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Short communication

Chilling requirements for hatching of a New Zealand isolate of *Nematodirus filicollis*

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ABSTRACT

The eggs of some species of the parasitic nematode *Nematodirus* require a period of chilling before they can hatch; *N. filicollis* is one such species. This study investigated this requirement for chilling in a New Zealand strain of this species. Eggs of *N. filicollis* were extracted from lamb's faeces and incubated at 20 °C to allow development to the third stage larvae within the egg. These eggs were then placed into tissue culture plates and incubated at: 2.7 °C (±0.99), 3.6 °C (±0.90), 4.7 °C (±0.35), 6.4 °C (±0.37), 8.0 °C (±1.54) or 9.9 °C (±0.14) for up to 224 days. At 14 day intervals until day 84, then every 28 days, one plate was removed from each temperature and placed at 13.1 °C (±0.44) for 14 days. Eggs were then assessed for hatching. From this data, chill units were calculated by subtracting the culture temperature from a constant threshold of 11 °C and multiplying by the number of days for which the sample was cultured; then the Gompertz model fitted. Even though hatching overall was low, a greater proportion of eggs hatched with chill accumulation. Maximum hatching of eggs required 800–1000 chill units. Consequently in the field, more than one season of chilling would be required before hatching. As such a generation time could take more than one year to complete. This is different to the hatching dynamics of *N. spathiger*, the other main species found in New Zealand sheep, which does not display this requirement for chilling and hatches immediately once the third stage larvae are developed.

1. Introduction

Nematodes of the genus *Nematodirus* are important disease-causing parasites of lambs (Thomas, 1959), on occasions causing severe pathology and even deaths (Kates and Turner, 1953; Thomas and Stevens, 1956; Brunsdon, 1961). A characteristic of this genus is that development to the infective third larval stage (L3) occurs within the egg, allowing them to persist on pasture for long periods (Thomas, 1959; Thomas and Stevens, 1960). In addition, in some species, hatching occurs soon after the completion of development, while in others a period of chilling is required before the eggs will hatch (Thomas and Stevens, 1960; Van Dijk and Morgan, 2008, 2009; Oliver et al., 2014).

New Zealand has two species of *Nematodirus*, *N. spathiger* and *N. filicollis*, which are commonly found infecting sheep. These species appear to have quite different population dynamics in that *N. spathiger* eggs hatch readily after the completion of development to the L3 (Kates, 1950; Kates and Turner, 1955; Oliver et al., 2014), whereas *N. filicollis* eggs will not hatch, following the completion of development, without a period of chilling (Thomas and Stevens, 1960; Van Dijk and Morgan, 2009; Oliver et al., 2014). Better management of *Nematodirus* infection in lambs is likely to be assisted by a better understanding of their dynamics on pasture (Barger, 1999) and yet little is known about the epidemiology of these parasites in New Zealand. To improve our understanding of the population dynamics of *N. filicollis* this study was conducted to define the chilling requirements necessary for eggs to hatch after the completion of development to the L3.

2. Materials and methods

2.1. *Recovery of* Nematodirus *eggs*

Faeces containing *N. filicollis* eggs were obtained from a pooled collection from approximately 150 naturally infected 4 month old lambs. Eggs were extracted from faeces within 6 h of being collected using a tiered sieve method (Onyango-Abuje, 1984). In brief, faeces were mixed into a slurry with tap water, and then passed through three sieves of decreasing pore size (150 μm, 100 μm and 64 μm).

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Table 1
Species specific and generic primers, with their associated sequences (Bisset et al., 2014), the amount added to the reaction mixture and product sizes used to identify larvae to species.

Target species	Primer	Sequence (5′-3′)	Amount added (pmol)	Product size (bp)
Nematodirus filicollis	NEFIRV1	GGGATTGACTGTTACGATGTAA	5	162
Nematodirus spathiger	NESPRV1	CATTCAGGAGCTTTGACACTAAT	3	213
Oesophagostomum venulosum	OEVERV1	CGACTACGGTTGTCTCATTTCA	2	323–7
Chabertia ovina	CHOVFD2	CAGCGACTAAGAATGCTTTGG	3	115–7
Generic	ITS2GFnest	CACGAATTGCAGACGCTTAG	3	
Generic	ITS2GRnest	GCTAAATGATATGCTTAAGTTCAGC	3	

Eggs were retained on the 64 μm sieve. To obtain eggs with minimal faecal debris this was mixed with saturated NaCl (sg1.2) in a volumetric ratio of 1:14 to float the eggs. These were then recovered with a suspended 18.5 cm glass plate and were subsequently washed over a 64 μm sieve to remove the saline and stored in water.

