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Potential anthelmintic properties of nitrogenous fertilisers

A Dissertation
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by
Jessica Elizabeth Cairns

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Agricultural Science with Honours.

Abstract

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by

Jessica Elizabeth Cairns

This dissertation describes a series of experiments designed to understand the potential anthelmintic properties of nitrogenous fertilisers for control of gastrointestinal parasites. Anthelmintic administration is no longer considered the sole method for control of gastrointestinal parasites. Due to the increasing levels of anthelmintic resistance, alternative methods of parasite control are required. Nitrogenous fertilisers produce a number of potentially toxic compounds during their conversion to plant available forms, and there are some reports they may have anthelmintic properties, although there is a paucity of specific evidence. Therefore, this series of experiments investigated the effects of nitrogen-containing fertilisers on *Trichostrongylus colubriformis* eggs hatching and larval development.

Experiment one (Chapter 3) consisted of topical application of either water or urea (Flow-Fert N, 20% concentration) onto 100g of faeces. The total number of larvae collected per 100g of faeces was 25,600 and 800 for water and urea respectively, a 97% reduction in larvae following treatment with urea. Visual assessment of the faeces showed a white fungus growing on the faeces sprayed with water that was not present on the faeces sprayed with urea.

The second experiment (Chapter 4) involved immersing *T. colubriformis* eggs in pH solutions ranging in whole number increments from 4 to 11, and in solutions of 20% urea (pH of 8). pH had a significant effect on egg hatching at pH less than 6 (P<0.001), whilst above pH 6 there was no effect on egg hatching. Urea solutions suppressed 90%+ of the eggs from hatching indicating that the effect on egg hatching was independent of pH.

Experiment three (Chapter 5) determined the optimum concentration of urea required to inhibit eggs from hatching. *T. colubriformis* eggs were immersed in solutions of urea at various concentrations (1%, 2%, 4%, 6%, 8%, 10%, 20%, 50%). There was a significant effect of urea concentration on egg hatching with hatching decreasing as urea concentrations increased with less than 10% of eggs hatching in concentrations greater than 10%. Optimum concentrations estimated using an LD90 and ROC analysis were determined to be 19.6% and 5.5%, respectively.
The fourth experiment (Chapter 6) compared five nitrogen-containing fertilisers: urea (46-0-0-0), sulphate of ammonia (21-0-0-24), potato fertiliser (15-10-10-8), potassium nitrate (13-0-44-0) and nitrophoska blue (12-5.2-14-0) at various concentrations (1%, 5%, 10%, 20% and 50%) and their effects on eggs hatching. There was a fertiliser type X concentration interaction with all fertilisers, at concentrations greater than 10% inhibiting eggs from hatching to less than 6% (P<0.001). At a concentration of 1%, sulphate of ammonia and potassium nitrate had the strongest effect on eggs hatched, viz 24% and 48%, respectively, in comparison with 90% in the control. Regression analysis credited the variation in percentage of eggs hatched could be explained by both nitrogen percentage and electrical conductivity, but not phosphorus, potassium, sulphur levels or pH.

The final experiment (Chapter 7) considered the implications of four of the fertilisers and whether exposure to the fertilisers was reversible. Following 24 hours of immersion in the solution, and a further 24 hours of incubation in water, no eggs had hatched under any of the fertilisers (20% concentration), indicating a strong, irreversible effect on the eggs (P<0.001).

Nitrogenous fertilisers have the potential to control ruminant GINs outside of the host, as an alternative to anthelmintics. These fertilisers break the lifecycles of the parasites, however more research is required regarding the components of the fertilisers that are toxic to the nematodes and whether these in vitro studies can be transferred into the field and remain successful.

**Keywords:** pH, nitrogen, fertiliser, gastrointestinal nematodes, *Trichostrongyles colubriformis*, egg hatching
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Chapter 1

Introduction

New Zealand is the largest global exporter of sheep meat and wool and has the seventh largest population of sheep in the world. Animal disease is a major constraint to sheep production, impacting animal productivity and acquiring financial costs due to preventative and curative measures upwards of $700 million per annum in both lost production and anthelmintic costs. New Zealand sheep are typically run on extensive, pasture-based systems and given the ubiquitous nature of gastrointestinal nematode parasites (GIN), exposure to GINs is common.

Typically, control of GIN’s relies on administration of chemical anthelmintics to the animal. However, due to the widespread extent of anthelmintic resistance in New Zealand’s parasitic populations, chemotherapy alone is now viewed as an unsustainable method of parasitic control (Greer, 2005). Furthermore, with an increasing global population and a change in consumer demand, consumer concern about potential exposure to chemicals and residues from compounds such as anthelmintics is prevalent. Increasing concern regarding the reliance on anthelmintics for parasite control has led to investigation of alternative strategies for controlling gastrointestinal parasites. One of these may be the use of nitrogenous fertilisers to interrupt nematode development outside the host and break the parasite’s lifecycle.

Relatively few studies exist specific to ruminant GINs, but there is evidence that both pH and nitrogenous fertilisers have negative effects on the development of parasites. In other species, the majority of studies show that at a pH < 6 six larval development is halted (Howell et al., 1999; Somerville and Murphy, 1983). However, research above pH 6 and understanding of the mechanisms acting on the parasites is lacking. Human gastrointestinal parasites found in sewage sludge, such as Ascaris suum, appear to be susceptible to both pH and the presence of nitrogen in the form of ammonia (Pecson et al., 2007). Further, unpublished observations have shown that sheep gastrointestinal nematode eggs, Trichostrongylus colubriformis and Teladorsagia circumcincta can be reduced when incubated in liquid urea (Greer, unpublished).

This dissertation explores the potential impact of pH and commercially available nitrogenous fertilisers on a common GIN found in New Zealand sheep. A combination of larval development, egg hatch assays, various concentrations of nitrogenous fertilisers and time lengths were used to ascertain the impacts on parasitic egg hatching and development. This research could potentially offer an alternative method to controlling parasites, outside of the host, and reduce the reliance on chemical anthelmintics in pastoral production systems.
Chapter 2

Review of Literature

2.1 Gastrointestinal parasites of sheep
Gastrointestinal nematode parasitism is a major disease of pastoral ruminants. Ruminant production in New Zealand suffers losses upwards of $700 million/annum due to reduced productivity and anthelmintic costs, caused by the presence of gastrointestinal nematodes (GINs). Environmental conditions in New Zealand favour pastoralism as well as free-living stages of the helminth (Sykes, 1997). The following parasites are of most concern to sheep production in New Zealand – the abomasal nematodes *Haemonchus contortus, Teladorsagia* spp., the small intestinal species *Trichostrongyulus* spp., *Nematodirus* spp., and to some extent *Cooperia* spp. (Vlassoff, 1994).

2.1.1 Anthelmintic alternatives
The use of anthelmintics to interrupt the parasite life cycle and reduce the production losses caused by parasites has now become an unsatisfactory control method (Greer, 2005). Despite the availability of chemical anthelmintics, parasitism remains a risk to ruminant production due to the prevalence of anthelmintic resistance (Brunsdon *et al.*, 1983). It is estimated that 90% of the parasite population is found outside the host (Familton and McAnulty, 1997), thus research into potential methods of breaking the lifecycle through interrupting larval development and controlling these parasites outside of the host could severely reduce the reliance on anthelmintics.

