



# Abomasal nematode species differ in their in vitro response to exsheathment triggers

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## Abstract

A crucial step in the infection process of grazing ruminants by gastro-intestinal nematodes is the exsheathment of the infective third-stage larva following ingestion. Recently, heat shock was shown to play an important role in the carbon dioxide (CO<sub>2</sub>)-dependent exsheathment response in *Haemonchus contortus*. The current in vitro study set out to evaluate the role of heat shock in other abomasal species. In rumen fluid, all species tested exsheathed rapidly and efficiently in response to heat shock and CO<sub>2</sub>. This response was significantly higher compared to slow temperature changes, supporting the hypothesis that heat shock plays an important role in vivo. However, in artificial buffer, the effect of heat shock was species-dependent. For *H. contortus* and *Ostertagia leptospicularis*, the response in artificial buffer was similar to rumen fluid. In contrast, *Ostertagia ostertagi* and *Teladorsagia circumcincta* exsheathment was significantly lower and/or slower in artificial buffer, and there was no benefit of heat shock. For these two species, it appears that there are co-factors in the rumen fluid, in addition to heat shock and CO<sub>2</sub>, contributing to exsheathment. Overall, the data indicate that there are significant differences between abomasal species in their response to exsheathment triggers.

**Keywords** Exsheathment · Temperature · Heat shock · Carbon dioxide · Parasite · Rumen fluid

## Introduction

Gastro-intestinal nematodes pose an important problem in grazing ruminants (Mavrot et al. 2015), yet surprisingly, little is known about the fundamental biology of the infection process. Following ingestion by a suitable host, the nematodes go through major changes transitioning from a free-living to a parasitic stage. The exsheathment of the infective third-stage larvae (L3) initiates this transition. The retained sheath of the second stage, which protects the free-living L3 from environmental factors (e.g. desiccation) whilst on pasture, is shed upon exposure to a stimulus from the host animal (Rogers 1960; Sommerville 1957).

For abomasal nematodes, two such stimuli are carbon dioxide (CO<sub>2</sub>) and temperature (Petronijevic et al. 1985; Rogers

1960; Silverman and Podger 1964; Taylor and Whitlock 1960). More recently, it has been shown that in *H. contortus*, a rapid temperature change or heat shock, rather than just elevated temperature, in addition to elevated levels of CO<sub>2</sub>, was required to obtain an efficient exsheathment response (Bekelaar et al. 2018). The research presented here extends this observation to the exsheathment of other abomasal nematode species.

## Methods

This study evaluated the role of heat shock in triggering exsheathment of four abomasal parasite species: the deer parasite *Ostertagia leptospicularis*, the cattle parasite *Ostertagia ostertagi* and the sheep parasites *H. contortus* and *Teladorsagia circumcincta*. All studies were carried out in the presence of saturated CO<sub>2</sub> and in two contrasting media, i.e. an artificial buffer system (Bekelaar et al. 2018) and rumen fluid. Since pH has previously been shown to influence exsheathment (DeRosa et al. 2005), the experiments were carefully designed to obtain a neutral pH.

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## In vitro exsheathment assay

Approximately 600 L3 in 1 mL of tap water (at 20 °C) were placed in 10-mL polypropylene tubes before 9 mL of medium (preheated to 40 °C for the heat shock treatments) were added to each tube. Six experimental conditions were compared. Larvae were exposed to one of three temperature regimes in either CO<sub>2</sub>-saturated artificial buffer (the “[Preparation of an artificial buffer medium](#)” section) or CO<sub>2</sub>-saturated rumen fluid (the “[Collection of rumen fluid](#)” section). The temperature regimes were (i) a rapid change from 20 to 40 °C and then maintained at 40 °C (heat shock), (ii) a gradual increase (5 °C per hour) from 20 to 40 °C and then maintained at 40 °C (slow temperature increase) or (iii) a constant 20 °C (control).

For each species, four replicates were tested for each experimental condition with the whole experiment being repeated using L3 from the same culture, to give a total of eight replicates (2 complete sets of 4 replicates each). Sampling to measure exsheathment was performed 0, 4, 8 and 24 h after the start of the experiment. At each time point, a subsample was collected from each replicate. Tube contents were thoroughly mixed before 1 mL (approximately 60 larvae) was transferred to a 24-well plate, larvae killed using 1 drop of 3% Lugol's iodine solution and exsheathment (complete or partial loss of the sheath) enumerated.

