *Nematodirus* infestation in the spring can be explained by the availability of infective larvae on pasture at time when the herbage intake of the suckling lamb becomes significant and the

lamb still is immunologically naïve. Studies have also shown a large peak of infective larvae in autumn, although this peak does not seem to be followed by a peak in faecal egg count, as seen following the spring peak of infective larvae, and may indicate development of immunity in lambs. In conclusion, two larval peaks have been recognised, although there are seasonal fluctuations, *Nematodirus* appears to be present on pasture all year around (Brunsdon, 1960). It has also been suggested that *N. filicollis* only produces one generation each year, and that the hasty accumulation of the infective larvae in early spring is due to the fact that a sudden activation and hatching of larvae occurs at this time, resulting in the rapid increase of large numbers of free larvae, which possibly developed to this stage the previous year. This approach gives the advantage that no period of development of the larvae in spring is required (Thomas, 1959b).

Survival on pasture

Remnants of the autumn larval peak and eggs passed in autumn have the ability to survive on pasture over winter, and are the major source of infection of new lambs the following spring (Vlassoff *et al.*, 2001). L_3 can survive on pasture but also in the soil up to several months and sometimes even longer, depending on temperature and humidity (Knapp-Lawitzke *et al.*, 2014). Results from a study performed in south-western Montana, with average temperatures of minus 3.9°C during winter, 16.7°C during summer, a low relative humidity and high evaporation, showed that, compared with other trichostrongyloids, *Nematodirus* eggs and larvae are particularly resistant to the environment and can survive the annual weather variations (Marquardt *et al.*, 1959). There is also evidence showing that during subarctic winters in Greenland, *Nematodirus* eggs and larvae have the ability to survive for over two years on pasture, which again present the parasite's resistance to cold temperatures (Rose & Jacobs, 1990). It has moreover been concluded that larval survival is higher in cool periods than under summer conditions, and that sunlight with hot and dry conditions are unfavourable for larval survival (Marquardt *et al.*, 1959; Kates, 1950). Similar results were presented by (van Dijk *et al.*, 2009), providing evidence that natural levels of UV irradiation increase mortality rates of infective nematode larvae.

Characteristics of adult, infective larvae and egg

The adult *Nematodirus* worm is white in colour, slender, and relatively long with a coiled appearance. The anterior region is thinner than the posterior. The approximate length for males is 10-15 mm and for females 15-24 mm. The adult has a small, but distinct, cephalic vesicle (blister like inflation over the head) and the cuticle (outer covering) hold about 14-18 longitudinal ridges. The male bursa (an external male copulatory appendage, used to grip the female during mating) has elongate lateral lobes and the spicules (needle-like mating structures) are long and slender, and the tips of the spicules are joined except terminate in small expansion. The spicules vary in shape and this is a useful feature for species differentiation. The worm also has ventral rays, which are parallel and found close together. The female worm has a short tail with a slender terminal appendage (Taylor, 2016).

The infective larvae is noticeably longer than other trichostrongyloids and they have eight large elongated intestinal cells and a prominently longer sheath tail with evident terminal

appendages (van Wyk *et al.*, 2004). The mean length is approximately 912-1018 μm (Thomas, 1957).

The *Nematodirus* eggs are distinctive and roughly twice the size of a typical trichostrongyloid egg, measuring approximately 210μm x 100μm (length x width) and they are easily distinguishable from other trichostrongyloid eggs. They are ovoid, colourless and thin-shelled (Taylor, 2016). When the eggs are passed in the faeces, they contain two to eight large granular cells, which are taking up nearly two thirds off the egg. The development of the larvae takes place exclusively within the egg membranes and the egg will not hatch before the larva has reached the infective stage, providing that the environmental requirements have been met. The morphological characteristics are microscopic and are used for identification (Kates & Turner, 1955).

Nematodirosis

Pathogenicity and clinical signs

Nematodirus spp. are found in the small intestines of ruminants. *Nematodirus battus* is known to cause disease of more gravity, compared with *N. spathiger* and *N. filicollis*. Low to moderate infections may not cause any clinical signs, although it is not excluding subclinical disease. With more severe infections clinical disease can emerge (Taylor, 2016). Clinical impact of *Nematodirus* spp. is typically only found in young lambs up to 10 months of age, though adult sheep can be affected during times of stress or periparturient loss of immunity (Vlassoff & McKenna, 1994). The pathogenesis of *Nematodirus* is related to the migration of larvae into the intestinal mucosa during their development (Thomas, 1959a). Following ingestion of the third-stage larvae, they invade the small intestines and penetrate the deep layers of the mucosa, moving into the intervillar crypts and, as the parasites mature into the fourth- or fifth-stage larvae, they emerge within the villi and finally, as adults, they are found mainly in the lumen of the intestines. Large numbers of larvae can cause villous atrophy, crypt dilation and elongation, which impairs the intestines ability to exchange fluid and nutrients, and the lamb quickly becomes dehydrated (Taylor, 2016). In conclusion, there is evidence that immature worms are more pathogenic, as they cause more severe tissue damage to the intestines than the adults. Heavy infecting doses of *N. spathiger* have shown that the migration of the developing larvae causes extensive damage to the wall of the small intestines, and within five days of infection, they are contributing to the clinical effects of diarrhoea, loss of appetite, dehydration and weight loss. Therefore, the major clinical signs can be seen during the prepatent period (Kates & Turner, 1955). Outbreaks of nematodirosis are often sudden, affect a number of lambs simultaneously, and can also cease with similar abruptness. An affected flock will have the characteristic appearance of "excrement cakes" on the hindquarters and legs. The wool loses its gloss, and dehydration causes the eye to become sunken and often with a discharge. The ears sink and the lamb moves with a stiff gait. The acute illness lasts for about five days but the period of recovery can last for months. Death can follow within two days and is likely to be caused by dehydration resulting from the diarrhoea. At the time of acute clinical signs, faecal egg counts will not reflect the degree of infestation, as the worms are still immature and have not produced any eggs yet. As such, at the time of disease, diagnosis relies on clinical signs or necropsy (Brunsdon, 1961).

Age and immunity

Substantial infestation affects almost exclusively lambs under the age of nine months and particularly lambs two to five months old, meaning that most of the infestation occurs around the time of weaning, which is explained by the lambs start of grazing fully at this time (Brunsdon, 1967). It has been seen that the infestation of *Nematodirus* spp. reaches its highest numbers in spring and then decreases to a low level within a month, and is usually ended during the autumn. The immunity stimulated in the lambs by the spring infestation appears to protect the animals against further infestation at the time of the autumn larval peak (Brunsdon, 1960). Less *Nematodirus* eggs are seen in lambs as they get older and eggs are rarely found in mature animals, therefore ewes do not usually contaminate pastures with *Nematodirus* eggs. Hence re-infection does not seem to occur in the autumn, which could be explained with a developed resistance, including a possibly age-related resistance (Vlassoff, 1973). Studies have shown that there is an age-related resistance *per se,* finding that 18 month old sheep, worm-free since birth, were more resistant to *Nematodirus* spp. infestation, than six months old lambs, also worm-free since birth. This age-related resistance to *Nematodirus* spp. infestation was revealed by an increase in the length of the prepatent period, a resistance to the establishment of patent infestation and reduced egg production per worm, which results in less clinical impact and disease. An interesting fact, in these studies, was that no age-related resistance to establishment of infestation was observed for other *Trichostrongyloids* investigated in the study (Brunsdon, 1962).

Control

Management of parasitism

Control of nematodes on sheep farms in New Zealand has over numerous of decades relied on a combination of routine preventative anthelmintic treatments to delay the seasonal build-up of parasite larvae on pasture. Further, to avoid high pasture challenge, supplementary treatments are often given when the worm challenge on pasture is high, plus integrated grazing with other herbivore classes is used (Vlassoff *et al.*, 2001). Although some farmers are trying to find new ways of minimising the impact of parasites, such as improved nutrition and breeding for resistant animals, they are still mainly reliant on the broad-spectrum anthelmintic drenches (Miller *et al.*, 2012). Broad-spectrum anthelmintics in sheep has earlier only been available as oral short-acting formulations with little persistence in the animal. Though, the last decade there has been an increase of long-acting anthelmintics. These can be divided into drugs that have high initial activity that successively declines logarithmically over time and controlled release capsules (CRCs), which are intraruminal devices that release drug at a constant rate for about 100 days (Leathwick *et al.*, 2001).

There are six broad-spectrum anthelmintic groups used today to control nematodes of grazing stock. These groups are benzimidazoles, imidazothiazoles (levamisole) and hydropyrimidines (pyrantel/morantel), the macrocyclic lactones (avermectins and milbemycins), amino-acetonitrile derivates and the spiroindoles (George *et al.*, 2012; Coles *et al.*, 2006). New Zealand has a diversity of dual and triple combination anthelmintic products registered and they are all extensively accessible to farmers. Based on a study, where one of the aims was to report farming practices relating to control of gastrointestinal nematodes on 74 sheep farms in

New Zealand, there seems to be an increase in the widespread use of long-acting anthelmintics. Most farmers in the trial drenched the lambs regularly, every 4-6 weeks, and various combined drenching protocols were used to control gastrointestinal parasites. The most common anthelmintic drench given to the lambs in 2004-2005 was a combination of benzimidazole-levamisole or moxidectin. If quarantine drenching of newly introduced sheep was practised, macrocyclic lactones (ivermectin, abamectin and moxidectin), benzimidazole-levamisole combinations or triple combinations were commonly used. Drenching of ewes was also common and pre-lambing treatment could include controlled-release capsules containing ivermectin, albendazole or moxidectin. At some of the farms, grazing management was used, e.g. moving lambs, following anthelmintic treatment, to pasture previously grazed by other stock, often cattle. In additional, several farmers seemed to use the same products year after year (Lawrence *et al.*, 2007).

Resistant versus resilient animals

Selecting animals that are either resistant or resilient are two strategies to reduce the requirements for anthelmintics. These are based on the host response and are considered to contribute to the ability of the sheep to limit the effects of nematode parasites on their health and productivity. Resistance is the capability to limit the establishment and a nematode burden when exposed to larval infestation, and therefore presenting a low FEC. Resilience is the capability to tolerate the effects of larval infestation, and maintain adequate productivity and need for a minimal use of anthelmintic treatment despite frequently having high FEC (Morris *et al.*, 2010). Breeding for resistance to helminthic infection has shown progress in reducing the parasite burden, weakening the impact of infection on production and reducing the need of anthelmintic substances (Sayers & Sweeney, 2005). However, several studies have shown resistant animals to have reduced productivity and increased breech soiling (dried faeces on the wool surrounding the tail and breech), with reduced live and fleece weights (Morris *et al.*, 2010).

