Potential anthelmintic properties of urea

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Abstract

Current gastrointestinal parasite treatment options rely heavily on chemical anthelmintic intervention which targets the nematode residing in the host. However, targeting these organisms during parts of their lifecycle outside the host may also provide a tool for aiding control. In particular, nitrogenous fertilisers produce several potentially toxic compounds, the strategic use of which may affect nematode development. This series of experiments investigated the potential of liquid urea to affect gastrointestinal nematode development. Hatching of *Trichostrongylus colubriformis* eggs when immersed in liquid urea reduced with increasing urea concentration from 90% at 0% urea to less than 6% in 20% urea solution. This effect was independent of pH and appeared to be nematocidal rather than nematostatic as unhatched eggs failed to hatch following washing and immersion in water. Further, topical application of liquid urea to faeces at a rate equivalent to 200 litres per ha (40 units N) on fresh faeces resulted in a 97% reduction in the number of larvae recovered following culturing compared with equivalent rates of water ($P<0.01$). Although further studies and refinement are required, these studies indicated targeted application of liquid urea may be a useful tool to assist with parasite control.

Keywords: pH; nitrogen; fertiliser; gastrointestinal nematodes; Trichostrongylus colubriformis; egg hatching

Introduction

Gastrointestinal nematode (GIN) parasitism is a major constraint to high levels of productivity in all grazing animals. Typically, control of GINs relies heavily on the use of chemical anthelmintics. However, due to the widespread extent of anthelmintic resistance in New Zealand's parasitic populations, chemotherapy alone is no longer viewed as a sustainable method of parasitic control (Waghorn et al. 2006) and alternatives for nematode control need to be sought. One such approach may be exploiting the fact that parasite larval development from egg hatching through to the infective L3 stage occurs outside the host, thus providing an opportunity where the nematode life cycle can be interrupted to potentially provide effective disease control.

Parasite development can be affected by both pH and the presence of nitrogen. Development of the human gastrointestinal parasite *Ascaris suum* in sewage sludge has been reported to show an interaction between pH and the presence of nitrogen in the form of ammonia (Pecson et al. 2007). Relatively few studies that are specific to ruminant GINs exist, but development of *Haemonchus contortus* has been shown to be halted at pH<6 (Somerville & Murphy, 1983) whilst larval survival was shown to be sensitive to several nitrogenous fertilisers (Howell et al. 1999). Further, unpublished observations have shown egg hatching of the sheep gastrointestinal nematodes *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* can be inhibited when exposed to solutions containing a variety of fertilisers and acidic pH ranges (AW Greer and RW McAnulty, unpublished). However, the importance of either pH or nitrogen concentration for preventing nematode egg hatching is yet to be fully investigated. Further, if the application of fertilisers were to provide a parasitological benefit, it is conceivable that liquid application may give a more even coverage of the treatment area. This paper investigates the potential impact of both pH and liquid urea on *Trichostrongylus colubriformis* egg hatching.

Materials and methods

A series of experiments were carried out to determine the potential of liquid urea fertiliser and solutions of variable pH on GIN egg hatching and development, all of which used nematode egg-containing faeces collected from the same source.

Nematode egg collection

Two parasite-free 10-month-old wether (Hampshire) sheep were mono-specifically infected with *Trichostrongylus colubriformis* and used for the collection of nematode eggs. Faeces were collected from each animal with a harness holding a collection bag around the peri-anal region from 21 days post-infection. For each day of faecal collection, harnesses were emptied in the morning at approximately 9 am. Faeces were then collected up to two hours later, ensuring that all collected faeces were less than two hours old prior to processing.

Egg Hatch Assays

Collection of nematode eggs for each day an egg hatch assay was run was as follows: A 100g sample of fresh faeces was combined with 200 ml of tap water in a plastic bag and homogenized for 30 seconds using a stomacher (Colworth Stomacher 400). The liquid slurry was filtered through a 150 μm and then 38 μm pore sieve using pressurized water,

with the material retained on the sieve containing fecal debris and nematode eggs. Following centrifugation at 800 g for five minutes the supernatant was removed with a vacuum line before saturated sodium chloride was added to the pellet fraction and then centrifuged again at 800 g for five minutes allowing all eggs to float to the surface. The supernatant, which contained the eggs, was collected using a vacuum line and then washed in a 20 µm sieved container for 20 minutes with tap water. The cleaned eggs were then collected in water and counted to obtain the concentration of eggs in suspension which was adjusted as needed.

