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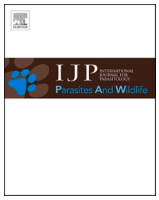

Environmental modulators on the development of the raccoon roundworm (*Baylisascaris procyonis*): Effects of temperature on the embryogenesis

Robin Stutz [a,b], Dorian D. Dörge [a], Anna V. Schantz [a], Norbert Peter [a], Sven Klimpel [a,b,c,d,*]

[a] Institute for Ecology, Evolution and Diversity, Goethe-University, Max-von-Laue-Str. 13, Frankfurt, Main, D-60438, Germany
[b] Senckenberg Biodiversity and Climate Research Centre, Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, Frankfurt, Main, D-60325, Germany
[c] LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), Senckenberganlage 25, Frankfurt, Main, D-60325, Germany
[d] Branch Bioresources, Fraunhofer Institute for Molecular Biology and Applied Ecology, Ohlebergsweg 12, Giessen, D-35392, Germany

A B S T R A C T

Members of the Ascarididae family are common zoonotic pathogens in humans and play an economic role in domestic and livestock animal husbandry. This family includes the obligatorily parasitic nematodes of the genus *Baylisascaris*, with the raccoon roundworm *Baylisascaris procyonis* being the most well-known representative. *B. procyonis* uses the raccoon (*Procyon lotor*) as its primary host and can utilise a broad range of mammals as paratenic hosts. Sexual reproduction of the adult nematodes occurs in the small intestine. Eggs are excreted into the environment through feces, where they develop into the infectious stage under suitable conditions within a few days to weeks. Infection of primary and paratenic hosts occurs through the oral ingestion of these infectious eggs. Raccoons can also become infected by ingesting infected paratenic hosts. Humans serve as accidental hosts and can suffer significant damage to organ tissues, the visual system, and the central nervous system after ingesting infectious eggs. The aim of the study was to investigate the effects of ambient temperature on embryonic development and to document the morphological changes during embryogenesis. Live specimens were collected from the raccoon intestine and incubated. Single-celled eggs were collected during this process. The eggs were decorticated and then preserved. To test the effects of ambient temperature, the eggs were incubated at 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, and 38 °C and monitored at 24-h intervals for their developmental stages. Detailed photographic documentation of the developmental stages was conducted. An increase in ambient temperature led to a reduction in development time. The temperature range within which embryogenesis proceeded to the L1 larval stage was between 10 °C and 30 °C. Incubation at 5 °C did not produce L1 larvae even after 11 months. Incubation at 35 °C and 38 °C resulted in the complete degeneration of the eggs before reaching the L1 larval stage.

1. Introduction

With over 25,000 species described to date, the phylum Nematoda is one of the most abundant and diverse monophyletic groups in the animal kingdom and the most species rich group of Metazoa (Bongers and Ferris, 1999; Hugot et al., 2001). The largest known part of the group is made up of parasitic species with over 8000 species parasitising in vertebrates, over 3500 species parasitising in invertebrates and over 4000 species parasitising in plants (Hugot et al., 2001). The actual number of existing nematode species is estimated to be between 500,000 and 1,000,000 (Hugot et al., 2001; Kiontke and Fitch, 2013). In addition to plants and wild animals, numerous species also can infest domestic animals, farm animals as well as humans. Nematodes of the family Ascarididae, which include highly prevalent pathogens of the genera *Ascaris* and *Toxocara*, are common zoonotic pathogens of humans (Abedi et al., 2021; Holland et al., 2022). This family also includes nematode species of the genus *Baylisascaris* with its most prominent representative the raccoon roundworm *Baylisascaris procyonis* (Sprent, 1968).

The genus *Baylisascaris* was first described by Sprent (1968) and today comprises 11 species which, apart from *Baylisascaris laevis*, utilise carnivores as their final hosts (Sprent, 1968; Camp et al., 2018). The first description of *B. procyonis* as *Ascaris procyonis* goes back to Stefanski and Zarnowski (1951). *Baylisascaris procyonis* uses the raccoon (*Procyon lotor*) as its final host and numerous other animals as paratenic hosts (Kazacos, 2001). Cases of both natural and experimental infections also have been detected in domestic dogs, other related procyonids and the Virginia opossum *Didelphis virginiana* (Kazacos, 2001; Bowman et al., 2005; Parkanzky, 2015), although there is uncertainty about the identity of the parasite species and the routes of infection in some cases (Parkanzky, 2015; Kazacos, 2016).

The life cycle follows a typical procedure for this group of parasites. It includes an adult phase inside the final host, a phase of embryonic development in the environment and a facultative larval phase in paratenic hosts. The adult *B. procyonis* nematodes parasitise in the intestinal tract of their final hosts where the oviparous females can excrete 115,000–179,000 eggs per day per worm (Kazacos, 1982, 2001; Snyder

* Corresponding author. Institute for Ecology, Evolution and Diversity, Goethe-University, Max-von-Laue-Str. 13, Frankfurt, Main, D-60438, Germany.
E-mail address: klimpel@bio.uni-frankfurt.de (S. Klimpel).

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and Fitzgerald, 1987; Reed et al., 2012). The eggs are released into the environment through the raccoons' feces, where infective egg stages develop after some time and can remain infectious for months and years even under extreme conditions (Kazacos, 2001; Pope et al., 2021). At this stage of the life cycle the eggs are directly exposed to the prevailing environmental conditions. There are several bioclimatic factors like ambient temperature, ambient humidity, soil type and moisture, pH, sun exposure or UV radiation, seasonal variation and aeration that are known to impact the viability of soil-associated parasitic nematodes (Boisvenue, 1990; Larsen and Roepstorff, 1999; Gamboa, 2005; Kim et al., 2012). The spread and transmission of *B. procyonis* takes place via the ingestion of infective egg stages by the raccoon or suitable paratenic hosts. Infected paratenic hosts are often subject to severe physical and neurodegenerative impairment due to tissue damage caused by the larvae migrating in the body (Tiner, 1953a, 1953b; Kazacos, 1982, 2001). *Baylisascaris procyonis* can utilise a wide range of vertebrates as paratenic hosts, particularly rodents and birds (Page, 1998; Page et al., 1998, 2001; Kazacos, 2016).

The infection of the raccoon occurs via two different routes. If the infected paratenic hosts are eaten by raccoons, the actual final host ingests the infectious larval stages through the body tissue of the paratenic hosts. In addition, oral ingestion of infective egg stages from the environment can lead to infection of the raccoon (Tiner, 1953a; Kazacos, 2001). Adult *B. procyonis* parasitise mainly in the small intestine of infected raccoons, where they reproduce sexually. For the final host *P. lotor*, no particular pathogenicity could be determined from the adult roundworms (Kazacos, 2001).

Under certain circumstances, humans can become infected with raccoon roundworm eggs as so-called dead end or accidental hosts. Infection of humans occurs exclusively through oral ingestion of eggs containing the infective larval stages from the environment (Kazacos, 2001; Graeff-Teixeira et al., 2016). Baylisascariasis can manifest itself in humans through a broad spectrum of symptoms with serious health consequences. Severe diseases are caused by the migration of the hatched larvae in the body where visceral organs, the visual system and the central nervous system are frequently affected (Kazacos, 1986, 2001; Graeff-Teixeira et al., 2016).

The ongoing spread of invasive raccoons in Europe is currently leading to a more widespread distribution of *B. procyonis* (Heddergott et al., 2020, 2023). The extremely opportunistic omnivorous diet and high adaptability of the final host *P. lotor* contribute greatly to its rapid and successful spread (Peter et al., 2024). The large and secure food supply of anthropogenic resources in urban habitats makes the raccoon a cultural successor, whereby the high concentration of resources can lead to an aggregation of raccoons in these habitats (Prange et al., 2004; Bartoszewicz et al., 2008; Cunze et al., 2023). In addition to economic damage, a high population density in urban areas also can increase the risk of disease transmission of raccoon zoonotic pathogens to humans, pets and livestock (Rosatte et al., 2010; Peter et al., 2023). Baylisascariasis represents the greatest risk of zoonotic transmission to date (Stope, 2019; Peter et al., 2023). In urban areas with high population densities of raccoons, a high density of latrines with infectious egg stages has already been detected (Roussere et al., 2003). In addition Ogdee et al. (2017) found that environmental factors such as rain or wind can disperse feces, including *B. procyonis* eggs, beyond the original latrine or defecation site. In addition to latrines where large numbers of eggs aggregate, raccoons also used isolated defecation sites. In central Germany, raccoon populations were found to have prevalence rates of the raccoon roundworm *B. procyonis* of up to 95 %, which suggests an increased potential for infection in these areas (Peter et al., 2023).

Despite the supposedly high prevalence of *B. procyonis* eggs in the environment, only a few cases of Baylisascariasis in humans are known in Germany, in contrast to the USA and Canada (Huff et al., 1984; Küchle et al., 1993; Graeff-Teixeira et al., 2016; Kazacos, 2016; Dunbar et al., 2019). It is assumed that asymptomatic courses account for a large proportion of actual infections. As a result, the actual prevalence of infection is probably underestimated (Conraths et al., 1996; Sapp et al., 2016; Weinstein et al., 2017). Furthermore, the variable clinical picture of Baylisascariasis is not yet widely recognised in Europe, so it is possible that disease outbreaks are misdiagnosed.

