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The Ability of Nitrogen Fertiliser to Break the Lifecycle of Gastro-intestinal Nematodes

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A Dissertation
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Jack Samuel Bennett

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

The Ability of Nitrogen Fertilisers to Break the Lifecycle of Gastro-intestinal Nematodes

by

Jack Samuel Bennett

Current gastrointestinal nematode (GIN) control relies heavily on chemical anthelmintic, targeting nematodes within the host ruminant. Most of the lifecycle occurs outside of the host so targeting the free-living population may be a successful approach to parasite control. Nitrogenous fertiliser application has been shown to reduce *T.colubriformis* egg hatch and larval development with evidence to suggest the effects may be universal, but little research has been conducted beyond the sheep host in ruminants. Further, the mechanisms through which this phenomenon occurs are not known, an understanding of which may assist in refining potential use of this approach to break the lifecycle of parasitic nematodes.

This series of experiments investigated the universality of urea treatment on egg hatch and larval development across several ruminant hosts. Hatching of deer and horse nematodes was reduced from 94% at 0% urea to less than 16% in 20% urea ($P<0.001$). Recovery of larvae in a development assay was reduced by up to 90, 100 and 94% following the topical application of liquid urea for deer, horse and *Nematodirus* parasite species, respectively, although there was variation between species regarding the amount required to achieve the desired effect. Despite apparent species variation in the sensitivity to urea, the inhibition of egg hatching and reductions in larval recovery appear to be universal across the ruminant GINs examined here.

Studies into the mechanisms included the time of exposure to urea, reversibility of the effects and the importance of osmolality. Although larvae still appeared to develop normally within the egg, the exposure time required for 50% of eggs to remain unhatched when exposed to a 10% urea solution for *Trichostronglyus colubriformis* was 7.5 ± 0.9 hours. Further, this effect was irreversable with no further hatching once eggs were washed and incubated in water for a further 168 hours.

It was postulated that osmolality may be a driver behind hatch inhibition instead of toxicity to components within urea fertliser. Solutions of urea, salt and glucose prepared to 1500 ± 50 mmol/kg (equivalent to 10% urea solution) reduced hatch by 90, 100 and 100%, respectively ($P<0.001$), suggesting the mechanism of inhibition of egg hatching may be associated with osmolality, rather than a direct toxic effect of urea. Although further studies are required to determine the suitability of this approach in the field, the results of this series of experiments suggests the application of urea or substances with a similar osmolality have the potenital to assist in breaking the parasite lifecycle through interrupting larval development outside the host.

Keywords: Urea, gastrointestinal nematodes, hatch percentage, larval development, Lethal Time, irreversibility, osmolality, hatch inhibition, *Trichostrongylis.colubriformis*,

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Chapter 1

Introduction

Gastrointestinal nematode (GIN) parasitism is a significant issue in global ruminant agriculture due to its negative impact on animal productivity, it's also increasingly harder to control. In New Zealand, the loss of production and cost of control associated with GIN parasitism is estimated to cost upwards of $300 million NZD per annum (Mcleod *et* al., 1995; Beef and Lamb NZ, 2017).

Traditional control relies on the administration of chemical anthelmintic however resistance is becoming a major problem globally. In New Zealand, there is high levels of resistance (>50%) developing against broad spectrum anthelmintic families (Leathwick *et al.,* 2000; Leathwick *et al*., 2001; McKenna, 1995) by various gastrointestinal nematode (GIN) species. Chemotherapy alone is now viewed as an unsustainable control method (Greer, 2005). As drench efficacy decreases and demand of the high-value consumer changes to favour more natural production methods, there has been increased investigation of alternative control methods.

Few studies specific to GIN parasites exist, but evidence suggests the application of nitrogenous fertilisers inhibit egg hatch and larval survival of sheep nematodes (Cameron and Gibbs, 1966; Goode *et al*., 1974a; Goode *et al*., 1974b; Howell *et al*., 1999; Cairns *et al.,* 2017). No research into the effect of N-fertiliser on GIN species of other ruminants was found, prompting the investigation of urea exposure and topical application on parasitic species of deer and horses, and members of the *Nematodirus* species. Previous studies indicated the ability of liquid urea to inhibit larval development and egg hatch (Cairns *et al*., 2017) but little is known about the mechanisms causing the effect. To be commercially viable control option the effect must be permanent. The observation of embryonation in unhatched eggs (Cairns *et al*., 2017) suggested hatch may be delayed instead of inhibited, and the effect was not one of toxicity but instead influenced egg hatch mechanisms, early research pointed to an osmolality effect (Wilson, 1958; Croll, 1974). The length of exposure time to the treatment is also unknown. This lead to the investigation of the required time of exposure to urea, hatch reversibility and effect osmolality had on *Trichostrongylus colubriformis* eggs.

This dissertation explores whether a urea treatment is universal across a range of GIN species found throughout New Zealand and investigates the mechanisms causing the effect on nematode hatch and larval development. A combination of egg hatch and larval development assays using various concentrations of urea, host species, exposure times and osmolality solutions have been utilised to determine the aforementioned effects. This research has the potential to offer an alternative parasite control, possibly reducing the rate of drench resistance and reliance on chemical anthelmintic.

Chapter 2

Review of Literature

2.1 Introduction

Gastrointestinal parasitism is a major problem for ruminant production systems throughout the world, with the farmer incurring both production losses and control costs. New Zealand's temperate climate provides many nematode species with ideal conditions to complete their respective lifecycles, which accentuates the problems faced by farmers trying to maximise growth and production as infection can result in significant loss and in severe cases death. Current anthelmintic control is becoming less sustainable with nematode resistance developing rapidly, driving the need for effective alternative control.

Within this review of literature, the cost of infection, the nematode lifecycle, factors affecting free-living larval development and the current and prospective methods of control have been outlined with the purpose of providing a background of the issue that is gastrointestinal parasitism.

2.2 Costs of parasitism

Infestation of parasites in the gastro-intestinal tract of ruminants has long been a problem in global agriculture.

Due to the habitation of infective larvae throughout the pasture profile, grazing ruminants are widely exposed to nematode infection. The relationship between parasite and ruminant host is complex with large variation expected between host phenotype and parasite genotype. Parasitism has been described a nutritional condition (Sutherland and Scott, 2009) highlighting the visual effects of parasitism on a ruminant host. The nature of the relationship is often viewed as open competition due to the parasite trying to overcome the immunity and resistance of its host and the host repelling the infection. For the host this means fighting off the infection and returning to health or facing considerable condition loss, metabolic issues and ultimately death. The parasite must overcome the resistance and immunity of the host, to grow, reproduce and excrete eggs to continue the lifecycle or be exterminated by the host. The compilation of many factors of physiological, genetic and environmental nature result in the favouring of either the host or the parasite to overcome one another with detrimental consequence for the losing party. A major driver for a ruminant's ability to fight off infection is its health and physiological state pre-infection (Kyriazakis *et al.,* 1996; Colditz 2008), its age and immune level (Waller and Thamsborg, 2004; Greer *et al*., 2008) and the level of nutritional available (Stear *et al.,* 1997; van Houtert and Sykes 1996; Sykes and Greer, 2003).

2.2.1 Feed Intake and Utilisation

Infection with nematodes reduces the voluntary feed intake (VFI) of grazing ruminants (Sykes and Greer, 2003), in particular, abomassal parasites (Sykes, 1994). A reduction in appetite due to parasite infection has obvious consequences on live weight, milk yield and wool production in domestic livestock (van Houtert and Sykes, 1996; Forbes *et al.,* 2000; Knox *et al.,* 2006), accounting for up to 90% of the cost of infection (Sutherland and Scott, 2009).

Reducing feed intake restricts the ruminant's intake of essential nutrients and minerals which has severe consequence on productivity and immune response, slowing down the development of resistance toward infection. The reasons for reduced feed intake during infection are not known but have been postulated to be a behavioural response to infection (Kyriazakis *et al.,* 1998) and could assist in recognising infected animals. The reduced intake may be to either allow greater diet selecting or to promote an immune response within the host. Alternately, damage to the gastrointestinal lining caused by internal parasites may induce pain for the host and in response an animal may decrease intake (Morton and Griffiths, 1985). More recently however, corticosteroid induced immune-suppression studies have indicated the reduction in voluntary feed intake may be a consequence of immune signalling (Greer *et al*., 2008). Ultimately, parasite infection is detrimental to animal productivity with VFI reductions driving the losses, however other factors play a role.

2.2.2 Metabolic cost

The metabolic cost is associated with the extent of the damage caused from infection and the energy costs associated with immune responses (Sutherland and Scott, 2009). Nutrients are diverted to repair (Butter *et al.,* 2000) and facilitating an immune response (Parkins and Holmes, 1989). Alterations in the metabolism of protein, carbohydrates, minerals and hormones were observed as a result of *T.colubriformis* infection (Nielsen, 1982). The efficiency of digestion was also impacted due to a rise in rumen pH, as a response or result of *T.colubriformis*, *T.circumcinta* and *H.contortus* infection (Sutherland and Scott, 2009). In particular, protein loss seemed to drive productivity losses. Reductions in the gross efficiency of ME utilisation appear to be dependent on diet quality, ranging from 20% in high protein diets to 50% in low protein diets.

2.2.3 Protein loss

GIT parasite infection results in a relative protein deficiency (Stear *et al.,* 2003), the extent of which varies depending on the type and severity of infection. Protein loss occurs from the leakage of plasma protein across damaged mucosa (Vaughan *et al.,* 2006), the diversion of protein to enhance the immune system and reparation of damaged epithelial tissue. The gastro-intestinal tract has a

high metabolic cost, which may double during infection due to the repair of damaged tissue (MacRae *et al.,* 1997).

Direct losses of plasma protein also occur into the gut lumen. *H.contortus* is responsible for the loss of 50µl of blood/worm/day equivalent to 20-125 g of protein (Poppi *et al.,* 1986). Similarly, *T.colubriformis* infection can result in endogenous protein losses between 20-125g/day amounting to 10-36% losses of endogenous nitrogen (Greer, 2005). The net effect is one where lost protein needs to be replaced. To prevent live-weight losses in sheep, 50 g/day (Bown *et al.,* 1991) and 17 g/day (Liu *et al.,* 2003) of metabolisable protein above maintenance was required, largely reflecting diversion of up to 30% of amino acids, meant for metabolism of periphery tissue, to use in GIT repairs in parasitized animals (Yu *et al*., 2000). Similarly, Jones and Symons (1982) reported an increase in the daily synthesis rate of liver synthesised protein from 0.346g.d^{-1} to 0.724g.d^{-1} during infection with *T.colubriformis* in lambs. Consequently, the net effect of infection being one where amino acids are diverted from the carcass to the liver and alimentary tract to replace blood protein and repair the intestinal tract. In conjunction with reduced protein intake, this overall protein deficiency can have some impact on the growth rates of livestock (Sykes, 1994).

2.2.4 Growth and Production

Although death as a consequence of GIN infection is rare, the cost of production can be substantial. Mcleod (1995) estimated the annual cost of sheep GIN was $222 million dollars in 1994 and Meat and Wool NZ (2000) reported a cost of $300M to the New Zealand sheep industry. With increasing drench resistance and similar meat output, it seems reasonable to assume the cost to the industry remains considerable.

Failure to control parasite populations reduces livestock growth rate, which is an important factor for the profitability of lamb and beef producer. Kyriazakis *et al.* (1994) recorded parasitized lambs taking 25.1 days longer to reach kill weights than uninfected lambs while on an ad.lib pasture diet. Sutherland *et al.* (2010) also reported 10% reductions in lamb growth rates when animals were treated with a 60% effective drench vs a drench with 98% efficacy, taking 17 days longer to reach target weights. Similarly, Macchi *et al.* (2001) found lamb live weights to differ by 1.4 kg when treated with an effective and ineffective anthelmintic. The reduced animal growth rates and resultant delays in the sale of product caused by a failure to control parasites is a major cause of lost productivity on-farm, while also increasing dag score and flystrike susceptibility, reducing wool yield and reducing body condition culminating in 14% losses in production (Sutherland and Scott, 2009). The high cost associated with failure to control parasite populations means that effective control regimes are critical to improve animal productivity which requires an understanding of the parasite lifecycle.

2.3 Gastrointestinal Parasites of New Zealand Ruminants

The nematode parasites of most importance to New Zealand pastoral agriculture belong in the super families of Thrichostrogyloidea and Strongyloidea (Pomroy, 1997; Balic *et al.,* 2000) and are outlined below.

2.3.1 Sheep

Small Intestine; *Nematodirus filicollis*, *Nematodirus spathiger*, *Trichostrongylus colubriformis* and *Trichostrogylus vitrines*.
Abomasum; *Haemonchus contortus*, *Ostertagia* (*Teladorsagia*) *circumcinta* and *Trichostrongylus axei* (Vlassof, 1994; Pomroy, 1997).

2.3.2 Cattle

Abomasum; *Trichostrongylus axei*, *Ostertagia ostertagi*.
Small intestine; *Cooperia* spp.
Lungs; *Dictyocaulus viviparous* (Pomroy, 1997; Charleston and Mckenna, 2002).

2.3.3 Deer

Lungs; *Dictyocaulus viviparous, Dictyocaulus eckerti*.
Small Intestine*; Cooperia* spp, *Nematodirus* spp.
Abomasum; *Haemonchus contortus*, *Ostertagia circumcinta* (Mason and Gladdon, 1983; Pomroy, 1997).

2.3.4 Horse

Cythosomes are the most prevalent nematode, followed by strongiloids, throughout horses in Australia (Bucknell *et al.,* 1995), Poland (Gawor, 1995) and England (Ogbourne, 1976). Of the cythosomes; *Cylicostephanus longiburstatus*, *Cylicocycles nassates*, *Cyathostomum cantinatum* were the most abundant. Common non-cythosomes included; *Trichostrogylus axei*, *Gastrophilus intestinalis*, *Strongylus vulgaris* and *Strongylus edentates* (Mathews, 2014).

