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ORIGINAL PAPER

Heat shock, but not temperature, is a biological trigger for the exsheathment of third-stage larvae of *Haemonchus contortus*

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Abstract

Gastrointestinal parasites are an important health issue in grazing ruminants. Understanding the processes involved in the transition from the free living to the parasitic life stage of these nematodes is one avenue to identifying new targets amenable to future intervention. The transition to parasitism is initiated by exsheathment and is triggered by the sudden change in environment after ingestion of the infective larva by the host. Two major changes in environment are the increases in temperature and carbon dioxide (CO_2) levels. For CO_2 a role in exsheathment has been described previously, but the exact role of temperature was unclear. The current study is the first to investigate the importance of temperature in triggering exsheathment of *Haemonchus contortus*. Carbon dioxide induced exsheathment in *H. contortus* proved to be temperature dependent, as no exsheathment was observed at room temperatures. However, the temperature requirement to trigger exsheathment was quite specific. A rapid change in temperature (heat shock) very efficiently induced high levels of exsheathment. In contrast, when the larvae were exposed to a slow increase in temperature, the exsheathment response was smaller and delayed. Further investigation revealed that timing of the heat shock in relation to the CO_2 administration was crucial, as well as the final temperature and magnitude of the heat shock. In conclusion, these data indicate that heat shock rather than temperature itself is a crucial aspect in triggering the biological exsheathment cascade, and thus infection process, of *H. contortus*.

Keywords Exsheathment · *Haemonchus contortus* · Temperature · Heat shock · Carbon dioxide · Parasite

Introduction

Nematode parasites pose significant health and welfare issues for grazing ruminants in farming systems throughout the world. While clinical manifestations of infections occur only in a minority of cases, the relevance of subclinical infections on animal health as well as their economic impact is well recognised (Emery et al. 2016; Mavrot et al. 2015). Traditionally, parasite control has largely relied upon the use of anthelmintics, but the utility of these compounds is increasingly under threat due to the development of resistance (Hodgson and Mulvaney 2017; Kaplan 2004; Kaplan and Vidyashankar 2012; Waghorn et al. 2006a; Waghorn et al. 2006b). Therefore, it is becoming increasingly important to find alternative approaches to controlling infections. A detailed knowledge of the biological processes involved in the nematode life cycle is one approach to identifying potential drug targets or other weaknesses amenable to intervention, whether these are inside or outside the host animal. The study described here is part of a larger body of work aiming to improve our understanding of physiological processes in a number of important nematode species in an attempt to identify targets suitable for future intervention.

For most direct life-cycle strongyle nematodes, the free-living third-stage larva (L3) is capable of surviving long periods on pasture, awaiting ingestion by a potential host. This is because the larva is protected from the external environment by the retained sheath from the second larval stage, which fully encapsulates the L3 (Rogers and Sommerville 1963; Sutherland and Scott 2010). Following ingestion by a suitable host, this sheath is rapidly shed to initiate the parasitic phase of the life cycle. The *exsheathment* process (Rogers and Sommerville 1963; Sutherland and Scott 2010) is triggered

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in response to the sudden and substantial change in environment which occurs with ingestion (Rogers 1960; Sommerville 1957).

For abomasal parasites such as *Haemonchus contortus*, exsheathment occurs in the rumen, and so it is expected that the triggers to which the larvae respond are present there (Sommerville 1954; Sommerville 1957). Two major differences between the pasture and the rumen environments are the elevated temperature and the high levels of carbon dioxide (CO_2) present in the latter. A role for CO_2, or a related compound (such as dissolved CO_2 species or compounds mimicking CO_2), has been described as one of the chief factors stimulating exsheathment in *H. contortus* (Petronijevic et al. 1985; Rogers 1960; Rogers and Sommerville 1963; Silverman and Podger 1964; Taylor and Whitlock 1960). In a number of other nematode species, including *Trichostrongylus axei, Labiostrongylus eugenii* and *Anisakis* sp., an elevated temperature, close to that of the host's body temperature, has been shown to play a role (Campbell and Gaugler 1991; Rogers and Sommerville 1960; Smales and Sommerville 1977; Sommerville and Davey 1976). However, to date, the exact role of temperature in the transition to the parasitic life cycle of *H. contortus* has not been studied. The current project was initiated in order to investigate the importance and role of temperature in *H. contortus* exsheathment.