2.2 Effects of parasitism on host
Grazing animals are inevitably exposed to parasites when consuming larval-infested grass. The parasite X host relationship is complex and it has been suggested that ruminant hosts co-evolved with parasites and developed a commensal relationship. However, with increased intensity of pastoral farming this balance has been interrupted, leading to pathological levels of worm burdens within the host. Parasitic infection can have numerous effects dependent on the parasite genera, infection rate and immunological state of the host (Greer, 2005). In susceptible animals there are severe reductions in the production of both wool and meat which are driven by a decrease in voluntary feed intake, reduced efficiency of nutrient utilisation and partitioning of resources to the immune response (Brunsdon, 1970). Typically, animals suffer through a combination of decreased voluntary feed intake and nutrient utilisation. Effects are temporal, typically broken into three distinct phases: hyporesponsiveness where an infection is established without detrimental effects on the host; acquisition, the phase in which protective development is established with symptoms of infection beginning to be expressed; and expression, where a mature immune response that prevents further infection has been established (Sykes and Greer, 2003; Kimambo *et al.*, 1988).
2.2.1 Feed intake

The reduction in feed intake during gastrointestinal nematode (GIN) infection is the major cause of reduced animal performance (Sykes, 1997; Greer, 2005). Despite 40-90% of production losses during infection attributed to inappetance, the reasons for decreased feed intake are unclear but appear to be associated with the presence of nematodes in the gut as intake is rapidly restored following anthelmintic treatment (Kyriazakis et al., 1998). In sub-clinical parasitism, feed intake reductions (10-30%) can be observed, and in clinical cases complete anorexia within 1-3 weeks of challenge can occur (Sykes, Poppi and Elliot 1988). The extent of reduction in feed intake appears to be partly dependent on the number of larvae ingested as Steel et al. (1980) showed intake reductions are greatest in lambs ingesting 30,000 T. colubriformis larvae per week, with a lesser reduction in lambs ingesting 3000-9500 larvae per week. Kyriazakis et al. (1998) suggested two functional hypotheses for the cause of inappetance in infected lambs, 1) Anorexia helps animals to promote an effective immune response and 2) Anorexia allows greater diet selectivity (provision of high protein supply to meet increased protein requirements). However, specific evidence of either of these hypotheses has not yet been provided.

Studies have shown a relationship between gastrointestinal hormones, the hypothalamus and decreased feed intake (Dynes et al., 1990), with the hypothalamus thought to play a role in appetite regulation. Dynes et al. (1990) showed that manipulating hormonal signals at the hypothalamus in lambs resulted in a short term proportional increase in feed intake, however, there was no long term effect observed following 22 hours of administration. Whilst the research with ruminants is lacking, studies in humans indicate pro-inflammatory cytokines, such as interleukin-1 and interleukin-6 may be a causing factor for anorexia (van Houtert and Sykes, 1996). Cortico-steroid based immune-suppression studies have provided strong evidence that components of immune response associated with the acquisition phase of immunity may have a causal role in the anorexia of infection (Greer et al., 2005; Greer et al., 2008; Greer et al., 2009). Manipulation of the hypothalamus and its receptors may be a method to encourage ruminants to graze during the course of a parasite infection, decreasing the effects of reductions in feed intake (Greer, 2005).

2.2.2 Protein losses and nutrient utilisation

Depression of feed intake is not solely responsible for reduced production seen in parasitized animals (Sykes and Coop, 1976; Sykes and Coop, 1977), with the remainder of lost production attributed to a reduction in nutrient utilization (Greer, 2005). In pair-feeding studies where animals un-infected with parasites and infected animals were offered the same amount of feed, DM digestibility was not largely affected by parasitic worm burden, however nitrogen digestibility was decreased (Sykes and Coop, 1976; Sykes and Coop, 1977). Physical damage caused by parasites causes increased leakage of
plasma proteins (Vaughan et al, 2006). Poppi et al (1986) reported protein losses into the alimentary tract of 20-125g/day in T. colubriformis infected sheep, and concluded that the decrease in overall nitrogen balance was due to the increase in endogenous nitrogen losses. Whilst some plasma protein is reabsorbed, Bown et al (1991) reported losses increased from 0.68gN/day in the controls to 1.97gN/day in animals infected with T. circumcincta and T. colubriformis, values comparable with those of Vaughan et al (2006). These plasma protein losses can amount to 10-36% of the endogenous nitrogen loss (Greer, 2005). Further, Jones and Symons (1982) reported an increase in the daily fractional synthesis rate of protein in the liver, from 0.346g/d⁻¹ to 0.724g/d⁻¹ in lambs infected with T. colubriformis. Consequentially, protein synthesis shifts from carcass to the liver and alimentary tract as energy is used for the replacement of blood proteins and repair of the gastrointestinal tract (Greer, 2005).

2.2.3 Production costs
The multiple effects that parasites have on their host translates to lost productivity in production systems. Direct production costs to the host, in terms of feed intake, protein losses and nutrient utilisation are difficult to discern especially in sub-clinical cases. However, liveweight gain and body condition score can be used to understand the actual cost to production (Macchi et al, 2001). Sutherland et al (2010) reported that failure to adequately control parasite infection, through the use of monthly anthelmintic treatment with an efficacy of less than 60%, reduced lamb liveweight gain by 10% with lambs taking 17 days longer to reach slaughter weight than their counterparts treated with an anthelmintic with an efficacy in excess of 98%. Furthermore, Macchi et al (2001) reported a 1.4kg difference in live-weight between lambs treated with an “effective” drench and “ineffective” drench. In addition to the reductions in liveweight gain, animals also suffer production losses attributed to the increased presence of dags. Sutherland et al (2010) credited a loss in wool production to the increased faecal scouring, which lead to an increased dag score, a consequential loss of wool and potential exposure to flystrike. Furthermore, a decreased LWG can also be associated with losses in BCS which can adversely affect carcass composition and value, a reported loss of 14% (Sutherland et al, 2010).
2.3 Life cycles of gastro-intestinal parasites

Effective parasite control relies on understanding the nematode lifecycle and parasitic epidemiology (Familton and McAnulty, 1997). The majority of the gastrointestinal parasites in ruminants in New Zealand have a similar, direct lifecycle (Figure 1).

![Figure 1: The life cycle of gastrointestinal nematode parasites of sheep. Sourced from Elanco, 2015](image)

Given the appropriate environmental conditions, the life cycle of a gastrointestinal parasite can be completed within 4 weeks. Parasitic eggs are ovoid, measuring 40-110μm, and are expelled from the host onto the pasture through faeces. Eggs hatch within the faeces, typically within 48 hours, to free-living L1 larvae which actively feed on microorganisms within the faecal pat. Within 3-5 days the L1 larvae moult, shed their cuticle and become free-living L2 larvae, which also remain in the faeces until a final moult to L3 larvae where they retain their cuticle. The length of time for L3 larvae to develop is strongly regulated by temperature and can take more than two weeks in temperatures of less than 5 degrees. The retained cuticle of the L3 infective larvae serves to provide additional protection from imperfect environmental conditions. However, L3 larvae are unable to feed, thus rely on stored metabolites (Familton and McAnulty, 1997). Once conditions are suitable, L3 larvae migrate out of the faecal pat through moisture films, onto the herbage where they are then ingested by the ruminant host. L3 larvae are then triggered to exsheath by changes in carbon dioxide, temperature and pH within the host, and develop into L4 larvae. After 8-10 days L4 larvae moult to form immature adults, which mature over the following week, reproduce and expel eggs out through the faeces and back onto the pasture. Typically, the time from L3 ingestion to egg-laying adults is 21 days.
2.3.1 Mechanisms of egg hatching
Reports on the mechanism of hatching of different species give emphasis to either mechanical or chemical processes for the breakdown of egg membranes leading up to hatching. Research of soil, plant and animal nematodes indicate three key components to egg hatching: 1) Increased levels of trehalose (a disaccharide) 2) Increased cell permeability and 3) Action of bio-chemicals both within and outside the egg.

Hatching appears to be initiated when carbon dioxide gas penetrates the permeable lipid layer of the egg. The lipid layer becomes hydrated and permeable, allowing the passage of trehalose (Perry, 1989). Not only is trehalose let in to the egg, but it can also be expelled from the shell, with the presence of trehalose causing thinning of the egg shell. The presence of the trehalose signals the movement of the larvae within the egg, further weakening the egg shell and increasing the cell permeability. Specific evidence of how GINs exit the egg is lacking, whether it is the movement of the larvae that affect the elasticity of the egg, the permeable layer gives way to the larvae, or the larvae have sharp body parts to burst out of the shell. Emergence is not completely independent of the surrounding environment, as temperature, moisture and oxygen all affect hatching and development of the larvae within the egg (Gates, 1987). It is unclear whether the permeability of the shell affects larval development, and what triggers the egg to develop into a L1 larva.