2.4 Nematode Life-Cycle

Most of the gastro-intestinal parasites found in New Zealand share a similar direct life cycle (Figure 2.1), with the exception being the *Nematodirus* spp.

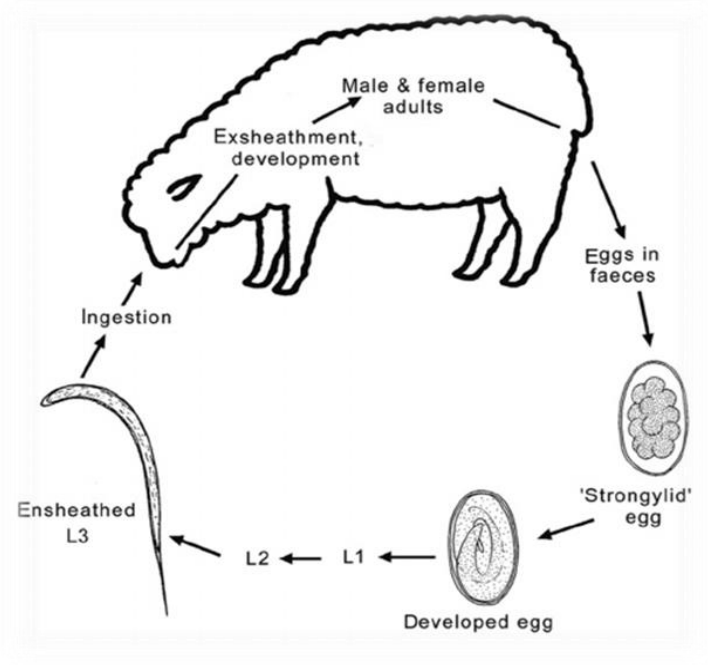


Figure 2.1. Life cycle representing gastrointestinal nematodes (Strongylid) of small ruminants. From Roeber *et al*. (2013).

2.4.1 Egg Hatch

Gastro-intestinal parasite eggs measure 40-110µm, are ovoid in shape and are released onto pasture through faeces. The time taken for hatching into L1 larvae varies according to temperature but typically occurs in 48h (Silverman and Campbell, 1959; Familton and McAnulty, 1997), in colder environments the hatch time may be increased to 40-160h (Silverman and Campbell, 1959; Young *et al.,* 1980) ranging from 1 day (25 °C) to 11.8 days (5 °C) in *T.colubriformis* (Salih and Grainger, 1982). Generally, temperature needs to be in excess of 9 °C, with adequate moisture (Silverman and Campbell, 1959) to permit larval activity and stimulate hatch, which is most successful when the environment is relatively constant (O'Connor *et al.,* 2006; Morgan and van Dijk, 2012). Ideal hatching conditions of GIN eggs have been extensively studied but few have researched the mechanisms involved specifically in egg hatching.

Early research suggested egg shell permeability plays a vital role in the hatching process, assisted by the action of chemicals in the surrounding environment and within the shell (Perry and Clarke, 1981; Perry, 1989; Behm, 1997). Typically, the shell weakens of prior to the larvae forcing itself from the shell (Perry and Clarke, 1981). Three key components have been identified: increased trelahose concentration, increased cell permeability and bio-chemical emulsification of the egg shell.

Behm (1997) observed the importance of trelahose in the circulatory systems of nematodes, suggesting species that can withstand extreme environments had high levels of the sugar, and stated it is important in the egg hatching process. The presence of trelahose is linked with the thinning of the shell and signals the movement of the larvae within, further weakening the egg shell, and increasing permeability reinforcing a positive feedback loop. The egg shell of typical nematodes ranges from 1-5 layers (Wharton, 1980; Bird and McClure, 1976) and is composed of the vitelline, chitin, lipid, uterine and operculum layers (Wharton, 1980). It was first though the egg shell is impermeable to water in its early stages and only becomes permeable after the hatching sequence is initiated (Perry and Clarke, 1981) supported by the fact eggs developed in a variety of hyper and hypo-osmotic solutions as well as toxic solutions (Arthur and Sanborn, 1969). Clarke and Perry (1980) showed that the water content of unstimulated, unhatched juveniles changed with the osmotic pressure of the medium in which the eggs were immersed, and that the effect was reversible. Thus, the egg-shell is permeable to water and acts as a semi-permeable membrane. Larval development is also aerobic, requiring exogenous O_2 (Hinton, 1969) suggesting semi-permeability throughout development. To prevent desiccation and allow sufficient O_2 for larval development the shell must have a low permeability to gas exchange ratio (Wharton, 1980), alternately active transport of O_2 would restrict water loss and facilitate O_2 supply limiting desiccation.

Osmolality of solutions may influence the egg hatch of nematodes. Matthew (1985) studied two species of hook worm (*Ancylostoma ceylanicum* and *A. tubaeforme*) found in cats and concluded that increasing osmotic stress from 0 to 600 mOsm/kg using various salt and sugar solutions caused a delay in hatching time but didn't significantly decrease hatching percentage, no difference in hatch time between treatments and water controls were observed until the osmolality exceeded 300 mOsM/Kg, after which hatch time was extended by 30-40 hours. Wilson (1958) found that low concentrations of NaCl (0.05 mol) delayed hatch without affecting final hatch percentage in *T.retortaeformis*. However, when osmolality was increased to 5-10 moles of sucrose or NaCl solutions, hatch percentage decreased from 95% (control) to below 20% after 36h soaking. Croll (1974) found that *Necator americanus* developed normally in distilled water and in solutions of up to 4% NaCl within 24 h, but larvae only emerged at lower tonicity's, below 1%. With a definite effect on hatching there is potential for further research to determine what osmotic stress level is required to reduce hatch percentage to a practical level for nematode control.

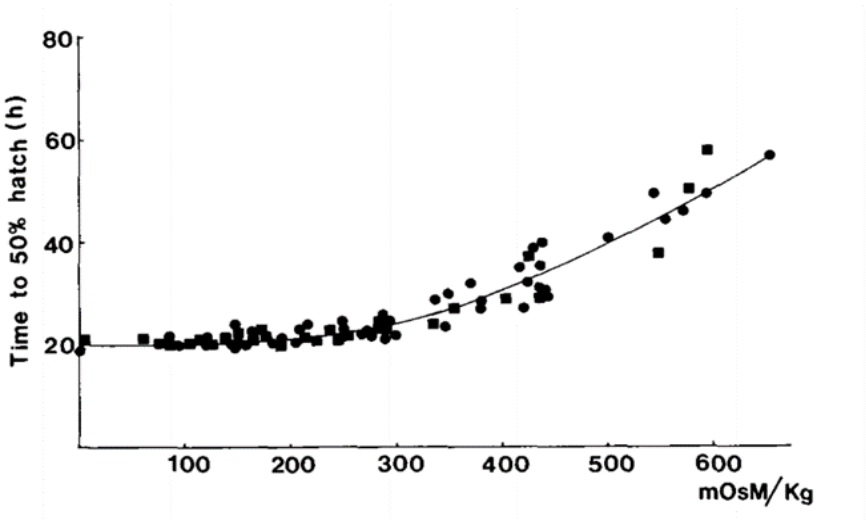


Figure 2.2: Influences of osmotic stress on the rate of hatch of *A.ceyanicum* ● and *A. tubaeforme* ■ eggs at 25°c. Osmotic stress was provided by incubation in 15 different organic salt or sugar solutions. From Mathews (1985).

The presence of enzymes facilitating egg hatch is unclear, as not all studies can confirm whether enzymes found are affecting the hatch process. In *Ascaris* eggs, Fairbairn (1961) and Rogers (1958) noted enzyme damage but Barrett (1976) did not. In *H.contortus* eggs Rogers and Brookes (1977) identified lipase and leucine aminopeptidase, enzymes suspected of deteriorating *T.colubriformis* egg shell. Chitinase enzymes were found within nematode eggs by Bone and Parish (1988), which weren't found within the ruminant host void of infection, suggesting these enzymes are harboured by the nematode and triggered under the correct conditions during the hatching process. The role of enzymes in the hatching process is not clear but internal egg pressure is known to facilitate mechanical hatching. After water is imbibed for *Necator americanis*, *Pratylenchus* spp. and *Trichuris muris* internal pressure increased adding stress to the egg shell. Larvae movement or stylet cutting of the shell weakened it to a point where the internal pressure forced a rupture, completing the hatching process (Croll, 1974; Panesar and Croll, 1981).

2.4.2 Larval Development

L1 larvae develop into infective L3 larvae in 3-5 days under optimal conditions (Veglia, 1915; Hsu and Levine, 1977) with Silverman and Campbell (1959) recording development times of 5, 9 and 15 days under temperatures of 21.7, 14.4 and 11° C respectively, temperature influences larval development as it does in most biological processes. Typical L1 larvae feed on coliform bacteria in the animal faeces and moult into L2 larvae within 3-5 days. The free-living L2 continue to feed on micro-organisms and develop within the faeces. The second moult is incomplete as the L3 larvae retain the cuticle of their previous stage, which ensheathes the individual and provides more protection then the sub-infective forms, but also prevents feeding. The L3 larvae migrate from faecal pats and up the pasture sward, becoming available for ingestion by a host species (Hsu and Levine, 1977; Familton

and McAnulty, 1997). Larval activity and development can cease in undesirable conditions, i.e. low temperatures but L3 larvae can remain on pasture for up to 12 months (Familton and McAnulty, 1997; O'Connor *et al.,* 2006) before dying allowing considerable time to be ingested and restarting the life cycle. Under optimal conditions of moisture and temperature, larvae can reach the infective stage in 5–6 days (Stromberg, 1997).

Nematodirus larval development is a unique process, where the development of the embryo to L3 larvae occurs within the egg shell detailed below (Section 2.4.3).

2.4.3 Nematodirus

The free-living development of *Nematodirus* to the L3 stage occurs entirely within the egg shell (Kates, 1950; Michel, 1969) protecting the susceptible developmental stages from environmental conditions. The species of most significance to New Zealand are *N.spathiger* and *N.filicollis* in sheep (Vlassof and McKenna, 1994). At 21° C, following egg recovery, *N.filicollis* reaches the first larval stage in 8-9 day. The first sheath becomes visible after 11 days and begins partially moulting in 16 days, becoming L2 larvae (Thomas, 1959). After 24-27 days incubation pre-parasitic development is complete the second moult begins, in comparison pre-parasitic development of *N.spathiger* is completed in 18-22 days at 21° C (Kates and Turner, 1955). The rate of development increased with temperature, up to 28° C, after which the success of development to L3 declined to less than 2% at 36° C (Thomas, 1959). Reducing temperature of incubation to 15° C, extended development to L3 to 40-45 days, the entire process occurs within the shell.

To stimulate development and hatching *N.filicollis* and *N.spathiger* required a chilling period of 800-1000 cold units (Oliver *et al.,* 2016) which can be longer than a winter season in many parts of NZ (Vlassof and McKenna, 1994; Oliver *et al.,* 2016), but effectively restricts *Nematodirus* spp. to the areas of Canterbury, Otago and Southland (Brunsden, 1967; Brunsden *et al.,* 1975). In fact, larvae of *N.filicollis* can survive temperatures of -64 to +74° C with repeated freezing and thawing to -6.5° C having little effect (Fallis, 1938; Poole, 1956).

Nematodirus infection in New Zealand is only evident in young lambs (Vlassof, 1973; Vlassof and McKenna, 1994) with contamination building up from deposition of eggs from the previous year's lamb crop. *Nematodirus* infection is likely to occur in spring (Zawadowsky and Zvjaguintzev, 1933; Brunsden, 1967; Brunsden *et al.,* 1975; Vlassof and McKenna, 1994) due to eggs over-wintering on pasture which provides the chilling requirement for hatching, then hatching occur synchronously during optimal development and survival conditions provided in spring (Morgan, 2008). In acute cases of *Nematodirus* infection, the onset of disease occurs suddenly, and death can follow within two days (Brunsdon, 1967).

2.4.4 General Lifecyle

A complete lifecycle of a GIN can be completed in as little as 4 weeks, under optimal environmental conditions. Two days after infection L3 larvae are established in the gastrointestinal tract, after 6-8 days post-ingestion the third moult occurs (Douvres, 1957), at this point the retained cuticle of the L2 stage is exsheathed, triggered by local changes in CO_2 Level, temperature and pH (Familton and McAnulty, 1997). Larval exsheatment typically occurs after ingestion by the ruminant, as the larvae passes through the rumen in trichostrongylid species (Sommerville, 1954; 1957). The CO_2: carbonic acid ratio, pH and the oxidation reduction potential are all involved in the exsheathing process (Lapage, 1935; Rogers and Sommerville, 1960; Taylor and Whitlock, 1960; Slocombe and Whitlock, 1970). Douvres (1957) recorded early L4's may be recovered 5 days after ingestion. Around 6 to 10 days post-infection the 4^{th} and final moult occurs (Douvres, 1957; Familton and McAnulty, 1997). CO_2 gas and organic acids such as H_2CO_3 are essential for the change from infective stages to parasitic stages of the nematode (Petronijevic *et al.,* 1985; Petronijevic *et al.,* 1986). In another 7 to 10 days full maturity is reached, adults breed, eggs are laid, and the fertile eggs are excreted onto pasture within faeces. The typical time from L3 ingestion to egg laying is 21 days (Familton and McAnulty, 1997). The remainder of the lifecycle occurs on pasture, in what is described as the 'free living stage' where eggs develop into L3 larvae which are ingested by ruminants.