Resistance to anthelmintics

Resistance could be defined as termed by Prichard *et al.* (1980): "resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species and is heritable". There has been an exclusive reliance on anthelmintic substances for parasite control, and the extreme and irresponsible use of these chemicals, has led to the parasites development of resistance against them (Brunsdon *et al.*, 1983). Since the 1980s, farmers in New Zealand have been advised to limit unnecessary use of anthelmintics, drench-test, use annual drench-group rotations, implement preventive-drenching practises, use of faecal egg counts to decide when to treat, and quarantine management as strategies for reducing the rate of progress of resistance. Despite of that, resistance has increased rapidly (Rhodes *et al.*, 2006). The standard for detecting anthelmintic resistance is the use of faecal egg count reduction test (FECRT), which compares faecal egg counts before and after treatment. Anthelmintic resistance is confirmed if reduction is less than 95% (Coles *et al.*, 2006). In New Zealand, there is a profound level of resistance in *Nematodirus* spp. to benzimidazole, with albendazole-resistance more common in *N. spathiger* than *N. filicollis (Oliver et al., 2016)*. In New Zealand, 2007-2009, the mean prevalence of resistance in *Nematodirus* was 82%, 24%, 16%, 6% and 44% to benzimidazole,

levamisole, benzimidazole/levamizole combination, to full dose of ivermectin and half dose of ivermectin, respectively. Resistance to triple-combination anthelmintics in *Nematodirus* spp. has rarely been reported in New Zealand (McKenna, 2010). In terms of identification of genetic markers linked to anthelmintic resistance among the strongylid populations, PCR has shown to be a useful tool as the method has the ability to efficiently identify parasites holding the resistant gene, and therefore PCR can be a crucial step in the struggle against this increasing problem of handling gastrointestinal nematodes (Gasser *et al.*, 2008).

Nitrogen's inhibitory ability

Several studies suggest that the development of nematodes can be affected by the presence of nitrogen. Rodriguez-Kabana (1986) summarize the direct relation between the quantity of nitrogen and the effectiveness as nematode population suppressant. It is proposed that the larvicidal action of the nitrogen compounds can be explained by the toxic qualities of ammonia. Urea is a compound that is readily converted to ammonia (Howell *et al.*, 1999). Ammonia in sewage sludge (biosolids) has been presented to have a dose-dependent ability to inactivate *Ascaris suum* eggs (Pecson *et al.*, 2007). Comparable results was seen when ammonia was added to wastewater sludge, where the viability of *Ascaris* suum eggs decreased, with the viability related to the quantity of ammonia added, and not the pH achieved (Ghiglietti *et al.*, 1997). Furthermore, the larval survival rate of *Haemonchus contortus* has been shown to decrease when nitrogenous fertilisers has been used. The nitrogen levels used in the study, were all less than 34kg N/ha, which is below commonly used levels on pasture in North Carolina, USA (Howell *et al.*, 1999). Similar outcomes were also seen in a comparable study performed in Odisha, India, looking at fertilisers with high urea concentration, and the effect on *H. contortus.* The fertiliser formula included urea, single super phosphate and muriate of potash. A significant reduced larval translation was seen when the fertilisers were administered at 80kg of urea/ha, 40kg/ha of single super phosphate and 40kg/ha of muriate of potash (Roul *et al.*, 2017). In a recent *in vitro* study, observing *Trichostrongylus colubriformis* eggs, urea at a concentration of 40kg N/ha, was topically applied to sheep faeces, and a 97% reduction in larvae recovery was seen. The study concluded that liquid urea, within the range of normal New Zealand farm practice using nitrogenous fertilisers, appears to have strong nematocidal qualities that reduce egg hatching and larval development (Cairns *et al.*, 2017). In a series of *in vitro* experiments, the treatment of ureas effect on egg hatching and larval development of some gastrointestinal nematodes, including *Nematodirus* spp., was investigated across several ruminant hosts. The result presented comparable evidence for urea's ability, at a rate of 40 kg N/ha repeated every week for five weeks, to inhibit *Nematodirus* egg hatching and reduction in larval recovery. Though, in contrast, the development was unexpectedly increased at a low rate of a single application of 40 kg N/ha. The author concluded that these *in vitro* result needs to be translated into the field and possible find a way of interrupting the *Nematodirus* lifecycle through targeting the nematode outside the host (J Bennett, 2017, unpublished honours dissertation, Lincoln University).

Methods measuring parasites

Faecal egg count

Faecal egg count (FEC) is used to determine the concentration of nematode eggs per gram (EPG) of fresh faeces. FEC is often utilized to provide an indication of the worm burden within the host, either to encounter the disease and limit the production costs, or to determine the efficacy of anthelmintic treatment through a FECRT. FEC is not an absolute number; it is a ratio, representing the number of eggs per gram of fresh faeces. Any factors that change the volume of fresh faeces, such as differences in dry matter intake, feed quality or faecal moisture, can affect FEC with no change in the total number of eggs excreted. For example, a count of total nematode eggs in faeces calculated as FEC × faecal DM, may provide little indication of numbers of nematode population in the host, as female fecundity varies considerably between nematode species. Additionally, an animal with an immature immune response and low nematode burden may conceivably produce as many eggs as an animal with a more mature immune response infested with a higher worm burden. Also, during the pre-patent period there will be no eggs present in the faeces, though the animal might undergo infestation. In conclusion, FEC on its own can simply just provide a reliable indicator that nematodes are present (Greer & Sykes, 2012; Thienpont *et al.*, 1986).

The most commonly used and standard quantitative technique for counting present eggs in faeces is the McMaster method. There are numerous modifications reported in the literature, which differ in the weight of faeces, the flotation solution used, the flotation time, whether a centrifugation step is included or not, the design and number of McMaster counting chambers, the counting method and multiplication factors employed, and whether any correction factors are used to allow for faecal consistency (Pereckiene *et al.*, 2007; Cringoli *et al.*, 2004).

Pasture larval counts

Pasture herbage samples are collected for the determination of the concentration of infective larvae on pasture. The results are expressed as number of L_3 larvae per kilogram of pasture dry matter (L_3/kg DM). The majority of larvae are found in the first 2 cm of the plant, in the first 1 cm of the soil and within 30 cm from the faecal mass (Familton & McAnulty, 1997). Pasture larval counts (PLC) are not ideal for the diagnosis of nematode infections, as it is difficult to relate pasture infectivity to a clinical outbreak because it takes approximately 3-4 weeks after high pasture infestations before the outbreak of clinical disease. Furthermore, several factors can contribute to not very accurate results with this method, for example that larvae are not randomly distributed on pasture and that infective larvae concentrate in the grass surrounding the faecal pats. Nevertheless, pasture larval counts are useful in studies on the population dynamics of nematodes, and also in epidemiological studies with a well-defined aim (Eysker & Ploeger, 2000). It is both a quantitative method, totalling the larvae and a qualitative method differentiating the larval species, and nearly all laboratories use their own techniques to process the herbage samples, although they can all be divided into three major stages: collection of infective herbage, separation and concentration of larvae from herbage and enumeration/identification of larvae. Each phase of which can be affected by a variety of factors which can influence the results (Couvillion, 1993). The washing and

cleaning procedure, often involve either a "sieve"-method or the modified Baermann method described below. A number of protocols have been described in the literature and there are difficulties to establish standardisation. Differences between sampling procedures and the counting of L_3 seem to be the factors that can affect the final result, and it is important that the user understands the variations within the technique and work towards a more standard protocol (Molento *et al.*, 2016).

There is a wide variation in the way herbage samples are collected, though to collect the samples in a W-shaped pattern over the experimental area is commonly used. Grass may be collected by plucking small pieces by hand or by the use of scissors (Eysker & Ploeger, 2000). Depending on sampling technique, various results has been presented, with plucks by hand simulating a grazing animal whereby cuts may provide a better indication of total larvae present (M Martin-Mckie, 2018, unpublished honours dissertation, Lincoln University). As larvae are concentrated nearer to the faecal mass and to prevent bias the immediate area around the faecal mass could be avoided during sampling (Couvillion, 1993). The Baermann apparatus is commonly used for the extraction of infective larvae from herbage, soil or faeces to estimate the number of larvae present. The Baermann apparatus consists of a glass funnel with the bottom ending with clamped rubber tubing. The funnel is filled with tap water of room temperature with a fine mesh on top. The sediment containing the larvae is placed on top of the fine mesh and left to settle. The larvae will then migrate through the fine mesh down into the funnel neck, where they are concentrated at the bottom and are easily collected through the opening of the clamp (Thienpont *et al.*, 1986).

Pasture herbage mass

Herbage mass of a grass paddocks can be measured with a Rising Plate Meter (RPM) expressed as kilogram of pasture dry matter per hectare (kgDM/ha). The RPM (figure 2) is an instrument, which measures the density of the grass and provides a cumulative measure for that pasture. When using the RPM, it is lowered vertically onto the herbage, with the shaft falling through to rest on the ground, leaving the horizontal plate supported by the grass. A measure of herbage height, in centimetres, is recorded on a counter attached to the shaft. After approximately 30 readings, an average height of the pasture can be recorded, and together with a formula calibrated for the area, kg DM/ha can be calculated (MacAdam & Hunt, 2015). A study investigating the accuracy of herbage mass estimates when using a RPM, concluded that calibrations should be made frequently over the growing season to increase reliable estimates of the herbage mass (Ferraro *et al.*, 2012).

Figure 2. *Rising plate meter used for measurements of herbage mass. Photo: private.*

Soil samples

Most parasitologists concentrate on the recovery of larvae from herbage, but studies have shown that a high proportion of the larval population on herbage migrates into soil. For example, *H. contortus* infective larvae have been seen to be able to survive beyond 21 days in the soil or mat and infest the herbage when climate conditions are favourable. There are several developed methods for successful recovery of larvae from herbage but a standardised method for the recovery from soil is still missing (Knapp-Lawitzke *et al.*, 2014; Amaradasa *et al.*, 2010). Similarly, it appears there is no standard method for detecting gastrointestinal worm eggs in soil. A paper, where *N. battus* and *N. filicollis* were involved in the investigation of the disintegration of sheep manure and the pre-parasitic stages, presented evidence that much of the preparasitic life is spent, not in dung, but in the surface of the soil. It was concluded that rain plays a role in the faecal patch disintegration process. When rain wets the dung immediately after being dropped on pasture, it becomes soft and subsequently disintegrate faster, and thus the faeces persists longer on pasture if it is dried rapidly immediately after deposition. Observations from the trial, showed that the persistence of sheep droppings was very limited, and a complete disintegration could be as short as 6 days (Christie, 1963). In a study investigating *Ascaris suum* eggs in soil, the aim was to develop a new method for more efficient detecting soil-transmitted helminths, and with this new method help scientists evaluate soil contamination. The study focused on the three main steps: sieving, flotation and microscopy, and modified these steps to come up with the new method, having an egg recovery efficiency of 72.7% (Steinbaum *et al.*, 2017).

Aim of the study

The aim of the study was to investigate if liquid urea could break the *Nematodirus* spp. life cycle outside the host by inhibiting hatching of the eggs and development of the free-living larvae, on pasture, and therefore decrease the parasitic burden on pasture and indirectly reduce the faecal egg counts of the grazing animals on the experimental plots.

