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Abstract

Cyathostomins are considered as the most prevalent and pathogenic parasites of grazing horses. The development on pastures of the free-living stages of these gastrointestinal worms is particularly influenced by outdoor temperature. Understanding the bionomics of free-living stages is an important prerequisite to implement mathematical models designed to assess the parasitic risk for grazing equids. The aim of this study was to assess the effect of 3 constant temperatures under laboratory conditions (10±1°C, 23±2°C, 30±2°C) and one fluctuating temperature under outdoor conditions (mean: 17±4°C) on the minimum time taken by cyathostomin eggs to develop into first/second stage larvae (L1/L2) then into infective third stage larvae (L3) in horse faeces.

According to the temperatures, the minimum time taken by eggs to develop into L1/L2 was between 1 and 3 days and into L3 between 4 and 22 days. At 10°C, the development time of eggs into L3 was the longest and at 30°C the fastest. The results were consistent with historically available data and their compilation should lead to the improvement of parameterised models assessing the parasitic risk period in grazing equids.

Keywords: Horse, cyathostomin, free-living stages, larval development, temperature

Introduction

Cyathostomins (Small Strongyles: Nematoda, Strongylida) are considered as the most common parasites in grazing equids and usually the most prevalent cause of disease, ill-thrift and poor performance (Collobert-Laugier et al., 2002; Corning, 2009). The cyathostomin group consists of around 50 species, but most infections comprise only 5–10 common species (Lyons et al., 1999). Heavy infections can cause clinical strongylosis, especially in young animals (up to 2-3 years of age), the spring larval cyathostominosis (due to the simultaneous mass emergence of encysted fourth-stage larvae from the intestinal mucosa) being the most clinically severe leading sometimes to death. Such clinical infections occur in heavily infected animals which have been exposed to high pasture larval levels (Corning, 2009). The severity of these infections is therefore related to the factors having an effect on the development and survival of the free living stages on pasture, temperature being considered as the most important natural factor affecting those pre-parasitic stages, followed by moisture and sunlight (ultraviolet light) (Nielsen et al., 2007; Van Dijk et al., 2009). The studies focusing on the effect of temperature on the development of cyathostomins were carried out several decades ago and for some of them with artificial culture media (e.g., De Blieck et al., 1923; Hummelinck et al., 1946; Ogbourne, 1970; Rupasinghe, 1975; Mfitilodze and Hutchinson, 1987). Furthermore, since the introduction of highly efficacious anthelmintic drugs, the biology of free-living stages of equines strongyles has received scant attention and more interest should be paid to the actual larval biology in relation to climatic factors (Nielsen et al., 2007).

This relationship between larval biology and climatic factors is essential to develop model that mimic the parasite life cycle and the accumulation of free-living stages on pastures, such model allowing to better understand and predict the epidemiology of these infections. A model was carried out to describe the dynamics on pastures of the free-living stages of equine cyathostomins using the aged data (Leathwick et al., 2015).

The aim of this study was therefore to assess the effect of temperature (constant at the laboratory or fluctuating under outdoor conditions) on the minimum time taken by cyathostomins eggs to develop

into first/second stage larvae (L1/L2) then into infective third stage larvae (L3) in horse faeces. These results were compared to the available data to determine whether it could be necessary to update the parameterization of existing models and/or to populate the data for the construction of new models.

Materials and methods

Faeces of two 10 years old mares (trotters) were collected in September 2020. These mares were naturally infected with cyathostomins and had been grazing since March at the station of the French horse and riding institute (www.ifce.fr) in Normandy. From individual faeces samples, cyathostomin egg count was carried out through the modified McMaster technique with 15 eggs per gram sensitivity (Raynaud, 1970). Both animals presented a number of eggs of cyathostomins per gram of faeces > 500 (705 and 1545, respectively) classifying them as high egg shedders according to the guidelines suggested by the American Association of Equine Practitioners (AAEP Parasite Control Guidelines, 2019). Subsequently, individual faecal samples were pooled and 80 Petri dishes containing each 5 g of faeces were prepared and divided into four batches of 20 Petri dishes.