2.3.2 Larval distribution
Once larvae develop to the L3 stage they become less susceptible to environmental conditions due to their retained sheath which provides some resistance to desiccation. This allows them to migrate around the pasture sward until consumed by a host. Active larval migration is dependent on the availability of a water film. Larvae require precipitation to move, however are generally not found any further than 30cm from their fecal mass, with larvae concentration decreasing the further afield from the pat (Gronvold, 1984). The majority of larvae (50%) are found in the lower 2cm of a plant with increasing numbers in the upper sward occurring in conditions of high humidity and temperature (Gronvold, 1984; Stromberg, 1997). In a typical ryegrass sward the top 2/3 of a sward are most exposed to environmental conditions such as high/low temperature, wind, UV radiation and humidity, conditions which are unfavourable to larval survival.

2.4 External factors affecting larval survival and development
Once parasitic eggs have contaminated pastures, their ability to survive and develop depends on a number of environmental conditions, the most important of which are temperature, oxygen and moisture. Due to the relatively small variance in average climate across New Zealand, most parasites are able to survive in the cooler temperatures and continue developing regardless of the region they are found (Brunsdon, 1970). Whilst 90% of the parasitic population is found outside of the host, only
1-17% of the eggs expelled onto the pasture reach infective larval stages (Familton and McAnulty, 1997), as their development and subsequent survival can be severely influenced by the external environment. Nevertheless, even with low development rates, parasites have an extensive biotic potential that can quickly lead to large larval challenges on pasture. In areas of high contamination ewes can consume as many as 27,000 larvae/kg of fresh herbage (Familton and McAnulty, 1997). Given the right environmental conditions larval populations can explode to several million larvae/ha/day.

2.4.1 Temperature
Ambient temperature has a profound influence on egg hatching, larval development and the subsequent survival of the pre-parasitic stages (Vlassoff, 1982). Development of parasitic populations will occur between 4°C and 35°C, provided other environmental requirements are being met, with the most activity seen between the optimal range of 15°C and 30°C (Vlassoff, 1982). Stromberg (1997) stated that temperature encourages larvae development until temperatures exceed the optimal range at which point net development is affected by increased desiccation. At temperatures greater than 40°C eggs survive for no more than 24 hours, however at temperatures lower than 4°C eggs can survive for up to 46 weeks (Familton and McAnulty, 1997). Typically, temperatures during NZ summers are unfavourable for larval development. Lower larval numbers are seen during December – February than other times of the year as exposure to high temperatures and lack of moisture leads to desiccation of the L3 infective larvae. Whilst larvae are able to survive the more extreme temperatures, if exposed for longer than 1-2 days mortality rates can rapidly rise as larvae metabolism increases leading to draining of body energy reserves. In temperatures greater than 35°C larval survival can be limited to less than two days. During exposure to cold temperatures metabolic rates are reduced and parasites may become dormant, and can survive for extended periods of time. Familton and McAnulty (1997) reported that at temperatures of less than 4°C, T. circumcincta can survive for longer than a year. The average ambient temperatures in Canterbury over winter ranges between 5.8°C and 7.6°C thus egg hatching and development still occurs but at a far slower rate than over the warmer months (Familton and McAnulty, 1997).

2.4.2 Moisture
Adequate moisture is essential for larval development (Stromberg, 1997). Water is essential for the development and maintenance of L1 and L2 larvae, which could be described as aquatic as they require a film of moisture in order to migrate in search of food (Familton and McAnulty, 1994). Moist conditions prevent the drying out of the faecal mass, which allows the development of the infective L3 larvae. In dry conditions a crust may form on the faecal pat thus preventing drying out of the pat and providing suitable conditions for larvae development. The L3 larvae migrate to the centre
of the pat until the presence of moisture, such as that from rainfall or irrigation breaks the surface of the faecal pat, allowing their release (Gronvold, 1984). If moisture events occur following extended dry periods, a sudden increase in larval numbers on pasture can occur. Little research has been performed on the importance of moisture, as measuring and controlling moisture levels is complex. However, a number of studies have shown the relationship between precipitation/moisture and larval development, reporting the development from free-living to infective stages is affected by the time for precipitation to exceed evaporation (Barger et al, 1972). Furthermore, Bullick and Anderson (1978) found irrigation affects development as soil temperature decreased but soil/ground moisture increased and was conducive to larval survival. Ultimately moisture in the faecal pats is key to parasite development.

2.4.3 Oxygen
Oxygen is necessary for the development of parasite eggs, as a lack of oxygen will inhibit egg hatching, larval development and larval activity (Farlston and McAnulty, 1994). In support of this, the proportion of eggs that develop near the surface of faeces is greater and more rapid than the proportion that develops in the centre. Furthermore, as faecal breakdown occurs from insect/microbial action and weather, egg development proceeds as a result of aeration of the dung, provided other environmental conditions are adequate (Gronvold, 1989). Similarly, if faeces are waterlogged and anaerobic conditions are created, egg hatching will be inhibited despite other factors such as temperature being adequate. However, in contrast, in the laboratory parasitic eggs hatch and larval development is often observed in water, possibly indicating larvae have some tolerance to low oxygen availability.

2.4.4 pH
pH can have negative effects on the development of a range of parasites, especially at low pH. However, relatively few studies specific for ruminant GIN exist. Khatun et al (2013) showed no Haemonchus contortus eggs hatched at a pH of 2, with nematode number decreasing after an optimum pH of 6. Somerville and Murphy’s (1983) study found no larvae present at pH 2, and the greatest amount of larvae exsheathment occurring at pH 6. Most importantly there were lower rates of larval development at the lower pH. While investigating the potential anthelmintic properties of effective micro-organisms (EM) Lewis (2013) reported a crucial pH of 4.5 for egg hatching of T. circumcincta and T. colubriformis larvae. This research gives some understanding of the effects of pH on larval development between 2-8 however there is a gap in the knowledge above pH 6 especially above pH 9 and little information available on the effects on egg hatching, with most of the studies focussed on larval development.
2.4.5 Fertiliser applications
Numerous studies have investigated the use of nitrogen containing fertilisers for the control of various soil nematodes and human parasites in effluent. However, there is limited information on the effects of nitrogen fertilisers, specifically on ruminant gastrointestinal parasites. In one of the few studies, Howell et al (1999) showed that with diluted nitrogen based fertilisers, as nitrogen concentration increased Haemonchus contortus larval motility decreased, with the greatest change seen between 6% and 12% nitrogen. Furthermore, these authors reported ammonium nitrate was the most effective (97%) at rendering the L3 larvae non-motile compared with urea (81%) and ammonium nitrate and urea in combination (89%). Levels of nitrogen were less than 34kgN/ha, below levels commonly used on pastures as a growth promotant in any single application. Goode et al (1974) reported ammonium nitrate (60% concentration) caused a 65% decrease in larval survival when sprayed on pastures at a rate of 67.4kgN/ha, although it is unclear what parasites were present. In unpublished observations liquid urea (Flow-Fert N, 20% nitrogen) has been shown to inhibit T. circumcincta and T. colubriformis egg hatching by more than 90% when diluted to 20% concentration (Wozniak, unpublished, 2013). These studies provide support for the potential of nitrogenous fertilisers to strategically target the parasite population outside the host. However, further research may be required to ascertain whether the observed anti-parasitic effects are a consequence of pH or the nitrogen components.

Nitrogen fertilisers can impact parasitic larval survival however evidence is lacking regarding the components of fertilisers responsible. Howell et al (1999) claimed the larvicidal action of the nitrogen compounds can be attributed to the toxic qualities of ammonia. Gonzalez et al (2010) also stated the ammonia created by the nitrogen in the fertiliser promotes larval exsheathment which results in direct larval exposure to the surrounding environment and kills the larvae. In a review by Rodriguez-Kabana (1987) it was suggested that ammoniacal nitrogen was the compound toxic to nematodes. Urea, for example, is a compound that is readily converted to ammonia whereas ammonium nitrate is a fertiliser already consisting of ammonia, potentially explaining the greater toxicity observed by Howell et al (1999). Whilst ammonia on its own appears to be highly toxic to parasites, when coupled with nitrates it can suppress up to 97% of soil and plant nematode larvae (Gonzalez et al, 2010). It remains to be determined if this extends to gastrointestinal nematodes.