## L3 collection and maintenance

Pure cultures were generated by isolating species from their preferential host based on morphological identification (MAFF 1986), and validated by PCR in the next passage (Bisset et al. 2014). Cultures of *H. contortus*, *T. circumcincta* and *O. leptospicularis* were individually maintained by passage through otherwise parasite-free lambs, while *O. ostertagi* was cultured in calves. All animals were housed indoors on an artificial diet of commercial stock feed pellets, Lucerne chaff and fresh water. Fresh faecal material containing eggs was mixed with vermiculite, cultured at 23 °C for 14 days, before L3 were collected using the Baermann technique (Hendrix 1998) and stored at 8–10 °C. One hour prior to use, L3 were cleaned by gravity migration filtration through nylon mesh (pore size 20 µm), ensuring larval viability and motility.

## Preparation of an artificial buffer medium

The artificial buffer (Bekelaar et al. 2018) was a 10 mM phosphate buffer solution at pH 6.6 made using 3.75 mM Na<sub>2</sub>HPO<sub>4</sub>, 6.25 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub> and 0.5 mM CaCl<sub>2</sub>. The buffer was supplemented with NaHCO<sub>3</sub> to a final concentration of 72.5 mM and bubbled with 100% CO<sub>2</sub> 1 h prior to use. Buffer pH after saturating with CO<sub>2</sub> ranged between 6.5 and 6.8.

## Collection of rumen fluid

Rumen content was collected from fistulated cattle and squeezed through a cheese cloth to remove large particles. The animals were pasture grazed and supplemented with hay to achieve a neutral rumen pH (measured pH of 6.34) (DeRosa et al. 2005). Equal amounts of rumen fluid from two animals were combined and centrifuged (20 min at 2000 g) and the supernatant collected. The supernatant was bubbled with 100% CO<sub>2</sub> for 1 h prior to use.

## Statistics

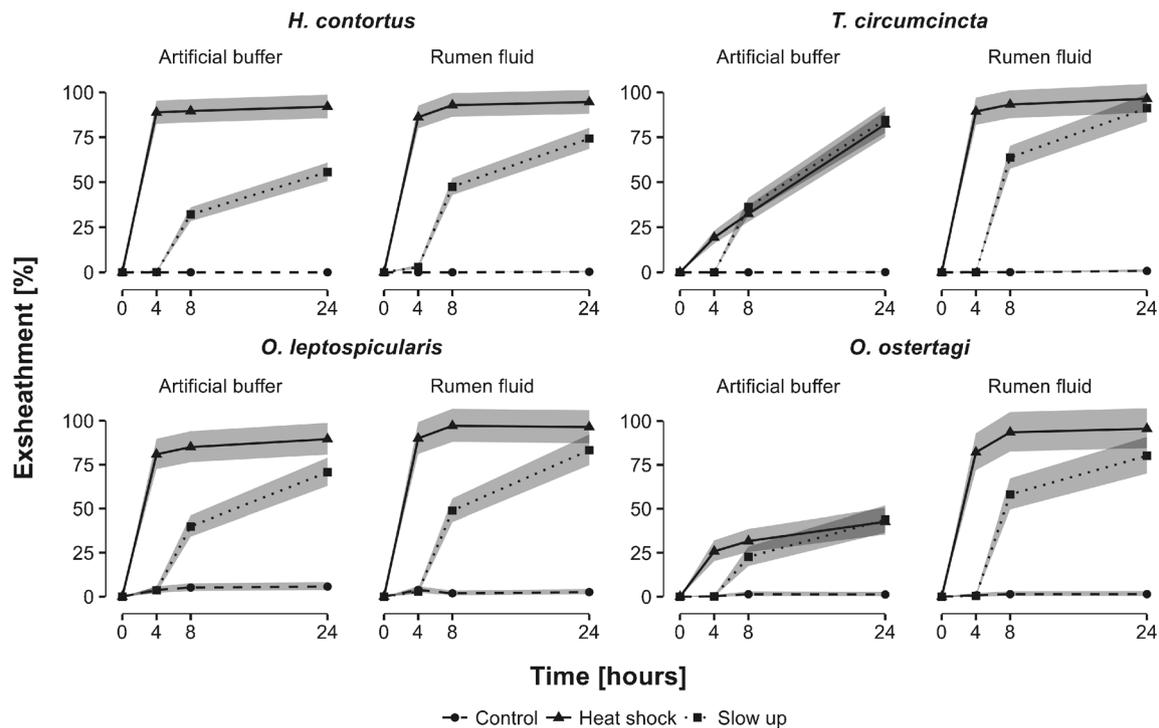
Analysis of variance (ANOVA) was carried out on the percentage of larval exsheathment. To meet the assumptions of ANOVA for normality and homogeneity of variance, the raw data was square-root transformed, and treatment combinations where all results were zero were omitted. The fitted model included the factors parasite species ( $n = 4$ ), medium ( $n = 2$ ), temperature regime ( $n = 3$ ) and time of sampling ( $n = 3$ ). “Experiment” was included as a blocking effect to correct for potential differences between experiments. Fisher's least significant differences post hoc test was used to compare means. Analysis was carried out using GenStat 18th edition (VSN International 2016).