To explore a wide temperature range, three batches were placed in three different incubators set at 10±1°C, 23±2°C and 30±2°C. These temperatures corresponded to the ovens available in our laboratory. Moreover, 10°C and 23°C are respectively close to the average temperatures in France at the beginning of the grazing season (March-April) and in the middle of the season (July-August) while daytime temperatures often reach 30°C (https://meteofrance.fr). The last batch was placed outside in a shelter protected from the rain and in indirect sunlight (starting date: 15/09/2020, Fig. S1). The temperature at the location of the samples was taken and recorded every 30 minutes.

In each Petri dish, a constant faecal humidity level was maintained using a pH 7 commercial mineral water (Volvic®) as previously described (Collobert-Laugier et al., 1999). Faeces were then slightly re-moistened each day with a few drops of water. In addition, jars of mineral water were deposited in the incubators next to the petri dishes.

Every day from days 1 to 16, then on days 20, 22, 24 and 28, one of the 20 Petri dishes was collected from each batch and faeces were sedimented for 24 hours at room temperature in Baermann apparatuse (Baermann, 1917) to collect potential larvae.

Microscopical examination was carried out to detect larvae of cyathostomins and differentiate L1/L2 from L3 (Russel, 1948; Belivaqua et al., 1993). To facilitate larvae identification under the microscope, 10 µL of Lugol's iodine solution was added to 100 µL of the resulting filtrate to immobilize larvae.

Results

In coproscopical analysis and larval cultures, only eggs and larvae of *Cyathostomum* spp. were identified.

According to the temperatures, the minimum time taken by cyathostomin eggs to develop into L1/L2 was between 1 and 3 days and into L3 between 4 and 22 days (Table 1). At 10°C, the development time of eggs into L3 was the longest and at 30°C the fastest. With the outdoor conditions (temperatures ranging from 11-25°C with a mean of 17°C, Fig.S1), the results obtained were similar to those with 23°C.

Figure 1 summarises the data published in various countries worldwide on the minimum time taken by strongyle eggs in faeces to develop into L3 at different temperatures. These data were published between 1923 and 1987, and have been supplemented by our own results. Infective larvae were recovered from faeces incubated at temperatures between 7.5 and 37°C. Globally, larval maturing time was very slow at the lowest temperature and fast at the highest temperature (33-48 days at 7.5-8°C *versus* 1.7 days at 37°C, Fig. 1). However, important differences between published data in terms of minimum time taken by eggs in faeces to develop into L3 were observed at low temperatures: 15 days at 8°C (min: 33 days, max: 48 days) and 10 days at 10 °C (min: 15 days, max: 25 days),

129 respectively (Figure 1). Besides, at high temperatures (around 26-27°C), a variation ranging from 1 to
130 8.5 days was also identified.
131
132 **Discussion**
133 In this study, we examined the effect of temperature on the development of cyathostomin eggs into
134 L3 in order to estimate the time of onset of infective stages, with three different constant
135 temperatures and fluctuating temperatures under outdoor condition. In our protocol, a long
136 incubation time was allowed, especially for the low temperature (10°C), in order to avoid mistakenly
137 concluding that larval development had been arrested. Indeed, it had already been observed
138 previously that the development of eggs into L3 could take 48 days at 8°C (Shagalin, 1960).
139 In our results, the long delay between the appearance of L1/L2 and L3 stages at 10°C suggests that
140 the slowing in larval maturing was linked to a blocking development during the L1/L2 stages rather
141 than a longer hatching time.
142 When considering together data from several previous studies culturing eggs in which their
143 development into L3 took place in faeces, in overall, no difference in development time was
144 observed according to i) the year of the study, ii) the climate of the country in which parasites were
145 collected (continental/oceanic/tropical/Mediterranean/temperate climates) and iii) the strongyle
146 species (*Cyathostomum* spp*. versus* other strongyle species + unidentified).
147 However, data from the literature would indicate that differences between species are possible. As
148 an example, *Strongylus vulgaris* (large strongyle) was found to require a much longer time to develop
149 in faeces from L2 to L3 and then to complete it's development than the other strongyle species
150 (Ogbourne, 1972). In the Figure 1, at around 27°C, *Strongylus vulgaris* was also the slowest to
151 develop from eggs into L3 (8.5 days; Baruš, 1958) in comparison to *Trichonstrongylus axei* (4 days;
152 Mirzayans, 1969) and *Triodontophorus Tenuicolli* (4 days; Ortlepp, 1925).
153 In an artificial culture medium, Rupasinghe (1975) also observed that *Cylicostomum nassatus* differed
154 from other *Cyathostomum* spp*.* and was the quickest strongyle to become infective.