In order to draw conclusions about the risk of infection with *B. procyonis* and to generate a reliable risk assessment, the influencing bioclimatic factors and their effects on the life cycle of the parasite must be analysed.

The present study therefore focusses on the section of the life cycle of *B. procyonis* in which the parasite stages are exposed to different environmental influences. The effects of ambient temperature on the embryonic development of *B. procyonis* were investigated to draw conclusions about ecological interactions and infectiological risks. Focussing on the bioclimatic modulator of ambient temperature, the aim was to find out how it affects the embryogenesis of *B. procyonis*. A further aim was the precise photo documentation of the distinct developmental stages in the life cycle of the raccoon roundworm. To our knowledge, this is the first study on this scale to investigate the effects of temperature on the embryonic development of *B. procyonis*.

2. Materials and methods

2.1. Sampling and dissection of raccoons

A total of 33 raccoons from the Main-Kinzig district in the Gelnhausen region in south-eastern Hesse were caught, euthanised and provided by persons authorised to hunt in the course of legally regulated hunting. The rectal temperature of nine animals was measured directly after death to obtain a reference for the incubation temperature of the nematodes. Dissections were performed by first making a diamond-shaped incision from the tail root around the sphincter muscle at the anus and then opening the abdominal wall from the sternum. Because *Baylisascaris procyonis* nematodes primarily parasitise the small intestine, the thoracic cavity was not opened, and the entire organ complex was not removed. The small intestine was separated from the stomach below the pylorus at the duodenum. The intestinal complex was then removed together with the anus after opening the pelvic bone. The pH in the small intestine was measured as a reference for the incubation medium of the nematodes. The dissections of the small intestine were carried out no later than 1 h after death.

2.2. Incubation of nematodes

The found adult *B. procyonis* nematodes were removed alive and washed several times in 38 °C saline solution (0.9 % NaCl) to remove digestive secretions and food components. They were then incubated in a Goodwin's solution (0.2 g calcium chloride, 5 g glucose, 0.1 g magnesium chloride, 0.2 g potassium chloride, 0.15 g sodium bicarbonate, 8 g sodium chloride and 0.5 g sodium hydrogen carbonate, 1 L demineralised water (Donahue et al., 1981; Nicolas and Acero, 2019) in cell culture vials (200 ml). The temperature and pH of the medium were adjusted to 38 °C–39 °C and pH 6.5–7 based on the intestinal environment of the raccoon. In order not to completely submerge the nematodes, the vials were filled with 25–50 ml of culture medium, depending on the number of nematodes used, and then placed down. In order to minimise contamination of the culture medium, no more than 5–10 nematodes were used per vial. Care was taken to ensure that all vials were filled with both males and females. Single-cell eggs were removed from the culture medium at 24 h intervals by targeted pipetting and transferred to centrifuge tubes (50 ml). To keep the incubated nematodes alive for as long as possible, the culture medium was changed daily during egg collection.

2.3. Preservation of eggs

The centrifuge tubes containing the 1-celled eggs were then

centrifuged for 5 min at 1500 rpm to form a sediment of eggs. The sedimented eggs were then transferred to 0.5 % sodium hypochlorite solution and cleaned by stirring for 3 min on magnetic stirrers during which the outer egg layers were decorticated (Fig. 1.) (Ward and Fairbairn, 1972; Brownell and Nelson, 2006). Decortication of the outer layers of the eggshell reduces the adhesive properties of the eggs and thus prevents them from sticking together, to working materials and to surfaces. The eggs were then cleaned of the sodium hypochlorite residues by triple centrifugation and decantation in tap water and preserved at 5 °C in tap water filled centrifuge tubes until use.

2.4. Incubation and monitoring of cell stages

For incubation, the eggs were transferred to well plates in tap water (9,6–10,3°dH). The eggs were checked for their stage of development so that 100 1-celled eggs were used per well resulting in 600 1-celled eggs per plate and incubation temperature (6-well plate). To test the influence of temperature on egg development, the well plates were incubated at eight different ambient temperatures (5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 38 °C). The incubation temperatures were monitored and recorded day and night using temperature loggers (HOBO ONSET 1-800-LOGGERS). Humidity was kept constant by incubation in liquid medium.

The controls were carried out in 24 h intervals until the L1 larval stage was reached by 95 % of eggs (5 °C in longer irregular intervals). On each control date, 40 eggs per well were randomly selected and categorised according to their stage of development. No eggs were removed during the control, as the plates were examined directly under the microscope. As a result, the total sample size was not changed during the controls. The categorisation was based on the work of Cruz et al. (2012), Sakla et al. (1989) and the observations from our own preliminary studies on the organism *B. procyonis*. Eleven developmental stages were classified as sufficiently recognisable and categorizable during microscopic evaluation and in accordance with Cruz et al. (2012) and Sakla et al. (1989) (Table 1).

The test plates were incubated until 95 % of the observed eggs had reached the L1 larval stage. The L1 larval stage was chosen as the end point as it can be precisely distinguished optically from the pre-larval stages. An inverted light microscope (Leica DMi1) was used to check the egg stages.

Table 1
Stages of development of *Ascaris suum* (as defined by Cruz et al., 2012) used for developmental characterisation of *Baylisascaris procyonis* in the course of standard light microscopy. Endpoint 1 reached when 50 % of the subsample (n = 240) reached early-morula stage. Endpoint 2 reached when 95 % of the subsample (n = 240) reached L1-larva stage.

Stage of development	Characteristics observed in the developing embryo
1-, 2-, 3-, 4-cell	1,2,3 or 4 cells that are clearly well defined
Early-morula	5-10 cells within the developing embryo **[Endpoint 1]**
Late-morula	11 or more cells within the developing embryo
Blastula	A spherical layer of cells surrounding a pseudo fluid-filled cavity
Gastrula	A layer of cells surrounding the embryo plus a kidney-shaped invagination in one side of the embryo
Pre-larva 1	A larva-like coiled structure creating no more than one concentric ring inside the ovum
Pre-larva 2	A larva-like coiled structure creating at least one and a half concentric rings inside the ovum
L1-larva	A larva without well-defined structures inside the larva body wall, with no moult, and with intense motility in response to light **[Endpoint 2]**

In order to compare the effects of different incubation temperatures on the rate of development, two comparable endpoints were defined. Endpoint 1 was reached as soon as 50 % of the eggs from the subsample (n = 240) were at least in the early morula stage. The need for endpoint 1 resulted from the stop in development at 38 °C. Endpoint 2 was reached as soon as 95 % of the eggs from the subsample (n = 240) were at least in the L1 larval stage. To compare the development time required to reach both endpoints at different incubation temperatures, the required time was averaged over six wells.

3. Results

3.1. Incubation of nematodes and preservation of eggs

The maximum lifespan of the nematodes in the culture medium was eight days. By changing the culture medium daily, an average lifespan of 5 days could be guaranteed. By removing the culture medium at 24 h intervals, mature 1-celled egg stages could be reliably collected. Storage in water-filled, closed centrifuge tubes at 5 °C largely prevented further

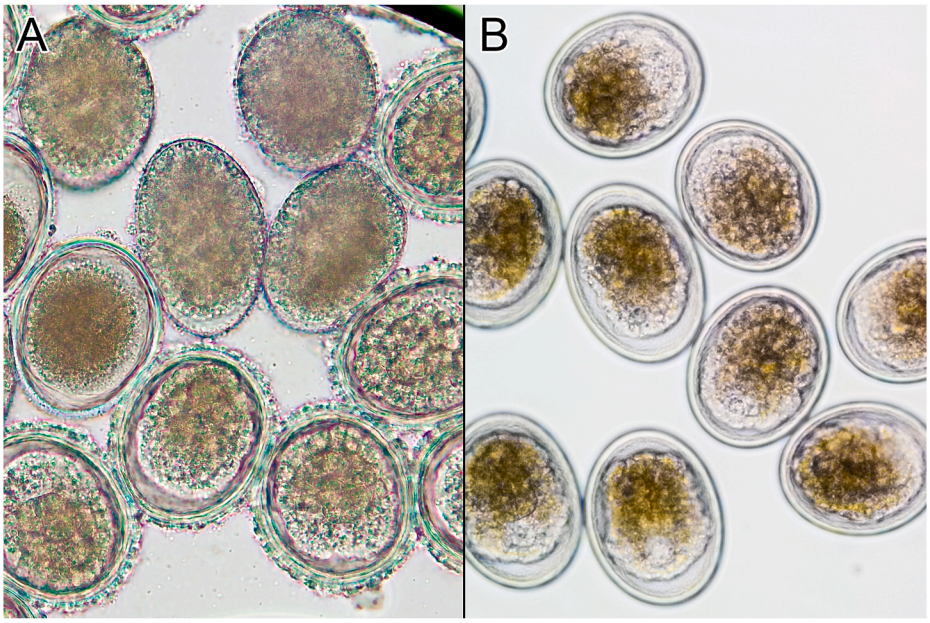

Fig. 1. Comparison of untreated *Baylisascaris procyonis* eggs (left) and decorticated eggs (right) after treatment with 0.5 % sodium hypochlorite for 3 min.

development of the eggs and was therefore sufficiently suitable for preservation.