2.5 Larval Distribution

Once developed to the L3 stage, the protective sheath makes larvae less susceptible to environmental factors and desiccation. The increased protection allows the L3 individuals to migrate through a pasture sward. Migration can be an active or passive process (Crofton, 1954; Grønvold and Høgh-Schmidt, 1989). Raindrop splash facilitates passive migration, distributing larvae up to 30cm from faecal pats (Familton and McAnulty, 1997). Active migration is dependent on the presence of a water film, with veined leaves providing channels for vertical migration (Crofton, 1954), the extent of which is also dependent on the ambient temperature and humidity. The majority of L3 larvae are horizontally distributed within 30cm of a faecal pat, and numbers decrease further from the source (Grønvold and Høgh-Schmidt, 1989; Familton and McAnulty, 1997; Almeida *et al.,* 2005). 80% of the L3 population is vertically distributed in the bottom 5cm of the sward, with the majority (50%) in the bottom 2cm of sward and top 1cm of soil with few individuals in buried faeces (Levine, 1963; Rose and Small, 1985; Familton and McAnulty, 1997; Pegoraro *et al.,* 2008; Gazda *et al.,* 2009).

2.6 Factors Effecting larval survival

Upon pasture contamination, the external environment determines the survivability of eggs and larvae. It is estimated that 90% of the parasite population is found outside of the host, but only 1-17% of the eggs expelled on pasture will reach the infective stage (Familton and McAnulty, 1997).

Temperature, oxygen and moisture level are the key determinants effecting the survival of the free-living nematodes in New Zealand. Due to the lack of climatic extremes and relatively small variance in climate throughout New Zealand a large variety of parasites can survive and develop outside of the host, regardless of what region they are found (Brunsdon, 1970).

Once the infective stage is reached, the influences of temperature and moisture on survival are less important (O'Connor *et al.,* 2006). The retention of the L2 larvae sheath results in high resistance to extremes in temperature and desiccation meaning that the survival time and therefore infective stage of nematode larvae is extended. The long survival is arguably the greatest factor contributing to the difficulty to control GIN's (Hsu and Levine, 1977, O'Connor *et al.,* 2006). The duration of the L3 survival is influenced by climatic factors (Vlassoff, 1982; Familton and McAnulty, 1994) as well as sward height (Rose and Small, 1984) and micro/macro climates (O'Connor *et al.,* 2006).

Table 2.1. Key differentiating features of environmental influences on the free-living stages of the major *Trichostrongylid* nematode parasites of sheep. From O'Connor *et al.* (2006).

Nematode Species	Stage of lifecycle			
	Unembryonated egg	Embryonated egg	Pre-infective larvae	Infective larvae
H. contortus	Highly susceptible to cold and desiccation. High mortality below 10°C.	Susceptible to cold and desiccation. Negligible hatching below 10°C. Low hatching rates in absence of moisture.	Highly susceptible to cold and desiccation.	Optimum survival in warm, moist weather. Poor survival in cool or warm dry weather and sub-freezing winters.
T.colubriformis	Intermediate susceptibility to cold and desiccation. High mortality below 5°C.	Intermediate susceptibility to cold. Low susceptibility to desiccation.	Susceptible to cold. High mortality below 5°C. Susceptible to desiccation.	Optimum survival in cool or warm moist weather. Poor survival over sub-freezing winters.
T. circumcincta	Low susceptibility to cold. High egg viability at 0-10°C. Intermediate susceptibility to desiccation	Low susceptibility to cold and desiccation. Hatching below 5°C.	Intermediate susceptibility to cold. Susceptible to desiccation.	Optimum survival in cool, moist weather and sub-freezing winters. Poor survival in warm, dry weather.

2.6.1 Temperature

Different climates have caused evolutionary differences between nematode species found throughout the world. *T. circumcincta* can develop at lower temperatures than *T.colubriformis* (Leathwick *et al.,* 1999), while both species develop at lower temperature than *H. contortus* (Gibson and Everett, 1972; Gordon, 1973; McKenna, 1998). Even with different species having environmental niches the optimal temperature range for GIN development is typically between 15° C and 30° C (Vlassoff, 1982), and will occur between 4° C and 35° C provided other conditions are met (Vlassof, 1982). In most New Zealand regions average daily temperatures exceeds 4° C, excluding the southernmost regions during winter for short periods of time, and the daily temperature rarely exceeds 35° C (MetService NZ, 2017), providing the ideal climate for larval survival.

Stromberg (1997) proposed that temperature rise encouraged larval development to a point and when exceeded, desiccation affects the development process, highlighting that development is dependent on both temperature and moisture.

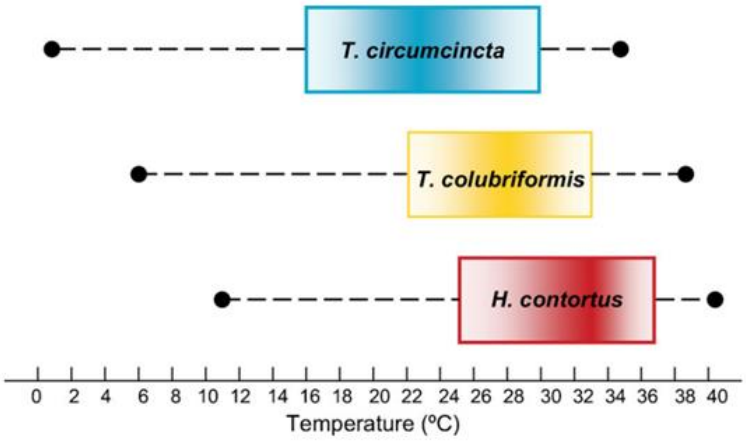


Figure 2.3. Temperature range for development of major Trichostrongylid species from unembryonated eggs to L3. Optimum temperature indicated with box, colour intensity indicting most optimum. Dashed lines extend to upper and lower limits for development. From O'Connor *et al.* (2006).

2.6.2 Moisture

Water is essential for the development of L1 and L2 larvae, which have been described as aquatic because they require a moisture film to migrate throughout the pasture sward or faecal pat (Crofton, 1954; Familton and McAnulty, 1994; Stromberg, 1997). A lack of moisture facilitates the desiccation of eggs and larvae when they are in the free-living stage, susceptibility to desiccation increases as the free-living nematode develops (Embryonated egg>pre-parasitic larvae>infective larvae) (O'Connor *et al*., 2006). In dry environments, a crust can form on faecal pats reducing the rate of moisture loss, allowing for a warm and moist environment within the pat, ideal for larval development.

During rainfall or irrigation events moisture breaks the pat crust releasing larvae onto the sward. Several studies have shown a relationship between moisture and larval development, with the time taken for precipitation to exceed evaporation affecting the rate of development from embryonic to infective larvae (Barger *et al.,* 1972; Bullick and Anderson, 1978). Moisture within faecal matter and the pasture sward has a direct impact on the survival of egg and larvae during the free-living development stage. On well-watered New Zealand pastures evaporation rates varied between 0.90 – 4.26 ml/day depending on the season (Scotter and Kelliher, 2004), calculated using estimates in Table 2.2. The high summer evaporation rates throughout New Zealand and quick desiccation of faeces negatively impacts nematode larvae and eggs within faeces and living in the pasture sward.

Table 2.2. Estimated average monthly and yearly evaporation from well-watered NZ pastures. From Scotter and Kelliher, (2004).

Site	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Year
Kaitaia	133	109	97	67	49	36	39	47	63	86	101	125	951
Rotorua	133	109	93	62	41	29	32	43	60	87	107	125	918
Tauranga	141	116	101	69	46	35	40	47	66	92	113	132	999
Napier	142	112	96	63	43	31	32	43	66	97	119	136	978
P.North	123	103	86	55	35	24	25	37	53	77	97	116	831
Blenheim	158	130	108	73	47	35	36	48	73	103	125	146	1080
Hokitika	101	84	70	45	32	27	28	38	48	67	86	101	727
Lincoln	133	107	85	53	34	25	25	36	57	90	109	128	881
Alexandra	131	104	81	43	21	11	10	23	50	84	110	129	795
Invercargill	104	85	69	45	33	22	23	35	52	75	90	107	741

2.6.3 Oxygen

Oxygen is necessary for the development of parasitic eggs (Familton and McAnulty, 1994). Grønvold (1989) found the proportion of eggs that developed near the surface of faecal pats was greater and quicker than in the centre. Further weathering and microbial/insect activity aerates the remainder of the faeces, facilitating further egg development, providing temperature and moisture aren't limiting. It assumed little development occurs in waterlogged soils and faeces due to anaerobic conditions but in laboratory experiments nematode eggs develop in water indicating a level of tolerance to anaerobic conditions. This occurrence allows the postulation that oxygen may be a secondary environmental driver of egg development, and if there is an adequate level, eggs and larvae will continue to develop. Upon stimulation of a dormant egg, the oxygen requirement increases (Passey and Fairbairn, 1955) which may be due to the increased requirement of oxygen during the utilisation of ATP as the larvae develops.

2.7 Nematode Control

Anthelmintics have been the traditional method for controlling worm burden in grazing livestock however anthelmintic resistance is becoming a major problem in livestock production systems. Over-use and ineffective drenching regimes have resulted in high levels of resistance (>50%) developing against broad spectrum anthelmintic families (McKenna, 1995; Waller, 1997; Leathwick *et al.,* 2000) specifically benzimidazole (Oliver *et al.,* 2016) by various nematode species. In horses, benzimidazole resistance in *Cyathosomes* is widespread globally, with increasing resistance to Pyrantel and Ivermectin (Mathews, 2014). The sole use of anthelmintic to interrupt the parasite lifecycle and reduce production losses has now become an unsatisfactory control method (Greer, 2005), but parasitism remains a risk to ruminant production (Brunsdon *et al.,* 1983).

2.8 Alternatives to Anthelmintics

Due to much of the nematode population developing outside of the host there is scope for control of parasites by disrupting the lifecycle during the free-living stage. If successful, the rate and reliance on modern anthelmintic drenches will be reduced. Current control strategies are 'protective' and need to be 'reductive' toward the parasite population to minimise animal production effects. Strategies to do so must prevent the build-up of dangerous numbers of larvae on pastures and remove susceptible animals from heavily contaminated pastures before these levels are reached (Brunsdon, 1980). The use of alternative plant species high in proteins (Kyriazakis *et al.,* 1994; Hutchings *et al.,* 2000) or containing plant secondary metabolites such as tannins (Athanasiadou *et al.,* 2000a; Athanasiadou *et al.,* 2000b; Athanasiadou *et al.,* 2001; Butter *et al.,* 2000; Villalba *et al.,* 2010) are known to reduce worm burdens in grazing livestock without the need for traditional anthelmintic. Farm management strategies such as alternative grazing between ruminant host species also reduced parasite burden, seen in New Zealand (Moss *et al.,* 1998), Australia (Southcott and Barger, 1975), Scotland (Bairden *et al.,* 1975) and other countries (Rocha *et al.,* 2008). Incorporating animal nutrition and management practices can successfully reduce parasite worm burden but this success relies on the animal's immune system and preferential selection of foods that replicate an anthelmintic effect. Alternately, treating pasture directly may inhibit development and survival of the free-living nematode with effects on parasite survival and development reported using nitrogenous fertilizers (Cairns *et al*., 2017; Goode *et al*., 1974a; 1974b)

2.8.1 Fertiliser and other inputs

Nitrogenous solutions may affect nematode development in the free-living stage. Addition of ammonia to wastewater sludge reduced egg activation of *Ascaris Suum* (Pecson *et al*., 2007) when combined with increasing pH and temperature. Treatment concentrations ranged between 0 and 5000 mg/L of NH_3-N, a maximum inactivation of 98% was recorded at the highest N loading rate. Previous investigations have also recorded inactivation of Ascaris eggs when 200 mg NH3-N/L was applied to pig sludge (Pecson and Nelson, 2005). These results are promising considering Ascaris eggs are resistant to many chemicals and environmental conditions that typically destroy pathogens (Krishnaswani, 1968; Barrett, 1976; Gaasenbeek and Borgsteede, 1998) and have been described as one of the most resistant biological structures by Wharton (1980). There is evidence to suggets that N fertilisers may help limit parasite populations.

The possibility that nitrogenous fertilizer could have larvicidal properties was suggested by Cameron and Gibbs, (1966) in a grazing study who though nitrogen application removed some parasites, when applied to pasture. Goode *et al.* (1974a; 1974b) reported a similar effect. More recent work from Howell *et al.* (1999) documented the effects various nitrogen sources and concentrations had on *H. contortus* larvae, finding larval motility decreased as N concentration increased with the greatest effect observed between treatment with 6 -12% Nitrogen. Of the N sources used, ammonium nitrate was most effective reducing larval motility by 96.8%, followed by liquid nitrogen fertiliser (93.3%), ammonia/urea mix (89.2%) and urea (81.3%) at a rate of 18g N/100mls. Goode *et al.* (1974) showed reduction in survival of the parasite population by 65% when subjected to ammonium nitrate spray at 67.4 kg N/ha. Wozniak (*unpublished observation*, 2013) showed a 90% reduction in *T.colubriformis* and *T. circumcinta* egg hatch when eggs were exposed to 20% nitrogen. Cairns *et al.,* (2017) observed a 97% reduction in larvae recovery when urea was topically applied to sheep faeces at a rate of 40 kg/ha.

An effect of nitrogen fertiliser on larval survival and development is clear, but little is known about the mechanisms involved or fertiliser components that effect parasite larvae and egg development. Gonzalez (2010) and Rodriquez-Kabana (1987) suggested nitrogen in the form of ammonia was the toxic component, explaining the results of Howell *et al.* (1999), as ammonium nitrate already contains NH_3 and had the largest effect, where urea is converted into NH_3 and any loss would have lowered toxicity, explaining the smallest effect. It's known that ammonia is toxic to ticks and other insects (Lees, 1947) and it appears these effects may occur in helminths. The use of nitrogen as a parasite control mechanism is becoming an increased possibility but further research into effects on gastro-intestinal nematodes are needed.