In our study, due to the absence of identification at the species level within *Cyathostomum* spp., it was not possible to compare the development between individual species in faeces. In addition, to establish the comparison of our results with the available published data, we did not include the data obtained from experimental studies using artificial culture medium (e.g., faecal agar, water) because the time of development of strongyle eggs/larvae has been showed to be slower on artificial culture medium than in faeces (Hummelinck, 1946; Ogbourne, 1970). Besides, in artificial medium, the lowest temperature at which the development into L3 took place was higher than in faeces (12°C *versus* 7.5°C respectively), probably due to an inadequate food supply (e.g., the bacteria added to the medium multiply too slowly) (Ogbourne, 1970; Rupasinghe, 1975).

Despite a strong relationship between temperature and development time, some variabilities between data might also be explained by differences in experimental protocols (e.g. eggs directly extracted from adult parasites *versus* from faeces, that is to say after a certain period of time after the laying of the female parasites).

In conclusion, despite the lack of replica our results are consistent with previously published findings and supplement current knowledge on the bionomics of cyathostomins. The use of two high egg shedders to collect eggs may have facilitated the observation of larvae in the filtrate/sediment from the Baermann apparatus. An important point revealed by this study is that the different species of strongyle/cyathostomin encountered throughout the world seem to share the same global relationship between incubation temperature and the time required for development into L3.

The compilation of these data should help to improve the parameterisation of models assessing the infective larval pressure on paddocks and then the parasitic risk period for grazing equids in order to help owners to optimise their use of anthelmintics.

Ethical statement: The procedures involving horses received approval from the Ethics Committee of Normandy (France), DGRI agreement APAFIS#2020041615267486. Animal studies were compliant with all the applicable provisions established by European directive 2010/63/UE. All the methods were performed by approved staff members in accordance with the relevant standard operating procedures approved by the abovementioned ethics committees. All the animals used in this study were handled in strict accordance with good clinical practices and all efforts were made to reduce animal stress.

Declaration of competing interest: The authors declare no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial and personal relationships that could inappropriately influence this work.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authors' contributions: AM conducted and supervised this study. All authors participated in the protocol development. MD and LB collected the faeces. CS and MB carried out the laboratory analysis. AM, LH and CS performed the data analysis. AM and LH drafted the article. All the authors critically revised the article and approved the final version before submission.

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Table 1. Minimum time taken by cyathostomin eggs to develop into first/second stage larvae (L1/L2) and then into infective third stage larvae (L3) at different temperatures

Temperature (°C)	L1/L2	Infective larvae (L3)
10	3 days	22 days
23	1 day	5 days
30	2 days	4 days
Outdoor condition[a]	1 day	5 days

[a]Mean: 17±4°C

Figure captions

Figure 1. Minimum time taken by strongyles eggs in faeces to develop into infective third stage larvae (L3) depending on incubation temperature. Data collected from the literature and supplemented by our own results.