By treating the eggs in 0.5 % sodium hypochlorite solution and sufficient washing processes, it was possible to successfully remove impurities from them. In addition, this process removed the outer sticky layers of the eggshell, which made handling of the eggs considerably easier (Fig. 1.). The adhesive properties of the outer layers were removed by this process. At an ambient temperature of 5 °C in tap water in centrifuge tubes, 1-celled egg stages could be reliably preserved for several months and kept ready for testing.

3.2. Stages of development

Eleven well-differentiated developmental stages of embryogenesis up to the L1 larval stage were observed. The first four clearly definable stages (Fig. 2 A - D) are subdivided into 1-, 2-, 3- and 4-cell stages with large, well-defined cells. From five to ten large cells and still clearly recognisable cell contours, the eggs were classified as early morula (Fig. 2. E). When ten cells were exceeded, the transition to the late morula stage was determined. This stage can contain eleven or more clearly differentiated and significantly smaller cells before the transition to the blastula takes place (Fig. 2. F). The blastula is characterised by a spherical ring of very small cells arranged in a circle around an unstructured intercalation inside the embryo. The cells arranged in a ring are more transparent and translucent than the interior, whereby the cells on one side of the egg are clearly lighter/more transparent than on the other (Fig. 2. G). The gastrula stage also shows a spherical arrangement of small transparent cells. The main feature, however, is the kidney-shaped arrangement of the cell structure, caused by a one-sided invagination (Fig. 2 H, Fig. 3. A). After completion of the gastrula stage, the first larva-like structure can be recognised in the egg, which is referred to here as pre-larva 1. A characteristic feature is the said larva-like structure, which cannot form more than one concentric ring in the egg (Fig. 3 B & C). The subsequent pre-larva 2 is a larva-like structure that

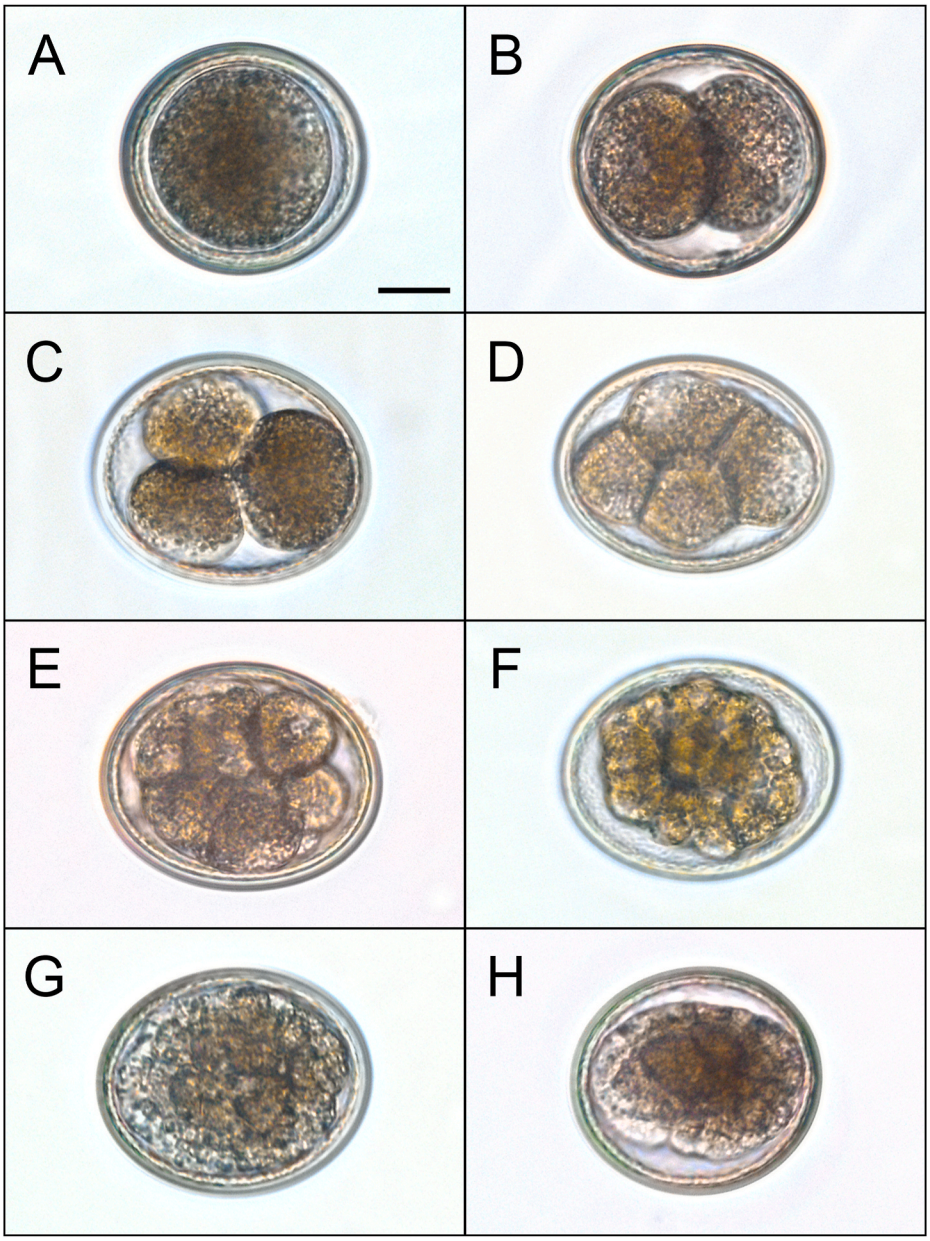

Fig. 2. Stages of development of *Baylisascaris procyonis* in-vitro incubated eggs 1. (A) 1-cell, (B) 2-cell, (C) 3-cell, (D) 4-cell, (E) early-morula, (F) late-morula, (G) blastula, (H) gastrula. Scale bar: 20 µm.