Methods

Two laboratory experiments were conducted. The first investigated the importance of the rate of change in temperature to both the triggering and exsheathment processes, in the presence and absence of CO_2, while the second investigated the importance of the magnitude of the change in temperature, and possible critical temperature thresholds.

L3 collection and maintenance

Pure cultures of *H. contortus* were maintained by regular passage through otherwise parasite-free lambs housed indoors. Fresh faecal material containing eggs was mixed with vermiculite and cultured at 23 °C for approximately 14 days. The resulting L3 were collected using the Baermann Technique (Hendrix 1998) and stored at 10 °C. Larvae from the same batch were used for all experiments in order to allow comparison of exsheathment percentages, as these are known to differ between batches (Petronijevic et al. 1985, 1986). Due to timing between experiments, L3 were 1–3 months old at time of use. Immediately prior to use, L3 were cleaned by gravity migration filtration through nylon mesh (pore size 20 μm), ensuring larval viability and motility.

Preparation of a 10-mM phosphate buffer

Three stock solutions were prepared: 100 mM Na_2HPO_4, 100 mM KH_2PO_4, and 50 mM $MgCl_2$ + 50 mM $CaCl_2$. Na_2HPO_4 (37.5 mL) and KH_2PO_4 (62.5 mL) were combined and made up to 990 mL with Milli-Q water. Then, 10 ml of the 50 mM $MgCl_2$ + 50 mM $CaCl_2$ solution was added. This resulted in a 10 mM phosphate buffer solution at pH 6.6. The buffer was freshly prepared before each experiment.

For experiments where CO_2-saturated buffer was required, 6.086 g/L of $NaHCO_3$ (final concentration 72.5 mM) was added to maintain pH 6.6 after saturation with CO_2. The CO_2-saturated buffer was bubbled for 30 min with 100% CO_2 at a high flow rate immediately prior to use.

Experiment 1—the nature of temperature change

Each sample (experimental unit) consisted of approximately 600 *H. contortus* L3 in 1 mL of buffer (at 20 °C, not CO_2-saturated) placed in 10 mL polypropylene tubes before 9 mL of buffer was added to each tube. The temperature (20 °C or preheated to 40 °C for heat shock experiments) and CO2 saturation (0% or 100%) of the added buffer was adjusted according to the experimental condition (see below) before adding it.

The treatment structure involved larvae being exposed to one of six temperature regimes (using fan-heated incubators) in the presence and absence of CO_2. Carbon dioxide-saturated buffer was administered at various time points to give a panel of combinations of temperature and CO_2 treatments (Fig. 1 an Table 1). For conditions where CO_2 administration was performed at a later time point, L3 were exposed to CO_2-free buffer at the start of the experiment (time point 0 (T0)) and at the appropriate time, the unsaturated buffer was removed and fresh CO_2-saturated buffer was added. The temperature of the CO_2-saturated buffer was adjusted to match that of the temperature pattern. If CO_2 administration preceded heat shock, the CO_2-containing buffer was removed, L3 washed twice with CO_2-free buffer and then given a heat shock using preheated CO_2-free buffer at T0.

Sub-sampling to measure exsheathment was performed at the initiation of the experiment (T0) and after 4, 8, 12, 16, 24, 36, 48, 72 and 120 h. Four replicates were tested for each treatment × sampling time combination with approximately 60 larvae being sampled per time point. Tube contents were thoroughly mixed before 1 mL was transferred to a 24-well plate and exsheathment enumerated. The complete set of treatment combinations (with 4 replicates) was repeated twice to give 8 replicates.