2.9 Conclusion

Gastrointestinal parasitism is a leading cause of production losses in New Zealand pastoral agriculture and a variety of species are found throughout New Zealand. Parasite control using chemical anthelmintic is becoming increasingly ineffective due to the development of drench resistance, observed in many common parasites. This creates a major problem for animal production systems who are reliant on drench as the primary source of control. However, there is some respite with alternative control methods providing some control without the need of chemical treatment.

One such control method is the use of nitrogenous fertiliser to disrupt the nematode lifecycle while in the free-living stage, reducing the number of infective larvae that grazing ruminants are exposed to, ultimately minimising the effect parasitism has on livestock productivity. Studies have shown the topical application of a range of fertilisers, and recently urea, can reduce the number of eggs and larvae recovered from pasture. There is evidence to suggest that the effect may not be nitrogen specific, but little research has been conducted on gastrointestinal nematodes, specifically those important to New Zealand agriculture.

There are gaps in research regarding the scope of the effects observed on different parasite species, more research is needed to determine if application is species specific or whether common mechanisms are occurring which disrupt normal larval development. The permanence of reported effects has also been overlooked, and is worthy of investigation because a control method must be irreversible for it to be considered an effective treatment of gastrointestinal parasites.

Chapter 3

Species Effect

3.1 Introduction

Interrupting the parasite lifecycle through targeting life stages outside the host are an attractive means of reducing the reliance on anthelmintics for the control of gastrointestinal nematodes. Previous studies have shown that N-based fertilisers or their associated compounds may have the potential to substantially reduce both parasite egg hatching and development. Incorporation of ammonia to sewage sludge has been shown to reduce egg hatching of *A.suum* (Pecson *et al*., 2007) providing a proof of concept. For sheep nematodes, immersion of *H.contortus* larvae in varying N sources was reported to reduce larval motility (Howell *et al.,* 1999). More recently, *T. colubriformis* egg hatching was observed to be reduced in a dose dependent manner when immersed in liquid urea solutions, with reductions in egg hatching from 90% hatched in water to less than 6% hatched in 20% urea (Cairns *et al*., 2017). In the same study topical application of liquid urea to sheep faeces at a rate equivalent to 40 units of N per ha reduced larval recovery after culturing by 97%. The findings for *A. suum*, *H. contortus* and *T. colubriformis* provide the possibility that the ability of N based fertilisers to interrupt nematode development may be universal across nematode species. However, to date there is limited information available for nematodes from other grazing ruminants to support this. In particular, if found to be a universal effect, *Nematodirus*, being a nematode of sheep, is one species that spends a lengthy period of time on pasture as an egg and for which timely urea treatment may provide a useful tool to assist control. This experiment investigates the effect of urea immersion and topical application on the egg hatching and recovery from faeces following culture on nematodes from both deer and horses, and the effect topical application of urea has on *Nematodirus* species in sheep.

3.2 Deer Materials and Methods

3.2.1 Faeces Collection

Deer faeces were collected from animals at slaughter from AgResearch Invermay which had been subjected to natural field challenge and had not been treated with anthelmintic for at least 8 weeks prior. The faeces from eight animals were bulked and sent, via overnight courier, to Lincoln University with chill pads. Upon arrival the sample was refrigerated and processed as soon as possible to reduce development.

Eggs were extracted from faeces described below (Section 3.2.3) and used for egg hatch assays (Section 3.2.4). Remaining faeces was used for larval development assays as described below (Section 3.2.5).

3.2.2 Faecal Egg Counts

For each cohort of faeces, the concentration of nematode eggs (eggs per gram; EPG), was determined. A 3.4g sample of fresh deer faeces was placed in an 80 ml jar, 10 ml of tap water was added and the slurry was rested for three minutes and then stirred. 50 mls of saturated sodium chloride (NaCl) was added to the solution and it was then homogenized for 60 seconds. A wide bore glass pipette was used to transfer a sample of the egg suspension to a McMaster slide which was viewed under 4x magnification and eggs counted. Three to four slides were counted for every sample, the average egg count was taken and multiplied by 100 giving an eggs per gram (EPG) of fresh faeces.

3.2.3 Faecal Egg Extraction

Based on the concentration of eggs recorded in the FEC the appropriate amount of faeces (generally 80-100g) was taken and mixed with tap water at the ratio of 1 g Faeces: 2.3 mls tap water in a plastic bag. The bag was then homogenized for 30 seconds using a stomacher (Colworth Stomacher 400).

The homogenate was filtered using a 150 µm sieve and collected, a spray hose provided pressure over the sieve. Course material remaining in the first sieve was discarded. The filtrate was then re-filtered with a 45 µm sieve and the slurry containing some debris and eggs remaining in the sieve was collected, this 45 µm sieving was repeated. The fine filtrate was centrifuged at 2000 rpm for 5 minutes, excess liquid was removed using a vacuum line. The tubes containing the remaining debris and faecal eggs were gently mixed and 35 mls of saturated NaCl was added. A second centrifugation at 2000rpm for 5 minutes was conducted and the remaining supernatant was collected using a vacuum line and washed with fresh water in a 20 µm sieve apparatus for 20 minutes. The washed eggs were then collected and the concentration of eggs in suspension was determined (eggs per millilitre).

3.2.4 Egg Hatch Assay

0.5 mls of egg solution was pipetted into wells of a 48 well plate in triplicate, with an average of 60-80 eggs per well. 0.5 mls of the pre-mixed urea solutions described below were added to the respective wells to expose eggs to urea concentrations of 2, 5, 10 and 20% with tap water as a control (0%). Plates were gently agitated to mix the contents, and incubated at 26° C. After a 48 hour incubation period a few drops of Lugol's iodine was added to each well to prevent any further

development and both larvae and eggs were counted using a reverse microscope at a x 100 magnification, obtaining the hatch percentage of each replicate.

15 ml batches of urea solutions were made up to concentrations of 4, 10, 20 and 40% by mixing granular urea and water. A warm water bath was used to speed up the dissolving process, and the samples were left to cool to room temperature before use in the EHA's.

3.2.5 Larval Development Assay

Two kilograms of fresh deer faeces was mixed for 5 minutes by hand to ensure a well-mixed bulk sample. The faeces was then pelleted and twenty-seven 1.7g pellets, were evenly distributed onto a plastic tray (300mm x 210mm) lined with a plastic bag.

Plate 3.1.Pelleted deer faeces for Larval Development Assay

A control treatment (0 kg N/ha) and a nitrogen treatment (40 kg N/ha) was administered topically. For the nitrogen treatment 10% Liquid urea was applied via spray bottle at a rate equal to 40kg N/ha (40kg N/ha x 46% = 87 kg Urea/ha, x 10% urea = 869.6L 10% urea/ha, ÷ 100 000 000 (cm2/ha) * 630cm2 * 1000 mls = 5.5 mls/tray).

Following urea application, trays were covered with a perforated plastic bag and stored in a 26° C climate room for 10 days. After the 10-day culture the content of tray was emptied, and the bag rinsed onto a sieve block lined with two-ply tissue paper, and gently topped up with tap water.

After 24 hours soaking at 26° C, the sieves were drained for 20 minutes and the solution containing larvae was washed into 10 L beakers. The beakers were stored at 4° C overnight. Following chilling the beakers were siphoned to 750 mls using a suction line without disturbing the settled debris, the

contents were then poured into a measuring cylinder which were left to stand at 4° C for at least 6 hours before another siphoning, this time leaving 2 inches of liquid above the debris line.

The remaining debris/larvae solution was gently mixed and washed into a Baermann funnel filled with warm water and covered with a piece of two-ply tissue paper. The funnels were left for 48h in a heated room, allowing any larvae to migrate through the paper and collect at the bottom of the funnel. After 48h, 100 ml samples were drained from the funnel into a glass jar and rested for a minimum of 4 hours at 4° C. The top 50 mls of solution was then siphoned using a vacuum line and the jars re-filled with a further 50 mls from the funnels. The jar was rested at 4° C overnight then siphoned to leave 20 mls of solution. The remaining 20 mls was shaken and a 200 µl aliquot was pipetted onto a slide, accompanied by 2-3 drops of Lugol's iodine. Four slides were prepared from each replicate with the jar shaken between each sample to ensure an even sub-sample was obtained. The slides were observed under a reverse microscope at x 100 magnification and the number of larvae per gram of faeces initially incubated was calculated (larvae per slide * dilution factor/ grams of faeces processed). The four sub-samples were averaged providing the larvae per gram of fresh faeces. The process was repeated for each tray.

3.3 Horse Materials and Methods

3.3.1 Faeces Collection

Robert Derrick provided all horse manure. Faeces was collected over a 12 h period from one standard bred and two Clydesdale horses which were subjected to normal field challenge and had not been treated with anthelmintic for 6 weeks prior. 370 g samples from each individual were bulked (1.1 kg) and mixed thoroughly.

3.3.2 Faecal Egg Counts, Extraction and Egg Hatch Assay

Faecal egg counts and EHA's were performed as described for deer faeces in Section's 3.2.2, 3.2.3 and 3.2.4 with the exception that 4.0g fresh samples of horse faeces were used for FEC's.

3.3.3 Larval Development

Larval development assays were performed as described for deer faeces in section 3.2.5 with the exception that faecal samples were homogenised in a plastic bag by hand for 5 minutes with 250 g of mixed horse faeces evenly distributed onto a plastic tray (300mm x 210mm) lined with a plastic bag, then incubated. Following an initial trial run, faeces were collected again and the LDA repeated with urea treatments equivalent to a water control (0 kg N/ha), 40 kg N/ha, 200 kg N/ha, 500 kg N/ha and 1000 kg N/ha.

3.4 Nematodirus Materials and Methods

3.4.1 Faeces Collection and Faecal Egg Count

One parasite-free Hampshire wether, located at JML, Lincoln University which was intentionally infected with a *N. filicollis* and *N.spathiger*, was used for the collection of *Nematodirus* eggs. The animal was penned on wooden grating with ad-lib access to fresh water and fed a diet of Lucerne pellet/chaff diet and was fitted with a harness to the peri-anal region which allowed faeces collection when needed. Large quantities of faeces were needed so various collections were required, the bag was emptied every 6 hours during collection and any faeces was collected and stored at 4° C, until four kilograms of faeces were obtained.

Faecal egg counts were performed as described for deer faeces in Section 3.2.2 with the exception that 1.7 g fresh samples of sheep faeces were used for FEC.

3.4.2 Nematodirus Larval Development

Two, two kg samples of faeces were collected and thoroughly mixed by hand resulting in a 4 kg bulk sample. 250 g of fresh pelleted faeces was thinly spread onto each of 14, 300mm x 210mm trays lined with a plastic bag. A perforated plastic bag was used to cover the trays preventing contamination and allowing aeration.

Due to the time required for *Nematodirus* eggs to hatch (Section 2.3.3), the initial assay was run for 5 weeks with the treatments for the topical application included a weekly water application (Water), a weekly nitrogen application (Nx5), a single nitrogen application at week 1 (N-W1), and for the following 4 weeks (N-W2, N-W3, N-W4 and N-W5). Each treatment was performed in duplicate.

For each N treatment liquid urea was applied at a rate of 40 kg N/ha equivalent to 5.5 mls of 10% urea per tray, the same volume of water was applied to non-N treated faeces. The trays were cultured at 26° C, at constant humidity, for 5 weeks (5-week culture). Following incubation, a sample from each tray was removed, weighed, and stored separately in a refrigerator set at +4.1°C and cultured for a further 5 weeks to replicate chill requirement for hatching (10-week culture). These chilled samples received no additional urea application and were watered weekly at the previous rate.

Following culture, the content in a tray was processed using the post-culture extraction methodology used in the earlier larval development assays (Section 3.2.5). After extracting and cleaning the larvae the number of *Nematodirus* larvae and other larvae were counted. This process was repeated for each of the 14 trays.

3.5 Statistical Analysis

The hatch percentages for egg hatch assays and proportion of larvae recovered during larval development assays were compared with a one-way ANOVA (MiniTab 17, MiniTab Inc., USA). The urea concentration required to inhibit 50, 90 and 95% of the eggs hatching and larvae developing was determined using a Probit analysis (GenStat 18th edition, version 16.2, Rothamstead, VSN International, UK).

3.6 Results

3.6.1 Deer

A mean of 80 ± 6 eggs or larvae in total were recovered from each well. The mean hatch percentage for deer nematodes exposed to varying concentrations of urea is given in Figure 3.1. The hatch percentage for the 0% urea treatment (control) was 94%. There was an effect of urea on nematode egg hatch percentage but was dependent on concentration with no effect at 2% exposure (P=0.124), whereas concentrations of 5% urea or greater resulted in significant reductions (P<0.001). There was no further significant effect on egg hatching beyond 10% (P=0.779). Embryonation of larvae and hatch failure was observed in eggs at 2, 5 and 10% urea, as urea concentration increased the level of in-shell development decreased (greater termination of unhatched larvae).