2.4.6 Fate of N sources
Nitrogen, in the form of fertilisers is applied to pastures to promote plant growth. Nitrogen fixation, through symbiotic bacteria causes the transformation of nitrogen molecules to ammonia. These bacteria contain the nitrogenase enzyme that combines gaseous nitrogen with hydrogen to produce ammonia, which is converted by bacteria to other organic compounds. Following ammonification,
the ammonia may then undergo nitrification, caused by nitrifying bacteria and the ammonium molecules are oxidised to form nitrates and nitrites. Fertilisers containing ammonia, such as ammonium sulphate bypass the nitrification stages and do not require the bacteria to be made plant-available. Similarly, fertilisers containing nitrates do not rely on bacteria to convert the nitrogen into a usable form.

2.4.7 Nitrogen and egg hatching
Support for the influence of nitrogen and pH on egg viability can be obtained from studies of sewage sludge and other biosolids which contain a wide range of pathogens. Of the classes of pathogens present in biosolids, helminths are the most resistant to inactivation. Alkaline stabilization is an option for inactivation of helminths, but the reported effectiveness of this varies in pH of 10-12 (Pecson et al, 2007; Katakam et al, 2014). To achieve 90% inactivation of helminth eggs, the reported minimum exposure times vary from 2 h to 4180 d, suggesting that alkaline stabilisation may not be the most successful way to control helminths (Pecson et al, 2007). Furthermore, evidence suggests that factors such as temperature, type and dosing of alkalinising agent, pH and the change in pH during storage may impact the level of inactivation. However, Pecson et al (2007) showed that ammonia causes inhibition of over 76% of Ascaris spp. eggs hatching in 3 days, and the level of inactivation was directly proportional to the activity of NH3. Pecson et al (2007) stated that the addition of nitrogen, in the form of ammonia, caused a significant increase in inactivation rates of Ascaris spp. eggs, effects which were independent to any pH effects. Fidjeland et al (2015) found Ascaris suum inactivation was not significantly affected by pH (pH range less than 12) but pH had a strong effect on the ammonia equilibrium, with an indirect effect on Ascaris egg inactivation. This suggests that coupled together, pH and nitrogen may successfully suppress the development and survival of Ascaris eggs. This provides support that both pH and nitrogen can impact on the development and survival of parasites. This appears to be an area worthy of investigation for ruminant GINs to assist in controlling parasite populations.
2.5 Summary
Gastrointestinal parasites in New Zealand are an ongoing problem in grazing ruminant production systems. Administration of chemical anthelmintics are the most common method of parasitic control in New Zealand, however the increasing level of anthelmintic resistance requires research into alternative control options to chemotherapy. One such approach is to target the parasite at a stage of its lifecycle outside the host. Studies show that pH and nitrogenous fertilisers can adversely affect soil and plant nematodes. However, few studies have investigated these effects on gastrointestinal nematodes. Investigation of the potential effects that both pH and nitrogen-containing fertilisers may have on GINs appears to have largely been an area overlooked previously and is one that is worthy of further investigation.
Chapter 3
Topical application of urea and water

3.1 Introduction
Manipulation of the parasite in the external environment through application of urea and other forms of nitrogen may negatively affect the hatching and development of T. colubriformis.

Unpublished work by Wozniak (2013) indicated that liquid urea (Flow-Fert N, Ravensdown) can inhibit T. circumcincta and T. colubriformis eggs from hatching by more than 90%. Pecson et al (2007) reported that larval development of Ascaris suum was also inhibited when ammoniacal nitrogen was applied to sewage sludge. Other studies (Howell et al 1999; Gonzalez et al, 2010) also support these findings, with larval development inhibited when nitrogenous fertilisers are applied in solution. This experiment looks at topical application of Flow-Fert N to faeces and tap water and the subsequent effects on T. colubriformis development through to the infective L3 stage.

3.2. Materials and methods

3.2.1 Fecal collection
Two parasite-free 10-month-old wether (Hampshire) sheep at JML, Lincoln University were mono-specifically infected with the parasite Trichostrongylus colubriformis and used for the collection of nematode eggs. Animals were held indoors on wooden slatted floors and offered a lucerne pellet/chaff diet with ad libitum access to water. Faeces were collected from each animal with an improvised harness holding a collection bag around the peri-anal region of the animal. Faecal collection occurred from 21 days after the animals had been infected with the parasites, with faeces used for faecal egg counts, egg hatch assays and faeces incubated for the development of larvae as described below. For each day of faecal collection, harnesses were emptied in the morning at approximately 9am. Faeces were then collected up to 2 hours later, ensuring that all collected faeces was less than 2 hours old prior to processing.

3.2.2 Topical application
Faeces were collected as per section 3.2.1. Faeces were homogenised by gentle mixing with 100 grams then spread evenly across each of four plastic trays measuring 0.3m by 0.2m, ensuring pellets were not lying on top of each other. The faeces containing trays were placed in the field and were sprayed with either Flow-Fert N (20% liquid urea, Ravensdown, New Zealand) or water with a held-held pump sprayer at a rate of 200l per ha, with each treatment replicated twice. The trays were then left in the field for 18 hours before being placed individually into a plastic bag which was punctured to allow airflow and incubated for 10 days at 26°C to allow larvae to develop. Following incubation, faeces were placed on Baermann funnels for larval recovery. Briefly, Baermann funnels were filled with water and covered with two-ply tissue. Faecal samples were inverted and washed
into plastic bags which were then transferred into Baermann funnels, resting on the tissue paper. These were then left for 48h at room temperature for larvae to migrate through the tissue paper and collect in the liquid at the base of the funnel. Funnels were kept topped up with water to ensure the tissue paper remained damp. After 48 hours, 100ml samples of the liquid was drained from the base of the funnels and refrigerated at 4°C overnight. The top 50ml from each bottle was siphoned off using a suction water pump, with care taken to avoid shaking the bottle and disturbing the larvae from the bottom. The remaining 50ml contained the larvae. The bottles containing the larvae were then inverted and shaken and four 50μl aliquots from each were pipetted onto slides, with the bottle shaken between each aliquot to ensure an even sub-sample was obtained. Larvae were then killed using 2-3 drops of Lugol's iodine solution and the number of larvae in each 50μl aliquot counted using a reverse microscope at X 100 magnification. The number of larvae in each 200μl aliquot was then multiplied by the dilution to give the total number of larvae recovered from each 100g of faeces.

3.2.3 Statistical analysis
All results are shown as the arithmetic mean. Mean total number of larvae recovered from urea and water treated faeces was compared using a one-sided T-Test on Minitab 16 (Minitab Inc., Version 6.1., 2016).
3.3 Results
Visual differences were observed in the faeces at the conclusion of the incubation period prior to bearmanisation. Faeces sprayed with water developed a white fungus, which was absent from the urea treated faeces (Figure 2). The mean number of larvae recovered from 100g of faeces topically sprayed with either liquid urea or water is shown in Figure 3. Overall, a total of 800 ± 100 larvae were recovered from faeces sprayed with urea compared with 25,600 ± 828 recovered from faeces sprayed with water (P<0.001) representing a 97% reduction in the number of larvae recovered from urea treated faeces.

Figure 2: Plate A - 100g faeces treated with tap water; Plate B - 100g faeces treated with Flow-Fert N after 10 days incubation

Figure 3: Total number of larvae recovered per 100g of faeces following topical application of 20% concentrated liquid urea and water. Values represent the mean ± s.e.m. of two replicates
3.4 Discussion

Topical application of urea inhibits larval development as seen in the 97% reduction in larval development following urea application. Howell et al (1999) reported similar results in H. contortus larvae that were topically applied with urea at 6-12%, with urea inhibiting 81% of larvae from hatching. These findings support those of Pecson et al (2007) and Fidjeland et al (2015) who reported that Ascaris suum larval development was inhibited when nitrogen, in the form of ammonia, was added to sewage sludge. Pecson et al (2007) stated that ammonification of the nitrogen in the urea created an unfavourable environment for the parasites, restricting them from further development.