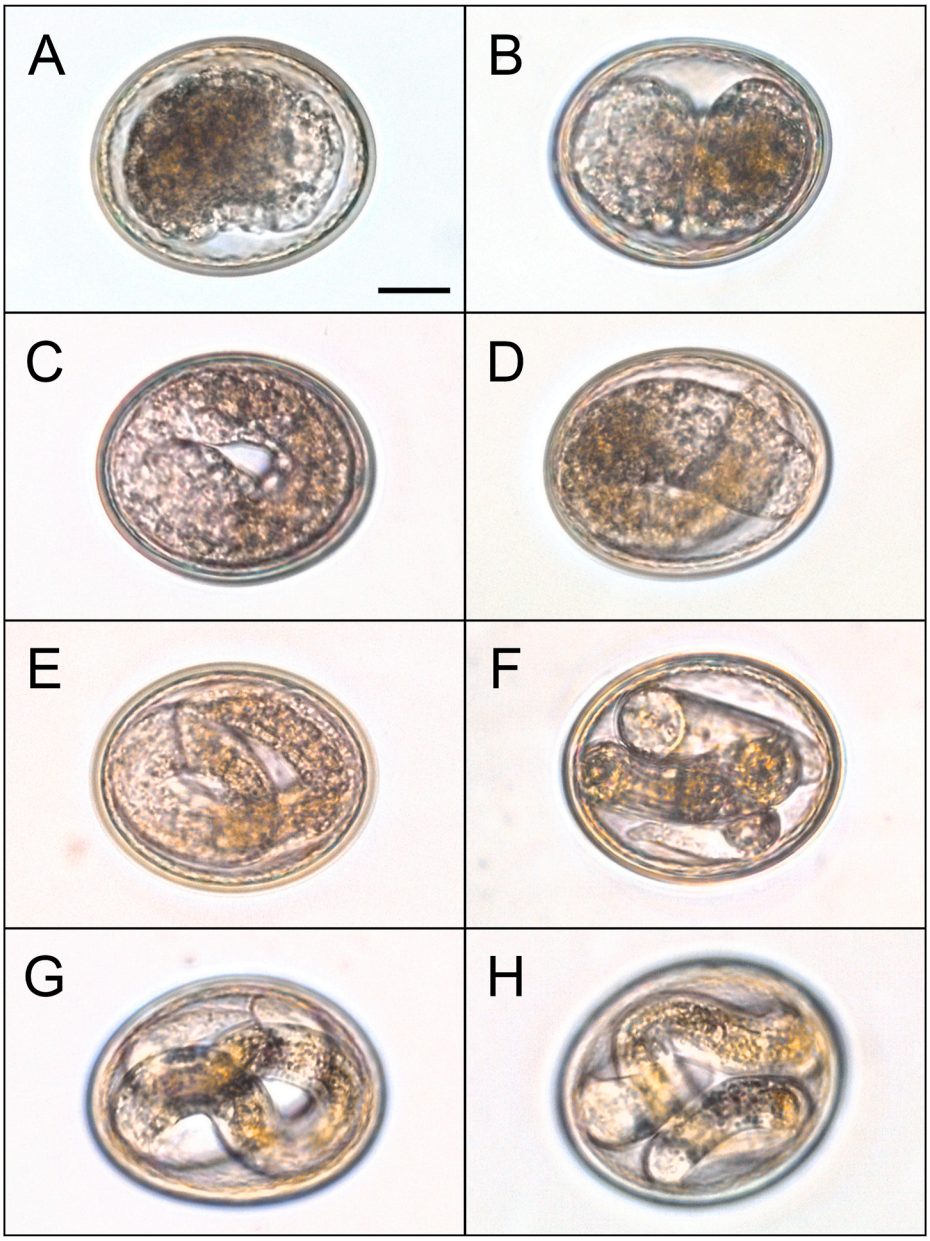

Fig. 3. Stages of development of *Baylisascaris procyonis* in-vitro incubated eggs 2. (A) gastrula, (B) & (C) pre-larva 1, (D) pre-larva 2, (E) early-L1-larva, (F) L1-larva, (G) & (H) potential L2-larva. Scale bar: 20 µm.

can form about 1.5 concentric rings and shows the first signs of motility. The pre-larva 2 is not able to wriggle in the egg and, due to its circumference, can hardly form any other shape than the ring shape (Fig. 3. D). The transition to the L1 larva is characterised by the ability to form two or more concentric rings in the egg. This is due to the decreasing thickness of the structure and the simultaneously increasing length. The L1 shows high motility, but no clearly defined structures inside the larval body (Fig. 3 E & F). As it was difficult to differentiate between the L1 and L2 larval stages using a light microscope and photographic documentation, Fig. 3 G & H show examples of possible appearances of the L2 larva. The differentiation of the L1 and L2 larvae presented here resulted from the size comparison and the filling of the ovum by the body of the larva. Differences in motility due to light stimulation could not be analysed precisely enough due to the large sample size and did not serve as a distinguishing feature.

In tests with low incubation temperatures (10 °C (Fig. 4.), 15 °C, 20 °C (Fig. 5.), 25 °C), all categorised developmental stages could be observed in their succession with a control frequency of 24 h intervals. For most stages, a peak could be observed in the form of the dominant stage at the control time. When the 30 °C mark was exceeded (Fig. 6.), not all developmental stages could be observed within the 24 h intervals.

3.3. *Effects of temperature on the embryonic development*

The incubations at 10 °C, 15 °C, 20 °C, 25 °C and 30 °C led to endpoint 2 being reached with 95 % of the eggs from the subsample (n = 240) in the L1 larval stage. Incubation at 5 °C led to a slow succession of the developmental stages over several months up to the late morula stage (no further development beyond the late morula stage after 11 months - data not shown). In comparison, preservation at 5 °C in closed centrifuge tubes did not lead to any recognisable development beyond the 1-celled stage. Incubation at 35 °C resulted in only 1.7 % of the eggs reaching the L1 larval stage. Development was almost completely inhibited in the late morula and pre-larval stages. Embryos that reached

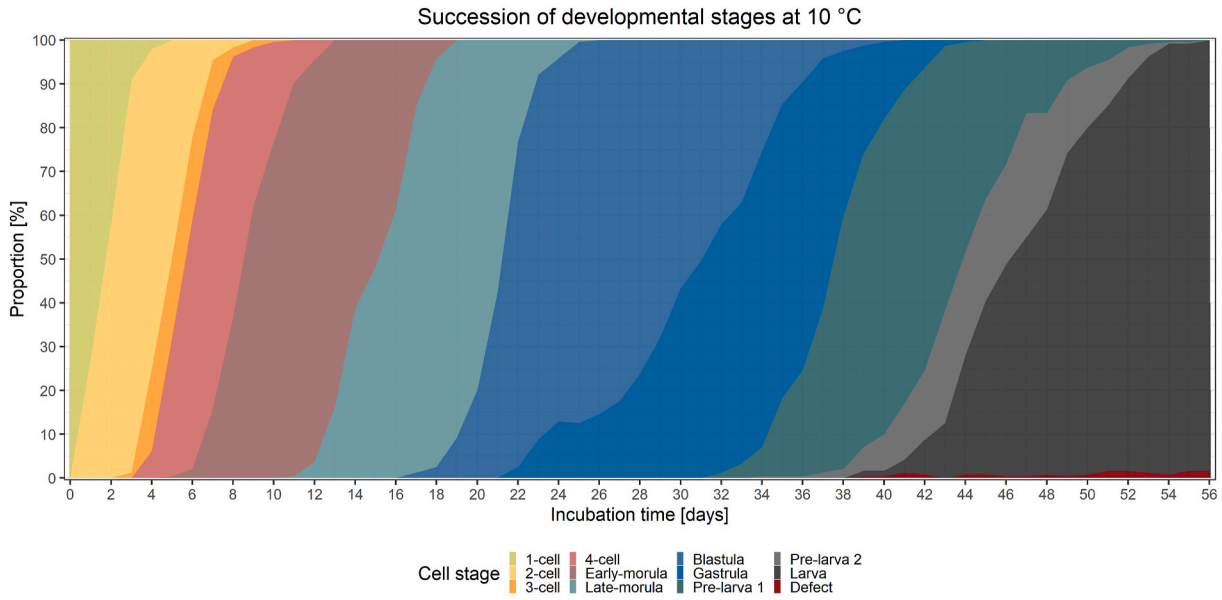

Fig. 4. Succession of *Baylisascaris procyonis* eggs developmental stages at 10 °C. Percentage of n = 240 at each control time (24 h intervals).

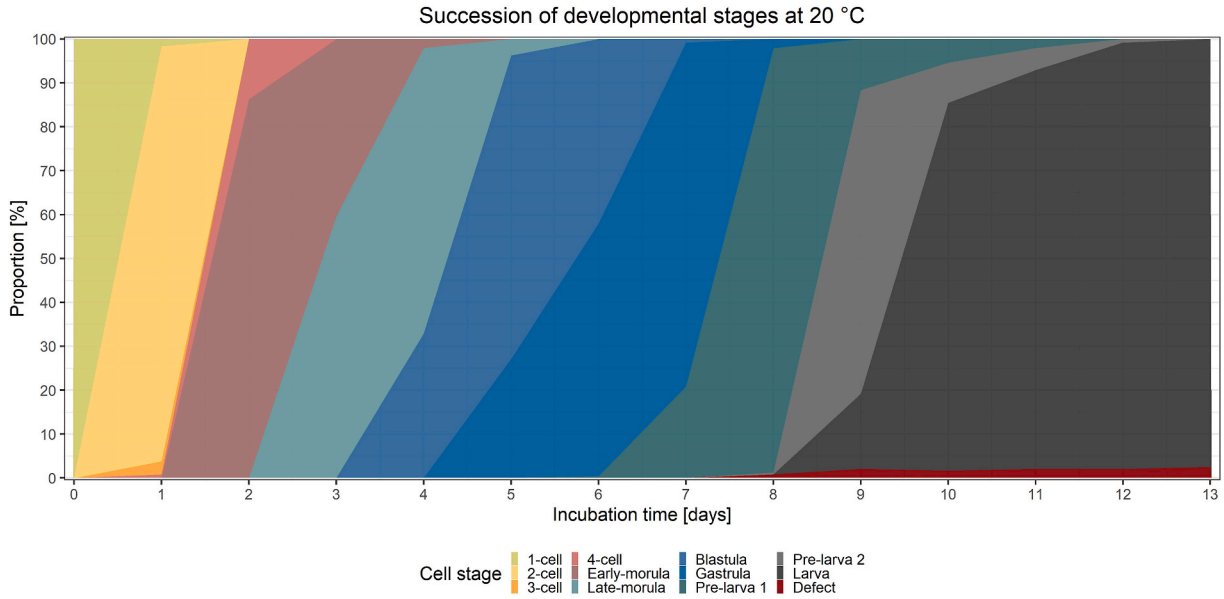

Fig. 5. Succession of *Baylisascaris procyonis* eggs developmental stages at 20 °C. Percentage of n = 240 at each control time (24 h intervals).

pre-larval stages showed atypical deformations and were visibly degenerated (Fig. 7 A - E). Incubation at 38 °C led to complete degeneration of the eggs by the time they reached the late morula stage at the latest. Development beyond the late morular stage was not observed (Fig. 7 F - H). Fig. 8 shows the course of embryonic development influenced by different incubation temperatures through the succession of dominant stages.