For each assay, 75 µl aliquots of the egg suspension were pipetted into the wells of a 24 multi-well plate, with an average of 60-80 eggs per well. Each test solution (described below for each assay in Experiment 2 and 3) was added to the egg suspension in 1 ml aliquots. Tap water was used as a control. This was repeated in triplicate for each test solution at each sampling time. The plate was gently agitated to mix the eggs and solutions, which were then incubated at 26°C for 24 hours. Immediately following removal from the incubator, further development of eggs was halted with the addition of several drops of Lugol's iodine into each well. The number of both unhatched eggs and L1 larvae in each well were recorded using an inverted microscope at X 100 magnification.

Experiment 1

The effect of topically applied liquid urea on parasite larval development was investigated. Faeces were collected as stated above. Faeces were gently mixed with care taken not to break up the faecal pellets. One hundred grams of faeces were then spread evenly across each of four plastic trays measuring 0.3 m by 0.2 m, ensuring pellets were not lying on top of each other. The trays containing faeces were placed on the ground in a paddock and were sprayed with either Flow-Fert N (20% liquid urea, Ravensdown, New Zealand) or water at a rate equivalent to 200 L per ha, with each treatment replicated twice. The trays remained outside for 14 h overnight before being placed in a plastic bag with several holes punched in the top for aeration and incubated at 26°C for 10 days. Following incubation, faeces were placed onto tissue paper in water-filled Baermann funnels for larval recovery for 48 h. One hundred ml of liquid that contained the larvae was collected from the base of the funnels. This was allowed to sediment then carefully reduced to 20 ml prior to counting by siphoning the upper layers. The number of larvae and unhatched eggs present were counted using four 50 µl aliquots from each using an inverted microscope at X 100 magnification.

Experiment 2

The effect of both pH and liquid urea on nematode egg hatching was evaluated using collected eggs and an egg-hatch assay (see above), with solutions of varying pH or urea added to each well. Solutions of varying pH were prepared at whole number intervals from pH 4 to pH 11 using 1% sodium acetate, 0.125M sodium hydroxide and acetic acid at either 1%, 4% or 100%. The pH solutions were stored at 4°C for three days to ensure stability and pHs were read with a Thermo-scientific pH probe (Thermo Scientific Orion Dual Star Meter). Liquid Urea was made up to a 10% concentration with water. Tap water was used as a control. Egg-hatch assays were performed in triplicate on each occasion then the whole assay process was repeated for three separate days.

Experiment 3

The optimum concentration of urea for inhibition of egg hatching was investigated using collected eggs and an egg-hatch assay as described above with the exception that the egg-hatch assays were performed using solutions of either 1%, 2%, 4%, 6%, 8%, 10%, 20% or 50% of urea solutions in water. Tap water was used as a control (0%).

Statistical analysis

All results are shown as arithmetic means. In Experiment 1, the mean total of larvae recovered from urea or water treated faeces were compared using a one-sided T-test on Minitab 16 (Minitab Inc, Version 6.1, 2016). In Experiments 2 and 3, the proportions of eggs that hatched were analysed by analysis of variance (ANOVA) with Minitab 16. Lethal doses (LD) were calculated using probit analysis (GenStat, 16th edition, Version 16.1, Rothamstead, VSN International). In experiment 3, the proportion hatched at each concentration underwent Receiver Operator Characteristic (ROC) analysis to determine the sensitivity and specificity at each fertiliser concentration (JROCFIT, John Eng, John Hopkins Hospital, 2016). Optimum concentration was determined from the maximum value of sensitivity plus 1 – specificity.

Results

Experiment 1

A clear visual difference was observed at the end of the incubation period with a white fungus present on faecal pellets sprayed with water but not on those sprayed with urea. Overall, 25,600±828 larvae were recovered from water-treated faeces compared with 800±100 larvae recovered from urea-treated faeces ($P<0.001$, Figure 1).

Experiment 2

The percentage of eggs that hatched relative to pH is shown in Figure 2. Overall, the percentage of eggs hatching was influenced by pH with less than 2% of eggs hatching at pH 5 or less. By comparison, at pH 6, 68±3.7% of eggs hatched and at pH 7 or above more than 90% of eggs hatched, an effect that was not influenced by further increases in pH ($P>0.05$). At comparable pH to tap water, egg hatching was not influenced by pH ($P>0.05$) but was less in urea ($P<0.001$). The mean percentage of eggs that hatched in tap water and urea was 90±2.28% and 6±1.76%, respectively. Visible differences were observed in the development of unhatched eggs of the urea compared with pH treatments. Unhatched eggs in the pH treatments showed no signs of development, whereas unhatched eggs in the urea treatment showed clear embryonic development with coiled larvae clearly visible.