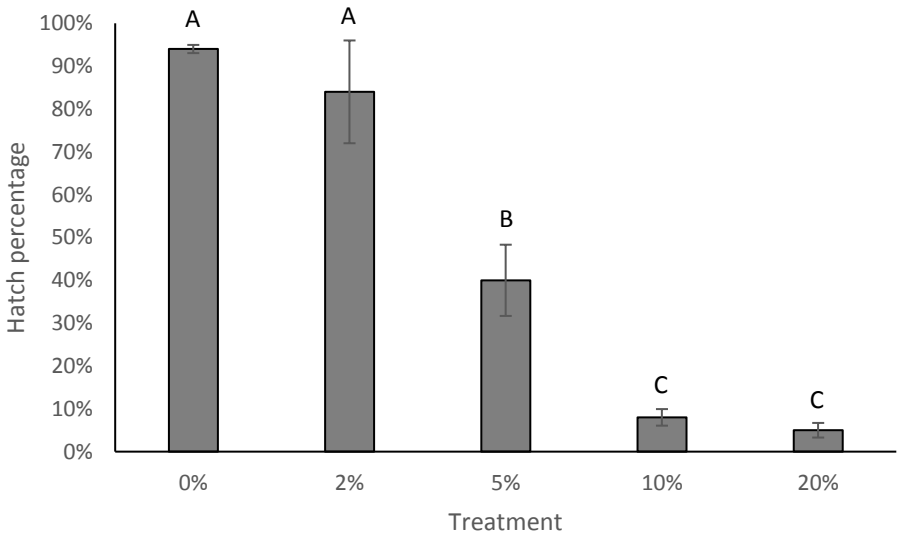


Figure 3.1. Hatch percentage of deer nematode eggs following a 24h immersion in urea solution of varying concentration. Different letters represent a significant difference in the mean, to a 95% significance

To achieve hatch inhibition of 50, 90 and 95% the urea concentration required was 10.9%, 20.8% and 23.7% urea respectively, equating to the nitrogen concentrations of 5.0, 9.6 and 10.9%, respectively. Lethal concentration (Lethal Dose; LD) estimates are displayed in Table 3.1.

Table 3.1. Lethal concentration estimates of urea for total population of deer nematode eggs.

LD	Urea %	s.e.	lower 95%	upper 95%
50	10.9	0.3	10.3	11.6
90	20.8	0.7	19.6	22.3
95	23.7	0.8	22.2	25.3

The number of larvae recovered (L.100g^{-1}) from deer faeces following urea application is given in Figure 3.2. Overall, there was an effect of urea application with the number of recovered larvae decreasing from 338 L.100g^{-1} after 0 kg N to 37 L.100g^{-1} when the equivalent of 40 units of N was applied (P=0.034), reflecting an 89% reduction.

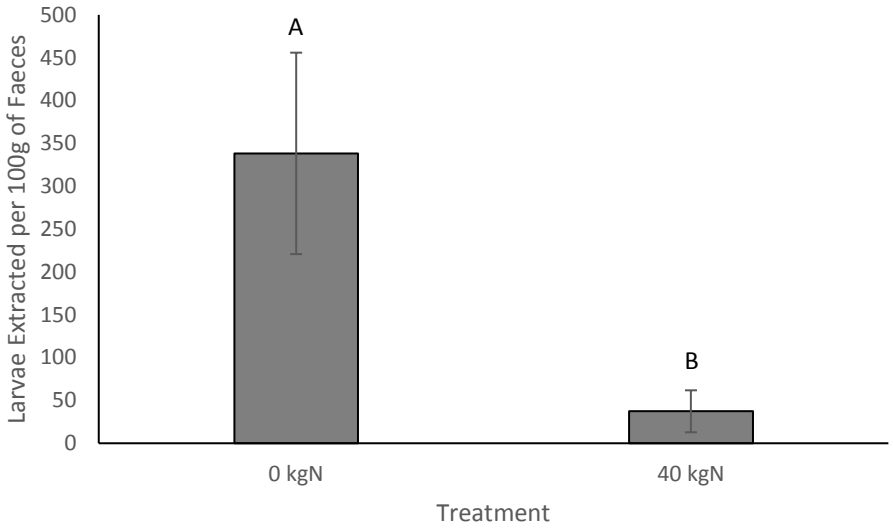


Figure 3.2. Number of larvae (per 100g) from deer faeces recovered following a 10-day incubation after the application of urea at the equivalent rates of 40 kg/ha on day 1 of culture.

3.6.2 Horse

A mean of 87 ± 4 eggs or larvae were recovered from each well. Overall, 89% of the eggs in 0% urea hatched. There was an effect of urea concentration on egg hatching with no difference in hatching at 2 or 5% urea, whereas hatch was reduced to 42% ($P<0.001$) and 16% ($P<0.001$) in urea solutions of 10% and 20% respectively, given in Figure 3.3. In-shell development of larvae was observed in eggs that failed to hatch at 2 and 5% urea, as urea concentration increased the level of in-shell development decreased (greater termination of unhatched larvae).

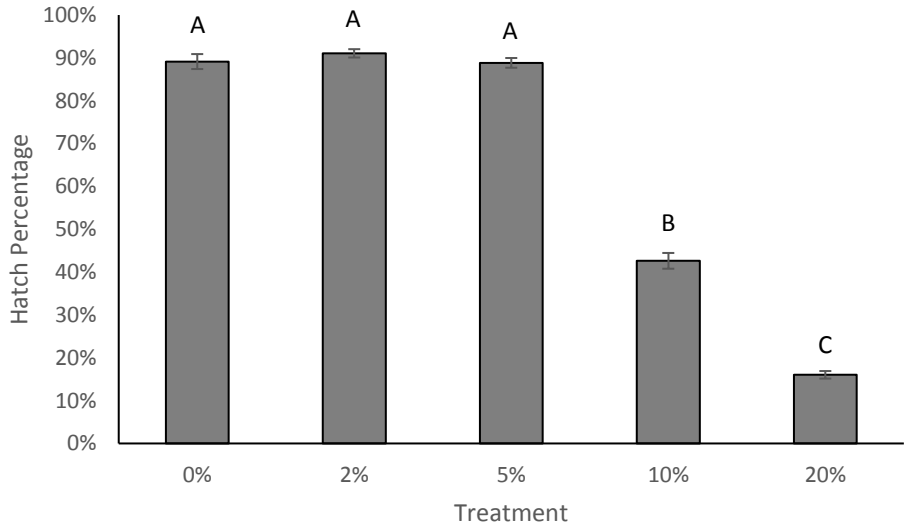


Figure 3.3. Hatch percentage of horse nematode eggs following a 24h immersion in urea solution of varying concentration. Different letters represent a significant difference in the mean, to a 95% significance.

Probit Analysis revealed the urea concentration to inhibit 50, 90 and 95% of egg hatching was 11.1, 21.1 and 29.3%, equating to nitrogen concentrations of 5.1, 9.7 and 11%, respectively, Lethal concentration estimated are given in Table 3.2.

Table 3.2. Lethal concentration estimates of urea for total population of horse nematode eggs.

LD	Urea %	s.e.	lower 95%	upper 95%
50	11.1	0.3	10.6	11.7
90	21.1	0.6	20.0	22.3
95	23.9	0.7	22.7	25.4

The number of larvae recovered at varying urea application rates is given in Figure 3.4. An initial larval development assay resulted in an increase in larval recovery following urea application when comparing the equivalent of 0 kg N/ha and 40 kg N/ha treatments, with the number of larvae recovered ($L.g^{-1}$ fresh faeces) increasing from 172 ± 0.8 $L.g^{-1}$ to 281 ± 43 $L.g^{-1}$, respectively, the difference was significant (P=0.036). A second assay conducted with higher urea application rates showed an effect with the number of recovered larvae increasing from 189 ± 14 $L.g^{-1}$ at 0 kg N/ha to 270 ± 23 $L.g^{-1}$ at 40 kg N/ha (P=0.019), whereas larval recovery was reduced by 99% or more when 200 kg N/ha or more was applied (P<0.001).

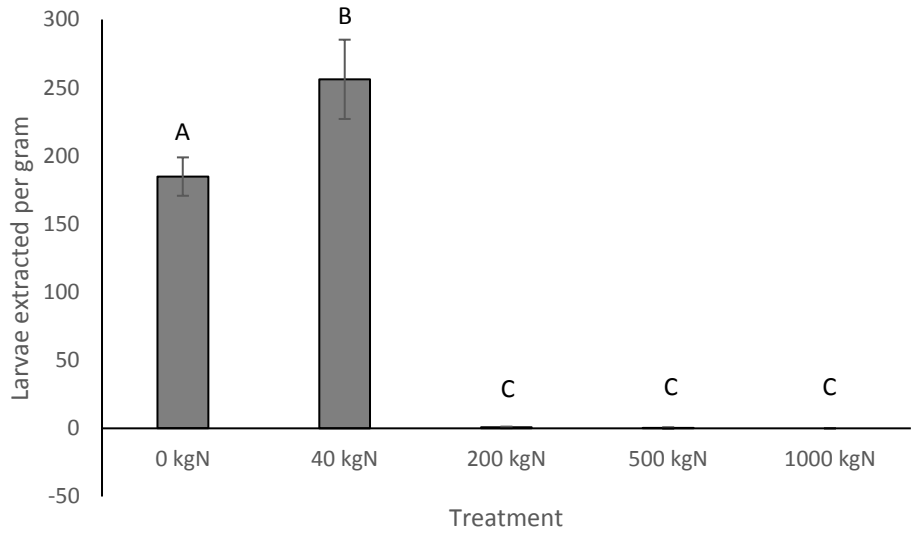


Figure 3.4. Number of Larvae per g fresh faeces ($L.g^{-1}$) from horse faeces recovered following 10 days of incubation after the topical application of urea at equivalent rates of 40kg/ha, 200kgha, 500kg/ha and 1000 kg/ha on day 1 of culture. The 0 and 40kg N/ha rates includes 4 replicates (two time periods) whereas other concentrations include three replicates (one time period).

3.6.3 Nematodirus

The number of Nematodirus larvae recovered per 100 g faeces is given in Figure 3.5, lettering indicates significant difference. The number of larvae recovered from the control (water) treatment was 439 L.100g^{-1} faeces, the timing of the 40 kg N/ha applications had no effect on larval development compared with the control except N-W1, where a 752% increase in L.100g^{-1} was recorded (P=0.002). The larval recovery from N-W1 was significantly higher than all other treatments (P=0.012). No Nematodirus larvae were recovered from the Nx5 treatment.

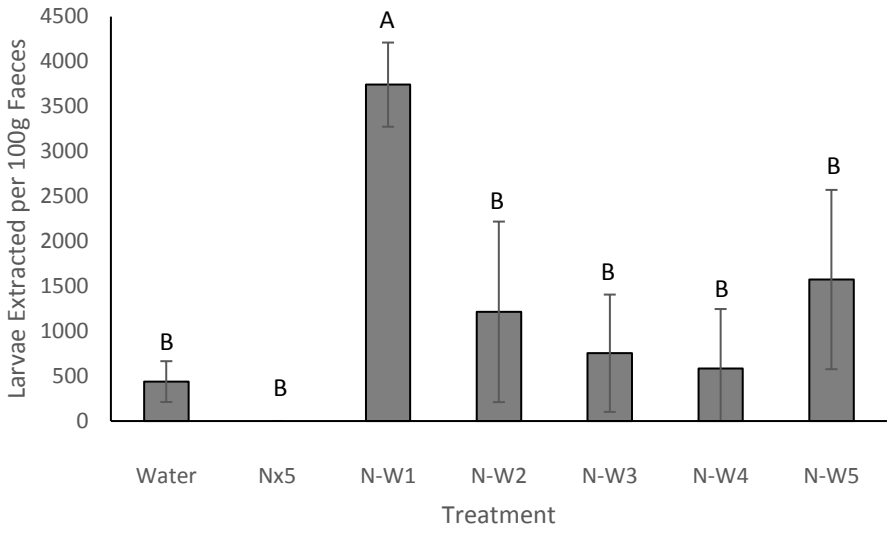


Figure 3.5. Number of Nematodirus larvae per 100g faeces following a 5-week culture.

Nematodirus species made up 69.5% of the total extracted larvae, the remaining 30.5% of larvae were analysed separately, and changes is recovery are displayed in Table 3.3. There was a significant effect of topical N application on non-*Nematodirus* larvae (P=0.016). The counts of the N-W1 treatment were significantly higher than any other treatment (P<0.012).

Table 3.3. Significance of 'Other' nematode larvae extracted in 100g Faeces, after a 5-week culture.

Treatment	Mean	s.e	Significance
Water	286	34	BC
Nx5	78	78	C
N-W1	1140	478	A
N-W2	1286	134	A
N-W3	1061	138	A
N-W4	799	146	AB
N-W5	1391	20	A

Overall, single N treatments increased larval recovery compared with the control and Nx5 treatments. The weekly N treatment (Nx5) resulted in the lowest L.100g^{-1} of non-*Nematodirus* at 78 L.100g^{-1} faeces.

The number of *Nematodirus* L.100g^{-1} incubated faeces after 10-weeks in given in Figure 3.6. Overall, there was no significant result of a topical nitrogen treatment (P=0.137) compared with the control. Although N application at N-W1, N-W2 and N-W5 resulted in an increase in larvae recovered when compared with the Nx5 treatment (P=0.020, 0.031 and 0.036) the larval counts following single urea application weren't greater than the control. Nx5 was not statistically different to the control (P=0.225).

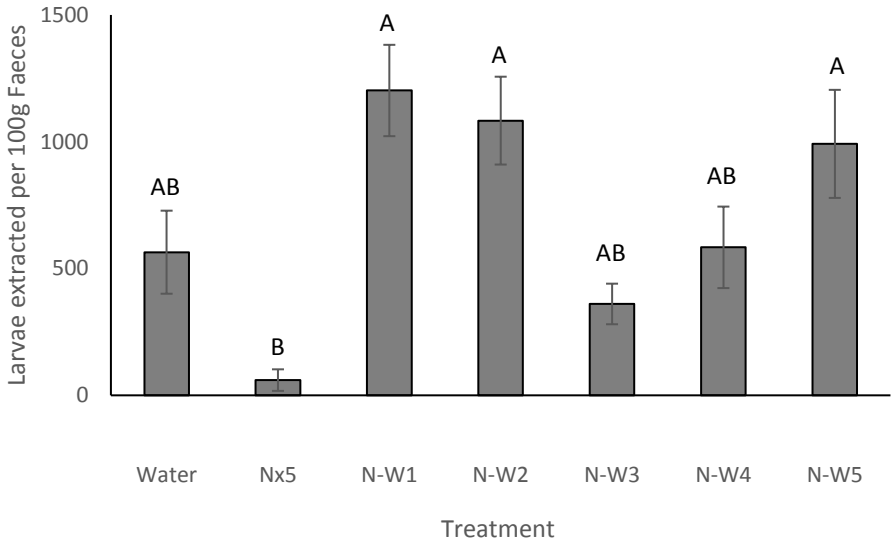


Figure 3.6. Number of Nematodirus larvae extracted after a 10-week culture including 5 weeks of cold exposure.

