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Research paper

Influence of physical and chemical factors on the embryonation, hatching and infectivity of *Spirocerca lupi*

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ABSTRACT

Spirocerca lupi is a parasitic nematode which causes spirocercosis, a severe disease of dogs. Its life cycle involves dung beetles as intermediate hosts and canids as definitive hosts. The effect of different physical conditions and chemical factors on the embryonation and hatching of *S. lupi* eggs were investigated in this study in order to understand the triggers for progression in the early development of this parasite.

Exposure to potassium dichromate significantly enhanced the embryonation of eggs compared to formaldehyde and controls ($p < 0.0001$), reaching the maximum embryonation level of 83% within 2 days of incubation. Hatching of eggs was significantly ($p < 0.05$) enhanced in the presence of 2.5% trypsin, pH 6.0 and 8.0, a temperature of 26 °C, 20% CO_2 and mechanical force by stirring with 3-mm beads. Dissection of *Onthophagus sellatus* beetles 8 h post-feeding with eggs showed that 13% of the ingested eggs hatched in the buccal cavity and the midgut. Finally, the pH range of the beetle's gut was 6.0-6.2 compared to 7.2 ± 0.4 in dog feces suggesting that this pH change may induce hatching in the beetle.

These findings contribute to the understanding of the early steps in the life cycle of *S. lupi* and may be used in the future to block the development of *S. lupi* and prevent dog infection and disease.

1. Introduction

Spirocerca lupi (Spirurida: Thelaziidae) is a parasitic nematode that infects carnivores predominantly of the Canidae family and causes the life threatening disease spirocercosis. Although this nematode can be cosmopolitan, it is usually found in tropical and subtropical regions with warm climates (van der Merwe et al., 2008).

The life cycle of *S. lupi* involves a coprophagous beetle and a dog or other carnivore as intermediate and definitive hosts, respectively. The adult worms develop in nodules found in the dog's esophagus, but aberrant migration can also occur to the stomach, intestine, mediastinum (Dvir et al., 2001), diaphragm (Harrus et al., 1996), heart (Garg et al., 1989), lung, kidney (Singh et al., 1999) and anal mucosa (Borah et al., 2015). Infected dogs can develop an esophageal tumor and approximately 25% of them will progress to a malignant neoplasm (Dvir et al., 2008). The worm eggs are shed from the esophageal nodules through feces or vomitus (van der Merwe et al., 2008). Infected feces with *S. lupi* eggs consumed by a dung beetle develop from L1 to L3 in the beetle (Austin et al., 2013). *Onthophagus* spp. (Scarabaeidae) beetles have been reported as intermediate hosts of *S. lupi* (du Toit et al., 2012) and in Israel, *Onthophagus sellatus* has been implicated as the most important intermediate host of the parasite (Gottlieb et al., 2011).

Obligatory parasitic nematodes require appropriate timing and conditions for the embryonation and egg-hatching processes. The embryonation of eggs can occur inside the uterus of the female worm, or outside the definitive host as observed for *Toxocara canis* (Azam et al., 2012) and *Capillaria obsignata* (Tiersch et al., 2013). Chemical compounds and physical conditions which promote embryonation, such as potassium dichromate, formaldehyde, sulfuric acid and specific temperatures, have been tested as embryonation promoters for several worms including *T. canis* (O'Lorcain, 1995; Azam et al., 2012), *Ascaris suum* (Oksanen et al., 1990) and *C. obsignata* (Tiersch et al., 2013). After embryonation, hatching from the eggs can occur following the secretion of fluids produced by the larval stages inside the eggs, or exposure to host triggers (Rogers, 1960). Several studies have evaluated hatching events in a number of worms including *A. suum* (Clarke and Perry, 1988; Jaskoski and Colucci, 1964), *Ascaris lumbricoides*, *Trichostrongylus axei* and *Toxocara cati* (Rogers, 1960). These studies have evaluated different conditions, such as temperature, pH, CO_2, lytic enzymes and reducing agents.

The conditions or triggers that favor the embryonation and hatching of *S. lupi* eggs remain unknown to date. It has been hypothesized that

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the beetle's mouthparts may play an important role in the hatching process inside the intermediate host (Miller, 1961; Chhabra and Singh, 1972). Therefore, the purpose of this study was to analyze the effect of different physical conditions and the influence of chemical factors in the embryonation and hatching of *S. lupi* eggs, observe the hatching of the eggs *in vivo* in their intermediate host, and to analyze the pH transition that eggs undergo in the passage from the dog feces to the beetle's gut and whether that change may be related to hatching.

2. Materials and methods

2.1. Beetle collection and offspring rearing

Dung beetles were obtained from dog fecal samples collected from a public park in Ramat Gan, located in Israel's coastal plain near Tel Aviv. The beetles were separated from the feces and taxonomically classified as *O. sellatus, Onthophagus similis* and *Onthophagus novaki*, as previously described (Gottlieb et al., 2011). They were maintained and reared in the laboratory in plastic buckets with autoclaved soil at 18% humidity. Rearing conditions were set at a constant temperature of 27 °C with light-dark cycles of 14 and 10 h, respectively. Beetles were fed twice weekly with dog feces free of parasites, as demonstrated by fecal concentration and flotation (Castro and Guerrero, 2006). After 14 days, the soil from the buckets was sieved to separate and obtain brood balls (potentially containing beetle eggs or larvae) from the soil. The brood balls were transferred to a new bucket with the same conditions as described above. The F1 generation adult beetles which emerged from the brood balls were used for further experiments.