Experiment 2—the effect of final temperature and the magnitude of the temperature increase

Approximately 600 *H. contortus* L3 in 1 mL of buffer (at 19 °C) were placed in polypropylene tubes and acclimated

overnight, before 9 mL of CO_2-saturated buffer (preheated to the appropriate temperatures, see below) was added at T0.

1. In part 1, L3 were exposed to a heat shock of the same size (20 °C) with different initial and final temperatures, all in the presence of CO_2. Larvae were acclimated overnight to either 10, 15 or 20 °C (start temperature) and heat shocked to 30, 35 or 40 °C (final temperature) respectively. Larvae maintained at the starting temperatures (exposed to CO_2 at T0) were used as controls.
2. In part 2, L3 were exposed to a heat shock of different sizes with the same final temperature, all in the presence of CO_2. Larvae were acclimated overnight to either 10, 15, 20, 30, 35 or 40 °C (start temperature). The higher start temperatures were reached by slowly (5 °C per hour) increasing the temperature to 30, 35 or 40 °C. After acclimation, samples were heat shocked at T0 to 40 °C (final temperature). Larvae maintained at the respective starting temperatures (exposed to CO_2 at T0) were used as controls.

Sampling to measure exsheathment was performed after 24 h. Four replicates were tested for each experimental condition, and from each of the tubes, approximately 60 larvae were sampled per time point. Tube contents were thoroughly mixed before 1 mL was transferred to a 24-well plate and exsheathment enumerated. As above, the complete set of treatment combinations was repeated, using the same source batch of L3, to give eight replicates (two complete sets of four replicates each).

Measuring exsheathment

At the selected time points for each experiment (see above), L3 were killed by adding one drop of 3% Lugol's iodine solution to each well and larvae were subsequently examined at × 40 magnification using an inverted microscope (Olympus IX 73). Sheathed and exsheathed (complete or partial loss of the sheath) parasites were enumerated to calculate the percentage exsheathment.

Statistical analysis

For experiment 1, the effects of the temperature regimes and/or CO_2 administration (see Table 1 for combinations) on percentage exsheathment were compared by repeated-measurements ANOVA. In experiment 2, ANOVA was used to compare the effect of the magnitude and final temperature of the heat shock (see 1 and 2) on percentage exsheathment. For both experiments, "trial" was included as a blocking effect to correct for potential differences between the repeat of treatments within each experiments. The percentage exsheathment was square root transformed to meet the assumptions of normality and homogeneity required for ANOVA. Where the F-test was significant, Fishers least significant differences post hoc test was used to compare means for comparisons of interest. All analyses were carried out using GenStat 18th edition (VSN International 2016).

Results

Experiment 1—the nature of temperature change

Rapid changes in temperature

In the absence of CO_2, there was minimal exsheathment irrespective of the imposed temperature regime (Fig. 2). When temperatures were increased to 40 °C, either rapidly or slowly, and maintained at this level, exsheathment never exceeded 20% even after 120 h exposure (treatments (ii a), (ii d) and (iv a) in Fig. 2). No exsheathment was observed when L3 were maintained at constant 19 °C (treatment [i]; Fig. 2).

The highest percentage exsheathment was found when L3 were simultaneously exposed to a rapid shift to 40 °C (heat shock) and CO_2. Subsequent maintenance of L3 at 40 °C resulted in a further small increase in exsheathment from 79% after 4 h to 94% after 120 h (treatment [ii b]; Fig. 2). When larvae were exposed to simultaneous heat shock and CO_2 for 30 min and then returned to 19 °C (treatment (iii b)), exsheathment was lower ($p < 0.001$) but still substantial with 65% exsheathment observed after 4 h and 77% after 120 h (Fig. 2).