Nematodirus species made up 90.9% of the total extracted larvae in the 10-week culture, the difference in recovery counts of the other extracted species have been given in Table 3.4. Total larvae (all species) recovered after 10 weeks of culture was 55% lower than the larvae count after 5-week culture. Total larvae recovery, inclusive of all treatments of the 10 week development assay were composed of *N.filicollis*, *N.spathiger* and *N.abnormalis* in the proportions of 56%, 29%, 6%, respectively, other *trichostrongylids* made up the remaining 9%. There is no distinct trend within the dataset

Table 3.4. Significance of 'Other' nematode larvae extracted in 100g Faeces, after a 10-week culture.

Treatment	Mean	s.e	Significance
Water	141	2	A
Nx5	18	9	B
N-W1	151	72	A
N-W2	89	11	AB
N-W3	30	10	B
N-W4	27	6	B
N-W5	18	18	B

There was a significant effect of topical nitrogen on non-*Nematodirus* larvae (P=0.048). The control and N-W1 treatments resulted in higher average larval counts than all other treatments, excluding N-W2 (P=0.078)

A comparison between total larval recovery of the 5-week and 10-week cultures is given in Figure 3.7, inclusive of all larvae species recovered. The only difference between cultures existed at the N-W1 treatment.

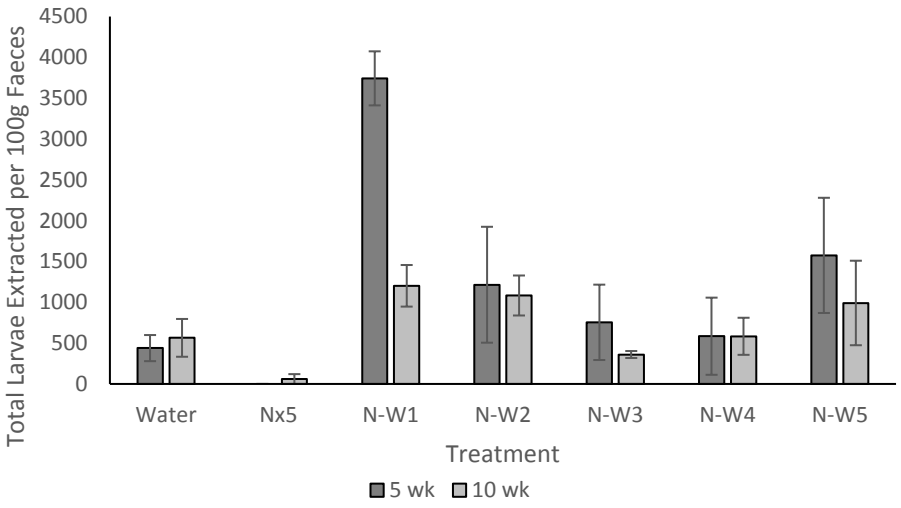


Figure 3.7. Total number of Larvae (L.100g^{-1}) from sheep faeces recovered following 5 and 10 weeks of incubation. Liquid urea was applied at the equivalent rate of 40kg N/ha.

3.7 Discussion

Overall, the ability of urea to interrupt the nematode lifecycle appeared to be universal across species. For deer and horse EHA and LDA's the proportion of eggs hatching was substantially reduced upon exposure to urea. The concentration of urea required to inhibit egg hatch by 90% in deer and horse nematodes was 20.8 ± 0.7% and 21.1 ± 0.6%, respectively, similar values to the LD_{90} of 19.6 ± 0.2% determined for *T.colubriformis* when exposed to urea (Cairns *et al*., 2017). A noticeable difference between the deer and horse EHA was the difference in inhibition after 5% urea exposure, with the horse nematode hatch being significantly higher (89%) than the deer (40%) and sheep (Cairns *et* al., 2017) counterparts indicating some differences in sensitivity. Unfortunately due to the workload of technical support, it was not possible within the time frame of this experiment for deer and horse species to be identified, which may have helped determine if the differences in sensitivity were common across all species of deer or horse nematodes. The topical application of urea resulted in reductions in larvae equivalent to the decrease in *T.colubriformis* reported by Cairns *et al*. (2017). There were exceptions observed following the horse LDA, with an unexpected increase in larvae recovered at 40 kg N (Figure 3.4) indicating variation between species. A secondary LDA concluded that increasing the application rate beyond 200 Kg N/ha decreased larval recovery by more than 99%, synonymous with the 81.3% to 96.8% reductions in *H.contorus* motility (Howell *et* al., 1991) and the reductions apparent in *T.colubriformis* (Cairns *et al*., 2017), suggesting the overall effect is common but the level of tolerance to urea differs between nematode species.

The tolerance to urea of *Nematodirus* appears to be higher than seen in sheep and deer nematodes further indicating variation in tolerance level. The *Nematodirus* LDA was reflective of the horse LDA, where low dosage caused an increase in larvae recovered following a single urea application. The Nx5 applications resulted in a significant decrease in larval recovery reducing larval count by 100% and 89% for the 5 and 10-week LDA, respectively, which was expected when considering the findings by Cairns *et al*. (2017) and earlier LDA's. The difference in effect between low and high N application was found in several other studies which investigated the effect on N on nematode hatch and recovery (Goode *et* al., 1974; Howell *et al.,* 1991; Pecson *et al.,* 2007; Cairns *et al*., 2017; Roul *et al*., 2017). One possible, but unlikely explanation was the presence of the fruiting body of an unknown fungus which grew in both N-W1 replicates (Appendix B1) and no other trays. The fungi may have diminished the effect nitrogen application had on developing larvae or improved the development conditions within the faecal pat explaining the higher recoveries of the N-W1 treatments. To determine if a direct relationship occurred more must be known about the fungal species and any relationship with nematodes or nitrogen use. Of the single urea application treatments larval recovery was higher at N-W1. This might be explained by an increase in ammonia within the faecal pats as culture time progressed, which was suggested to have a toxic effect to nematodes

(Rodriquez-Kabana, 1987; Gonzalez, 2010). There was obvious condensation inside the cover bags even though they were perforated, which suggests some of the volatised nitrogen and moisture was returned to the faecal pats. Microbial activity within manure denitrifies nitrates within the soil, which are lost to the environment as volatile gases (McLaren and Cameron, 1996), if these were condensed and returned to the trays more nitrogen, beyond the treatment, may have been applied, intensifying the effect as time increased because nitrification and denitrification cycles are not instant. If the *Nematodirus* LDA was to be re-attempted, a concentration LDA would allow the determination of the lethal concentration needed to reduce hatch and larval recovery, which would make the relationship between urea and *Nematodirus* clearer. Increasing the technical capability when working with *Nematodirus* would also strengthen results by removing some human error that occurred around culturing.

The phenomena of increasing larval recovery at a low nitrogen dose isn't fully understood but there seems to be some variation between species. Low N application (40kg N/ha) increased larvae recovered in horse and *Nematodirus* LDA's but not in deer or sheep LDA's. There was no evidence from EHA's in this study or by Cairns *et al*. (2017) to suggest a low nitrogen dose would have a positive effect on egg hatch, bearing in mind the control hatches in both studies were above 90% so an increase would be hard to achieve. When comparing the horse EHA to deer EHA, the horse nematodes appeared to be more tolerant to 5% urea than deer nematodes due to a higher hatch (Figure 3.1 and Figure 3.3), when urea concentration was equal to or greater than 10% the level of inhibition was similar. Upon further investigation of horse nematodes, 85.4% and 15.6% of the recovered larvae were *Cyathostomum* spp. and *Strongoloides westeri*, respectively, the deer nematodes were undifferentiated but likely to be *Ostertagia*, *Cooperia* or *Haemonchus* species (Pomroy, 1997). Speciation of *Nematodirus* samples found the presence of *N.filicollis, N.spathiger* and *N.abnormalis*. The decrease in larvae recovwered from the deer EHA following 40 kg N/ha is comparable to Cairns *et al*., (2017) due to the likeness of the *Trichostrongylus* species and common deer nematodes listed above (Brunsdon, 1980; Familton and McAnulty, 1997). *Nematodirus* and *Cyathostomum* species are very distinct from the common ruminant species in terms of structure and develop (Kates, 1950; Michel, 1969, Vlassof and McKenna, 1994; Mathews, 2014) which may help to explain the difference in hatch inhibition or larval recovery noticed. However, analysis of 'other' non-*Nematodirus* larvae, given in Table 3.3 and Table 3.4 suggested another factor could be driving the change. Most 'other' larvae were *Trichostrongylus* nematodes and increased by an average of 235% and 57% for the 5-week and 10-week LDA's, respectively, when urea was topically applied. Cairns *et al.* (2017) suggested a 40 kg N/ha application reduced recovery by 97%. A micro-climate effect may explain the increase in recovery of both *Nematodirus* and other larvae. Additional nitrogen may have been insufficient to reduce larval hatch but instead possibly increased protein

supply for growing nematodes and microbial organisms, in turn increasing the temperature or moisture levels within the pat, which favours egg hatch and larval development (Silverman and Campbell, 1959; Stromberg, 1977; Familton and McAnulty, 1997; O'connor *et al*., 2006). The covering of the trays trapping condensation may have accentuated this problem.

Low larval recovery was an issue in the *Nematodirus* and deer LDA's which may have influenced the findings and weakened the strength of results. The hatch of any nematode egg is dependent on the penetration of CO_2 gas through the permeable shell layer, which signals permeation of the lipid layer, allowing the passage of trelahose (Perry, 1989). Oxygen is equally as important as a deficiency will inhibit egg hatch, larval development and activity (Familton and McAnulty, 1994), moisture is also essential for larval development and survival (Stromberg, 1997). There was minimum agitation of the trays to simulate a faecal pat sitting on pasture, which may have reduced aeration throughout the samples. However the trays were loaded at one pellets depth and spread evenly, which would minimise that effect, the presence of insect larvae would have also improved aeration. It is possible the pelleted deer faeces may have been too compact and prevented oxygen penetration into the centre of each pellet, which in turn would have reduced the hatch and subsequent larval development. The plastic bag covers were permeated to allow air flow, but there may have been an oxygen or carbon dioxide deficiency potentially reducing egg hatch percentage, explaining the lower than expected recovery, which may have influenced the observed effect as larvae with low oxygen tolerance could have been over represented. Significant weight loss was observed over the duration of all LDA's, especially the 5-week LDA, with an average loss of 35.7 g presumably through the evaporation of water, after 10 weeks water loss may have been so extreme hatch was inhibited. Garfield and Walker (2008) observed water content in dog excrement and found any moisture to be completely lost between 50 to 200 hours, dependant on evaporation rate, so it is understandable that the sheep faeces may have dried out, however the weekly application of 5.6 mls of water should have negated this loss, under the constant humidity. It may be that water loss was so rapid, watering more than once every seven days was required to optimise egg hatch, improve larval survival conditions and ultimately increase recovery. This would help reduce the effect of unstudied variables like relation to water level or oxygen and strengthen the data set.

There is some suggestion that tolerance to treatment varies among species but the overall effect was universal between gastrointestinal nematode species. General variation of treatment susceptibility or tolerance is expected in biological experimentation, which may help to explain why these changes were apparent. However, when nitrogen application was high enough the same effect on egg hatch and larval development was witnessed over a range of nematodes and host, supporting the notion that the effect is universal. This extends the suitability of a topical urea application throughout New Zealand agriculture as any ruminant farmer could utilise the application of urea to improve pasture

growth, while reducing the effects of parasites and ultimately reducing the current reliance on anthelmintic. If the effect is broader, and other plant and soil nematodes are impacted the scope of treatment is even greater, however more research is required regarding exact concentration application for specific species and to see if results *in vitro* are translated onto the paddock.

Chapter 4

Mechanisms

4.1 Introduction

Previous studies along with the results of Chapter 3 indicate that the ability of liquid urea to reduce larval development may be common across many gastro-intestinal nematode species. However, little is known about the mechanisms through which this may be occurring. Given the experiments to date have been run *in vitro* under controlled conditions and for a definite time period, this information is critical before the suitability of this approach to provide a meaningful benefit can be assessed. More specifically, the minimum contact time is not known. Given the observations in the previous chapter that unhatched eggs in the urea treatment were embryonated but failed to hatch suggests that the effect is not immediate. It is possible that the urea may have a direct toxic effect on the larvae or that it may be causing structural changes to the egg shell. This then raises the question as to whether such changes are reversible, which may limit the applicability of this approach in the field or if they are specifically associated with urea. Cairns *et al.* (2017) demonstrated a significant association between electrical conductivity and the reduction in egg hatching when comparing different N based fertilisers, however the effect is still evident for urea which has a very low electrical conductivity due to its lack of dissociation into anions and cations when in solution. However, differences in osmolality do exist between the urea concentrations and to date this has not been explored as a possible mechanistic cause.

Using the sheep nematode *T.colubriformis* as a model, this series of experiments explores the mechanisms of this phenomenon through establishing the minimum contact time of the urea solution with eggs to prevent hatching, whether this effect is permanent and whether the effect of failure to hatch is dependent on N compounds or a general effect of osmolality.

4.1.1 Faecal Collection, Egg Count and Egg Extraction.

Faecal collection, egg extraction and FEC are described earlier (section 3.2.1, 3.2.2 and 3.3.3).