Urea, when applied to soil can be transformed to other compounds given the right bacteria are present. Symbiotic bacteria containing the nitrogenase enzyme will transform the nitrogen to ammonia. The ammonia may then undergo nitrification, forming nitrate and nitrates, or may volatilise into ammonia gas. Although faeces were kept in the open air for 18 hours prior to being placed in the incubation room, the plastic bag covering the plates in this experiment may have created a micro-climate that amplified the effects of the urea on the parasites. It is possible this unique micro-climate may have led to increased levels of nitrogen volatilisation, with the plastic bag trapping the noxious ammonia gas in the bag, causing the effects on larval development to be over-estimated. Volatilisation levels are influenced by several environmental factors including temperature, pH and water content. In an enclosed environment, as seen in this experiment, the warm temperatures and moisture content would encourage the rate of nitrogen volatilisation. Furthermore, alkaline soils, with a pH greater than 8, are proven to increase nitrogen hydrolysis.

Whilst the pH of the faeces is unknown, liquid urea has a pH of 8-9, thus may increase the level of volatilisation.

Studies of Ascaris suum have indicated that nitrogen, when coupled with pH, has a stronger effect on parasitic egg development than either of the two factors on their own (Pecson et al, 2007; Fidjeland et al, 2015; Katakam et al, 2014). Liquid urea, with a pH of 8-9, clearly inhibits eggs hatching, however whether this is a nitrogen effect or pH effect is unclear. Previous studies indicate that acidic solutions are most effective at inhibiting larval development (Howell et al, 1999; Goode et al, 1974), however there is a lack of information regarding alkaline solutions. Therefore, it cannot be concluded which component of the urea is inhibiting the eggs, whether this is a nitrogen effect, pH effect or components of the transformed nitrogen that impact on the GINs. Furthermore, it is unclear which stage of the parasite’s lifecycle was disrupted following application of urea.
Chapter 4

pH and egg viability

4.1 Introduction
Chapter 3 indicated a strong effect of urea application on *T. colubriformis* egg hatching. While urea reduced eggs hatching the underlying mechanisms were not able to be determined. Previous studies of both ruminant and human parasites indicate a relationship between pH and nitrogen application on egg hatching and larval development (Pecson *et al.*, 2007; Katakam *et al.*, 2014; Khatun *et al.*, 2013). However, many of the studies focussed on pH primarily report on the acidic range. Somerville and Murphy (1983) reported that at pH less than 6, larval motility of *Haemonchus contortus* is decreased, with no larvae present at pH 2-3. Other studies that support this statement (Khatun *et al.*, 2013; Greer, unpublished) provide little evidence on alkaline pH. Pecson *et al.*, (2007) reported alkalinisation on its own may not be the best method of inhibiting *Ascaris suum* development, however when coupled with nitrogen appears to have significant effect. This requires clarification of whether pH plays a role in the interaction nitrogen in the form of urea has with parasites, or whether the negative effect caused by urea applications is independent of pH.

Thus, this experiment will investigate the effects of pH 3-11 and urea application on *Trichostrongylus colubriformis* egg hatching and consider the link between pH and urea application.

4.2 Materials and methods

4.2.1 Fecal egg counts
Confirmation of the presence of eggs in the faeces was determined using faecal egg counts. Faecal egg counts were carried out 21 days after the animals were infected. Faeces was collected as per section 3.2.1. A 1.7 g sample of faeces was taken and placed into a glass bottle. 7mls of tap water was added and the slurry was homogenized for 20 seconds. 50ml of saturated sodium chloride solution was added to the suspension and homogenized for a further 1 minute. A wide bore glass pipette was used to transfer the solution into each chamber on a McMaster slide and the numbers of eggs were then enumerated under a microscope. 3 slides were counted for each animal; the average was taken and multiplied by 100 to estimate the number of eggs per gram (epg) of fresh faeces.

4.2.2 Egg Hatch Assay
On each measurement day, a 100g sample of fresh faeces that was less than 2 hours old, was combined with 200ml of tap water in a plastic bag and homogenized for 30 seconds using a stomacher (Colworth Stomacher 400). The liquid slurry was filtered through a 150μm pore sieve using pressurized water. The solution was then re-filtered through a 38μm sieve with the material retained on the sieve containing fecal debris and nematode eggs. This process was then repeated to
remove the majority of the fecal debris. The solution containing the nematode eggs was placed into 50mL centrifuge tubes and centrifuged at 2000rpm for 5 minutes. The supernatant was then removed with a vacuum line, leaving sediment at the bottom, containing the remaining faecal debris and eggs. The tubes were shaken to loosen the sediment and 35mls of saturated sodium chloride was added before centrifugation again at 2000rpm for 5 minutes allowing all eggs to float to the surface. The supernatant, which contained the eggs, was collected using a vacuum line and then washed in a 20μm sieved container for 20 minutes with tap water. The cleaned eggs were then collected in water and counted to obtain the concentration of eggs in suspension.

75μl aliquots of the egg suspension were then pipetted into the wells of a 24 multi-well plate, with an average of 60-80 eggs per well. 1ml of each test solution (described below for each assay) was added to the egg suspension. Tap water was used as a control. This was repeated in triplicate for each test solution at each sampling time. The plate was gently agitated to mix the eggs and solutions, which were then incubated at 26°C for 24 hours. Immediately following removal from the incubator further development of eggs was halted with the addition of several drops of Lugol’s iodine into each well. The number of both unhatched eggs and L1 larvae in each well were counted using a reverse microscope at X 100 magnification.

### 4.2.3 pH solutions

Solutions of varying pH were prepared at whole number intervals from pH 4 to pH 11. 200ml of 1% Sodium Acetate was prepared and a pH reading taken with a Thermo Scientific pH probe (Orion 2Star pH Benchtop). Sodium Acetate had a pH of 8.2, thus, 0.125M Sodium Hydroxide was added to increase the pH. Acetic Acid at 0.1% and 4% was used to decrease the pH of the sodium acetate solution, with 100% Acetic Acid used to reach the lower pH’s of 4 and 5. pH solutions were left at room temperature for two hours before being re-read and adjusted to whole numbers if necessary. The pH solutions were then stored at 4°C for 3 days to ensure stability and re-read. Liquid Urea was made up to a 10% concentration (Urea: water), with the pH recorded. Tap water was also used as a control and a pH reading taken. Egg hatch assays were prepared as per section 4.2.2. EHA were performed in triplicate each day, repeated on three separate days.

### 4.2.4 Statistical Analysis

All results, unless stated, are shown as arithmetic means. The proportions of eggs that hatched was analysed by analysis of variance (ANOVA) with Minitab 16 (Minitab Inc., Version 6.1, 2016). Lethal doses (LD) were calculated with GenStat, 16th edition, (GenStat, Version 16.1, Rothamstead, VSN International) using Probit analysis.
4.3 Results
The sensitivity of egg hatching to pH is given in Figure 4. Overall, in sodium acetate the percentage of eggs that hatched was influenced by pH. At pH 4 and 5, 0% ± 0% and 2% ± 0.7% of eggs hatched, respectively. At pH 6, 68% ± 3.7% of eggs hatched whereas at pH 7 or above greater than 90% of eggs hatched, an effect that was not influenced by further increases in pH. For pH, LD50 and LD90 values were observed to be at pH 6.3 ± 0.11 and 4.0 ± 0.20, respectively. At comparable pH to water, egg hatching was not influenced by sodium acetate (P>0.05) but was reduced in urea (P<0.001). The mean percentage of eggs that hatched in water and urea was 90% ± 2.28 and 6% ± 1.76, respectively.