MATERIAL AND METHOD

Study design

The field trial, a proof of concept study (a realization of a theory in order to demonstrate its feasibility), was carried out at the Ashley Dean sheep Farm located in Lincoln, Canterbury, New Zealand from May 2019 to February 2020 (still in progress as writing this thesis). Seven paddocks, respectively measuring 0.3 ha, were used for the trial. These paddocks were already included in a long-term study, where the animal productivity in resistant and resilient genetic lines of Romney sheep is investigated. The lines were established by AgResearch in 1979 (Hamie *et al.,* 2019) and in 2008 they were transferred to Lincoln University. The lines have been grazing the same areas for the past five years, and therefore useful data of faecal egg counts were available. Previous pasture samples from the paddocks have been shown to have an existing population of *Nematodirus* spp. Only resistant animals have grazed paddocks 1-3. Only resilient animals have grazed paddocks 4-6. While a mixture of resistant and resilient animals have grazed paddock 7. None of the animals used have been drenched with anthelmintic treatment. The paddocks are grazed by the Romneys August to March each year, with a single time grazing in the middle of the winter. The length of the single winter grazing,

usually performed in May, was around seven days, though depended on the pasture mass each year. Previous years approximately four ewes with six lambs grazed each paddock per season, all-depending on grass quality. This season, to maintain grass quality, five to ten ewes with lambs grazed paddocks 1-6, and four ewes with lambs grazed paddock 7. The ewes used in the study were all born between 2015-2016.

To investigate the effect of urea on *Nematodirus* larvae development and inhibition of egg hatching on pasture, this study included three different treatments of liquid urea sprayings (46% nitrogen) of the different paddocks. The paddocks used for control were left untreated. The protocol of sprayings is each described below and presented in figure 3 (including a map over the different paddocks used). On the 2nd of September 2019, prior to lambing, the ewes were put out in the paddocks. The lambing went on for approximately three weeks. The effect of treatments was evaluated utilising three methods, i.e. observing pasture larval concentration, faecal egg counts and concentration of eggs and larvae in soil, each of which are described below. The study had a main focus on *Nematodirus* spp., although "other" *Strongyles* were included to give a better understanding of the overall effects of the treatments. As urea stimulates pasture growth, consequently there is a dilution effect on each paddock considering larvae per kg DM, and because of this dilution effect, herbage density gives the ability to count larvae per paddock. As there was no present routine protocol for processing the soil samples, the used method went through a validation step in the lab to get the technique correct and confidence in the recovery of eggs.

Treatment of the paddocks

Sprayings were applied only on a dry day and with no rainfall the following 24 hours. No irrigation was applied during the time of treatment. To apply the urea on to the paddocks a turbo hind sprayer (a type of agricultural sprayer with a tank) was used, with a four-meter spray boom attached to a quad-bike. The application rate was fixed so the correct volume of solution was evenly and accurately sprayed on each of the paddocks.

A single spraying of 200 units N per ha (200N) was applied in the end of May 2019, on paddock 1 and 5. The single sprayings consisted of 130kg urea (Ravensdown Limited, NZ) diluted in 300 litres of water per paddock. On paddock 2 and 6 five repeated sprayings of 40 units of N per ha (5x40N) were applied, with the first spraying in the end of May 2019 and repeated every three weeks until prior to lambing, and was finished with the last and fifth spraying applied last week of August 2019. The repeated sprayings each consisted of 30kg urea diluted in 60 litres of water per paddock. Paddocks 3, 4 and 7 were used as controls and left untreated. Although paddock 7 was not comparable

Paddock **1** *Resistant sheep 200N*	Paddock **4** *Resilient sheep Control*	(Paddock 7) *Mixed sheep Control*
←	←Raceway	←
Paddock **2** *Resistant sheep 5x40N*	Paddock **5** *Resilient sheep 200N*	*(Paddock with sheep used in other trial)*
←	←Raceway	←
Paddock **3** *Resistant sheep Control*	Paddock **6** *Resilient sheep 5x40N*	*(Paddock with sheep used in other trial)*

Figure 3. *Protocol of the sprayings and map over the paddocks. 200N=single treatment of 200 units of urea and 5×40N=repeated treatment of 40 units of urea.*

with any of the other paddocks, as it had a mix of resistant and resilient animals, it was used as a control paddock and a subject for discussion.

Faecal collection and faecal egg counts

Faecal sampling and faecal egg counts were carried out from the ewes on the first day grazing the plots, prior to lambing, and again after six weeks and also of the lambs at time of weaning. The first samplings of faeces from the ewes were collected approximately 24 hours post start of grazing and pooled within paddock. The samples were collected from fresh faeces on the ground, knowing that, from a new plot after winter, the faeces will be as fresh as they needed to be for the FEC. For each paddock, 10 pinches of faeces were collected from 10 different piles of faeces. The samples from each paddock were mixed and put in plastic bags, giving one mixed sample of faeces per paddock. The additional samples for FEC were taken directly from the rectum of all ewes and lambs grazing the experimental plots and these samples were not pooled. The samples were stored at 4°C until processing, which occurred within 72 hours.

A Modified McMaster technique, similar to the method described by Thienpont (1986), was used to process the faecal samples. Eggs that are present in the McMaster chambers float to the surface of a salt solution and stick to the cover glass, where they could be counted. For each sample 1.7 g of faeces was placed in a glass jar, then 7 ml of water was added and soaked overnight to soften. Next day, 46 ml of saturated sodium chloride (NaCl) solution was added and the sample was mixed for 25 seconds, with an electrical stirrer, until the faecal pellet was completely broken up. A Pasteur pipette was used to fill both chambers of a moistened McMaster slide (0,15 mL x two cell volume) with the faecal suspension. The number of *Strongyle* eggs present in both chambers of the slide where counted under a microscope, totalled, and multiplied by 100 to give the number of eggs per gram (epg) for that sample. Eggs of *Nematodirus* were recorded separately.

Pasture sampling and counting of pasture larvae

Pasture herbage samples were collected for the determination of the concentration of *Strongyle* larvae, including *Nematodirus* spp. larvae, on each paddock. The pasture samplings were performed every two weeks for six weeks, with the first sampling on the first day of grazing. A hand plucking method was used and the samples were plucked at ground level with samples taken every ten steps crossing each of the respective experimental plots in a W-shaped pattern, irrespective of any faecal deposit. The samples were immediately placed into plastic bags and stored at 4°C until processing. All samples were processed within two days of collection.

Process of herbage samples

During processing, the plastic bags containing the herbage samples were weighed and each sample was put into a bigger bag and placed in an electrical "pasture larvae washing machine" (Easy pressure, Banmix Food Machine (NZ) LTD, Auckland). The little bag was rinsed with 1400ml of ambient tempered water and then rinsed with 300ml of lukewarm water, all poured in to the big bag containing the herbage. Then the bag was sealed and the machine was operated for 1800 revolutions during three minutes. After washing, a small incision was made in the bag and the washings water were allowed to pass through a coarse

mesh sieve (aperture size 2mm) into a 5-liter beaker. The remaining herbage in the bag was then removed and washed gently with a jet of water and as much fluid as possible was recovered from it by hand squeezing. The herbage was then spread on a tray and dried in an oven at 70°C for 48 hours. When thoroughly dry the herbage was weighed again. The fresh grass weight was used in the final estimation of numbers of larvae per kilogram of fresh herbage. The resultant suspension in the beakers was allowed to settle over night at 4°C and then the fluid was siphoned off leaving sediment and larvae which were then transferred to a 250 ml measuring cylinder for a second cold sedimentation overnight. Again fluid was siphoned off leaving sediment and larvae. The volume of sediment was recorded and the contents were agitated until mixed. The larvae suspension was then poured onto a 150 mm diameter filter paper and allowed to dry until all the surface water had disappeared. The filter paper was inverted and placed on a Baermann filter funnel filled with water. After a minimum of 36hours, 100ml of fluid was withdrawn from the rubber tube on the Baermann funnel and stored in glass bottles. After storage at 4°C the samples was reduced in volume to 20ml by siphoning and larvae present in four 0.5ml samples were counted and differentiated into *Strongyle* and *Nematodirus* species using a method similar to those described by Van Wyk *et al.* (2004) and results expressed as number of larvae per kilogram of herbage dry matter. A single and blinded operator carried out all pasture larvae counting and differentiation. Calculation of larvae per kilogram of dry herbage (larvae/kg DM) is as follows; number of larvae counted × 10 / weight of dry grass sample in kilogram.

Herbage density measurements

The measurements were carried out immediately prior to the start of grazing and post-grazing pasture mass was recorded every two weeks for six weeks. The total pasture herbage was estimated by performing measurements every three steps in a W-shaped pattern within each plot, giving approximately 30 readings per paddock. The number shown on the RPM before the first reading and the number after all of the readings in each plot were used to get the difference between the readings. The difference was then divided by the number of readings to generate the average compressed pasture height per sample, and herbage mass could be estimated using the formula (mean RPM height (cm) × 200). The formula used had been calibrated last year for the same area (Hamie, 2018, Doctoral Thesis, Lincoln University). With this method it is possible, together with the amount of pasture larvae/kg DM, to count the cumulative larval challenge in each paddock. Calculation of larvae/paddock is as follows; larvae/kg DM × pasture mass in kg DM for each 0.3 ha paddock.

Soil samples

Soil samples were collected for the determination of the concentration of *Strongyle* eggs, including *Nematodirus*, in each paddock. The soil samplings were performed monthly for two months, with the first sampling on the first day of grazing. A soil sampler was used to collect approximately 20 samples in each paddock. The sharp soil sampler was pressed five cm into the ground every 10 steps, crossing each of respective experimental plot in a W-shaped pattern, giving cylinder-shaped samples measuring about 5×2 cm. The samples were immediately placed into plastic bags and stored at 4°C until processing. The first soil samples, collected the 2nd of September, were processed nine weeks after sampling, the soil samples

collected the 30th of September were processed five weeks after sampling and the soil samples collected the 28th of October were processed one and a half week after sampling.

Faecal egg extraction and validating of method

To validate the method, eggs of *Nematodirus* and other *Strongyles* were extracted from fresh faeces collected from lambs with a known infestation of *Nematodirus*. The samples were stored at 4°C until the extraction process was performed 24 hours after sampling. Approximately 120 grams of faeces were used. 50ml of water was added to the bag with faeces and the bag was then homogenized using a stomacher (Stomacher 400) and mixed for one minute. The content of the bag was then poured through a stainless steel sieve, size 500µm, to remove large particles, with the filtrate draining into a fine stainless steel sieve, size 38µm, and with a spray hose provided pressure over the sieve. To capture the eggs, the filtrate was re-filtered through the 38µm-sized sieve and the content of this sieve was then rinsed through a 25µm-sized sieve. The remaining fine filtrate was rinsed into a 500ml beaker with a settling volume of 200ml. One ml of the solution was pipetted and put on a petri dish. The solution was examined using a microscope under 10x magnification to count *Strongyle* eggs and *Nematodirus* eggs, which resulted in 187 and 17 eggs, respectively. Multiplied by 200 gave in total 37400 and 3400 eggs, respectively, in the solution. The solution was once more rinsed through a 38µm sieve to reduce the volume to 60ml, estimating the total of *Nematodirus* and *Strongyle* eggs in the solution, to approximately 3000 and 36000 eggs, respectively, after this step. Three soil samples of 15 grams of soil were each seeded with 20ml of solution containing approximately 1000 eggs of *Nematodirus* and 12000 eggs of *Strongyles* each, and left to settle for 48hours. Then these samples went through the process for soil samples following the protocol described below. With the method used, the recovery of *Nematodirus* and *Strongyle* eggs were approximately 56% and 95%, respectively. With focus on *Nematodirus* eggs, the sensitivity of this method was approximately 37 eggs per gram soil as the count per McMaster slide varied between 0-2 eggs.