## Results and discussion

All four species investigated here exsheath in the rumen (Hertzberg et al. 2002), and cross infection between different host species can occur (Borgsteede 1981). It could therefore reasonably be expected that they would respond similarly to exsheathment triggers. However, this appears to be not strictly the case.

In rumen fluid, efficient exsheathment (> 80% in under 4 h) was achieved in all four species following exposure to heat shock and elevated CO<sub>2</sub>, whereas exsheathment following a slow increase in temperature was significantly ( $p < 0.001$ ) delayed and/or reduced (Fig. 1). These findings support the hypothesis that heat shock plays an important role in vivo, and are consistent with earlier in vitro work in that heat shock rather than temperature itself is an important component of the exsheathment trigger (Bekelaar et al. 2018). This is not entirely surprising, given that susceptibility to exsheathment following slow temperature changes would put third-stage larvae at risk of exsheathment on pasture, as they are potentially exposed to elevated CO<sub>2</sub> levels (due to bacterial activity in soil and faeces) and high temperatures, especially in faecal pads (Berbigier et al. 1990).

For *H. contortus* and *O. leptospicularis*, a similar exsheathment response was obtained in artificial buffer compared to rumen fluid, although the response to slow



**Fig. 1** Heat shock and CO<sub>2</sub>-triggered exsheathment responses are species-specific in artificial buffer, but not in rumen fluid. Mean exsheathment response (%) (black data points and lines) ± 95% confidence intervals (grey shading) for each species in artificial buffer (left panel) and rumen fluid (right panel). Each species was subjected to three

temperature patterns in both media: a rapid change from 20 to 40 °C and then maintained at 40 °C (heat shock, solid line), a slow increase in temperature from 20 to 40 °C and then maintained at 40 °C (slow temperature increase, dotted line), or a constant 20 °C (control, dashed line). All presented data was back transformed

temperature increases (but not heat shock) was significantly lower in the artificial buffer (Fig. 1). It seems that for these two species, a combination of heat shock and elevated CO<sub>2</sub> is all that is required to trigger exsheathment.

In contrast, for *O. ostertagi*, < 45% of larvae exsheathed in artificial buffer in response to heat shock and CO<sub>2</sub>, and there was no benefit of heat shock over a slow temperature increase (Fig. 1). The exsheathment profile of *T. circumcincta* in artificial buffer showed a gradual increase in exsheathment over time regardless of whether the larvae were exposed to heat shock or a slow temperature increase (Fig. 1). The reduced exsheathment of *T. circumcincta* and *O. ostertagi* and the lack of response to heat shock in artificial buffer, but not rumen fluid, suggest that an undetermined factor present in rumen fluid is required to obtain high and rapid exsheathment of these species. Some potential suspects are a higher ionic strength, differences in calcium or salt contents, presence of micro minerals such as copper, manganese or iron or the presence of volatile fatty acids (Petronijevic and Rogers 1987; Saleem et al. 2013; Sykes and Coop 2001; Taylor and Whitlock 1960). Further investigation to determine the co-factors involved is required.

Interestingly, these data highlighted some important differences between abomasal nematode species. To date, findings on the exsheathment of one species have often been extrapolated to nematodes with the same predilection site, but

potential differences between them have not been thoroughly investigated. Two earlier studies reported mild differences in response to exsheathment stimuli between the abomasal species *T. axei* and *H. contortus* (Rogers 1960; Rogers and Sommerville 1960). Given the variation that is known to exist between different cohorts of larvae (Petronijevic et al. 1985; Petronijevic et al. 1986; Rogers 1960) (Sauermann et al. unpublished), it is unclear whether these findings constitute differences between species, or differences between cohorts. In the current study, all species were equally capable of efficient exsheathment, yet their response to a combination of two triggers (i.e. heat shock and CO<sub>2</sub>) was markedly different.

## Conclusion

This in vitro study found important differences between the four abomasal species in their response to exsheathment triggers. Heat shock and CO<sub>2</sub> were sufficient to elicit a strong exsheathment response in all species when conducted in rumen fluid but not in artificial buffer. In addition, the effect of heat shock in artificial buffer proved species dependent. These differences suggest that the required exsheathment triggers are not the same for all abomasal species, and that there are co-factors in rumen fluid, in addition to heat shock and CO<sub>2</sub>, which contribute to triggering exsheathment in some species.

Why there should be differences in the prerequisites for triggering exsheathment between abomasal species remains unclear.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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