Figure 4: Percentage of Trichostrongylus colubriformis eggs that hatched following immersion for 24h in solutions of varying pH that consisted of Sodium acetate (open circles) urea (open triangles) and tap water (closed squares). Each data point represents the mean ± s.e.m. of three replicates.
4.4 Discussion

Egg hatching was sensitive to pH, with pH less than 6 strongly inhibiting egg hatching, but with no effects seen at pH greater than 7. This agrees with Khatun et al (2013) who found an optimal pH of 6 for *H. contortus* larval development, with larval motility decreasing as pH decreases with no larvae present at pH of 2. Somerville and Murphy (1983) also reported no larvae present at pH 2 with high levels of exsheathment at pH 6, similar to that seen in this experiment. Alkaline pH are ineffective at inhibiting *T. colubriformis* egg hatching. There appears to be limited information to the alkaline effects on GIN survival. However, Pecson et al (2007) and Katakam et al (2014) reported that alkalinisation was ineffective at disturbing the lifecycles of *Ascaris suum*.

Acidic pH was effective at inhibiting egg hatching. Mapes (1969) reported that carbon dioxide levels at acidic pH is the primary effect on egg hatching. At a low pH, carbon dioxide saturation levels are low, which is related to the low percentage of eggs hatching (Mapes, 1969). Without measuring carbon dioxide levels it is difficult to conclude whether or not this was the case here.

Urea application effected eggs hatching irrespective of pH. At a pH of 8, egg hatching was unaffected, however urea, also with a pH of 8 inhibited more than 90% of eggs from hatching, indicating the negative effect is independent of pH. In agreement, Howell et al (1999) showed that diluted nitrogenous fertilisers of between 6-12% inhibited eggs hatching by 81% (urea). Whilst clear there is a relationship between urea and eggs hatching, it is independent of pH thus it may be surmised that the effect is relative to the nitrogen in the urea, or other nitrogen compounds that may be present.
Chapter 5
Liquid Urea

5.1 Introduction
Chapters 3 and 4 show urea effects in hatching and developing. Howell et al. (1999) stated that as nitrogen concentration increases, the larval motility of *H. contortus* decreases, with urea (concentration 6-12%) most effective (81%) at inhibiting larval development. The same results were also seen in experiments on biosolids containing *Ascaris suum*, where the addition of urea inhibited larval development (Pecson et al., 2007; Katakam et al., 2014). Chapter 4 showed that urea at a concentration of 20% inhibited eggs from hatching to less than 10%. This experiment elaborates on these observations of the negative effects urea had on egg hatching and aims to find the optimum concentration at which *Trichostrongylus colubriformis* eggs were prevented from hatching by testing a range of urea concentrations from 1% to 50%.

5.2 Materials and methods
Egg collection and egg hatch assays occurred as per section 3.2.1 and 4.2.2 respectively, with the exception that the egg hatch assays used the following treatments: Urea granules were used to make 50ml solutions of eight concentrations of liquid urea (urea: water), viz., 1%, 2%, 4%, 6%, 8%, 10%, 20% and 50%.

5.2.1 Statistical analysis
All results, unless stated, are shown as arithmetic means. The proportions of eggs that hatched were analysed by analysis of variance (ANOVA) with Minitab 16 with concentration as the factor. Least significant differences were calculated at the 5% level and used to compare hatch percentage. The concentration required to prevent 90% of eggs from hatching (Lethal Dose, LD90) was calculated using Probit Analysis. The proportion hatched at each concentration underwent Receiver Operator Characteristic (ROC) analysis to determine the sensitivity and specificity at each fertiliser concentration (JROCFIT, John Eng., John Hopkins Hospital, 2016). Optimum concentration was determined from the maximum value of sensitivity + 1 - specificity.
5.3 Results
Mean percentage of eggs that hatched relative to urea concentrations are given in Figure 5. Overall, egg hatching was sensitive to urea concentration, with no apparent effect on hatching at 1 or 2% concentration compared with water (0%) (P>0.05). Hatching was then reduced to 23% at 6% (P<0.001) from which point further reductions in egg hatching with increasing urea concentration were not significant (P>0.05). Observed values indicated the LD90 for urea concentration was 19.6% ± 0.20. ROC analysis of the data showed an area under curve of 0.893, indicating successful discrimination between true positives and false negatives. The biological optimum, where sensitivity plus 1 less the specificity, was at a maximum of 5.5% urea concentration.

![Figure 5: Percentage of Trichostrongylus colubriformis eggs that hatched following immersion for 24hrs in solutions of various concentrations of liquid urea. A and B represent the concentration interactions; those with the same letter have similar interactions whilst those with different letters have no interaction.](image)

5.4 Discussion
An increase in urea concentration causes a decrease in the number of T. colubriformis eggs that hatch. This is supported by Howell et al (1999) who reported that as nitrogen concentration increases the larval motility of H. contortus decreases, with the greatest change seen between 6% and 12%. Urea is applied to pastures as a growth promotant at rates of 40kgN/ha to 300kgN/ha. 10% Urea is equivalent to 4.6kgN/ha, well within the common levels of nitrogen applied in New Zealand. At 50% urea concentration, 23kgN/ha would be applied in a field trial, again well within recommended levels in New Zealand. In a practical sense, this enables these concentrations of urea: water to potentially be used in field trials to inhibit egg hatching, without compromising environmental performance. Furthermore, the 50% concentration of urea could not only act to inhibit eggs hatching but also promote pasture growth.
Whilst there is an obvious effect of nitrogen concentration on egg hatching, the causing effect of this is unclear. Rodríguez-Kabana (1987) suggested that ammoniacal nitrogen was the compound toxic to the nematodes, however there is not enough evidence in this experiment to support this. Evidence from Chapter 4 indicates that it is not a pH effect however it could be the presence of nitrogen. This raises the question of whether other nitrogenous fertilisers may have the same negative effect on eggs hatching.
Chapter 6

Different forms of Nitrogen sources

6.1 Introduction
Chapter 5 showed a relationship between urea and eggs hatching with decreased egg hatching as urea concentration increased. Howell et al (1999) tested a number of nitrogenous fertilisers and found ammonium nitrate was the most successful at inhibiting parasite development (97%), compared with urea (81%) and ammonium nitrate and urea in combination (89%). This suggests that rather than nitrogen, it is other nitrogenous compounds formed through nitrogen fixation, ammonification and nitrification that have the larger effects on the parasites. Thus, this experiment looks at a range of common fertilisers that contain not only nitrogen but other elements, at a range of concentrations, and their effects on T. colubriformis eggs.

6.2 Materials and methods
The impact of various forms of nitrogenous fertilisers on egg viability was evaluated using an EHA as described in section 4.2.2. Eggs were collected from faeces as previously described (section 3.2.1) and placed into solutions of one of five nitrogenous fertilisers of varying forms and supply of other elements. These were urea (46-0-0-0); sulphate of ammonia (21-0-0-24); potassium nitrate (13-0-44-0); potato fertiliser (15-10-10-8); and nitrophoska blue (12-5.2-14-0). Solutions of each fertiliser were made to concentrations of 1%, 5%, 10%, 20% and 50% (water: fertiliser).

The electrical conductivity of the 50ml solutions of each concentration of each fertiliser type was measured with a Mettler Toledo electrical conductivity probe at room temperature. The pH of the 50ml solutions was measured with an Orion Two-star pH probe at room temperature. For pH and electrical conductivity readings, care was taken to rinse and de-ionise, respectively, the probes between each solution.

6.2.1 Statistical analysis
All results, unless stated, are shown as arithmetic means. The proportions of eggs that hatched was analysed by analysis of variance (ANOVA) with Minitab 16, with fertiliser type and concentration as factors. Least significant differences were calculated at the 5% level and used to compare hatch percentage. The concentration required to prevent 90% of eggs from hatching (Lethal Dose, LD90) was calculated from the proportion hatched at each concentration using Probit Analysis on GenStat. The proportion hatched at each concentration for each fertiliser type underwent Receiver Operator Characteristic (ROC) analysis to determine the sensitivity and specificity at each fertiliser concentration. Optimum concentration was determined from the maximum value of sensitivity + 1-
specificity. Regression analysis was carried out on Minitab16, using best subsets regression and general regression.