Process of the soil samples

On the first day the soil samples were mixed using a "Stomacher 400", with repeated 20-second sessions until they were evenly mixed. The soil from the experimental paddocks was generally of a dry, easily mixed, texture. Then 15g aliquot of soil was added to a 50mL centrifuge tube for each sample. From each paddock, two sub-samples were taken, giving 14 samples in total for each date. Surfactant, 1% 7X (MP Biomedicals, Auckland, NZ), was added to each sample, bringing the volume up to the 35mL line, and the samples were vigorously shaken by hand for two minutes. Then the sides and cap of the tube were rinsed with 1% 7X, and also added to the 45 mL line on the centrifuge tube, and left to soak overnight in 4°C. On the second day each sample were hand shaken for one minute and poured through a stainless steel size 50-mesh sieve (500µm) to remove large soil particles. The sieve was rinsed with 1% 7X to capture any eggs stuck to the sieve, followed by a second rinse through a stainless steel 500-mesh sieve (150 µm) which was inverted and rinsed with 1% 7X into the final fine stainless steel 500-mesh sieve (38 µm), which also was inverted and rinsed with 1% 7X into a beaker. The settling volume was around 200 mL. The samples were left to settle for 30-60 minutes and then the supernatant was vacuum aspirated off each sample, with a final volume of approximately 20ml. To the remaining sample, saturated

sodium chloride (NaCl) was added as flotation solution and poured into one 50ml-glass jar, and filled up so the volume in each jar was 50ml. Each sample was mixed for 25 seconds with an electrical stirrer. A Pasteur pipette was used to fill both chambers of three moistened McMaster slides (0,15 mL x two cell volume) with the soil suspension. The number of *Strongyle* and *Nematodirus* eggs visible in the chambers of the slides where counted under a microscope, totalled, and multiplied by 56 to give the number of eggs for that sample, and then divided by 15 to give eggs per gram soil. A single operator carried out all processing of the samples and a single operator carried out all counting and differentiation.

Data handling and statistical analysis

The statistical analyses aimed to investigate if there was a significant association between treatment and response variables, accounting for potential effects of genetic line and date of sampling. The response variables were a) group means of faecal egg counts of ewes 6 weeks after let out on pasture and of lambs at weaning; b) pasture larval counts over time; and c) eggs and larvae in soil, for *Nematodirus* spp. and other *Strongyles,* respectively. The analysis of variance was performed using the General Linear Model (GLM) procedure in Minitab18, where an optimal box-cox transformation was performed to achieve approximately normally distributed residuals (λ=0.5 was selected when not possible to interpret in regression equation). The main factors in the model were treatment (200N, 5x40N and control), genetic line (resistant and resilient), and date (pasture and soil samples). To be able to do optimal transformation of the data including values of zero, the constant value of "1" was added to all values in the group. To provide an interpretation of the transformed results from the ANOVA's, estimated means on the actual scales were calculated after the regression equation of each analysis. Paddock 7 was excluded from the statistical analysis, as this paddock does not categorically qualify as a control.

BACKGROUND INFORMATION

Faecal egg counts prior treatment

Figure 4 and 5 shows the FEC for the total of *Nematodirus* and "other" *Strongyles (*total amount of *Strongyle* eggs − *Nematodirus* eggs) from lambs grazing the experimental paddocks the season before the start of the trial. The number of sampled lambs (n) per paddock (1-7) at the end of spring were n(1, 3, 4, 5, 7)=5 and n(2, 6)=6, and in the autumn n(1, 2, 4, 6)=3 and n(3, 5, 7)=5. At the end of spring (figure 4) the lambs were passing *Nematodirus* eggs. The resilient animals (paddock 4-6) and the mix of resilient and resistant animals (paddock 7) were passing a higher quantity of *Nematodirus* eggs compared to the resistant animals (paddock 1-3). The presence of other *Strongyle* eggs followed the same pattern. In the autumn (figure 5) the presence of *Nematodirus* eggs had declined to zero in most of the paddocks, though the amount of other *Strongyle* eggs were nevertheless noticeable. Origin of the data was from the laboratory manager.

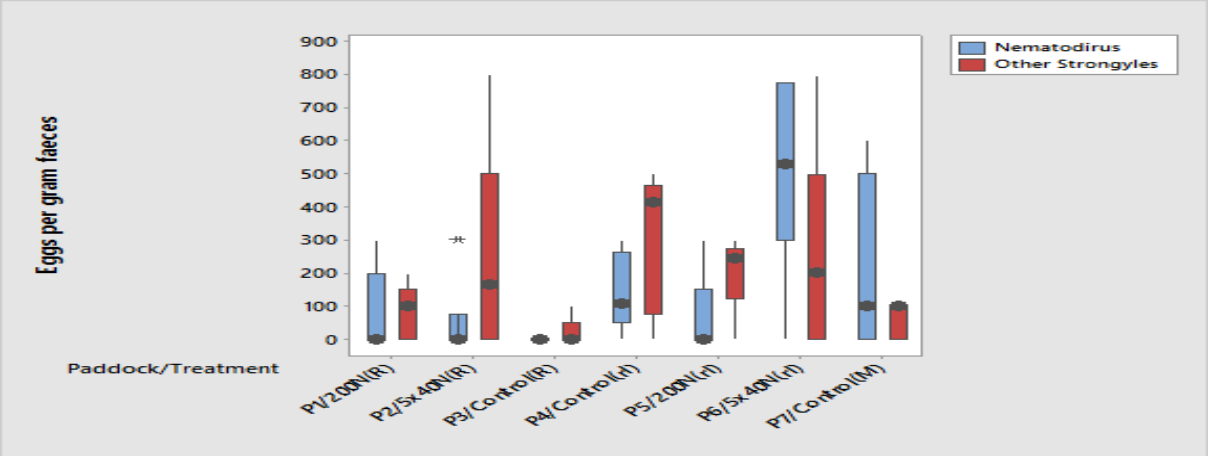

Figure 4. *Faecal egg counts (eggs per gram of fresh faeces) of Nematodirus and "other" Strongyles (total amount of Strongyle eggs − Nematodirus eggs) from lambs grazing the experimental paddocks in the end of spring the season before the start of the trial (27/11/2018). Number of sampled lambs (n) per paddock (1-7) were n(1, 3, 4, 5, 7)=5 and n(2, 6)=6. Black dots represent the median. 200N=single treatment of 200 units of urea and 5×40N=repeated treatment of 40 units of urea. R=resistant animals, rl=resilient animals and M=mix of resistant and resilient animals.*

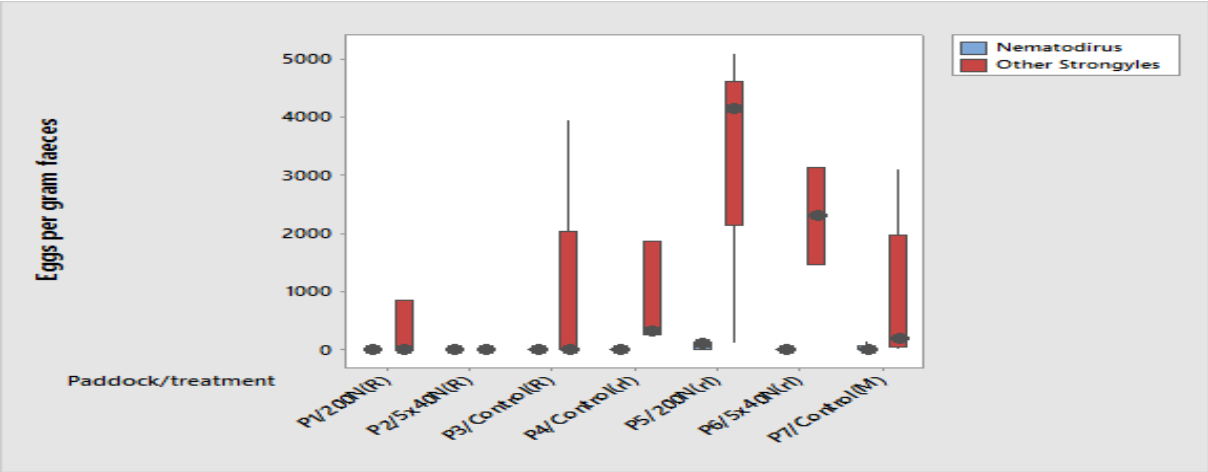

Figure 5. *Faecal egg counts (eggs per gram of fresh faeces) of Nematodirus and "other" Strongyles (total amount of Strongyle eggs − Nematodirus eggs) from lambs grazing the experimental paddocks in the autumn before the start of the trial (20/03/2019). Number of sampled lambs (n) per paddock (1-7) were n(1, 2, 4, 6)=3 and n(3, 5, 7)=5. Black dots represent the median. 200N=single treatment of 200 units of urea and 5×40N=repeated treatment of 40 units of urea. R=resistant animals, rl=resilient animals and M=mix of resistant and resilient animals.*

RESULTS

Faecal egg counts

Ewes prior to lambing

Pooled FEC from ewes taken immediately prior to lambing (02/09/2019) for paddocks 1-6 (paddock 7 was not included this sampling) are shown in Figure 6. Number of ewes (n) per paddock (1-6) were n(1, 6)=9, n(2)=8, n(3)=6, n(4)=5, n(5)=10. There were no eggs of *Nematodirus* spp. detected in the samples, though other *Strongyle* eggs were present, with a higher quantity in samples from paddock 4-6, i.e. paddocks with resilient sheep.