Sequential, as opposed to simultaneous, administration of triggers (heat shock and CO_2) did not stimulate exsheathment when the first trigger (either CO_2 or heat shock) was not maintained (treatments [ii d], [iii c], [iii d]; Fig. 2). When L3 were exposed sequentially to heat shock and then CO_2 while maintaining them at 40 °C during CO_2 administration (treatment [ii c]), an exsheathment response was observed (Fig. 2), but this only amounted to 22% exsheathment after 4 h which was significantly lower ($p < 0.001$) than the 80% measured when both were administered simultaneously.

Slow changes in temperature

When the temperature was increased slowly to 40 °C (treatment [iv]) in the presence of CO_2, a low initial exsheathment response was obtained which gradually increased over time. Four hours after reaching 40 °C, about 5–6% exsheathment was observed (T8) but this had increased to 65–70% after 120 h (Fig. 2). This contrasted with heat shock-stimulated exsheathment ($p < 0.001$), where the bulk of responding L3 did so within the first 4 h.

When the temperature was slowly cycled between 19 and 40 °C (treatment [v]; as opposed to maintained at 40 °C (treatment [iv])), such an increase with time was no longer

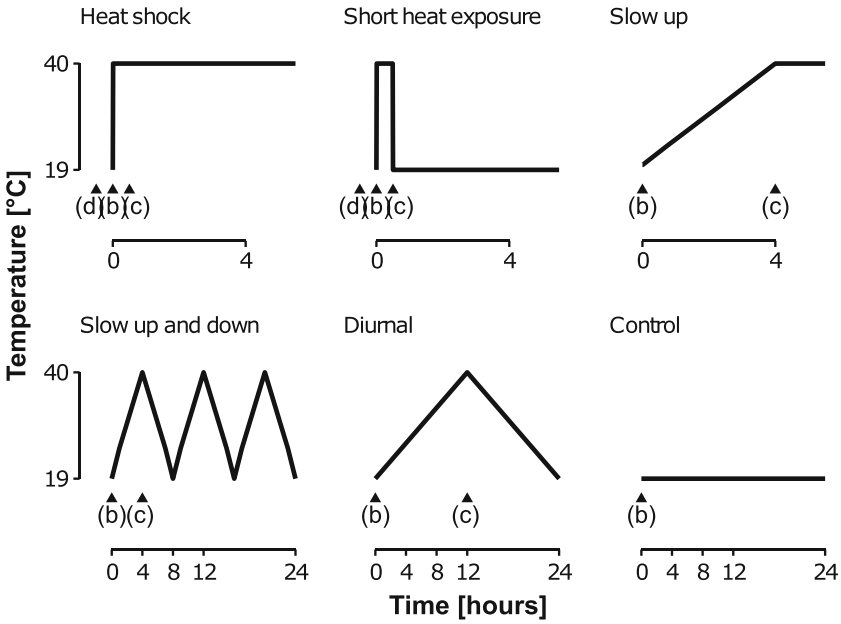

Fig. 1 Visual representation of the different temperature and CO_2 administration regimes. Parasites were exposed to temperature changes between 19 and 40 °C according to six different temperature regimes (Table 1). Temperature patterns were continuously cycled (bottom panels) or maintained at final temperature (top panels) for the remainder of the experiment (total duration 120 h). For each temperature regime, the CO_2 administration time points for the different treatment conditions are indicated (labelled arrowheads, see also Table 1)

observed, and exsheathment levels remained below 10% (Fig. 2). For both temperature regimes, CO_2 was added either at the start (treatments [iv b]; [v b]) or once 40 °C was reached (treatments [iv c]; [v c]). No substantial differences were observed between either condition. When the temperature changes were slower, the effect on exsheathment was similar when CO_2 was added at 40 °C (treatment [vi c]), but no exsheathment was observed when CO_2 was added at the start (treatment [vi b]; Fig. 2).