2.2. Incubation of eggs

Eggs were incubated at 20 °C, in a small amount of distilled water for 116 days to allow development to L3 and hatching of non *N. filicollis* eggs. The eggs in a suspension were then placed on a 40 μm sieve for 24 h to remove any larvae that had hatched. Approximately 500 larvae were removed and 55 of these were identified by a PCR-based assay (see below). These were predominantly other trichostrongyle species (69.1%) or *N. spathiger* (27.3%), with the residual 3.6% identified as *N. filicollis*. It was initially assumed the remaining eggs were *N. filicollis* based on the typical *Nematodirus* morphology and because *N. spathiger* hatch readily once developed. To confirm this at the end of the experiment, L3 were identified by PCR to confirm if any *N. spathiger* were present (see below). The remaining eggs were recovered into distilled water and 1 mL was dispensed into the middle 4 wells of a 24-well tissue culture plate (Falcon BD) delivering approximately 25 eggs into each well. Water was added to the unused wells of the plate to provide humidity and the lid placed on the culture plate.

2.3. Study design

The developed eggs were exposed to one of six constant temperatures i.e. 2.7 °C (±0.99), 3.6 °C (±0.90), 4.7 °C (±0.35), 6.4 °C (±0.37), 8.0 °C (±1.54) and 9.9 °C (±0.14). Temperatures were monitored (Squirrel 1000 data loggers, Grant Instruments Ltd, Cambridgeshire, GB) throughout the experiment. At every temperature and for each sampling interval, one plate was incubated with each well representing one replicate, giving four replicates per temperature. At intervals of 14 days up to day 84, then every 28 days until day 224 one randomly assigned plate was removed from each temperature and placed at 13.1 °C (±0.44) for 14 days to allow hatching. The number of hatched L3 and the number of un-hatched eggs were then recorded for each well. At day 28, 100 μL of Amphotericin B was added to all the wells at a concentration of 1.5 μg/mL after fungi were found to be growing in some wells.

2.4. Identification of larvae

To confirm the hatched larvae were *N. filicollis* they were individually identified using a PCR-based assay that identifies species-specific sequences in the second internal transcribed spacer (ITS-2) of rDNA (Bisset et al., 2014; Oliver et al., 2014). In brief, larvae were lysed using10 μL of lysis solution (1 mL Viagen Direct PCR (tail), Viagen Biotech Inc., Ca, USA and 0.25 mL of PCR grade Proteinase K recombinant, Roche, Basel, Switzerland) in a thermal cycler for 16 h at 55 °C, 1 h at 90 °C, and 10 min at 4 °C, then placed at −80 °C for at least 30 min, followed by a 1:3 dilution with high performance liquid chromatography (HPLC) water.

The reaction mixture for the multiplex PCR assay consisted of 4 species specific primers and 2 generic primers (Table 1) that were added to: 1.5 mM $MgCl_2$, 1 μL 10 x *Taq* buffer, 100 μM of each dNTP, and 0.25 units Platinum®*Taq* DNA polymerase (Life Technologies) and HPLC water. Lastly 1 μL of template DNA was added for a total volume of 10 μL. The thermal profile used for the PCR was: 95 °C for 8 min, then 12 cycles of: 94 °C for 15 s, 60 °C reducing 0.5 °C/cycle for 15 s, 72 °C for 30 s; followed by 25 cycles of: 94 °C for 15 s, 54 °C for 15 s and 72 °C for 30 s. There was an additional extension of 72 °C for 7 min and cooling at 10 °C for 10 min.

PCR products were electrophoresed through a 2% agarose-TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH8.0) gel, containing SYBR Safe DNA gel stain (Life Technologies) and visualised using the electronic documentation program Gel.doc 2.6.4 (Bio-Rad) under ultraviolet *trans*-illumination conditions. The resulting bands were assigned to species based on their size (Table 1).

2.5. Statistical analysis

In order to fully evaluate the effect of different times and temperatures on egg hatching, a variable designated as chill units (CU) was calculated, using

$$CU = (11°C - t)\,d,$$

where t is temperature and d is number of days of storage at that temperature. The constant 11 °C was chosen as it approximates the threshold for development within the egg (Van Dijk and Morgan, 2009) and so it is unlikely that chilling requirements would be met at this temperature. Assuming similarity between isolates from different countries it seemed a reasonable estimate for a threshold temperature above which no chilling would occur. There were altogether 66 CU values generated.

The proportion of eggs hatching was averaged over the four replicate wells of each plate (representing the CUs) and the natural log of this mean plotted against CU values. The graph indicated a trend, supported by examination of residuals plots, with an asymptote with increasing CU values. A choice of weighted non-linear (asymptotic) regression models were then evaluated for goodness of fit. The weights were defined as proportional to the inverse of standard deviation over the 4 replicates. Seven CU values were removed from the analysis as the proportion values for all 4 replicates were zero. Of the models tested, the Gompertz model, defined as

$$y = Ae^{Bc^x},$$

gave the best fit (based on AIC criterion) with A = −1.8795, B = −0.8558 and C = 0.9985. The asymptote and the pseudo R-squared (measure of goodness of fit) were calculated. All statistical analyses were carried out using the R software (R Core Team, 2014).

3. Results

The majority (approximately 75%) of *N. filicollis* eggs did not hatch, even after 224 days storage at temperatures between 2 °C