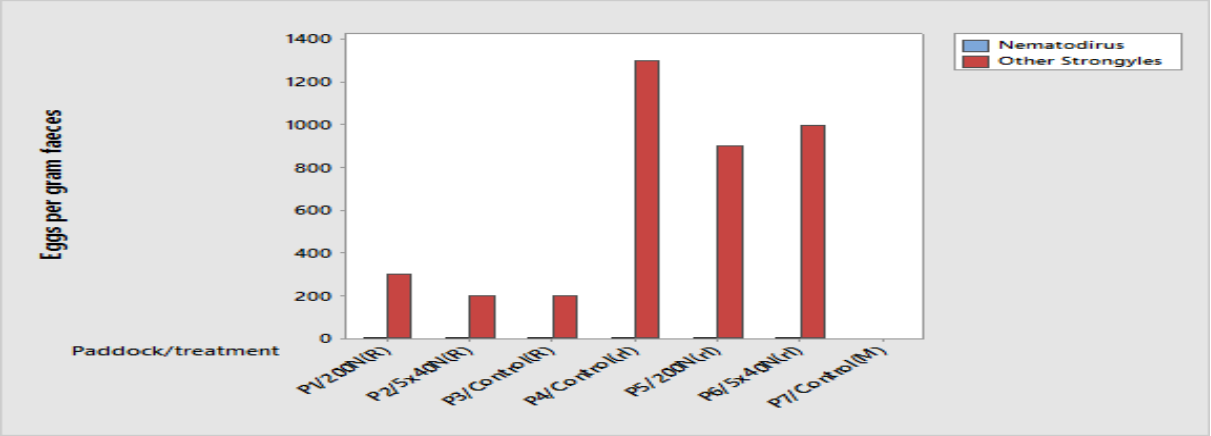

Figure 6. *Faecal egg counts (eggs per gram of fresh faeces) of Nematodirus and "other" Strongyles (total amount of Strongyle eggs − Nematodirus eggs) in pooled samples from when put out on experimental paddock 1-6 (paddock 7 not sampled), in the spring after treatments (02/09/2019). Number of ewes (n) per paddock (1-6) were n(1, 6)=9, n(2)=8, n(3)=6, n(4)=5, n(5)=10. 200N=single treatment of 200 units of urea and 5×40N=repeated treatment of 40 units of urea. R=resistant animals, rl=resilient animals and M=mix of resistant and resilient animals.*

Ewes six weeks post grazing start

The mean FEC from ewes six weeks after being put onto pasture are shown in Figure 7. The FEC are representing paddock 1-7 and all of the ewes were sampled. Number of ewes (n) per paddock (1-7) were n(1, 6)=9, n(2)=8, n(3)=6, n(4)=5, n(5)=10, n(7)=4. Overall, for *Nematodirus* spp. there was an effect of treatment (P=0.015), with fewer eggs found in the 200N-treatment than the control (P=0.028) and there was an effect of genotype (P=0.019), reflecting a greater number of eggs in resilient-line than resistant-line animals (Table 1). No effect was seen of the 5x40N-treatment. For other *Strongyles,* there was no effect of treatment (P=0.680), but there was an effect of genetic line (P<0.001), with a higher FEC in resilient animals.

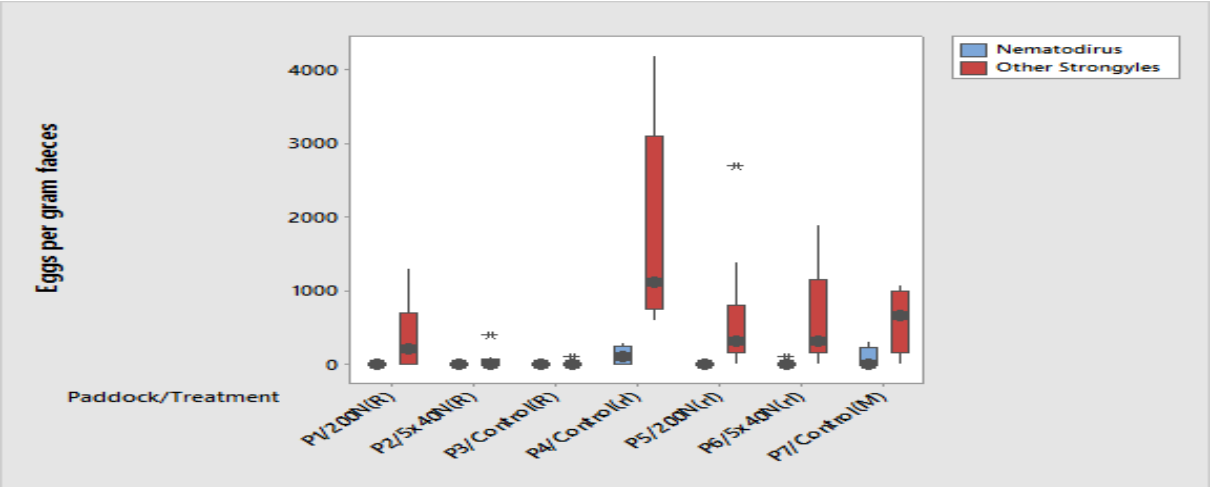

Figure 7. *Faecal egg counts (eggs per gram of fresh faeces) of Nematodirus and "other" Strongyles (total amount of Strongyle eggs − Nematodirus eggs) from the ewes six weeks after they were put out on experimental paddocks 1-7 (17/10/2019). Number of ewes (n) per paddock (1-7) were n(1, 6)=9, n(2)=8, n(3)=6, n(4)=5, n(5)=10, n(7)=4. Black dots represent median. 200N=single treatment of 200 units of urea and 5×40N=repeated treatment of 40 units of urea. R=resistant animals, rl=resilient animals and M=mix of resistant and resilient animals.*

Table 1. *Coefficients for transformed response (including estimated means on actual scale) for faecal egg counts from ewes six weeks after start of grazing*

	Nematodirus[a]				Other Strongyles[b]			
	Coeff	SE[c] Coef	P-value	Mean[e]	Coeff	SE Coef	P-value	Mean[f]
Treatment			0.015[d]				0.680[d]	
200N	-0.1146	0.0506	0.028	1.00	0.362	0.531	0.499	34.78
5x40N	-0.0564	0.0519	0.283	1.03	-0.416	0.544	0.448	15.97
Control	0			1.06	0			24.22
Genetic line								
Resilient	0.0900	0.0369	0.019	1.12	1.825	0.388	<0.001	150.2
Resistant	0			1.06				24.22
Constant	-0.8901	0.0379			3.817	0.398		

[a]Box-cox transformation: -(faecal egg count)$^{-2}$, [b]Box-cox transformation: ln(faecal egg count); [c]SE=standard error; [d]Overall p-value for the effect of treatment; [e]Estimated means on actual scale, derived from regression equation $\sqrt{[(coeff+const)\times(-1)]}/(\sqrt{[(coeff+const)\times(-1)]}\times\sqrt{[(coeff+const)\times(-1)]})$; [f]Estimated means on actual scale, derived from regression equation $e^{(coeff+constant)}$

Lambs at weaning

Mean FEC of lambs at weaning, when lambs age varied between 9 and 12 weeks-of-age, are given in Figure 8. The FEC are representing paddock 1-7. All of the lambs were sampled, although not all could provide enough faeces for the analysis, resulting in samples from five to six lambs/paddock, except for paddock 6 were only one of eight lambs could provide a representative faecal sample. Sampled lambs (n) per paddock (1-7) were n(1, 3, 7)=6, n(2, 4, 5)=5 and n(6)=1. Overall there was no effect of treatment for *Nematodirus* spp. or other *Strongyles* (P=0.558, P=0.734; Table 2), but there was an effect of genetic lines (P<0.001 and P=0.007 for *Nematodirus* spp. and other *Strongyles* respectively), with a higher FEC in resilient animals (Table 2). No *Nematodirus* eggs were detected in FEC from lambs representing paddock 1 and 3.

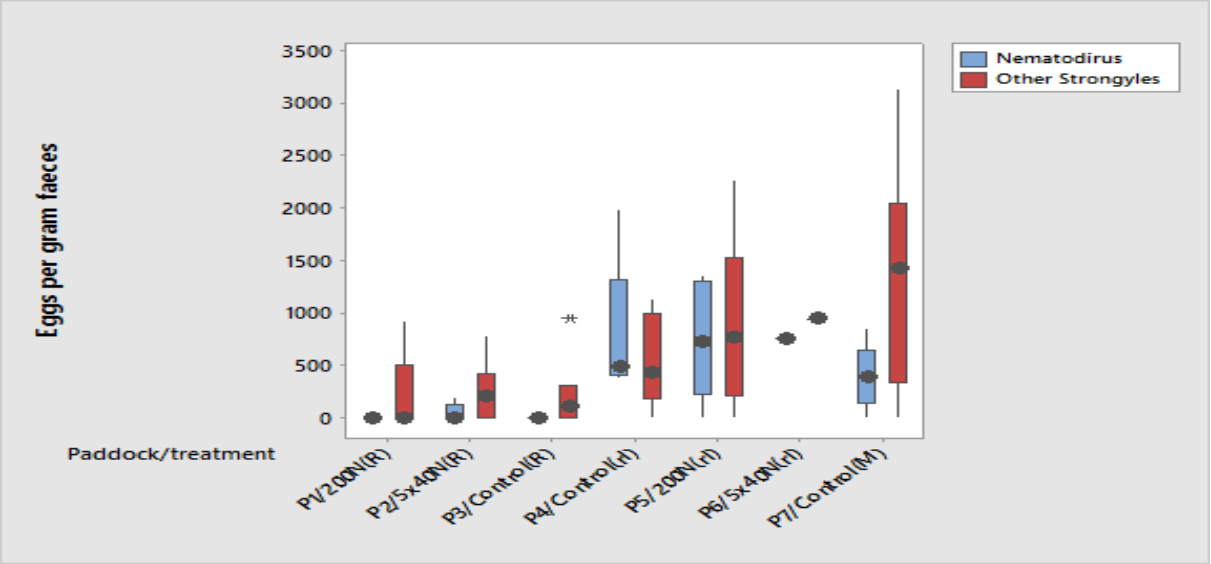

Figure 8. *Faecal egg counts (eggs per gram of fresh faeces) of Nematodirus and "other" Strongyles (total amount of Strongyle eggs − Nematodirus eggs) from the lambs at time of weaning (22/11/2019, representing experimental paddocks 1-7. Sampled lambs (n) per paddock (1-7) were n(1, 3, 7)=6, n(2, 4, 5)=5 and n(6)=1. Black dots represent median. 200N=single treatment of 200 units of urea and 5×40N=repeated treatment of 40 units of urea. R=resistant animals, rl=resilient animals and M=mix of resistant and resilient animals.*

Table 2. *Coefficients for transformed response (including estimated means on actual scale) for faecal egg counts from lambs at time of weaning*

	Nematodirus[a]				Other Strongyles[b]			
	Coeff	SE[c] Coef	P-value	Mean[e]	Coeff	SE Coef	P-value	Mean[e]
Treatment			0.558[d]				0.734[d]	
200N	-1.61	1.81	0.381	156.5	-0.24	2.99	0.938	322.6
5x40N	2.15	2.09	0.313	264.7	2.39	3.46	0.496	423.9
Control	0			199.4	0			331.2
Genetic line								
Resilient	11.99	1.40	<0.001	681.7	6.70	2.31	0.007	620.0
Resistant	0			199.4	0			331.2
Constant	14.12	1.40			18.20	2.31		

[a]Box-cox transformation: (faecal egg count)$^{0.5}$, [b]Box-cox transformation: (faecal egg count)$^{0.5}$; [c]SE=standard error; [d]Overall p-value for the effect of treatment; [e]Estimated means on actual scale, derived from regression equation (coeff+constant)^2

Pasture larvae

Arithmetic mean of pasture larvae for *Nematodirus* spp. and other *Strongyles* in the experimental paddocks over time, are given in Figure 9, representing the four dates of sampling. Over all, there was no effect of treatment for *Nematodirus* spp. (P=0.286). Although not significant, the 5x40N-treatment had a tendency of a stimulating effect (P=0.142), with estimated means being 1.8 times higher for the 5x40N-treatment compared to the control (Table 3), and there was an effect of genetic lines (P=0.019) with a higher PLC in

paddocks with resilient animals (Table 3). There was no effect of treatment for other *Strongyles* (P=0.056). However, the P-value being close to 0.05, was explained by the effect of the 200N-treatment (P=0.018), which presented a higher concentration of other *Strongyles*, and 1.7 times higher estimated means, suggesting a stimulating effect (Table 3). There was no effect of genetic lines (P=0.549), with a higher PLC in the paddocks with resistant animals, although the estimated means showing comparable values (Table 3).