Figure 1 Total number of *Trichostrongylus colubriformis* larvae recovered per 100 g of faeces following topical application of 20% concentrated liquid urea and water. Values represent the mean ± s.e.m. of two replicates (Experiment 1)

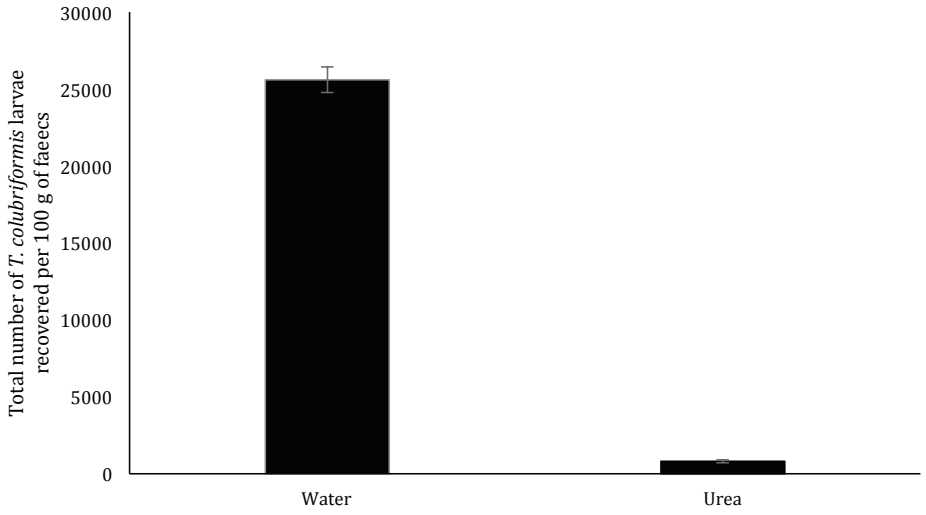


Figure 2 Percentage of *Trichostrongylus colubriformis* eggs that hatched following immersion for 24 h in solutions of varying pH that consisted of Sodium acetate (open circles) urea (open triangles) and tap water (closed squares). Each data point represents the mean ± s.e.m. of three replicates (Experiment 2)

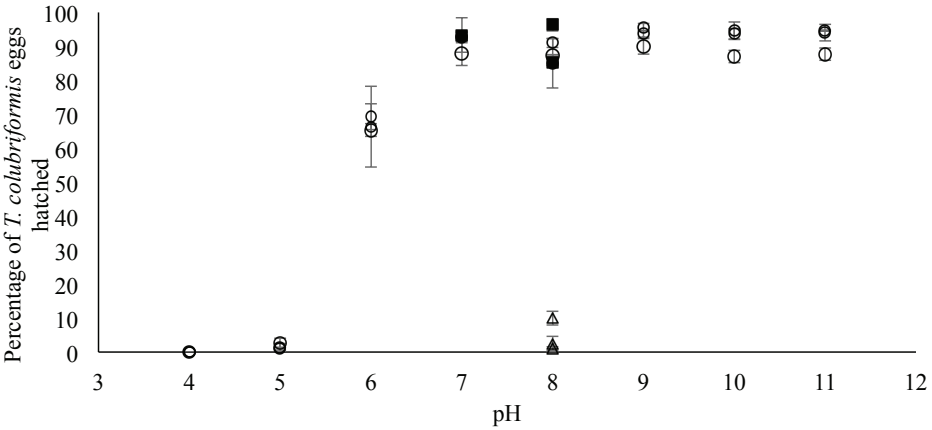
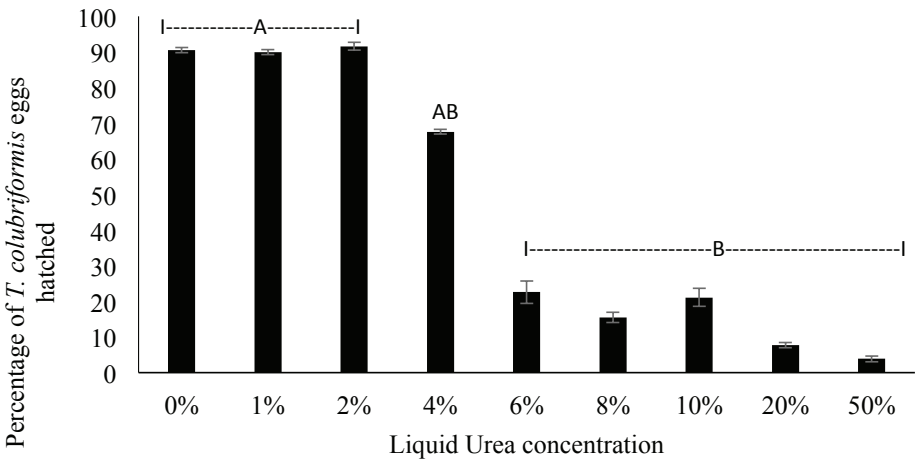