Experiment 2—the effect of final temperature and the magnitude of the temperature increase

The response of L3 exposed to CO_2 and heat shock depended on the final temperature. Over 60% of L3 exsheathed after 24 h when transferred from 20 to 40 °C (Fig. 3). However, when the temperature changes were from 15 to 35 °C or from 10 to 30 °C, exsheathment was significantly lower ($p < 0.001$) at 28 and 4%, respectively (Fig. 3). In no cases did prolonged

Table 1 Description of different conditions used in experiment 1

Temperature pattern	Description	CO_2 administration
[i] Control	Continuously kept at 19 °C	[a] No CO_2 [b] CO_2 added at the start (T0)
[ii] Heat shock	Rapid change from 19 to 40 °C, maintained at 40 °C	[a] No CO_2 [b] CO_2 concurrent with heat shock (T0) [c] CO_2 added 30 min post heat shock, while L3 were at 40 °C (T0.5) [d] CO_2 added 30 min prior to heat shock. No CO_2 was present during heat shock (T-0.5)
[iii] Short heat exposure	Rapid change from 19 °C to 40 °C environment (heat shock), maintained at 40 °C for 30 min and then cooled down to 19 °C	[a] No CO_2 [b] CO_2 concurrent with heat shock (T0) [c] CO_2 added 30 min post heat shock, while L3 were at 19 °C (T0.5) [d] CO_2 added 30 min prior to heat shock. No CO_2 was present during heat shock (T-0.5)
[iv] Slow up	Slow increase from 19 to 40 °C (5 °C per hour), maintained at 40 °C	[a] No CO_2 [b] CO_2 added at the start while L3 were at 19 °C (T0) [c] CO_2 added after 4 h just as L3 reach 40 °C (T4)
[v] Slow up and down	Cycling between 19 and 40 °C (5 °C per hour increase and decrease)	[a] No CO_2 [b] CO_2 added at the start while L3 were at 19 °C (T0) [c] CO_2 added after 4 h just as L3 reach 40 °C (T4)
[vi] Diurnal	Cycling between 19 and 40 °C over 24 h (3.5 °C per 2 h)	[a] No CO_2 [b] CO_2 added at the start while L3 were at 19 °C (T0) [c] CO_2 added after 12 h just as L3 reach 40 °C (T12)