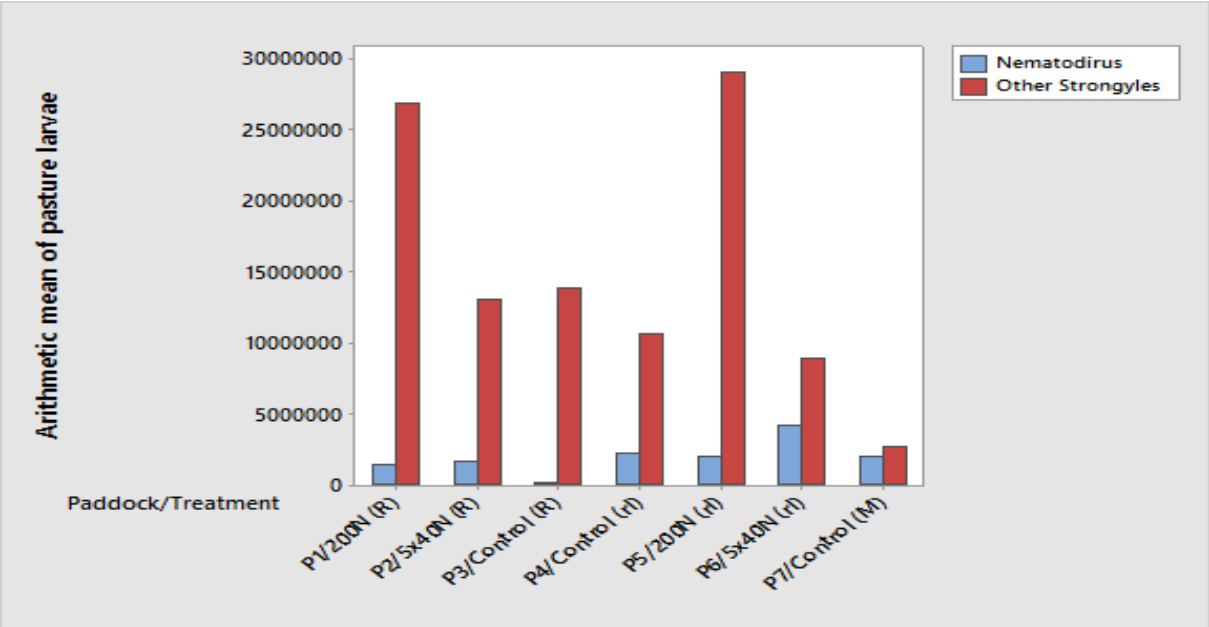

Figure 9. *Arithmetic mean of pasture larvae for Nematodirus and other Strongyles in the experimental paddocks over time, representing the four dates of sampling. 200N=single treatment of 200 units of urea and 5×40N=repeated treatment of 40 units of urea. R=resistant animals, rl=resilient animals and M=mix of resistant and resilient animals.*

Table 3. *Coefficients for transformed response (including estimated means on actual scale) for pasture larval counts from herbage samples over time*

	Nematodirus[a]				Other Strongyles[b]			
	Coeff	SE[c] Coef	P-value	Mean[e]	Coeff	SE Coef	P-value	Mean[e]
Treatment			0.286[d]				0.056[d]	
200N	-62	226	0.787	980,100	1159	445	0.018	23,561,316
5x40N	347	226	0.142	1,957,201	-681	445	0.144	9,084,196
Control	0			1,106,704	0			13,653,025
Genetic			0.019[d]				0.549[d]	
Resilient	412	160	0.019	2,143,296	-192	314	0.549	12,271,009
Resistant	0			1,106,704	0			13,653,025
Date			0.070[d]				0.036[d]	
2nd Sept	-583	276	0.050	219,961	-1184	545	0.044	6,305,121
16th Sept	-113	276	0.687	881,721	-708	545	0.211	8,922,169
30th Sept	715	276	0.019	3,122,289	1465	545	0.016	26,625,600
14th Oct	0			1,106,704	0			13,653,025
Constant	1052	160			3695	314		

[a]Box-cox transformation: (pasture larval count)$^{0.5}$, [b]Box-cox transformation: (pasture larval count)$^{0.5}$; [c]SE=standard error; [d]Overall p-value for the effect; [e]Estimated means on actual scale, derived from regression equation (coeff+constant)^2

Total amount of *Nematodirus* and *Strongyle* L_3 larvae per paddock, over the four dates of sampling, are given in Figure 10 and 11. The weather on the four sampling dates were dry, between 15-18°C and in general sunny, except for the 16th of September having more clouds, although with a similar temperature to the other dates. There was no effect of sampling date for *Nematodirus* spp. (P=0.070). For other *Strongyles* an effect of sampling date could be seen, showing a difference in the concentration of pasture larvae (P=0.036; Table 3). At 30th of September there was an evident peak of *Nematodirus* larvae in paddock 5 and 6, and also a peak of other *Strongyles* in paddock 5 the same date.

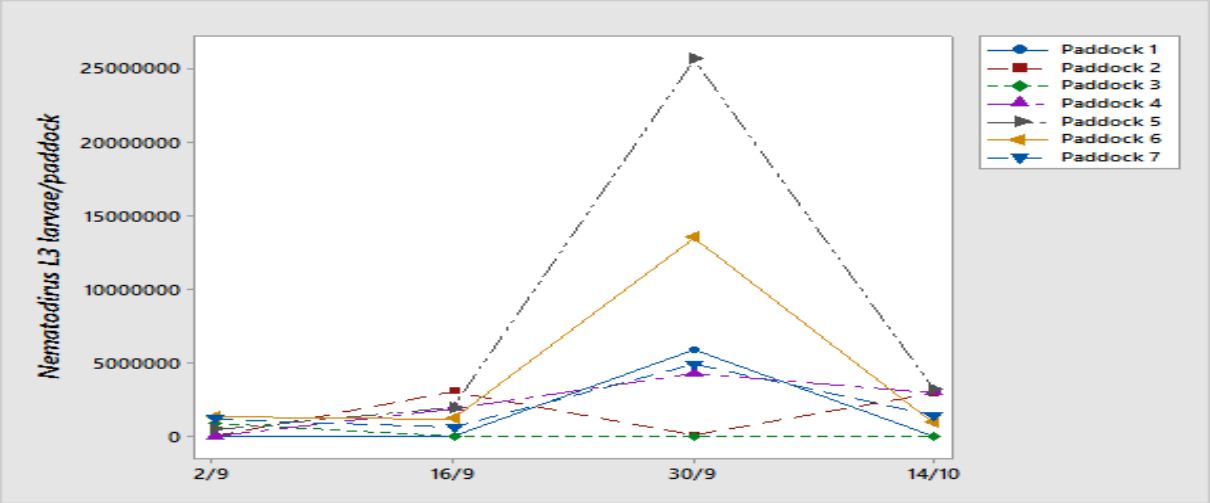

Figure 10. *Pasture larval count after treatments (total number of L_3 Nematodirus per paddock) for paddock 1-7 over time, representing the four dates of sampling.*

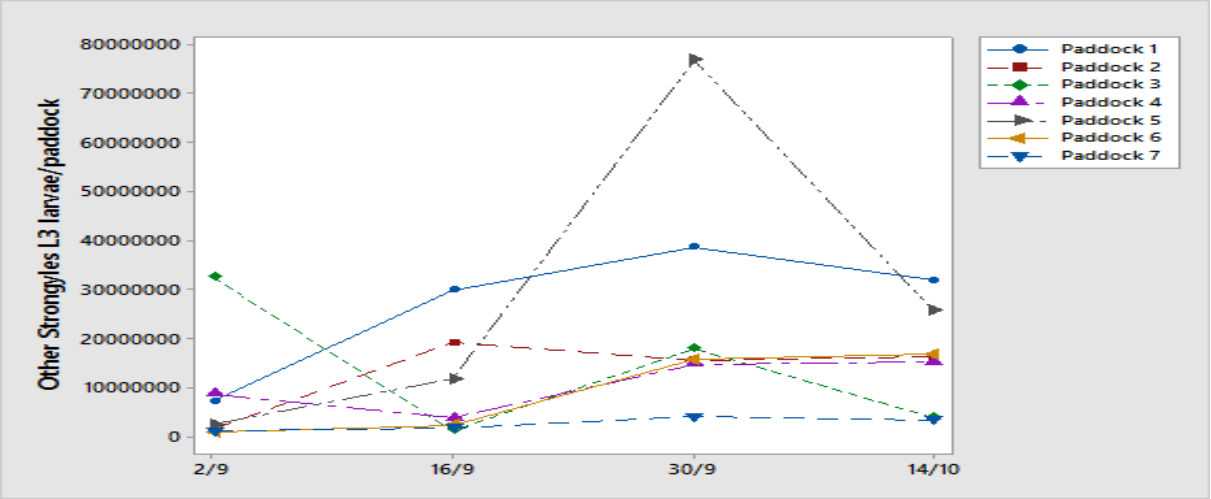

Figure 11. *Pasture larval count after treatments (total number of L_3 of other Strongyles per paddock) for paddock 1-7 over time, representing the four dates of sampling.*

Eggs and larvae in soil samples

Eggs in soil samples

The concentrations of eggs in the soil samples (eggs per gram soil) are given in Figure 12. No *Nematodirus* eggs were detectible in the samples, although other *Strongyle* eggs were present. Regarding other *Strongyle* eggs, there was no effect of treatment or sampling date (P=0.622, P=0.069). There was no effect of genetic lines (P=0.914), with comparable values (Table 4).