6.3 Results
Mean percentage of eggs hatched for different nitrogenous fertilisers relative to concentration are given in Figure 6. Overall, for egg hatching there was an interaction between fertiliser type and concentration (P<0.001) reflecting an increase in the percentage of eggs that hatched as all fertiliser concentrations decreased, the extent of which varied between fertiliser types. Regardless of fertiliser type, few eggs hatched when fertiliser concentration was 10% or above with all fertiliser at these concentrations having less than 6% of worms hatch. At fertiliser concentrations of 5% and 1% greater variability between fertiliser types was observed, with the hatching percentage of all fertilisers except sulphate of ammonia increasing as fertiliser concentration decreased, with maximum hatching at 1% observed to be 84% ± 3.46, 83% ± 2.59, 48% ± 2.45, and 84% ± 2.31 for urea, potato fertiliser, potassium nitrate and nitrophoska blue, respectively. By comparison, the hatching percentage for sulphate of ammonia showed no further increase as the concentration decreased from 5% to 1%, viz, 20% ± 0.27 and 24% ± 1.02, respectively. The fertiliser concentration required to prevent 90% of eggs hatching (LD90) was 11.2% ± 0.0097, 11.6% ± 0.032, 10.3% ± 0.0095, 10.3% ± 0.021 and 17.4% ± 0.017 for urea, sulphate of ammonia, potato fertiliser, potassium nitrate and nitrophoska blue, respectively. Receiver operator characteristic analysis indicated successful discrimination between true positives and false negatives with area under the curve (AUC) of 0.9364, 0.899, 0.9361, 0.9309, 0.9086 and the optimum concentration, as determined by the maximum value of sensitivity plus 1 less the specificity, being 6.67%, 3.70%, 14.45%, 2.70%, 5.72%, for urea, sulphate of ammonia, potato fertiliser, potassium nitrate and nitrophoska blue, respectively.
Figure 6: Percentage of *Trichostrongylus colubriformis* eggs that hatched after immersion for 24hrs in solutions of variable concentrations of urea (46-0-0-0), sulphate of ammonia (21-0-0-24), potato fertiliser (15-10-10-8), potassium nitrate (13-0-44-0) and nitrophoska blue (12-5.2-14-0). A, B, C, D, and E represent the concentration and fertiliser interactions; those with the same letter have similar interactions between concentrations and fertiliser whilst those with different letters have no interaction.

6.3.1 Concentration of nitrogen, phosphorous, potassium and sulphur, electrical conductivity and pH

The effects of each of nitrogen, phosphorous, potassium and sulphur concentration at each concentration of each fertiliser and the percentage of eggs hatched is shown in Figure 7 (A, B, C, D). Overall, nitrogen concentration has a significant effect on the number of eggs hatched (P<0.001) while all other elements were non-significant (P<0.05). The logged value of the electrical conductivity of the five fertilisers at each of the five concentrations is shown in Figure 7 (C). Overall, four of the five fertilisers had similar levels of electrical conductivity, however, all had various percentages of eggs hatched. Urea had a far lower electrical conductivity than all of the other fertilisers but had similar percentages of eggs hatched. Best subset regressions, with all of the individual components assessed (N, P, K, S, electrical conductivity and pH), showed electrical conductivity ($R^2=15.5\%$) and nitrogen ($R^2=12.4\%$) had the most significant effect on eggs hatched. Further regression analysis showed nitrogen had a significant effect on eggs hatched ($y=26.2-222N$, $R^2=12.4\%$, $P=0.047$) as did electrical conductivity ($y=28.6-0.110EC$, $R^2=15.5\%$, $P=0.029$). Regression analysis of natural N values and EC values showed a strong relationship between these variables and eggs hatched ($y=33.5-179N-0.0927EC$, $R^2=22.5\%$, $P=0.023$). Further log analysis of these two variables combined indicated the
relationship \( y = 52.9 - 693 \log_{10} N - 15.9 \log EC \), \( P < 0.005 \) was the strongest with 34.7% of the variation in eggs hatched explained. The other components, \( P \), \( K \) and \( S \) did not have significant effects on eggs hatching \( (P > 0.05) \). The pH of each fertiliser at each concentration and the effects on eggs hatched is shown in Figure 7 (F). Regression analysis showed pH did not have a significant effect on eggs hatched \( (P = 0.898) \).

**Figure 7:** Comparisons of nitrogen, phosphorous, potassium and sulphur concentrations, electrical conductivity and pH and percentage of *Trichostrongylus colubriformis* eggs hatched for each fertiliser at each concentration (1%, 5%, 10%, 20%, 50%). Graphs A-E have been log-transformed. Closed circles – urea; Rhombus – sulphate of ammonia; Triangle – potato fertiliser; Open circles – potassium nitrate; Square – nitrophoska blue. A: Concentrations of nitrogen in each fertiliser and percentage of *Trichostrongylus colubriformis* eggs hatched. \( y = 4.88 \ln(x) - 41.243, R^2 = 0.5588 \). B: Concentrations of phosphorus in each fertiliser and percentage of *Trichostrongylus colubriformis* eggs hatched. Urea, sulphate of ammonia and potassium nitrate contain 0% phosphorous. \( y = 22.27 \ln(x) - 93.676, R^2 = 0.8682 \) C: Concentration of potassium in each fertiliser and percentage of *Trichostrongylus colubriformis* eggs hatched. Urea and sulphate of ammonia contain 0% potassium. \( y = 22.27 \ln(x) - 71.625, R^2 = 0.8682 \). D: Concentration of Sulphur in each fertiliser and percentage of *Trichostrongylus colubriformis* eggs hatched. Urea, potassium nitrate and nitrophoska blue contain 0% Sulphur. \( y = 20.72 \ln(x) - 83.341, R^2 = 0.7366 \). E: Electrical conductivity of each fertiliser and percentage of *Trichostrongylus colubriformis* eggs hatched. F: pH of each fertiliser at each concentration and percentage of *Trichostrongylus colubriformis* eggs hatched.
6.4 Discussion

Nitrogenous fertilisers effect eggs hatching regardless of fertiliser type. There is a lack of supporting research, as many studies are carried out on larvae, with few studies investigating the effects of fertiliser on GiN parasites. Sulphate of ammonia was the most successful fertiliser at inhibiting egg hatching, even at low concentrations of 1% and 5%. This is supported by Goode et al (1974) who reported ammonium nitrate (60% concentration) caused a 65% decrease in larval survival when topically applied to pastures. Furthermore, a review by Rodriguez-Kabana (1987) suggested that ammonia, a compound present following ammonification of nitrogen from urea, is the compound toxic to parasites. A strong correlation between nitrogen content and eggs hatched existed. Studies have shown that nitrogen compounds, especially ammonia and nitrate are more successful at inhibiting egg hatching than straight urea (Goode et al, 1974; Gonzalez et al, 2010; Gates, 1987). Gonzalez et al (2010) reported that the ammonia created by the nitrogen in the fertiliser promotes larval exsheathment, resulting in direct larval exposure to the surrounding environment and kills the larvae. Whilst this study was carried out in the laboratory, it is possible that the bacteria containing the nitrogenase enzyme required for ammonification may not be present, however, given eggs were washed and contained within faecal debris, it is plausible that these bacterial populations were indeed present. Sulphate of ammonia, at 1% and 5% had less than 25% of eggs hatched, thus it could be assumed that the presence of ammonia was inhibiting the eggs from hatching. Furthermore, potassium nitrate at 1% and 5% had similarly low percentages of eggs hatching that could be attributed to the nitrate compounds in the solution. Due to the differences in egg hatching at each concentration between the fertilisers (Figure 6) it could be speculated that different components of each fertiliser are working differently on the eggs.