Figure 3 Percentage of *Trichostrongylus colubriformis* eggs that hatched following immersion for 24 hrs in solutions of various concentrations of liquid urea. A and B represent significant effects whilst those sharing letters have no significance (P>0.05) (Experiment 3)



Experiment 3

The average percentage of eggs that hatched following application of urea at varying concentrations is shown in Figure 3. Overall, egg hatching was sensitive to urea concentration, with no apparent effect on hatching at 1 or 2% urea concentration compared with the control (0%) ($P>0.05$). Hatching was then reduced to 23% at 6% ($P<0.001$) from which point further reductions in egg hatching with increasing urea concentration were not significant ($P>0.05$). The concentration required to prevent 90% of eggs from hatching (LD90) was 19.6±0.20%. ROC analysis of the data showed an area under curve of 0.893, indicating successful discrimination between true positives and false negatives. The biological optimum, where sensitivity plus 1 less the specificity, was at a maximum of 5.5% urea concentration. The electrical conductivity of urea solutions at concentrations 1%, 10%, 20% and 50% was 152.9 us/cm, 198.19 us/cm, 2289 us/cm and 237.19 us/cm respectively.

Discussion

Application of liquid urea has the potential to inhibit the development of the GIN *Trichostrongylus colubriformis*. The 97% reduction in the number of larvae recovered in Experiment 1 when urea was topically applied to fresh faeces provides strong evidence for the potential for urea treatment to add to GIN control programs through inhibiting larval development. This was achieved with the application of liquid urea at a rate of 40 units of N per ha, levels which are within the range in which urea may typically be applied on-farm. Perhaps surprisingly, despite their widespread use, there are few studies that have investigated the effect of fertilisers on GIN development. Howell et al. (1999) reported a decrease in

larval motility of *H. contortus* as nitrogen concentration increases, with the greatest change seen between 6% and 12% N. Relationships between urea applications and larval development have been indicated previously (Goode et al. 1974; Gates 1987) although these did not specifically investigate which larval stages were affected. Although further work is required to determine the sensitivity of other larval stages, it is clear from the current studies that urea can have a major effect on egg hatching. Receiver operator characteristic analysis indicated the optimum concentration at 6% urea at which point 80% of eggs failed to hatch. Translating even this relatively modest level of urea application into the field has the potential to provide a substantial benefit to GIN control programs through breaking the lifecycle outside the host.

In this study, liquid urea was specifically chosen as it is commercially available in liquid form which facilitates even application across the surface of the faeces. Given their aggregated nature, it remains to be determined if the application of solid urea granules would have the same efficacy. On the one hand, the ammonification process is similar for both solid and liquid urea, with the same potentially toxic forms of N produced which may have localised toxicity for GIN (Rodriquez-Kabana 1987). But on the other hand, it is possible the liquid form is more readily absorbed into the surface of the faecal pellet, allowing direct contact with the egg, although despite direct contact in the egg-hatch assays having an apparent nematocidal, rather than nematostatic effect, the importance of this cannot be substantiated from the current series of experiments.

Urea appeared to interrupt the hatching process. Clear visual differences in egg development were apparent between pH and urea treatments, with a developed larvae clearly visible in the urea treatments, indicating that the failure of these eggs to hatch was due to interruption of the hatching process *per se*, rather than a direct toxic effect on the egg. This effect was independent of both pH and electrical conductivity for urea and a range of other N-based fertilisers (Cairns, 2016). Stimulation of egg hatching is triggered by three things – increased levels of trehalose; increased cell permeability, caused by the trehalose; and biochemical action both inside and outside the egg (Perry 1989; Rogers & Brooks 1977; Gates 1987). Perry (1989) reported that as eggs developed, the shell of the egg became more permeable, allowing more biochemical agents to cross the biological membrane. However, as urea treatment did not appear to prevent larval development inside the egg, it can be speculated that the inability of larvae to hatch may be due to changes in the properties of the egg shell itself. This may be an important area for future research to assist in understanding the mechanisms involved in egg hatching and refine approaches aimed at preventing GIN egg hatching across a wide range of species.

Conclusion

At application rates that are within the range of normal farm practice, liquid urea appears to have strong nematocidal properties that reduce egg hatching and larval development. Although further work may be required to elucidate the mechanism and overcome environmental considerations, these results provide strong evidence to suggest that the targeted use of liquid urea may be a useful tool to assist with gastro-intestinal nematode control

Acknowledgements

We would like to thank Ravensdown for donating the urea for these studies. During the time of these studies Jessica Cairns was in receipt of a scholarship from Lincoln University Future Leader scholarship.

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