4.1.2 Exposure Egg Hatch Assay

Wells of a 24-well plate were filled with 0.5 mls of a 20% urea solution in triplicate, 0.5mls of extracted egg solution were then added into a capsule (Plate 4.1), a plastic barrel lined at one end with 20 µm gauze material, subjecting eggs to 10% urea solution. Capsules containing eggs were placed into wells with the urea mix for either one minute, 1 hour, 6 hours or 24 hours with a tap water as a control.

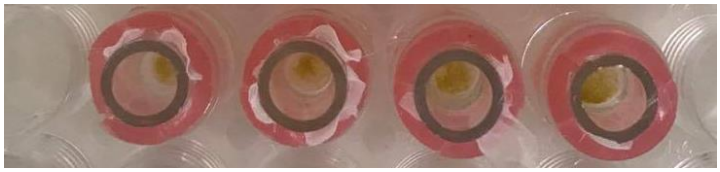


Plate 4.1. Exposure assay capsules

After the allotted exposure time for each treatment, the capsules containing eggs and larvae, were removed from the wells and rinsed in a water bath for 1 minute with the water changed after each wash. Each replicate capsule was then gently washed into a 15 ml centrifuge tube. After centrifugation at 2000 rpm for 5 minutes excess liquid was decanted down to 3 mls using a vacuum line. The remaining egg solution was gently mixed and counted with eggs per ml obtained, which was then used to determine the required aliquot for 80 eggs per well.

The calculated volume was pipetted into wells of a 48 well plate, and 1.5 mls of tap water was added. The plate was incubated at 26° C with counts of the number of larvae and eggs taken at 24 h, 48 h, 96 h and 168 h from the end of each soak.

4.1.1 Reversibility Egg Hatch Assay

Collection of eggs and allocation into wells for an EHA was performed as described previously (Section 4.1.2) with the exception that no iodine was added to wells. Post soak, counts of larvae and eggs were taken at 24, 48, 96 and 168 hours for the treatments; control, 1min, 1h, 6h, and 24h soaks. The hatch percentage was calculated for each well at each post soak time.

4.1.1 Osmolality Egg Hatch Assay

An osmotic standard curve was established for NaCl and glucose, equivalent to the osmotic potential of 10% urea being 1500 ± 50 mmol/kg. Solutions of glucose were made to 10, 20, 30, 40 and 50%, and solutions of sodium chloride (Agsalt) were made to 2, 4, 6, 8 and 10% along with a 10% urea solution. 30 µl samples were analysed using an osmolality machine (Wescor 5520 Vapro. Wescor Inc. Utah, USA) to determine the mmol/kg of solution. 1500 ± 50 mmol/kg was the equivalent of 10% urea, 4.9% NaCl and 30% glucose. The osmolality standard curves of salt and sugar are given in appendix A (A1 and A2). Once the concentration of aqueous solution of salt, sugar and nitrogen was determined, a 48h soak assay was completed with the treatments of a water control, 4.9% NaCl, 30% glucose and 10% urea performed in triplicate.

In wells of a 48 well plate 1ml of pre-mixed 40% glucose solution was diluted with 333 µl of egg solution resulting in the wells having a 30% glucose solution. For salt 1ml of 6% NaCl was diluted to 4.9% NaCl by adding 224 µl of egg/water solution.

The urea treatment was applied as described earlier by dilution of 0.5ml of 20% urea with 0.5ml of egg solution and water. A tap water control was included.

4.1.2 Statistical Analysis

Percentage hatched were analysed with ANOVA using MiniTab 17, the treatments (soak time) acted as the factor, the hatch percentage being the response variable. The time of urea exposure to achieve hatch inhibition of 95, 90 and 50% of *T.colubriformis* eggs was determined using Probit analysis on GenStat (GenStat 18th edition, Rothamstead, VSN International, UK).

The results of the reversibility EHA, given in percentage hatched were analysed with a repeated measures ANOVA and Post-hoc Tukey tests using MiniTab 17, the treatments acted as the factor, the hatch percentage being the response variable. A Post-hoc Tukey tests was used to determine any reversibility in hatch following urea exposure. The significance level was set at 95%.

The hatch percentages of the osmolality EHA were analysed using a one-way ANOVA in MiniTab 17, and a post Hoc Tukey test used to distinguish any difference between treatment means.

4.2 Results

4.2.1 Exposure

To achieve hatch inhibition of 50, 90 and 95% the exposure times, given in Lethal Time (LT), required were 7.5 ± 0.9 h (450 ± 52 min), 14.3 ± 1.8 h (858 ± 107 min) and 16.2 ± 2.1 h (973.7 ± 124 min), respectively. Probit analysis results are displayed in Table 4.1. Embryonation and egg hatch failure was observed following 6 and 24 hour urea exposure.

Table 4.1. Lethal time estimates of exposure (minutes) to achieve hatch inhibition of 50, 90 and 95% in a 10% urea solution.

LT	Estimate (min)	s.e.	lower 95%	upper 95%
50	450.1	26.36	404.8	507.8
90	858.1	54.79	765.8	980.4
95	973.7	63.29	867.3	1115.2

The mean hatch percentage of *T.colubriformis* eggs following varying exposure to 10% urea solutions is given in Figure 4.1. Overall there was an effect of exposure time on egg hatch inhibition. The mean hatch percentage for the control was 87%, with no difference in hatch following the 1min ($P=0.309$) and 1h ($P<=0.502$) treatment. Reductions in hatch occurred after 6h to 58% ($P<0.001$) with complete inhibition after 24h ($P<0.001$).

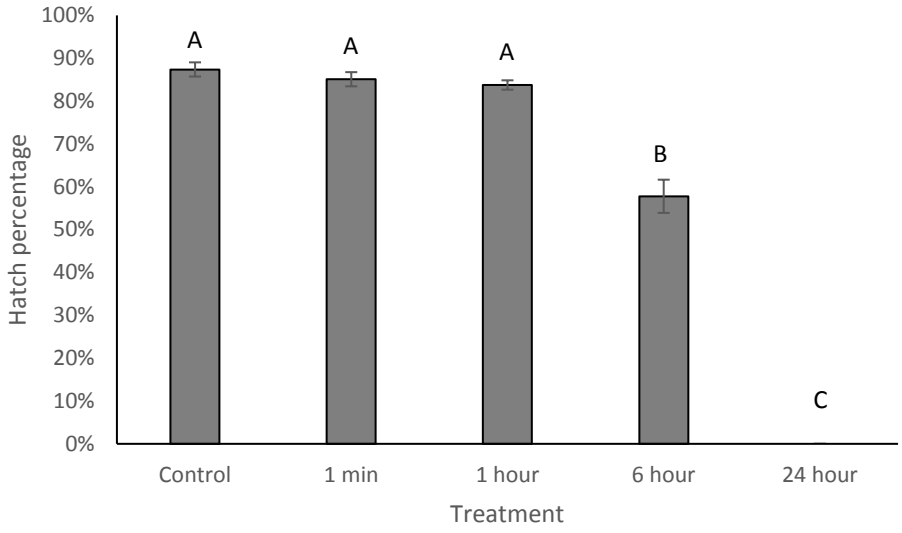


Figure 4.1. *T.colubriformis* egg hatch percentage following a 1 min, 1h, 6h and 24h soak in 10% urea. Letters represent significant difference between hatch percentage at 24 of water incubation following a given exposure treatment.

4.2.1 Reversibilty

Figure 4.2 gives the percentage hatched in relation to time for eggs exposed to 10% urea solutions for varying lengths of time. The hatch percentage at 24h for control, 1min and 1h hatch percentages were similar, being 87% (P=0.50 and P=0.309, respectively). The hatch percentage was reduced by 58% after 6h of exposure (P<0.001) and was further decreased to 0% after a 24h soak (P<0.001).

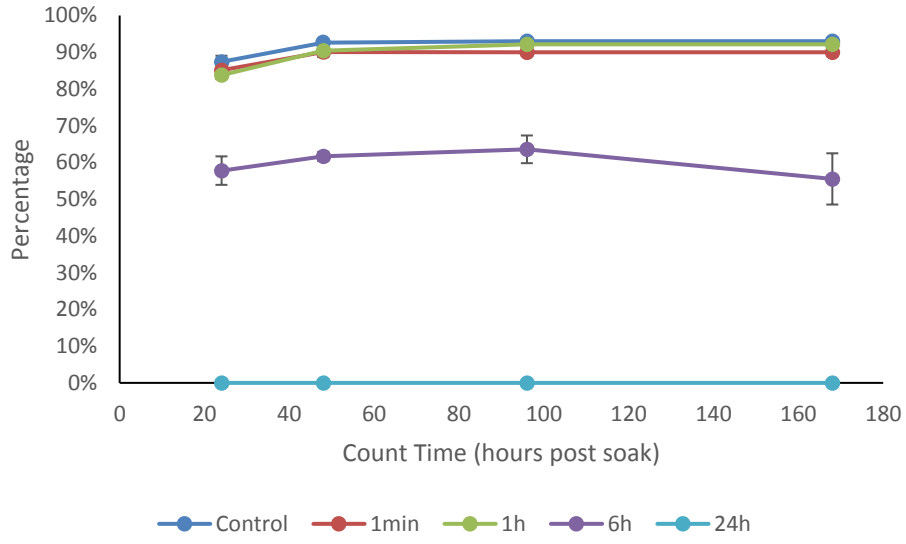


Figure 4.2. Temporal Egg Hatch Percentage of *Trichostrongylus colubriformis* after 10% Urea immersion. The key represents the primary treatments. The x-axis represents the points in time when counts were conducted to determine reversibility of hatch percentage.

There was a small increase in hatch of the control, 1 min and 1 h treatments beyond 24 h, given in Table 4.3, although no changes occurred with time in the 6 h and 24 h treatments.

Table 4.2. Reversion of egg hatch following 10% urea reversibility assay.

	Hours post-soak				
	24	**48**	**96**	**168**	**P-value**
Control	0.87 A	0.93	0.93	0.93	0.016
1min	0.85 A	0.90	0.90	0.90	0.028
1h	0.84 A	0.90	0.92	0.92	0.009
6h	0.58	0.62	0.64	0.56	0.782
24h	0.00	0.00	0.00	0.00	-

4.2.1 Osmolality Egg Hatch Assay

The effect of solutions of either salt or glucose at comparable osmolality to 10% urea on egg hatching is given in Figure 4.3. Overall, 89% of control eggs hatched which was reduced by 90% (P<0.001) in the Urea and 100% (P<0.001) in both salt and glucose solutions.

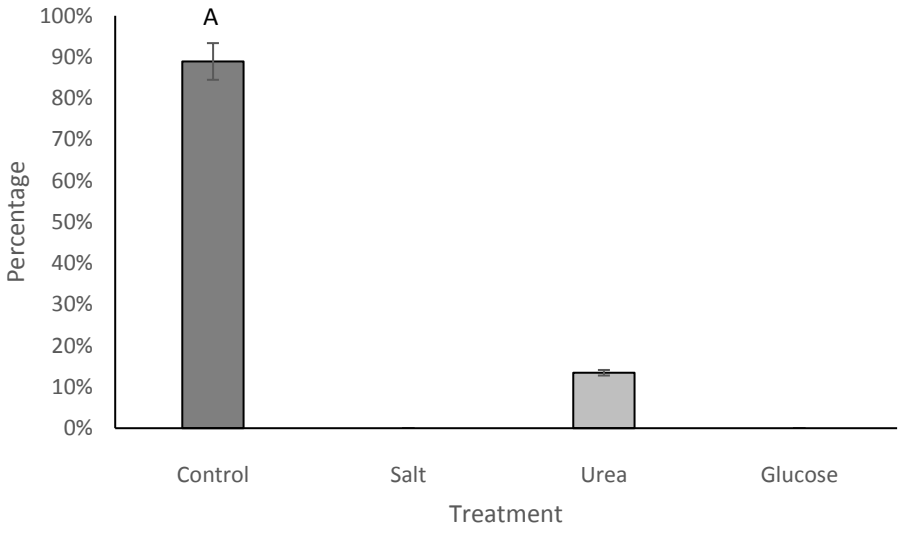


Figure 4.3. Nematode Hatch Percentage after standard osmolality EHA when exposed to solutions of salt, urea and glucose at an osmolality of 1500 ± 50 mmol/kg.

4.3 Discussion

The ability of urea solutions to prevent nematode egg hatching was dependent on time of exposure, the changes appear to be irreversible, although the mechanisms causing the effect remain unclear. There was no immediate effect of urea exposure as the time required to prevent 50% hatching (Lethal Time; LT_{50}) was 7.5 ± 0.9 h, hatch inhibition increased as exposure time increased. The fact egg hatch after one minute and one hour was unaltered (Figure 4.1) indicates the washing process was effective and a true effect was established. As such, the changes appeared to be irreversible after washing as there was no hint of increased hatch over an extended time period of 168 h, which is a period of time well in excess than normally expected in terms of egg viability (Familton and McAnulty, 1997). Increasing time of exposure or 'dose rate' resulted in greater egg hatch inhibition (Table 4.1) following a similar trend to drench administration. Kaminksy *et al*., (2009) found efficacy of monopantel against *Haemonchus*, *Trichostrongylus* and *Nematodirus* increased from 91% to 94% when dose rate increased from 1.25 mg to 5.0 mg, respectively, while Leathwick and Luo (2017) recorded an increase in efficacy from 69% to >99% when the proportion of recommended dose rate increased from of 0.5 to 1.4. Overall, it is clear the dose rate or exposure time is important to induce and effect, potentially indicating that the effect of urea on egg hatching is not an immediate toxic effect, but at the same time is irreversible.