[1] Ortlepp, 1925; Ogbourne, 1970

[2] Mfitilodze and Hutchinson, 1987

[3] Mikačić, 1953

[4] Our results

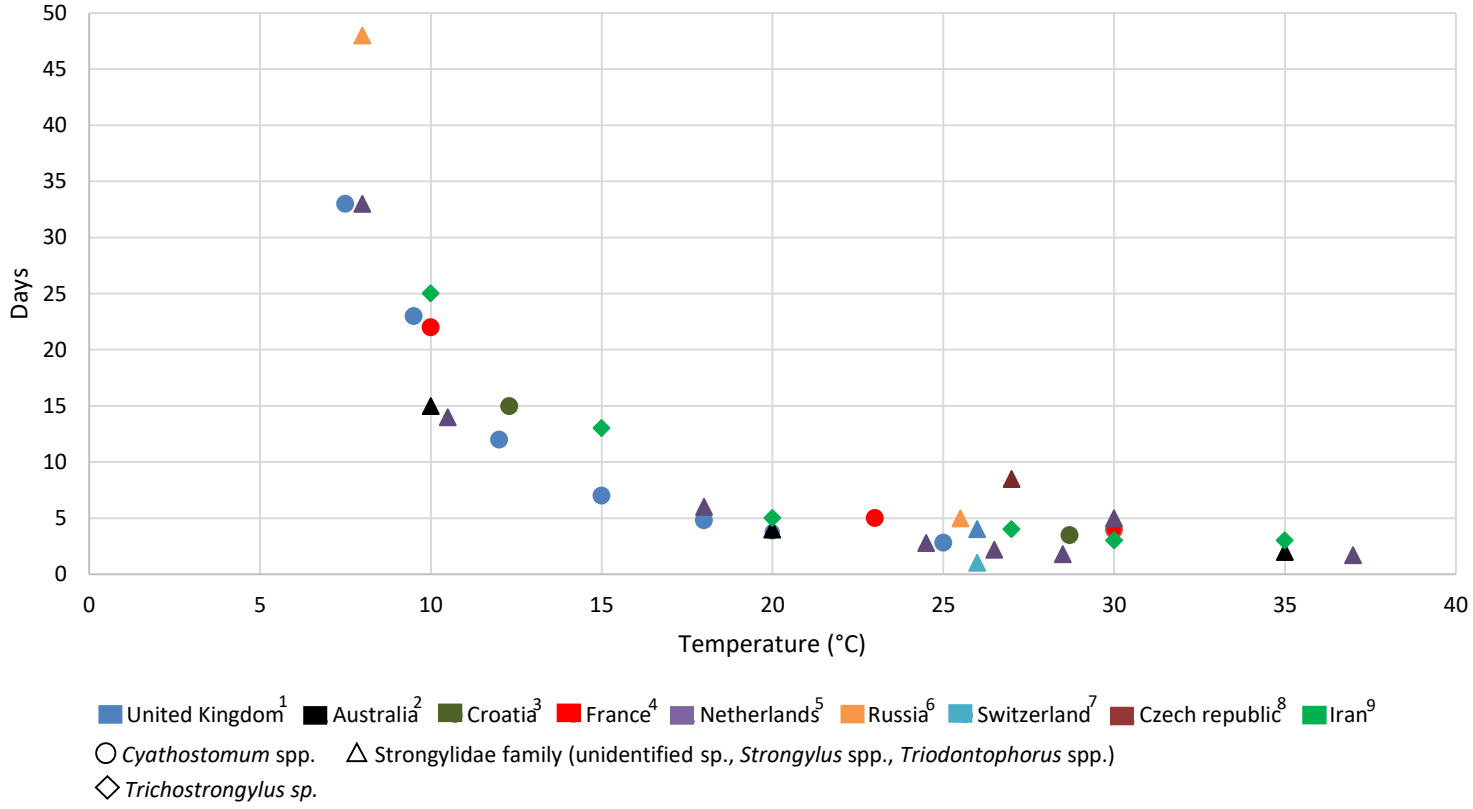
[5] De Blieck, 1923; Hummelinck, 1946

[6] Velichkin, 1954; Shagalin, 1960

[7] Wagner, 1938

[8] Baruš, 1958

[9] Mirzayans, 1969


50
45
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Days
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Temperature (°C)
United Kingdom[1]
Australia[2]
Croatia[3]
France[4]
Netherlands[5]
Russia[6]
Switzerland[7]
Czech republic[8]
Iran[9]
Cyathostomum spp.
Strongylidae family (unidentified sp., *Strongylus* spp., *Triodontophorus* spp.)
Trichostrongylus sp.