3.4. Speed of development

3.4.1. Endpoint 1 (50 % early-morula, Fig. 9.)

The duration averaged over six wells (Table 2) to reach the early morula stage for 50 % of the eggs from the subsample (n = 240) was 54.67 days at 5 °C, 9 days at 10 °C, 5 days at 15 °C, 2 days at 20 °C, 1.83 days at 25 °C, 1 day at 30 °C, 1.17 days at 35 °C and 2.60 days at 38 °C. This resulted in a difference of 52.07 days between the lowest (5 °C) and highest (38 °C) incubation temperatures. The largest difference between two tests was 53.67 days between the test with an incubation temperature of 5 °C and the test with an incubation temperature of 30 °C. When the incubation temperature of 35 °C was exceeded, a slowdown in the rate of development was observed regarding reaching the early morula stage.

3.4.2. Endpoint 2 (95 % L1-larva, Fig. 10.)

Only five of the eight incubation temperatures tested led to endpoint 2 being reached during the development tests. Incubation at 5 °C, 35 °C and 38 °C did not lead to 95 % of the subsample reaching the L1 larval stage during the test period. The duration averaged over six wells (Table 2) to reach the L1 larval stage for 95 % of the eggs from the subsample (n = 240) was 52.83 days at 10 °C, 27.33 days at 15 °C, 11.83 days at 20 °C, 5 days at 25 °C and 4 days at 30 °C. This resulted in a difference of 48.8 days between the lowest (10 °C) and the highest

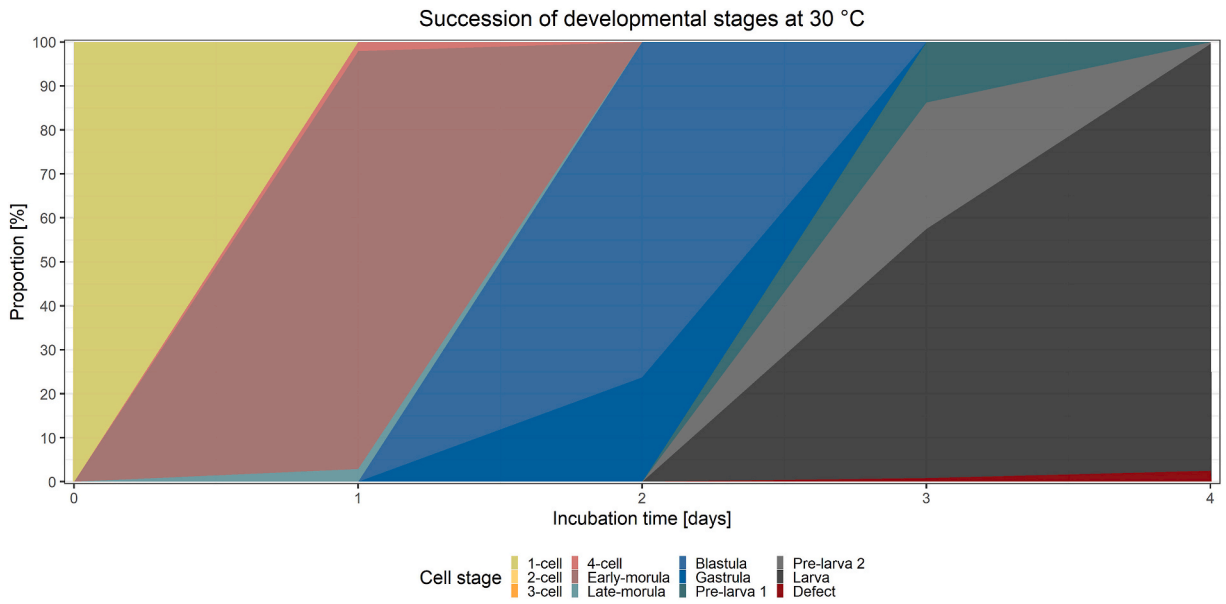

Fig. 6. Succession of *Baylisascaris procyonis* eggs developmental stages at 30 °C. Percentage of n = 240 at each control time (24 h intervals).

(30 °C) incubation temperature, which led to the L1 stage being reached. The maximum difference between directly adjacent tests on the temperature scale was 25.5 days (10 °C–15 °C), the minimum difference was one day (25 °C–30 °C).

On average, a temperature increase of 5 °C led to a doubling of the development speed (factor 1.96 on average) with regard to reaching the L1 larval stage. Overall, increasing the incubation temperature from 10 °C to 30 °C led to a reduction in the required development time by a factor of 13.21.

4. Discussion

4.1. Stages of development and life cycle (Fig. 11.)

The results of the present study show that the embryogenesis of *Baylisascaris procyonis* follows the sequence of developmental stages typical for roundworms of the Ascarididae family (Sakla et al., 1989; Cruz et al., 2012). The developmental stages predefined by Sakla et al. (1989) and Cruz et al. (2012) could be observed in their succession during the study. With regard to the categorisation according to Sakla et al. (1989), the developmental stage known as the tadpole stage was divided into two pre-larval stages. According to the definition by Cruz et al. (2012), these are the pre-larva 1 and pre-larva 2 stages, which differ mainly in size and motility. Pre-larva 1, which appears first, is characterised by little structure and very low motility and forms the first larva-like structure in the course of embryogenesis. The subsequent pre-larva 2 is significantly larger and more motile and typically forms 1.5–2 concentric rings in the egg. Due to the large body diameter compared to the body length, the pre-larva 2 is typically unable to form a shape other than the ring shape. The second stage larva (L2) shown in Fig. 11 presents a potential form of appearance based on the length and structure of the larva as well as the filling of the ovum. Sakla et al. (1989) and Donelly et al. (1989) identify the second stage larva (L2) as the infective stage and only migration in the tissue of hosts lead to the second moult and thus to the development of the third stage larva (L3). In paratenic hosts, the third stage larva (L3) acts as the infective stage for the final host. The development via further larval stages (L4) to the adult only takes place in the final host and some alternative hosts. This represents the widespread consensus of many authors (Kazacos, 2001; Bauer, 2013; Sapp et al., 2017; Gu et al., 2024). In contrast, the third stage larva (L3) is specified as the infective stage inside the egg for the related ascarids *Ascaris suum* (Greenen et al., 1999) and *Toxocara canis* (Bruňaská et al., 1995).

4.2. Speed of development

Our study shows a clear effect of incubation temperature on the development rate of *Baylisascaris procyonis* egg stages. With regard to reaching the L1 larval stage, an increase in temperature within the temperature range between 10 °C and 30 °C led to an increase in the rate of development and a decrease in temperature led to a reduction in the rate of development. *Ascaris suum* and *T. canis* are related species to *B. procyonis*. Their development follows the typical course of development for parasitic nematodes of the family Ascarididae, which includes a soil-associated free phase (Mehlhorn and Piekarski, 1981; Nadler and Hudspeth, 2000). Therefore, there is some comparability between the life cycles and the effects of abiotic factors such as ambient temperature. The findings on the temperature-dependent rate of development are consistent with those of other studies on the embryonic development of nematodes of the Ascarididae family in this respect (Arene, 1986; Gamboa, 2005; Kim et al., 2012).

With regard to reaching endpoint 1 (50 % early morula), there was a continuous decrease in the time required for development into the early morula stage in the temperature range between 5 °C and 30 °C. When the 30 °C mark was exceeded, an increase in the number of days required to reach the early morula stage for 50 % of the eggs was observed. Observation of the temperature effects regarding endpoint 2 (95 % L1-larva) revealed a continuous decrease in the development time required in the temperature range between 10 °C and 30 °C. Inhibition of development into the larval stage was observed in this study at incubation temperatures of 35 °C and above. These findings indicate an optimum temperature for embryonic development in the range between 25 °C and 35 °C. Regarding the speed of development at 5 °C there is some uncertainty concerning the exact time of reaching Endpoint 1 resulting from the irregular control dates. The results from incubation at 5 °C are only used to show development is taking place and to compare with results from preservation of eggs in centrifuge tubes at the same temperature.