Phosphorous, potassium, sulphur levels and pH had no effect on the eggs hatching however electrical conductivity and nitrogen were directly correlated with the percentage of eggs hatched. It could be speculated that electrical conductivity and the osmotic potential of the fertilisers caused the eggs to be inhibited however due to urea having far lower levels of electrical conductivity than the other fertilisers, but similar levels of egg hatching, this may not be true. Urea does not dissociate like the other fertilisers as it does not contain cations or anions. Thus, for urea it could be the nitrogen that is the causing factor whilst osmotic potential, caused by the presence of these cations and anions may be inhibiting the eggs from hatching in the other fertilisers. However, if electrical conductivity was the influential factor, at each concentration it would be expected that percentages of eggs hatched would be the same across all fertilisers however this was not the case, with a large amount of variability seen between fertilisers. Potential for further research involves understanding the osmotic potential of the fertilisers and whether it is a salt effect, caused by the cations and anions, causing the eggs to become dehydrated thus increasing mortality rates.
Chapter 7
Nematocidal or Nematostatic

7.1 Introduction
The previous experiments have indicated a clear effect of nitrogenous fertilisers on egg hatching. If the effects seen are temporary then there is limited application of this study to the field. However, should the effects be nematocidal and irreversible there would be potential for development into use on large-scale commercial farms. This experiment aims to explain whether the effects caused by these fertilisers are reversible.

7.2 Materials and methods
The toxic activity of the fertiliser solutions was assessed following incubation and washing of eggs and subsequent hatching. Solutions of urea, sulphate of ammonia, potassium nitrate, and potato fertiliser at 20% concentration were prepared as per Chapter 6. Egg hatch assays were prepared as per section 4.2.2 but were not added to 24 well plates. Instead, plastic tubes (2cm) were inserted into each well. Fastened over the end of each tube with an adjustable hose clamp was a 25μm precision woven nylon mesh. The eggs were pipetted onto the mesh and suspended into 24-well plates, containing 1ml of the 20% concentration of the nitrogenous fertiliser. They were then incubated for 24 hours at 26°C. The tubes were removed and inverted and eggs were washed into sterile 24well plates containing 1ml of water and were then incubated for a further 24 hours. Following removal from the incubator all the unhatched eggs and L1 larvae in each well of both plates were counted using a reverse microscope at X 100 magnification.

7.2.1 Statistical analysis
All results, unless stated, are shown as the arithmetic means. This data was analysed on Excel. No formal statistical comparisons were made.

7.3 Results
All fertilisers completely inhibited eggs from hatching. No larvae were observed in any treatment. By comparison, the control had 94% of eggs hatched.
7.4 Discussion

Fertilisers above the optimum concentration have a strong negative effect on the hatching of parasitic eggs. Fertiliser treatments on egg hatching appear to be non-reversible and on the surface seem to be nematocidal. However, visual observations of the eggs may be able to offer some clue as to the mechanisms that may be in play. While hatching was irreversible, larval development inside the egg was still evident, possibly indicating that rather than a toxic effect, the compounds may alter the physical properties of the egg shell. Further exploration of this phenomenon and the effect of nitrogen on physio-chemical properties of the eggs could be an avenue of further research.

Stimulation of egg hatching is triggered by three things – increased levels of trehalose; increased cell permeability, caused by the trehalose; and action of biochemical both inside and outside the egg (Perry, 1989; Rogers and Brooks, 1977; Gates, 1987). Figure 8 shows the varied development of larvae within the eggs, following treatments of water (A), fertiliser (B) and solution of pH 4 (C). This questions the phenomenon of why these effects are irreversible and whether it is associated with egg permeability or the biochemical properties of the fertilisers.

![Figure 8: Photographs of eggs following treatments of water (A), fertiliser (B) and solution of pH 4 (C)](image)

There is little information available regarding the length of time of exposure of GINs to fertilisers for the effects to be irreversible. This experiment was carried out over 24 hours and provides an opportunity to further test the efficacy of the fertilisers, and whether exposure can be effective through topical application in the field. Furthermore, further avenues of potential research could involve investigating the length of time the parasite be exposed to the solution for results to become irreversible and whether topical application is sufficient or soaking is required to see the desired levels of inhibition.
Nitrogenous fertilisers have an effect on *Trichostrongylus colubriformis* eggs, irrespective of pH. As fertiliser concentration increased, the percentage of eggs hatched decreased with a strong interaction seen between the two. Previous studies have indicated a relationship between urea applications and larval development (Goode *et al.*, 1974; Gonzalez *et al.*, 2010; Gates, 1987; Howell *et al.*, 1999), however there has been a lack of research regarding the effects on eggs hatching. Chapters 5 and 6 clearly support the notion that nitrogenous fertilisers negatively impact on eggs hatching as fertiliser concentrations increase, an effect which is most likely caused by the presence of nitrogen, with 34.7% of the variation in the results explained by nitrogen. However, evidence is lacking into the underlying mechanisms of nitrogen that causes the eggs to be inhibited. Sulphate of ammonia and potassium nitrate, with ammonia and nitrate molecules present, had higher levels of egg inhibition thus, it could be assumed that these compounds are more toxic to the parasites than straight nitrogen. Given that these experiments were carried out *in vitro*, it could be assumed that the bacteria required for the nitrogen in the urea, potato fertiliser and nitrophoska blue fertilisers to be converted to ammonia would not be present. Therefore, it could be that whilst not as toxic to parasites as other nitrogenous compounds, nitrogen itself provides unfavourable conditions for development and hatching of the egg, as these fertilisers still inhibited egg hatching, especially as concentration increased.

Fertiliser treatments appear to be irreversible however Figure 8 shows the varied levels of development within eggs following applications of water, fertiliser and a solution of pH 4. This makes it unclear how the *T. colubriformis* eggs are inhibited and how the lifecycle of the parasite is disrupted. Perry (1989) reported that as eggs developed, the shell of the egg became more permeable, allowing more biochemicals to cross the biological membrane. If this was the case it could be assumed that in these experiments, the greater concentrations of fertiliser cross through the shell of the egg and disrupt the lifecycle and development of the parasite. Furthermore, it could be speculated that the lower concentrations of fertiliser are not toxic enough to the parasites to effect development and function. Clearly this is an area that requires further investigation.

Whilst nitrogen had the most significant effect on the eggs in Chapter 6, electrical conductivity also inhibited egg hatching. Due to the lack of dissociation of cations and anions, urea had far lower electrical conductivity than the other four fertilisers all of which had relatively similar levels. The osmotic potential of these fertilisers, caused by the dissociation of cations and anions, require further research. Electrical conductivity can be associated with levels of salinity. These high levels of
electrical conductivity and high levels of salinity may be crossing the biological membrane of the eggs, as previously discussed and dehydrating the egg, preventing further development. Sodium chloride (NaCl) is a common salt used to preserve food, and has chemical properties that inhibit the development of bacteria and fungi. It could be assumed that the salinity of the fertilisers may be similar to sodium chloride and may also act in the same way on parasites. This provides further avenues of research into the potential of other salts such as potassium chloride (also a common fertiliser sold by Ravensdown) and its effects on GINs, and whether these are similar to the effects of the nitrogenous fertilisers in this study. Furthermore, this may conclude whether electrical conductivity or nitrogen is more effective to inhibit parasitic egg hatching.

Conclusion
Gastrointestinal is an ongoing problem facing pastoral farmers, with anthelmintic administration no longer a sufficient control method. Breaking the lifecycles of GINs to decrease the chances of animals contracting the parasites, through control of the parasite outside of the host appears to be the best way forward to decrease the reliance on chemotherapy. The potential anthelmintic properties of nitrogen-containing fertilisers that have been examined and described in this dissertation suggest that the incorporation of nitrogenous fertilisers into parasite management schemes may be a potential alternative to chemotherapy in the animal. There is strong evidence to suggest that nitrogen is toxic to parasites and has a strong effect on both egg hatching and larval development. Investigation of the toxic components of the fertiliser is required, whether the same results are seen in various larval stages and species of GINs, alongside field studies and whether small-scale fertiliser applications can be made practical for large-scale commercial farms.
References