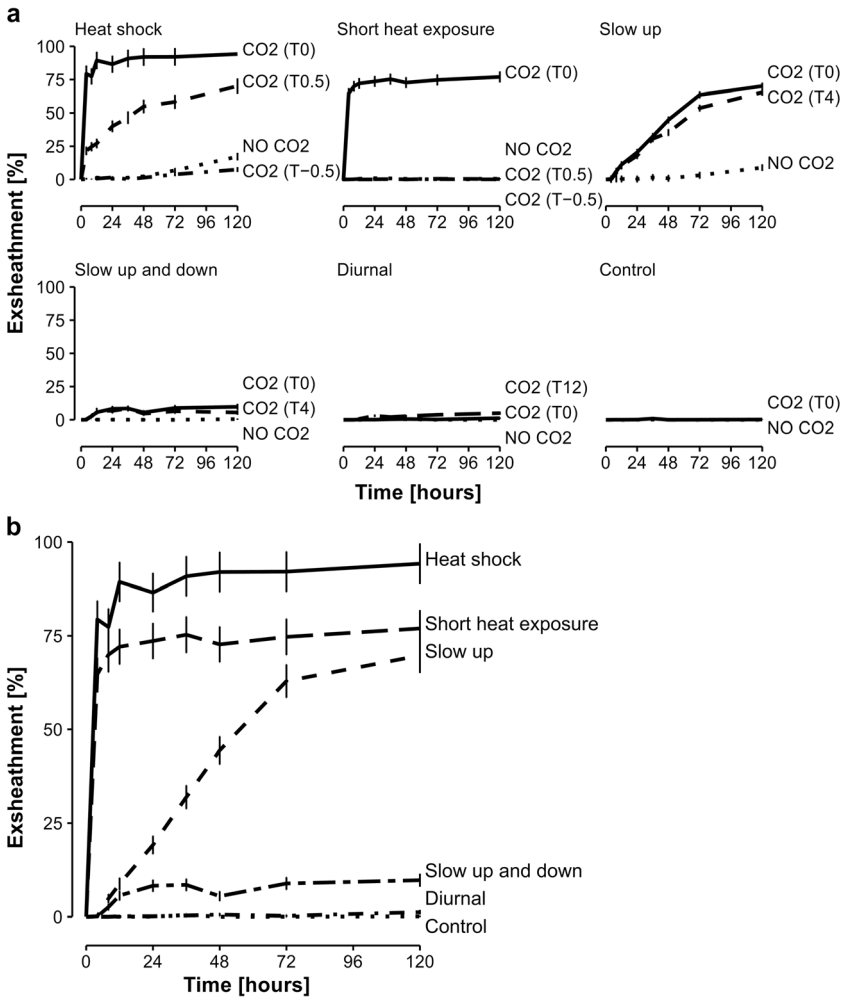

Fig. 2 The CO_2-dependent exsheathment response depends on the nature of the temperature change. **a** Mean exsheathment response (%) (± SEM) over time for each temperature pattern. For each pattern, various CO_2 administration conditions were used (see labels; see also Fig. 1 and Table 1). **b** Mean exsheathment response (%) (± SEM) over time when CO_2 was administered at the start of the experiment (CO_2 (T0); treatment [b]). The various temperature patterns are depicted (see labels; see also Fig. 1 and Table 1). All presented data was back transformed

exposure to the starting temperatures in presence of CO_2 induce exsheathment (Fig. 3).

The magnitude of the temperature increase during heat shock also influenced exsheathment rate. A heat shock with a total temperature difference of 10 °C or less (with a final temperature of 40 °C) elicited < 30% exsheathment after 24 h. Significantly higher ($p < 0.001$) exsheathment was obtained with temperature differences of 20, 25 or 30 °C (also with a final temperature of 40 °C), resulting in 60, 62 and 70% of larvae exsheathing (Fig. 3), respectively. Exsheathment after prolonged exposure to the starting temperature in the presence of CO_2 was only observed at 40 °C (Fig. 3) similar to the observations in Experiment 1 (treatment [iv c]).

Discussion

This work presents the first study focussed on understanding the role of temperature in triggering the exsheathment of *H. contortus*. As with other nematode species (Petronijevic et al. 1985; Rogers 1960; Rogers and Sommerville 1963; Silverman and Podger 1964; Taylor and Whitlock 1960), the CO_2-induced exsheathment in *H. contortus* was a temperature-dependent process. However, our experiments showed the temperature requirements to trigger exsheathment to be dependent on the rate and magnitude of the temperature change. A rapid change in temperature (heat shock) to a minimum threshold of 35–40 °C is far more effective at triggering exsheathment than constant or slow-changing elevated temperature alone.

This refutes the hypothesis underlying all earlier work, which assumed an elevated temperature to be a side-requirement for the CO_2 trigger. The current results imply that CO_2 and heat shock are two independent triggers working synergistically to stimulate exsheathment. This is an important distinction when investigating the biological mechanisms underlying the exsheathment process and might shed new light on some puzzling results seen by others (Davey et al. 1982). For example, the release of noradrenaline after stimulation of exsheathment might be triggered by heat shock, and thus entirely independent of CO_2 (Davey et al. 1982; Rogers and Head 1972). Future work on the biological pathways activated by heat shock might enable identification of drug targets