The results are similar to those of Arene (1986), who located an optimum temperature for the embryogenesis of *A. suum* at 31 °C ± 1 °C. Inhibition of development into the larval stage was localised at a temperature of 36 °C. Boisvenue (1990) found the shortest development

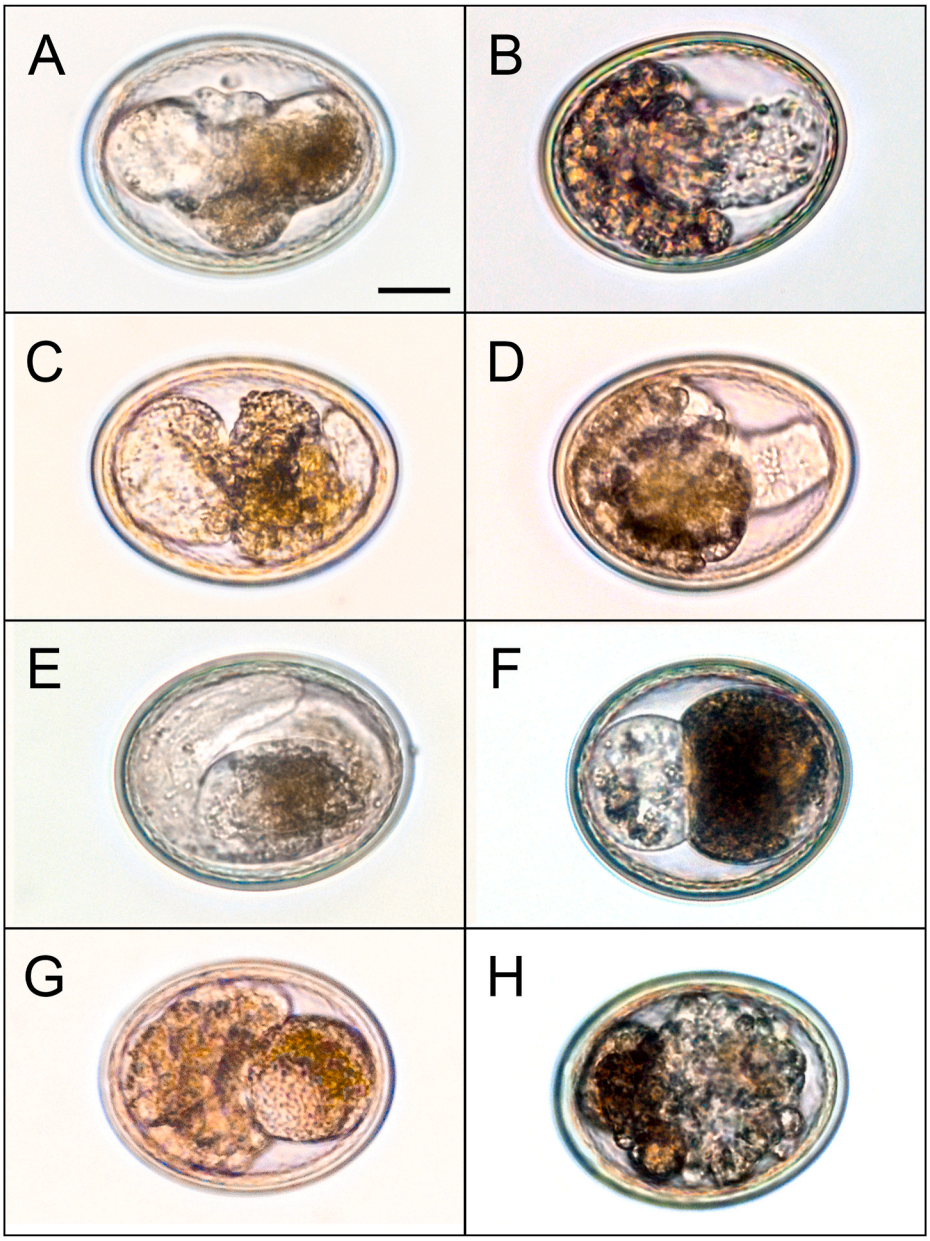

Fig. 7. Defective stages of development of *Baylisascaris procyonis* in-vitro incubated eggs. Incubation at 35 °C and 38 °C. (A)–(E) pre-larval stages at 35 °C, (F) & (G) = 2-cell stage at 38 °C, (H) morula stage at 38 °C. Scale bar: 20 µm.

time for *A. suum* eggs to the infective larval stage at 30 °C, followed by suboptimal development at 32 °C with an increase in L2-larva with reduced motility. Gamboa (2005) also reported an acceleration of the embryogenesis of *T. canis* eggs at increasing incubation temperatures with a subsequent reduction in the survival rate from an incubation temperature of 34 °C. In their study on *T. canis*, Kim et al. (2012) reported an increase in the rate of development when incubated at 35 °C compared to incubation at 25 °C.

The slightly different results regarding *T. canis* may be due to the choice of incubation medium and its moisture content. In addition to the ambient temperature, ambient humidity is one of the most influential abiotic environmental factors regarding the development of soil-associated nematodes (Larsen and Roepstorff, 1999; Maya et al., 2010; Kim et al., 2012). In addition, it has been shown in the past that the influence of temperature is modulated by humidity. The effects of temperature are amplified by a decrease in humidity (Gaasenbeck and Borgsteede, 1998; Hawksworth et al., 2010; Maya et al., 2010). It is speculated, that *B. procyonis* also does require sufficient humidity conditions and that hot temperatures in combination with low ambient humidity could lead to the desiccation of the eggs in the environment (Kazacos, 2001; Ogdee et al., 2016b). As a liquid incubation medium was used in this study, the results must be interpreted with caution regarding their transferability to terrestrial ecosystems and urban areas. The fact, that eggs in natural environments are protected from environmental influences by the insulating properties of the outer layers of the eggshell also should be taken into consideration. The microclimate at latrines also might play an important role considering possible insulation and protection from exposure to extreme temperature and humidity conditions due to fecal matter and substrate covering eggs. In order to generate useable information on embryonic development in terrestrial habitats in addition to embryogenesis in liquid matter such as water, incubation tests must be carried out in sediments of different moisture saturations. In addition, the effect of temperature on the actual rate of development can be influenced by the exposure time of the egg

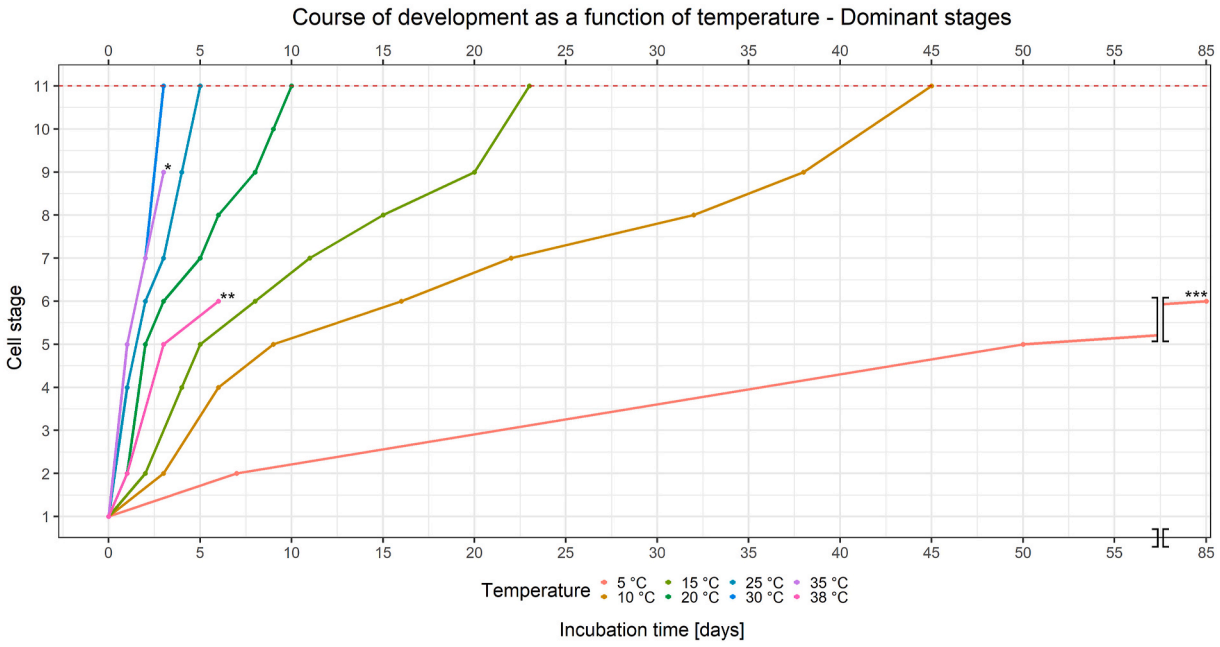

Fig. 8. Dominant stages of *Baylisascaris procyonis* eggs developmental succession. Data points: Dominant stages at the time of control. Red dashed line: Reaching the L1 larval stage as dominant stage. Cell stages: 1: 1-cell, 2: 2-cell, 3: 3-cell, 4: 4-cell, 5: early-morula, 6: late-morula, 7: blastula, 8: gastrula, 9: pre-larva 1, 10: pre-larva 2, 11: L1-larva. *Stopped development at 35 °C. **Stopped development at 38 °C. ***Stopped development at 5 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