Embryonation and hatch failure was evident in deer and horse nematodes. This has been observed previously in *T.circumcinta* (Cairns *et al.,* 2017)*, Cooperia oncophora* (Peña-Espinoza *et al*., 2017) and *Caenorhabditis elegans* (Anthony Page, *unpublished Observation*), though the mechanisms are unclear. A possible explanation could be the inactivation of Chitinase when N availability is excessive, in concordance with Olander and Vitousek (2000). Chitinase and other enzymes appeared to damage the egg shell prior to hatch (Rogers and Brookes, 1977; Bone and Parish, 1988), if deemed inactivate, the shell may be too hard for larvae to physically break reducing hatch success.

The cause of embryonation and hatch failure may be independent of urea application as there was commonality when using chicory extract (Peña-Espinoza *et al*., 2017) and urea (Cairns *et al.,* 2017). The fact that the same observation was made in multiple nematode species and ruminant hosts strongly supports the notion that the mechanisms influencing hatch are common, which appear to be related to osmolality. Increasing osmolality through the use of salt, urea and glucose solutions to the equivalent osmolality of 10% urea resulted in reductions in egg hatch of more than 90% (Figure 4.3), again embryonation in unhatched eggs was observed. Croll (1974) reported embryonation and hatch failure in 4 % salt solutions, the hatch percentage decreased as salt concentration increased and eggs only hatched when tonicity was below 1%, comparative to the effect seen in horse and deer EHA's with hatch occurring at the lower concentrations. Mathews (1985) and Wilson (1958) found

increasing the osmolality of organic salt and sugar solutions extended hatch time to 30-40 h. These findings favour the hypothesis that an osmolality effect is taking place, the exact effect is unknown.

The effect of osmolality may help to explain the reduction in hatch when urea concentration increased in the horse and deer EHA's, the change in hatch response to differing fertilisers at the same nitrogen concentration (Cairns *et al*., 2017) or the reduction in hatch when chicory extract increased (Peña-Espinoza *et al*., 2017) but does not answer how larvae continue to develop within the shell or the level of variation between replicates and treatments. Osmolality may play a critical role in the permeability of the egg-shell. The permeability of the shell immediately prior to hatch is important as it dictates the level of trelahose exposure the outermost, and subsequent layers receive which is critical for hatch success (Perry and Clarke, 1981; Perry, 1989; Behm, 1997). Originally it was believed the shell was impermeable until the initiation of the hatching sequence, which we would assume is near a typical *in vitro* hatch time of 24h, here an LT_{50} of 7.5 h was determined during the exposure EHA, suggesting earlier egg-shell permeation supported by Clarke and Perry (1980) who found water content of unhatched juvenile larvae were affected by changes in external osmolality disproving that hypothesis.

A certain level of biological variation within the hatch and development process is expected. Individual differences in the rate of egg development, time to hatch initiation and hatch and susceptibility to treatments may help explain some variation seen between the LT_{50}, LT_{90} and LT_{95} of different treatments or the fact some eggs hatched, while others failed to do so. The effect urea exposure appeared to be irreversible in *T.colubriformis* after a minimum exposure of 7.5 hour, the increase of exposure time resulted in exponential increases in inhibition (Figure 4.2). This is very important for the practicality of topical urea application to control parasite populations on pasture as unknown natural factors that aren't replicated *in vitro* might extend the required exposure time, and if lethal time isn't achieved hatch isn't reduced so the treatment would fail to break the free-living lifecycle.

Chapter 5

General Discussion

Overall, there was a promising effect of nitrogen based fertiliser as a tool for the control of free-living GIN nematodes, but further information is required before any direct conclusions can be made. The results of the reversibility EHA suggest that exposure, if in high enough concentration for a long enough time, reduced hatch permanently preventing the continuing of the free-living lifecycle, which would ultimately reducing the number of infective L3 larvae on pasture. This is important for the practicality of using topical treatments to control larvae populations. Preliminary, *in vitro,* results suggest that urea application requires a minimum of 7.5 hours and beyond to inhibit more than 50% of eggs, in a pastoral system where eggs and larvae exists in faeces and throughout the pasture sward (Crofton, 1954; Familton and McAnulty, 1997; Pegoraro *et al*., 2008; Gazda *et al*., 2009), the LT_{50} might be extended due to various realistic factors restricting the amount of contact between nitrogen and nematode egg.

A realistic factor that might alter required exposure time would be the occurrence of rainfall or irrigation after fertiliser application. Irrigation is common practice in drought prone or summer dry areas like Canterbury and won't be sacrificed for the benefit of parasite control. If irrigation events or rainfall dilute topically applied urea and limit the effects seen *in vitro* then the time required for action to occur is very important. Urea fertiliser is generally applied shortly before rain/irrigation events (Black *et al*., 1987) to prevent ammonia volatilisation which can result in significant loss of plant available nitrogen, Holcomb *et al*. (2011) found a 14.6 ml water application directly after urea fertilisation limited volatilisation to 10%. However, this might provide a problem when using a topical application of urea to control free living parasites, if these early rainfall events reduce the level of nitrogen exposure by nematode eggs and larvae in the field. Bouwmeester *et al*., (1985) found 8 mls of water, applied 3 days after fertiliser application reduced volatilisation losses to 19%, which fits in with results from the exposure assay where exposure time of 16.2 ± 2.1 hours which reduced egg hatch by 95%. Ideally, exposure of larvae and eggs beyond 16.2 hours should be implemented, before irrigation or rainfall. A balance between reducing volatilisation and increasing egg hatch inhibition would exist which opens the potential for further study. In field studies of topical nitrogen application on egg hatch inhibition would be required to ensure the effect seen *in vitro* is replicated in real farm scenarios. Investigation of the timing of rainfall events in relation to the effect N application has on nematode populations should also be researched to determine the withholding time of water application to allow permanent hatch inhibition while reducing volatilisation.

Topical urea application, when applied at high enough concentration, reduced larval recovery in horse, deer and sheep nematodes highlighting a universal effect, this is an important practical consideration of on-pasture control using a urea application as treatment could be utilised by various pastoral farmers, extending the range of control and ultimately reducing the amount and frequency of anthelmintic use. The observations of embryonation in unhatched eggs in the EHA and LDA experiments of this study and by other researchers (Cairns *et al*., 2017; Peña-Espinoza *et al*., 2017; Anthony Page, *unpublished Observation*) implied the effect wasn't specific to urea or nitrogen application. The resulting effect of osmolality using a range of toxic and non-toxic mediums confirmed that hypothesis but further research into the exact mechanisms are required. Regardless, urea is an important tool to improve pasture production, N fertiliser is most efficient when applied between 20 to 40 kg N/ha per application (Cameron *et al*., 2005; Moir *et al*., 2003; Ball and Field, 1982; McLaren and Cameron, 1996; Rownlings *et al*., 2016) in terms of improving pasture response while minimising nitrate or nitrous oxide losses. 40 kg N/ha was selected as the urea treatment for all topical application as it aligns with farm practice optimums and best practice recommendations of fertiliser use (Cameron *et al*., 2005; Fert.Research, 2013), however increasing the rate toward 200 kg N/ha would increase the likelihood of reducing nematode hatch. It appears the recommended rate stops the lifecycle of some common nematodes, but a greater application rate is needed to treat *Nematodirus and Cyathostomums*, with a low dosage worsening the problem by promoting larval activity. To confirm the exact rate further LDA's and field trials are required, they would also help to determine if the effect is mirrored on-farm, which would determine the suitability of urea as a parasite control method.

The mechanisms and effect appeared to be universal across gastrointestinal nematodes, however there were limitations. The technical capability to separate deer and horse species means it is hard to determine if the variation between species was caused by differences in species biology and function or an unexplained effect is occurring. There were issues around optimization of egg hatch and development, especially *Nematodirus*, in part this was due to a lack of experience of staff influencing the strength of results. The hatch and development conditions of most nematode parasite larvae (9°<x<35°c, with adequate moisture, carbon dioxide and oxygen) (Silverman and Campbell, 1959; Familton and McAnulty, 1997; O'Connor *et al*., 2006; Sutherland and Scott, 2009) can be replicated *in vitro,* with full hatch typically within 24h (Familton and McAnulty; 1997) but the cold unit requirement of 800-1000 units for *N.spathiger* and *N.filicollis* (Oliver *et al*., 2016) and the limited technical capability of *Nematodirus* were the likely cause of poor recovery, and may have influenced the strength of results.

An effect of nitrogen application has been established but the mechanisms driving reduced hatch and development are still unclear, further work to determine the exact cause and the appropriateness of treatment in the field are still required before a suitable control can be recommended.

5.1 Summary

The effects of urea exposure on GIN egg hatch and larval recovery were consistent over a range of parasite species found within grazing ruminants, however there was some variation with higher recovery seen at lower fertiliser rates. There is strong support for the notion that urea application disrupts the nematode lifecycle of multiple species. There was an effect of urea exposure on egg hatch that required a minimum contact time and appeared to be irreversible and associated with osmolality.

The potential to inhibit parasite development using a topical fertiliser application remains, but further work to understand the mechanisms and assess the suitability of application in a realistic environment are required.

Appendix A

Osmolality

A.1 Osmolality of Sodium Chloride derived from AgSalt

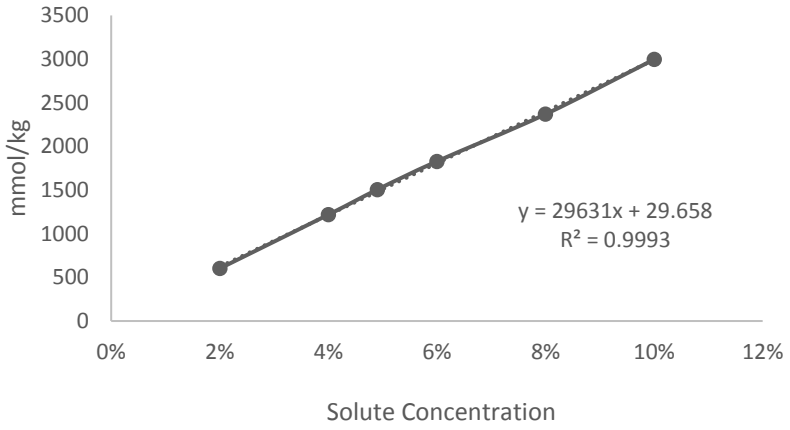


Figure A 1: Osmolality of a range of NaCl concentrations determined using the Wescor 5520 Vapro Osmolarity machine.

A.2 Osmolality of Glucose

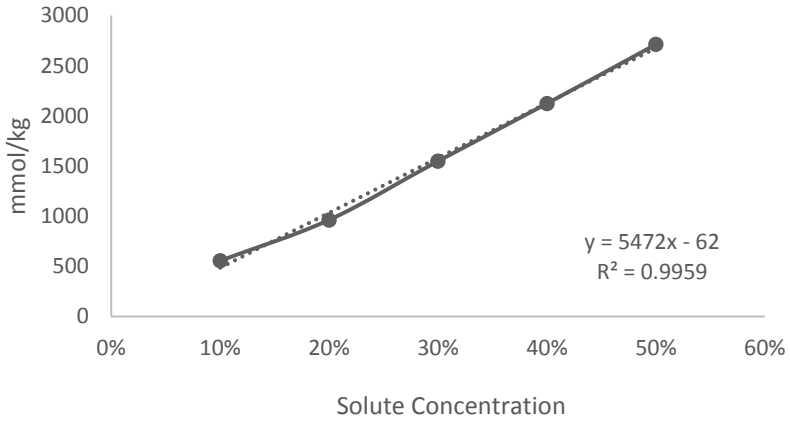
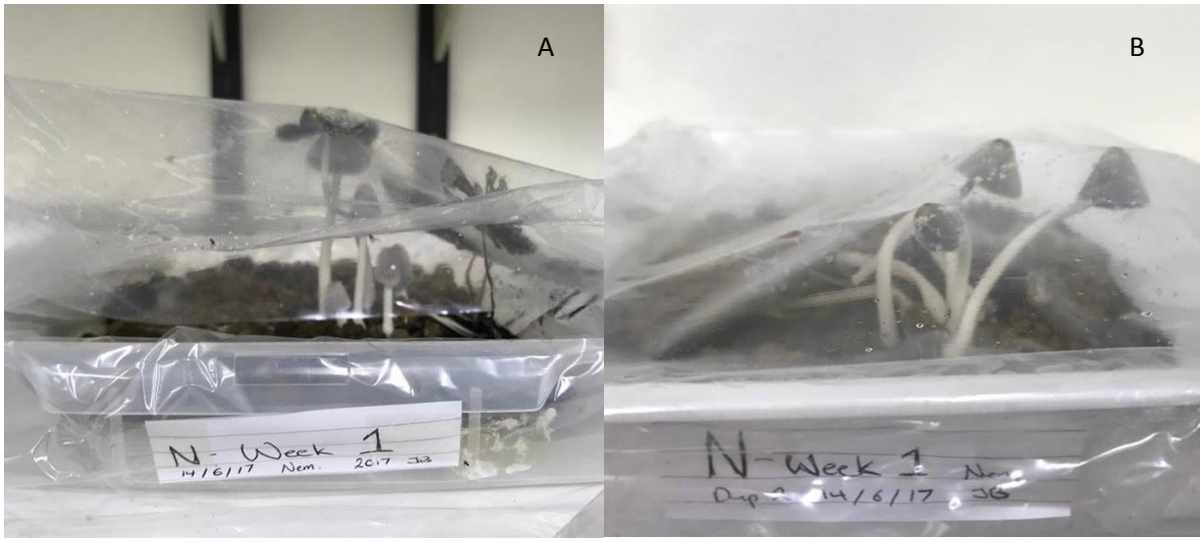


Figure A 2: Osmolality of a range of glucose concentrations determined using the Wescor 5520 Vapro Osmolarity machine.

Appendix B

Observations

B.1 Fungal Growth



B.1.1 Fungal Growth following N-W1. A: replicate one and B: replicate 2

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