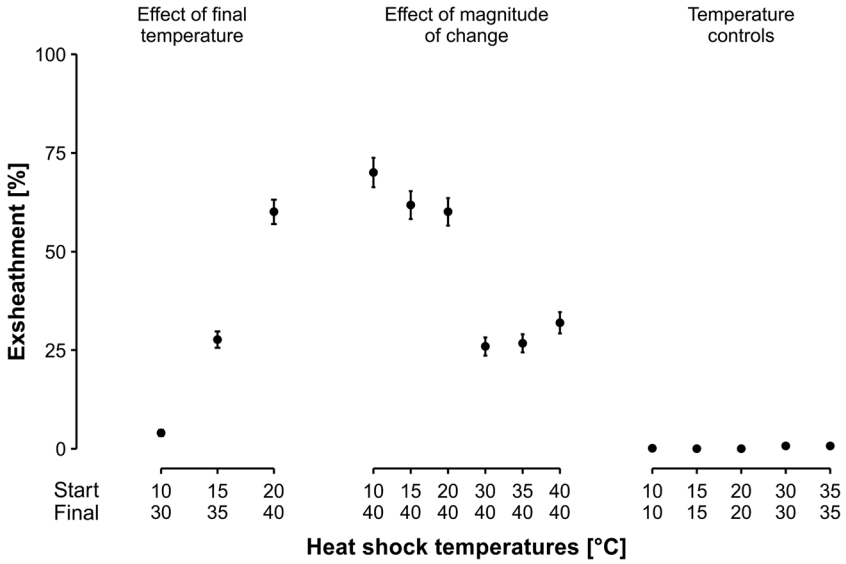

Fig. 3 The effect of the magnitude and final temperature of the heat shock. The mean (± SEM) percentage of larvae exsheathing 24 h after exposure to different heat shock regimes with CO_2 administered concurrently. Temperatures L3 were exposed to before (start) and after (final) heat shock are shown on the x-axis. Left: L3 were exposed to a heat shock of the same magnitude (20 °C) but with a different final temperature. Centre: L3 were exposed to a heat shock with variable magnitude (30, 25, 20, 10, 5 or 0 °C) to a final temperature of 40 °C. Right: L3 were maintained at the starting temperature throughout the experiment. All presented data was back transformed

inhibiting exsheathment and subsequent maturation and establishment of the parasites.

Heat shock in combination with CO_2 resulted in a rapid and efficient response, which was not prevented by a subsequent decrease in temperature. Given that few L3 exsheathed in the first 30 min (i.e., before the temperature was returned to 19 °C), this indicates that an elevated temperature was necessary to trigger exsheathment, but not for the exsheathment process itself. Once triggered, the L3 were capable of completing exsheathment at 19 °C.

These findings are similar to a number of studies in other nematode species. Recognition of the importance of an elevated temperature in the exsheathment of ruminant parasites was first indicated in an early publication that investigated the formation of refractile rings (Rogers and Sommerville 1960), one of the key features during exsheathment in the abomasal species *T. axei* and *Ostertagia* (*Teladorsagia*) *circumcincta*. Those authors found that refractile ring formation in *T. axei* did not occur at temperatures below 25 °C or above 60 °C, and the highest level of refractile ring formation was observed at temperatures around 40 °C. Further investigation on the timing of this temperature requirement showed that *T. axei* and *O. circumcincta* needed an elevated temperature during the triggering of exsheathment, but subsequent reduction of the temperature did not inhibit exsheathment (Rogers and Sommerville 1960). No in-depth description of the temperature transitions was provided for any of these experiments. Given the results of the current study, we speculate that the parasites were likely transferred rapidly from the storing temperature (2 °C) to the experimental temperature, exposing them to a heat shock in the process.

The data presented in this work revealed that the timing of the heat shock relative to CO_2 exposure played an important role in determining the magnitude of the exsheathment response. When CO_2 was administered 30 min after the heat shock (while maintaining L3 at 40 °C), the number of L3 that exsheathed was reduced compared to when both triggers were administered simultaneously. However, the response was faster than when L3 were exposed to CO_2 in combination with a slow temperature increase. Overall, the data imply that concurrent exposure to CO_2 and heat shock is an efficient exsheathment stimulus, but that sequential exposure to the same stimuli is far less effective. It is not at all clear why this should be the case, but the data suggest some kind of linkage between the response mechanisms such that the triggers are less effective when administered separately.