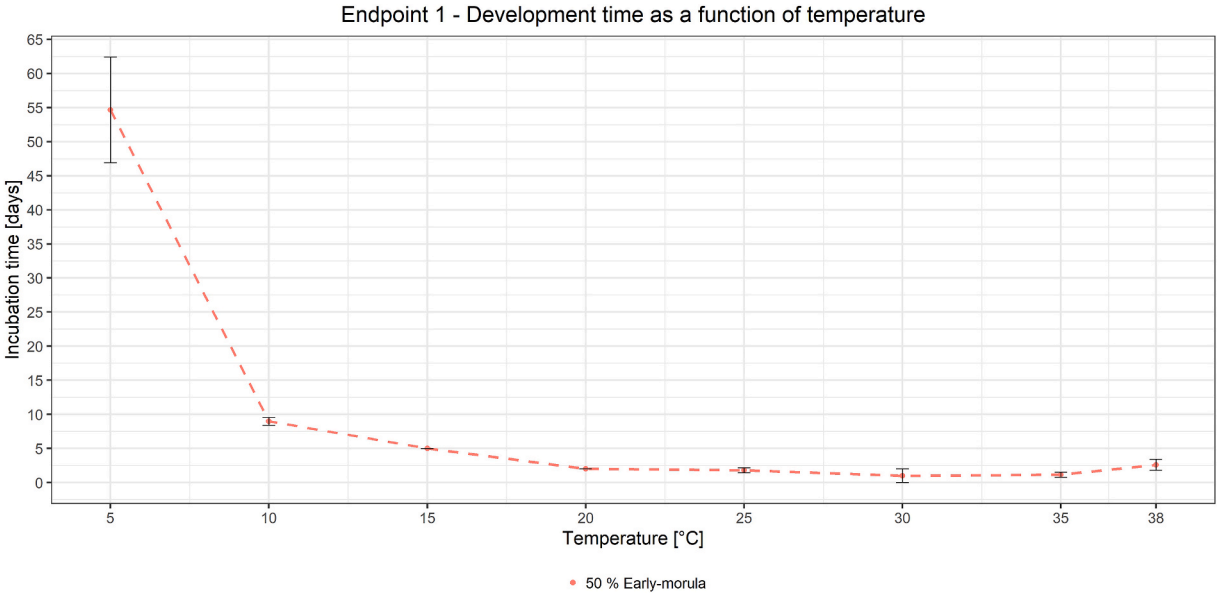

Fig. 9. Endpoint 1. Time for reaching early-morula stage for 50 % of in-vitro incubated *Baylisascaris procyonis* eggs. Data points: Duration to reach endpoint 1 averaged over 6 wells.

stages (Capizzi-Banas et al., 2004; Maya et al., 2010), which means that the data collected can only serve as an approximation of real conditions with regard to fluctuating temperatures. In addition to the type of sediment, the moisture saturation and the duration of exposure, the oxygen supply during embryogenesis may have to be considered as a further modulating factor. The comparison of the slowly progressing embryogenesis in the incubation test at 5 °C with the lack of embryonic development at 5 °C in closed centrifuge tubes in this study indicates a modulation by the availability of oxygen. Both a lack of oxygen in tightly sealed centrifuge tubes and the exposure of the eggs to atmospheric oxygen during transfer to the test plates could play a role here. Brownell and Nelson (2006) describe the 1-celled stage as the dominant stage in waste and drinking water with regard to *A. suum* eggs. Further developed eggs were only found after a significant time in an aerobic environment.

In addition, results from Shafir et al. (2011) show a high discrepancy between the temperature range in which embryonic development can take place and the temperature range in which larvae can remain viable. The temperature required to inactivate living larvae was 62 °C. Repeated freezing at −15 °C did not lead to inactivation of the eggs. These results show a high discrepancy between the temperature range in which embryonic development can take place and the temperature

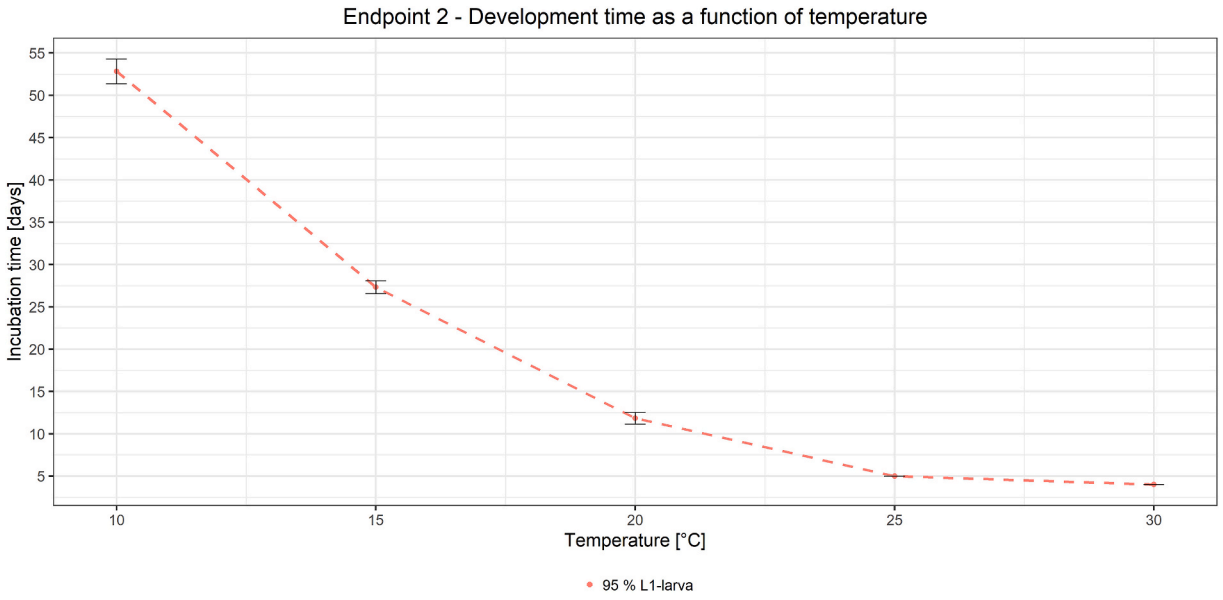

Fig. 10. Endpoint 2. Time for reaching L1 larval stage for 95 % of in-vitro incubated *Baylisascaris procyonis* eggs. Data points: Duration to reach endpoint 2 averaged over 6 wells.

Table 2
Days to reach the endpoints at different temperatures. Time to reach endpoint 1 (50 % early-morula) and endpoint 2 (95 % L1-larva) averaged over 6 wells.

Temperature Endpoint	5 C	10°C	15°C	20°C	25°C	30°C	35°C	38°C
50% Early-morula	54.67	9	5	2	1,83	1	1,17	2,60
95% L1-larva	–	52.83	27.33	11.83	5	4	–	–

range in which larvae can remain viable. However, it must be taken into account that the differences just mentioned also could be due to the removal of the outer protective shell coating, as it was removed here in comparison to Shafir et al. (2011). There are several studies that already discussed the environmental conditions that can influence *B. procyonis* eggs at latrines and how these conditions effect the viability of larvated eggs with all layers of the eggshell (e.g. Ogdee et al., 2016a, 2016b, 2017; Pope et al., 2021). In order to be able to assess the effects of decortication on the sensitivity of the eggs to environmental influences such as temperature, humidity, pH value and UV radiation, further tests must be carried out on the basis of this study. Here, the resilience of eggs during embryogenesis with and without outer shell coating should be compared.

4.3. Outlook regarding climatic conditions and future climatic changes

The results of the study show that months with an average temperature above 10 °C (May–October in Germany (Deutscher Wetterdienst, 2024)) offer the best conditions for complete embryonation of the eggs up to the infective larval stage. Months with a monthly mean temperature below 10 °C (November–April in Germany (Deutscher Wetterdienst, 2024)) are outside the temperature range of 10 °C–30 °C that was determined to be sufficient for embryonation in this study. Since a slow development of the eggs at 5 °C was observed and the preservation of single-cell eggs at 5 °C showed no visible degeneration even after several months, it is reasonable to conclude that monthly mean temperatures below the 10 °C mark have no negative effects on the viability of the eggs. Further evidence for this was provided by Larsen and Roepstorff (1999) in their study of *A. suum* nematodes. They observed a lack of development in cold months with subsequent development of the accumulated eggs in the summer months. Numerous other studies, including the present study, have previously provided evidence suggesting good preservation of unembryonated egg stages at cold temperatures (Annen et al., 1975; Larsen and Roepstorff, 1999; Brownell and Nelson, 2006; Kim et al., 2012). This could mean that eggs that do not undergo embryogenesis in cold months nevertheless develop rapidly into infective stages in warmer months. Maturation of eggs laid in cold months could significantly increase the burden of infectious stages in raccoon latrines in warmer months.