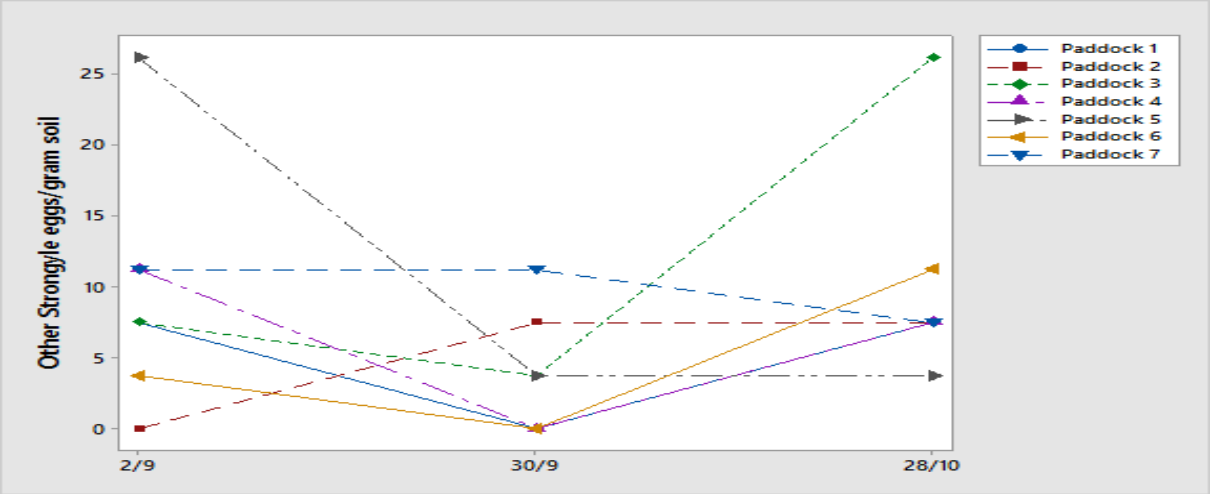

Figure 12. *Other Strongyle eggs in soil (eggs per gram soil) for paddock 1-7 over time, representing the three dates of sampling. No Nematodirus eggs were detectible.*

Table 4. *Coefficients for transformed response (including estimated means on actual scale) for eggs in soil*

	Epg other Strongyles[a]			
	Coeff	SE[b] Coef	P-value	Mean[c]
Treatment			0.622[d]	
200N	0.077	0.329	0.818	5.92
5x40N	-0.314	0.329	0.359	4.01
Control	0			5.48
Genetic line			0.914[d]	
Resilient	-0.026	0.232	0.914	5.34
Resistant	0			5.48
Date			0.069[d]	
2nd Sept	0.236	0.329	0.486	6.94
30th Sept	0			5.48
28th Oct	0.593	0.329	0.096	9.92
Constant	1.702			

[a]Box-cox transformation: ln(epg soil); [b]SE=standard error; [c]Estimated means on actual scale, derived from regression equation $e^{(coeff+constant)}$; [d]Overall p-value for the effect

Larvae in soil samples

The concentration of infective larvae (L_3 per gram soil) of *Nematodirus* spp. and other *Strongyles* in the soil samples, are given in Figure 13. Over all, there was no effect of treatment for *Nematodirus* larvae (P=0.219), or effect of sampling date (P=0.052), although the P-value close to 0.05, was explained by the sampling date 28th of October having no detectable larvae in the samples (P=0.020), and showing a reduction over time (Table 5). Regarding other *Strongyle* larvae, there was no effect of treatment (P=0.904), but there was an effect of sampling dates (P<0.001), showing a reduction over time (Table 5). No effect of genetic line was seen for *Nematodirus* larvae and other *Strongyles* (P=0.450 and P=0.214, respectively), showing comparable values (Table 5).

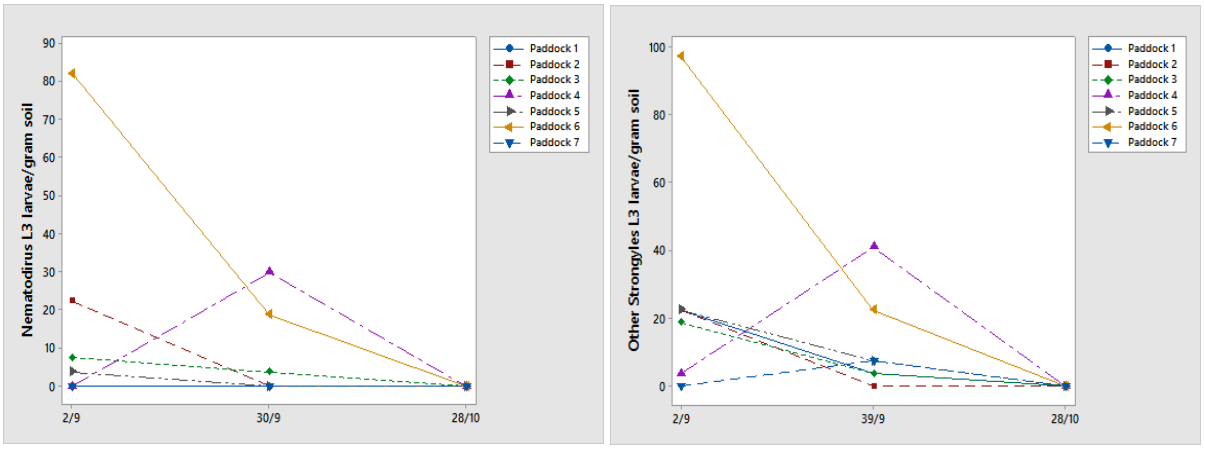

Figure 13. *Concentration of Nematodirus and other Strongyle larvae in soil (larvae per gram soil) for paddock 1-7 over time, representing the three dates of sampling.*

Table 5. *Coefficients for transformed response (including estimated means on actual scale) for larvae in soil*

	L_3 Nematodirus[a]				L_3 other Strongyles[b]			
	Coeff	SE[c] Coef	P-value	Mean[d]	Coeff	SE Coef	P-value	Mean[d]
Treatment			0.219[e]				0.904[e]	
200N	-0.189	0.104	0.095	1.21	0.0212	0.0651	0.751	3.48
5x40N	0.131	0.104	0.231	2.87	-0.0283	0.0651	0.672	2.92
Control	0			1.92	0			3.22
Genetic line			0.450[e]				0.214[e]	
Resilient	0.0575	0.0736	0.450	2.27	0.0605	0.0461	0.214	4.05
Resistent	0			1.92	0			3.22
Date			0.052[e]				<0.001[e]	
2nd Sept	0.201	0.104	0.077	3.69	0.3228	0.0651	<0.001	18.17
30th Sept	0			1.92	0			3.22
28th Oct	-0.278	0.104	0.020	1.00	-0.4426	0.0651	<0.001	1.00
Constant	-0.722				-0.5574			

[a]Box-cox transformation: $-(L_3\text{per gram soil})^{-0.5}$; [b]Box-cox transformation $-(L_3\text{per gram soil})^{-0.5}$; [c]SE=standard error; [d]Estimated means on actual scale, derived from regression equation $1/(\text{coef}+\text{constant}\times-1)^2$; [e]Overall p-value for the effect

DISCUSSION

This study is limited to the aspect of the aim and will not include or discuss the potential environmental effect of nitrogenous fertilisers. Though the concentrations of nitrogen used in the trial were all within approved levels for the area they do represent levels which exceed what may be commonly used. Best practise recommendations in NZ, are between 20-40 kg N/ha per application, as the fertiliser will improve pasture response the most efficient at this level, and also minimising nitrate or nitrous losses (Cairns *et al.*, 2017). In this study one of the chosen levels of urea was about five times higher than commonly used levels on farms in New Zealand and it was also in the upper level of what was approved for the experimental area. Because of the proof of concept study, the levels of urea needed to be beyond commonly applied levels, so that depending on the results there could be refinement studies following this study. Or the fact that the levels did not have an effect, there would be a certainty that the levels did not needed to go higher to receive an inhibiting effect.

Considering the FEC from the lambs grazing the experimental paddocks in the end of spring before the start of treatments (Figure 3), there is a noticeable concentration of *Nematodirus* and other *Strongyle* eggs passed in the faeces, giving the information that there will be eggs and larvae present on pasture while the different treatments are taking place. Although, there were no *Nematodirus* eggs detectable in samples representing paddock 3, and also a low concentration of other *Strongyles*. This could explain certain results and perhaps make paddock 3 a less desirable control paddock.

No effect of urea

In general, the use of urea on pasture does not appear to affect FEC significantly regarding *Nematodirus* spp. and other *Strongyles.* Although for *Nematodirus* spp. and FEC from the ewes, an inhibiting effect could be seen of the 200N-treatment, which will be discussed later. What is worth taking in consideration is that the FEC for *Nematodirus* spp. from the ewes may however not be truly representative as outcomes of the different treatments, as the adult ewes already have a developed immunity against the parasite (Brunsdon, 1962), resulting in lower FEC, and perhaps not detectable with the used method. Considering the FEC from the lambs at weaning, these results may not either be fully representative for the effect of the different treatments as most of the infestation occurs around the time of weaning (Brunsdon, 1967). Additionally, as the age difference between the lambs could be as much as three weeks, the amount of pasture ingested would probably differ individually. The final FEC would be recommended to be performed three weeks after weaning, as probably more eggs are passed at this time (Thomas, 1959a), and would be more appropriate for evaluation of the treatments. Unfortunately, one single lamb represented paddock 6, and although the single value does not differ particularly from paddock 4 or 5, one could not know if the FEC of the other lambs in the same paddock would change the outcome. There should be some certainty in that the *Nematodirus* spp. detected in the FEC, originates from the pasture, as the ewes did not pass *Nematodirus* eggs when let out on pasture. Something to also consider is that FEC on its own is not a reliable variable as the concentration can change depending on faecal volume (Greer & Sykes, 2012).

Overall, the treatments did not seem to create any clear effects on the pasture larvae for *Nematodirus* spp. or other *Strongyles,* although a possible tendency of a stimulating effect of urea will be discussed later. Because of the noteworthy fluctuations in the pasture larval counts, and the known coarseness of the method (Molento *et al.*, 2016), the arithmetic means should present a more reliable outcome following the treatments, although it did not change the general conclusion. Unfortunately, paddock 3 only showed a low number of *Nematodirus* spp. larvae on the first sampling, but nothing in the rest, and the role as control could again be questioned. Additionally, the FEC six weeks after start of grazing does not reflect the pasture larval counts from the sampling six weeks and four weeks before (Figure 8-9), as the paddocks with the highest concentration of infective larvae from 2nd of September were paddock 6 for *Nematodirus* and paddock 3 for other *Strongyles*, and from 16th of September were paddock 2 for *Nematodirus* and paddock 1 for other *Strongyles,* versus the highest FEC being paddock 4 for both *Nematodirus* and other *Strongyles*. Furthermore, as the pasture larvae are not randomly distributed on pasture, with a higher concentration surrounding the faecal pats (Eysker & Ploeger, 2000), the outcome could perhaps be affected, concerning

sampling technique or bias in avoiding the faecal pats. To secure the comparability between the paddocks one single person performed all the samplings, performing the procedure as identical as possible over the experimental areas, irrespective of any faecal deposit, and only avoiding collecting herbage if in the midmost of manure. Similar, one single person performed all the processing of the herbage samples, treating the samples as identical as possible, and one blinded single person performed all of the larval counts and differentiation. Moreover, when observing the results from the pasture larval counts over time, there was no well-defined pattern visible for the different paddocks, except on the 30th of September there was a more or less increase of *Nematodirus* spp. and of other *Strongyles* in majority of the paddocks, which could perhaps be explained of the rise in temperature as spring proceeded (Van Dijk & Morgan, 2009). Although, the rise in temperature does not explain the general decrease in pasture larval count from the last date of sampling. Possibly the decline could be explained of ultraviolet irradiation killing the larvae, supporting the results presented by van Dijk *et al.* (2009) or that intensive sunlight can influence the larvae to make a downward movement along the plant down into the soil (Familton & McAnulty, 1997), although no increase of larvae were seen in the last soil samples.