In contrast to the larval response to heat shock and CO_2, only a limited number of L3 exsheathed when the transition to a host-like temperature (40 °C) occurred slowly, even in the presence of CO_2. Moreover, to achieve more than 15% exsheathment, L3 needed to be continuously maintained at 40 °C for a protracted period, with at least 3 days of exposure required to reach 50% exsheathment. Given these extended periods required to achieve exsheathment with just elevated temperature alone, it seems unlikely that temperature itself, without heat shock, is a biological trigger. The flow of digesta through the gastrointestinal tract of a sheep is relatively fast, with a total transition time of 3–4 days (Grovum and Williams 1973). Infective L3 which do not exsheath in less than 3 days are therefore unlikely to still be in a position to establish, especially for species that inhabit the proximal gut. This hypothesis is also consistent with findings from other parasite

species, which have shown that L3 exsheath within a few hours of ingestion (Isenstein 1963). Thus, while elevated temperatures in conjunction with CO_2 can lead to exsheathment in vitro, it is much more likely that heat shock is the specific trigger in vivo.

Further investigation of the heat shock itself showed that there was a minimum temperature threshold below which triggering of exsheathment did not occur, i.e. no exsheathment occurred at or below 30 °C. In addition, a temperature shift of 20 °C (heat shock) was less efficient if the final temperature was below 40 °C. The magnitude of the temperature difference between initial and final temperature also affected exsheathment, with larger temperature differences resulting in greater exsheathment ratios. These data imply that the larvae were capable of responding to both the shift in temperature and the final temperature range, and that both are essential in determining whether exsheathment will subsequently take place.

Under temperate weather conditions, heat shock is an excellent candidate for an exsheathment trigger. However, in warmer climates, its role seems less obvious. Tropical parasites such as *H. contortus* (O'Connor et al. 2006) are often exposed to higher CO_2 levels (due to bacterial activity in soil and faeces), as well as environmental temperatures that could easily surpass 30 °C, and even reach 55 °C inside faecal pads (Berbigier et al. 1990). If parasite exsheathment were dependent on elevated temperature and CO_2, these pasture conditions might be able to trigger exsheathment outside the host. The heat shock requirement therefore provides the parasites a level of protection from premature exsheathment. On the other hand, heat shock and CO_2-induced exsheathment rates proved to be significantly reduced when parasites were first exposed to high environmental temperatures. This is not consistent with the prevalence of *H. contortus* in tropical environments. It is therefore likely that additional host factors are involved to increase exsheathment when the magnitude of the heat shock is smaller. In addition, the eating pattern of host animals (grazing when temperatures are lower in the morning or evening) could provide a natural mitigation of poor exsheathment when environmental temperatures are high. Further investigation into these hypotheses is required.

The only compound that, to our knowledge, has been shown to induce exsheathment at room temperature is sodium hypochlorite. However, sodium hypochlorite has been determined as a desheathment chemical rather than a compound that induces active L3 exsheathment (Campbell and Gaugler 1991; Lapage 1935; Silverman and Podger 1964). Currently, sodium hypochlorite is often used to obtain exsheathed larvae for research purposes (Chan-Perez et al. 2017; de Oliveira et al. 2011; Oliveira et al. 2011; van Wyk 1998), but its use may also damage the larvae themselves (van Wyk 1998). Based on the results presented here, a 30-min exposure to heat shock at 40 °C in combination with CO_2 followed by a 4-h incubation period might be a better alternative, at least for some parasite species. It is a quick and non-labour-intensive method to obtain larvae that are exsheathed in a biological manner, and which does not risk damage to the larval body.

In conclusion, the current results indicate that heat shock, rather than temperature itself, is a crucial trigger for in vivo exsheathment of *H. contortus* L3. Combined with elevated CO_2 concentrations, the two triggers act synergistically to generate an exsheathment response. Further elaboration of these findings may provide insights into potential novel avenues of antiparasitic treatments, inside as well as outside of the host.

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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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