Regarding climate forecasts for this century, an increase in ground-level air temperatures, summer days (min. temp. 25 °C) and hot days (min. temp. 30 °C) as well as tropical nights (min. temp. 20 °C) can be expected (Pfeifer et al., 2020). Based on the data collected, it can be assumed that embryonic development is rapid in these temperature ranges and that under these conditions a few days to a few weeks are sufficient to produce infective stages. However, the results also show that exceeding 35 °C for several consecutive days could lead to the degeneration of exposed eggs. This threshold could be even lower considering that only 30 °C and 35 °C have been tested in this study. Looking at studies by Shafir et al. (2011) and Ogdee et al. (2016b), there is evidence to suggest that the actual upper limit of viability is much higher. Ogdee et al. (2016b) were able to measure ambient temperatures of up to 48.9 °C in Southern Texas latrines, which did not lead to the degeneration of eggs embryonated with larvae. Here it is again important to note that the absence of the outer shell layers in this study could lead to significantly reduced temperature resilience. However, it also should be noted that in this study embryogenesis was investigated under the influence of ambient temperature in comparison to eggs that were already larvated at the time they got exposed to high temperatures in the study by Ogdee et al. (2016b) and Shafir et al. (2011). As mentioned earlier the microclimate at latrine sides also could alternate the real influence of ambient temperature on eggs. It is known that latrines play a major role in the transmission of *B. procyonis* especially for young raccoons and possible paratenic hosts that forage at latrine sites (Kazacos, 2001; LoGiudice, 2001; Page et al., 2001). The transmission dynamics of *B. procyonis* fluctuate in a yearly cycle, influenced by host

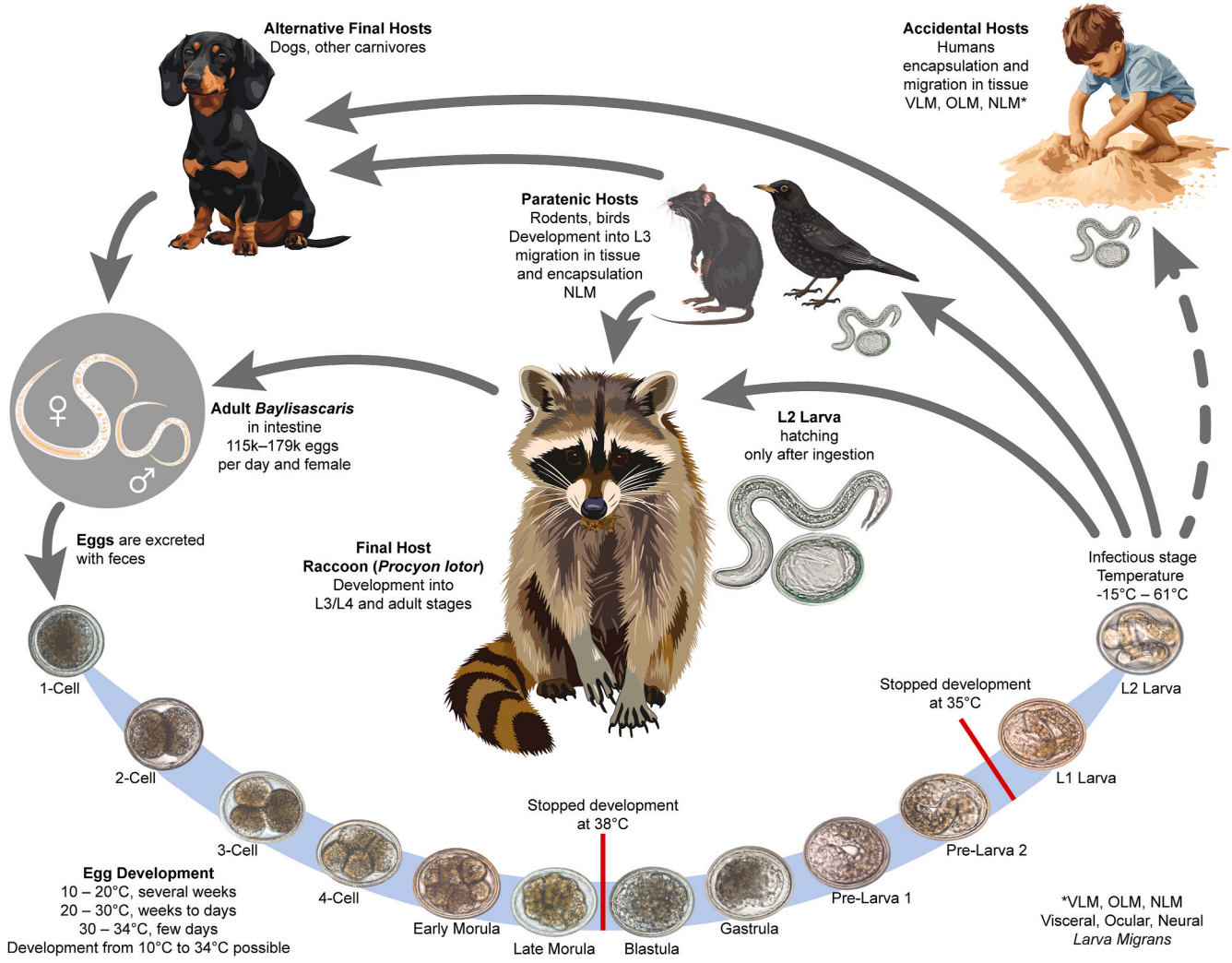

Fig. 11. Life cycle of *Baylisascaris procyonis* with focus on the succession of developmental stages outside the host. Also shown: Hatching of infectious larval stages inside paratenic host, accidental host, alternative host and final host. L2-larva shown as infectious stage for paratenic, accidental and final hosts. L3-larva in tissue of paratenic hosts also functions as infectious stage for final and alternative host. Sexual reproduction of adult stages in raccoon mid gut and dogs as potential alternative final host. Shedding of 1-celled egg stages through feces of final and alternative host. Development of infectious egg stages in the environment.

population attributes, landscape features as well as by season (Kidder et al., 1989; Kazacos, 2001; Page et al., 2005, 2016; Jardine et al., 2014). Differences in prevalence of *B. procyonis* and worm burden of raccoons are explained by self-curing of raccoons during winter (Kazacos, 2001), as well as nutritional stress during cold months followed by reinfection of *B. procyonis* during warmer months due to shifts in food habits and change in latrine use (Kazacos, 2001; Page et al., 2016). Another explanation for seasonal differences in prevalence and worm burden based on the collected data could be a faster development of the eggs into the infective larval stage in warm months. Together with the preservation during cold months and the possible aggregated development of eggs when temperatures start to increase, this could result in a higher risk of infection of paratenic hosts and raccoons at latrines. Climate forecasts for cold seasons suggest a decrease in days with a minimum temperature of 0 °C (frost days), a decrease in days with a maximum temperature of 0 °C and an increase in days with a daily mean temperature of over 5 °C (Pfeifer et al., 2020). Consideration of the data collected in this study suggests that temperature changes in the low ambient temperature range can have a strong effect on embryonic development of *B. procyonis*. The reduction of cold days could make a difference between embryonic development occurring or not occurring at all.

The development times observed in this study cannot be directly transferred to the average monthly temperatures, as fluctuating temperatures prevail under natural conditions. The fluctuation between cooler and warmer temperatures impacts the actual development time, which was only tested here under optimal conditions and constant temperatures. Thus, the increased rate of development in warm periods can be significantly altered by temperature differences between day and night temperatures or cold and warm days (Kazacos, 2001).

As an endoparasite that is very specific to raccoons, the regional distribution of *B. procyonis* is heavily dependent on the raccoon's dispersal behaviour. Starting from the German founder populations, it has been spreading unrestrainedly throughout Germany and the surrounding European countries for 90 years now (Stope, 2019; Boscherini et al., 2020). Alongside those in the North American areas of origin, the German populations are now among the largest in the world (Salgado, 2018). The complementary spread of the raccoon roundworm into previously uninfected populations has already been demonstrated several times (Al-Sabi et al., 2015; Maas et al., 2022; Heddergott et al., 2023) and the prevalence of some German populations is already similar to that of North American populations (Peter et al., 2023). Based on predicted climatic conditions, nothing stands in the way of the raccoon's further spread and expansion into previously unexplored regions of the northern latitudes (Louppe et al., 2019). Ambient temperature is considered one of the most influential bioclimatic factors affecting

raccoon distribution (Louppe et al., 2019) and also was identified in this study as a strong influencing factor for *B. procyonis*. The development of new geographical latitudes of the parasite-host network can lead to new ecological and infectiological dangers and risks, which also must be countered with specifically adapted management concepts in the already established areas. The ongoing spread of *B. procyonis* creates new infection risks for humans, domesticated pets and livestock as well as wild animals in the newly developed areas (French et al., 2019, 2022; Peter et al., 2023).

5. Conclusion

Our results show a clear effect of temperature on the developmental speed of *Baylisascaris procyonis*. Embryogenesis is strongly limited by low ambient temperatures (below 10 °C) and very high ambient temperatures (above 35 °C). Increasing ambient temperatures in the range of 10 °C–30 °C leads to an acceleration of embryonic development and thus to a shortening of the life cycle of the raccoon roundworm. Our results suggest that the temperature increase caused by climate change may increase the prevalence of infective stages of the raccoon roundworm by accelerating embryonic development. This study of the effects of temperature on the embryogenesis of *B. procyonis* provides a methodological approach for future studies on the effects of abiotic environmental factors on the development of *B. procyonis* and other related parasitic nematodes.

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CRediT authorship contribution statement

Robin Stutz: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Dorian D. Dörge:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Funding acquisition, Conceptualization. **Anna V. Schantz:** Writing – review & editing, Writing – original draft, Validation, Methodology, Funding acquisition, Conceptualization. **Norbert Peter:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Sven Klimpel:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

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