No effect was seen of the different treatments on eggs and larvae in the soil samples. Interestingly, the samples from the 28th of October had no detectable larvae in them and overall there was a reduction over time, which may indicate that the eggs in the earlier samples (processed nine and five weeks after sampling), possibly could have hatched while in fridge at 4°C. A fact explained by Familton & McAnulty (1997), where some parasites could reach the L_3-stage in 65 days in 0.6°C, so theoretically possible. This could also explain why there were no *Nematodirus* eggs in the samples, but *Nematodirus* L_3. Another explanation to why there were no detectable larvae in the last sample, or overall a low concentration in the samples, could perhaps be that the conditions were optimal for the larvae, and that all larvae were on the herbage (Knapp-Lawitzke *et al.*, 2014), although this was not possible to compare with the pasture larval count missing from the same date. A possible explanation to the low concentration of eggs and larvae in the soil samples could be that the eggs and larvae are expected to be in or close to faecal pats (Familton & McAnulty, 1997), and therefore there might be a sampling bias occurring. The procedure of the sampling were irrespective of any faecal deposit (although the samples were not collected directly in manure) with the indication that complete disintegration of the faeces could have happened (Christie, 1963) and faecal pats from the previous season being disintegrated, leaving eggs and larvae in the soil. Therefore, a sampling bias avoiding faecal pats could explain the low concentration. Regarding other *Strongyles,* comparing the EPG in soil with the pasture larvae on the 30th of September, there was a decline in epg for paddock 1 and 3-6, and for the pasture larvae there was an increase in the same paddocks. There could be a possibility that eggs on pasture the 2nd of September had hatched and contributed to a higher concentration of pasture larvae on the 30th of September. To secure the comparability between the paddocks one single person performed the processing of the soil samples, treating the samples as identical as possible, and one single person performed the counts and differentiation. The used method was validated, giving confidence in the egg recovery, although the method was not validated for larvae recovery, and could be giving unreliable results. In general, the findings in the soil samples provided results difficult to explain, and as it is not routine to analyse soil samples, further

elaboration of methods and collecting technique, together with analysing the samples close after collecting, is recommended.

Effect of urea

There was some suggestion of a positive effect with the higher concentration of N applied. FEC from the ewes six weeks after start of grazing indicated an inhibiting effect on *Nematodirus* spp. of the 200N-treatment compared to the control. The estimated means on actual scale shows different values, but the difference is minimal so the relevance could be questioned. Also, while statistically significant the biological relevance and importance is not completely clear as this benefit did not appear to extend to the lambs which can be expected to be immunologically naïve and grazing the same areas. The immunological naïvety are clearly demonstrated when comparing the *Nematodirus* spp. FEC at the end of spring with the FEC in autumn, where there was a clear pattern showing how the spring peak of infective larvae on pasture, results in higher FEC at end of spring and how it almost decline to zero in the autumn following the development of immunity in the lambs, as described by Brunsdon (1960). Additionally, the FEC may be affected by the post-parturient rise in FEC in the lactating ewes, when their immune response may be compromised by the stresses imposed by pregnancy and lambing, resulting in a peak in FEC, 6-8 weeks after parturition (Vlassoff *et al.*, 2001). This might influence the ewes individually and give skewed results, as the rise will not occur simultaneously. Regarding other *Strongyles,* there is a possibility that the ewes also could be re-infected with the eggs they were passing 6 weeks before. Although theoretically possible but not likely, as the development of free-living larvae and prepatent period would be rather fast completed in 42 days (Vlassoff *et al.*, 2001; Familton & McAnulty, 1997).

There was a tendency of a stimulating effect on *Nematodirus* spp. from the 5x40N-treatment when observing the results of the pasture larvae, although not statistically significant. The estimated means on actual scale for the 5x40N-treatment showed almost double the amount of pasture larvae compared with the control, so the stimulating effect is nevertheless a subject for discussion. The result is comparable to the *in vitro* results seen in the unpublished dissertation of J Bennett (2017), where a single treatment of 40kg N/ha caused a stimulating effect, but five repeated treatments every week of the same concentration showed an inhibiting effect on *Nematodirus* egg hatching and larval recovery. In contrast, using the same concentration every third week in this trial, gave a stimulating effect, possibly because it worked as repeated "single" treatments, due to the longer time in between treatments. On the other hand, the 200N-treatment seemed to stimulate egg hatching and larval recovery for other *Strongyles*. These results were not comparable to the conclusions presented by Cairns *et al.* (2017), where urea at a concentration of 40kg N/ha, topically applied to sheep faeces, showed an evident reduction in larval recovery for *Trichostrongylus colubriformis.*

Further support for a tendency of a stimulating effect can be seen from the peak of *Nematodirus* larvae in the soil samples from 2nd of September, in paddocks 2 and 6. As these paddocks were both treated with the 5x40N-treatment, this may indicate that the treatment stimulated hatching during the winter, although not statistically significant. In contrast, the greater number of larvae were not present by the 30th of September, which may indicate either consumption of the larvae by the ewes, death during this time or again that the larvae were on

the herbage (Knapp-Lawitzke *et al.*, 2014). The theory of the possibility that the eggs could have hatched in the fridge remains, as the samples were not processed until several weeks after the sampling.

Genetic lines

Overall the genetic lines presented results as expected, with higher parasitic burden in paddocks grazed by resilient animals and greater FEC from the resilient lines, which is comparable to the conclusions of Sayers & Sweeney (2005). This was also comparable with the FEC prior to treatments, where there was a considerable lower concentration off eggs being passed by the resistant animals compared to the resilient animals. In contrast, one would expect the genetic lines to present a significant difference in pasture larval count, which is the case for *Nematodirus* spp., but not for other *Strongyles,* where the paddocks with resistant animals had a greater, but similar concentration of pasture larvae compared to the paddocks with resilient ones. Also the genetic lines did not result in a significant difference of the concentration of *Nematodirus* or other *Strongyles* larvae in the soil. These results were not comparable with the history of the paddocks with resistant animals, where the FEC for other *Strongyles* had a noticeable lower concentration of eggs compared to the paddocks with the resilient animals. Thus, breeding for resistance has in this trial, presented ambiguous evidence in reducing the parasite burden on pasture.

Reflections

The hatching requirements appear to involve an adversity of behaviours (Van Dijk & Morgan, 2009) and one would presume that the timing of the treatments plays an enormous part in the effect, as climate conditions regulate hatching and development. If the field trial would be repeated, it would be interesting to see if a more intense frequency of the sprayings, e.g. once every week, could give the inhibiting effect seen in the *in vitro* study by J Bennett (2017). Additionally, in the laboratory it is far easier to be certain of the concentrations applied on the eggs and larvae, compared to a field trial. In the field there are many factors, such as pasture mass or humidity, which could affect the actual concentration reaching the eggs and larvae. Moreover, even if a non-controllable factor, such as weather, could affect the different treatments or the experimental paddocks with the eggs and larvae, the paddocks would have been affected in the same way and thus not affect the comparable outcomes from the different paddocks. Although, if a non-controllable factor would affect the eggs and larvae to a high inhibiting grade, such as intense sunlight, it could possible make the detection methods not sensitive enough and the values perhaps too low for comparison.

Conclusion

Overall the results were equivocal, with absence of strong support that liquid urea could break the life cycle of *Nematodirus* spp. in the field, unlike the *in vitro* results presented by Cairns *et al.* (2017) and J Bennett (unpublished dissertation 2017). However not significant, although a subject for discussion, was that urea may have a stimulating effect on egg hatching and larval development, which are results similar to some of the *in vitro* results seen in the unpublished dissertation of J Bennett (2017).

In summary, the results from this field trial have demonstrated poor evidence that liquid urea could provide epidemiological benefits in reducing larval contamination on pasture. It has been shown that it is difficult to translate the *in vitro* results into the field, and with the lack of delicate measuring methods it is, to some extent, problematic to interpret the results. The epidemiology approaching these gastrointestinal nematodes are involving complicated dynamic changes, which occur both within the host and within the environment. If we could understand the complexity of it, we will probably be able to develop efficient control methods.

POPULAR SCIENCE SUMMARY

Nematodirus is a common parasitic roundworm that causes disease in the small intestines of sheep and subsequently reduced productivity for sheep farmers around the world. Today, the control of these parasites depends mainly on anthelmintic treatment (substance against intestinal worms). Unfortunately, there is a widespread and emerging resistance among the gastrointestinal parasites towards these substances, and there is an urgent necessity for alternatives.

It is the young lamb that becomes most affected of *Nematodirus,* as the adult sheep develops immunity by being exposed and with age. The life cycle of *Nematodirus* comprises of stages both within and outside the animal, with a great part of the development taking place on pasture, where the animals become infected through grazing. When inside the animal, the parasite invades the small intestines and the lamb often suffers from reduced appetite, diarrhoea and decreased growth. Parasite eggs are secreted with the faeces and hatch when the conditions are optimal, and therefore give rise to the parasite burden on pasture. The hatching of the eggs is mainly dependent on moisture and a temperature rise, and therefore the peak of infective parasites (larvae) on pasture coincide with spring and often at the time when the lambs start to graze fully. Since it has been estimated that more than 90% of the total parasitic population is present in the stage outside of the host, a suitable strategy to combat the problem may be targeting this stage. This is especially the case for *Nematodirus,* which may spend many months, and also have the ability to overwinter, on pasture. There is evidence suggesting that nitrogenous fertilisers could have the ability to inhibit egg hatching and survival of gastrointestinal worms. Thus the aim of this study was to investigate if urea, a nitrogenous solution commonly used as fertiliser, could break the life cycle of *Nematodirus* in the field and hopefully find an alternative to anthelmintic treatment.

The field trial was performed through targeted application of liquid urea to pastures at times when *Nematodirus* eggs are expected to be present. Seven paddocks were used, grazed by sheep with an existing population of *Nematodirus*. Additionally, the sheep were of two different genetic lines; resistant and resilient, which should affect the tolerance against gastrointestinal worms, thus this aspect was also included in the overall results. None of the animals used had been given anthelmintic treatment. The study included different sprayings of liquid urea during the winter, when there were no animals present on pasture. The sprayings consisted of one single treatment of a high nitrogen concentration, one repeated treatment of a “in field” commonly used nitrogen concentration, and the control paddocks were left untreated. The effect of treatments was evaluated utilising three methods, i.e. observing pasture larval concentration, faecal egg counts and concentration of eggs and larvae in soil.

In summary, the results from the field trial did not demonstrate convincing evidence that liquid urea could break the lifecycle of *Nematodirus*. Although, some of the results are suggesting that urea on the contrary may have a stimulating effect on egg hatching and larvae development, which is a less desirable effect considering the search for anthelmintic alternatives. Overall, the genetic lines presented results as expected, with higher parasitic burden in paddocks grazed by resilient animals. With this trial it has been shown that it is

difficult to translate laboratory results into the field, and that the measuring methods may not be delicate enough to present reliable results. The hatching requirements comprise an adversity of behaviours as the environment plays an important role in regulating hatching and development, and this is making the timing of the treatments problematic. In conclusion, because the lifecycle of this parasite involves complex dynamic changes, which occur both within the host and within the environment, it is difficult to develop efficient control methods.

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