2.2. In vitro growth of Spirocerca lupi adults and collection of eggs

Spirocerca lupi worms were collected from esophageal nodules of a naturally infected dog at necropsy and placed in PBS with 200 U penicillin and 0.2 mg streptomycin (termed PBS supplemented with antibiotics). Then, the adult worms were processed externally by 10-sequential washes, each of 10 min, in PBS supplemented with antibiotics. The adult worms were separated according to sex and placed in 6-well plates (Corning® Costar®, Sigma-Aldrich, USA) in RPMI-1640 media (Biological Industries, Israel) supplemented with 2% D-glucose (Sigma-Aldrich, USA), 10 mM L-glutamine (Biological Industries, Israel) 200 U/ml penicillin, 0.2 mg/ml streptomycin (Biological Industries, Israel), 0.2 mg/ml gentamycin (Biological Industries, Israel), and 0.25 μg/ml amphotericin B (Sigma-Aldrich, USA). The plates were incubated at 37 °C in 5% CO_2 for 30 days. The liquids were harvested after 24 h of incubation and the eggs shed into the media were maintained at 26 °C for further analysis. The eggs were observed under a light microscope at 100× and 400× magnifications to confirm the full embryonation development of the juveniles inside the egg and rate of hatched eggs. These eggs were used to test the effect of different physicochemical conditions on hatching.

Eggs from the proximal portion of the uterus of a *S. lupi* female were obtained by removing and washing the uterus with PBS supplemented with antibiotics. The recovered eggs were concentrated by centrifugation at 5500 rpm for 10 min. The pellet was washed five times in PBS with antibiotics. The numbers of non-embryonated and embryonated eggs (Supplementary Fig. S1A and B in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.05.026) were registered with means and standard deviation of 73.7 ± 9.4% and 26.2 ± 8.3%, respectively. These eggs were used to test the effect of different chemical conditions on embryonation.

2.3. Induction of S. lupi eggs embryonation

The effects of potassium dichromate, formaldehyde and CO_2 on the embryonation of *S. lupi* eggs were evaluated as done in previous studies with other nematodes (Oksanen et al., 1990; Tiersch et al., 2013). Firstly, solutions of *S. lupi* eggs collected from a female uterus were incubated at 26 °C in microcentrifuge tubes with a solution of either 0.1% potassium dichromate (group no.1) or 0.5% formaldehyde (group no. 2). A third group (group no. 3) was incubated in sterile PBS as a control. Observations were made at days 0, 1, 4, 7, 12, 14 and 16. The effect of CO_2 was evaluated by incubating a suspension of non-embryonated eggs in PBS at 37 °C with 5% CO_2 or under room atmosphere (approximately 0.04% CO_2) as control. All conditions investigated were tested in triplicate. The number of free larva, non-embryonated, embryonated and empty eggs was registered in each experiment. Segmented embryos to fully developed larva inside the egg, as well as empty egg shells or free larva were classified as eggs that underwent embryonation. The highest number of either free larva or empty eggs was used to calculate the percentage of embryonation in order to avoid overrepresentation of hatching events. The percentage of embryonation per observation day was calculated as the mean of the replicates.

2.4. Induction of S. lupi eggs hatching

The effects of pH, temperature, CO_2, trypsin, lipase and mechanical force on the hatching of embryonated *S. lupi* eggs were evaluated as follows. A solution of female growth media with approximately 5 embryonated eggs/μl was exposed to different conditions relating to each of the above factors. All experiments were tested in triplicate. After incubation of different times for each factor as detailed below, an aliquot of 50 μl of each suspension was observed under the microscope and the numbers of closed eggs, empty eggs, free larva and releasing larva were registered (Fig. 1 and Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetpar.2017.05.026). A hatching event was defined when any of the latter three were observed. Since an observed free larva and an empty egg could represent a single hatching event, only the highest number of either free larva or empty eggs was used to calculate the percentage of hatching in order to avoid overrepresentation of hatching events. The hatching percentage was calculated by the sum of either empty eggs or free larvae and releasing larvae divided by the total counted eggs and multiplied by 100. The mean of each replicate was calculated on each observation day for each tested condition.

To evaluate the pH effect, the suspended eggs were concentrated by centrifugation as described above and the supernatant was discarded. Eggs were resuspended in PBS and the pH was adjusted to 4.0 (used as control), 6.0 or 8.0 with HCl or NaOH, using a pH meter (PL-700PV MRC Ltd, Israel). The eggs were then incubated at 26 °C in room atmosphere. Hatching events were recorded from each replicate at days 0, 2, 5, 8, 12, 15 and 20, as described above.

The effect of temperature on hatching was tested by exposing *S. lupi* egg suspension to 4 °C (used as control), 26 °C or 37 °C. Hatching events were recorded from each replicate at days 0, 2, 6, 9, 12, 15 and 20, as described above.

The effect of CO_2 was evaluated at 26 °C in either 5% or 20% CO_2. The results in each CO_2 concentration were compared to the result of a control sample in room atmosphere at 26 °C. Hatching events were recorded from the 20% CO_2 environment at days 0, 3, 7, 10, 13 and 17 and from the 5% CO_2 environment also at days 0, 3, 7, 10, 13 and 17.

The effects of lipase and trypsin on the hatching of *S. lupi* eggs were evaluated as follows. A suspension of *S. lupi* eggs was concentrated as described above and the supernatant was discarded. Five hundred microliters of 2.5% trypsin (Sigma-Aldrich, USA), 0.25% trypsin, 5% lipase (Sigma-Aldrich, USA) or 1% lipase were added in separate tubes. Each experiment had a separate PBS control. The suspensions were incubated in room atmosphere at 37 °C, corresponding to the optimal temperature for the activities of these enzymes according to the manufacturer's instructions. Hatching events were recorded for each replicate at days 0, 1, 4, 9, 10, 15, 17 and 19 for lipase and days 0, 1, 4, 7, 11 and 19 for trypsin.

The effect of mechanical stirring on hatching was tested by exposing

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