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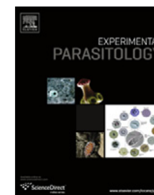

Parasitological and morphological study of *Schistosoma mansoni* and diabetes mellitus in mice

Maarten Hulstijn [a], Lucas de Andrade Barros [d], Renata Heisler Neves [b], Egberto Gaspar de Moura [c], José Roberto Machado-Silva [d,*]

[a] *Biologia Parasitária, Departamento de Ensino, Pavilhão Arthur Neiva, Instituto Oswaldo Cruz, Fiocruz, Avenida Brasil 4365, Manguinhos, 21045-900 Rio de Janeiro, RJ, Brazil*
[b] *Laboratório de Helmintos Parasitos de Vertebrados, Instituto Oswaldo Cruz, Fiocruz, Avenida Brasil 4365, Manguinhos, 21045-900 Rio de Janeiro, Brazil*
[c] *Departamento de Ciências Fisiológicas, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Avenida 28 de Setembro, 87 fundos, 20551-030 Vila Isabel, Rio de Janeiro, RJ, Brazil*
[d] *Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rua Professor Manoel de Abreu, 444 5° andar, 20551-170 Vila Isabel, Rio de Janeiro, RJ, Brazil*

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ABSTRACT

Schistosomes are blood-dwelling flukes which are highly dependent on the host metabolism. The aim of this study was to investigate possible relationship between streptozotocin-induced diabetes and the outcome of acute murine schistosomiasis mansoni. Male and female SW mice were treated by a single intraperitoneally injected dose of streptozotocin (180 mg/kg). Seven days after induction, both control and diabetic animals were infected with 70 *Schistosoma mansoni* cercariae (BH strain). Diabetics and their controls were weighed 45 days after birth and for the last time prior to killing. Susceptibility to infection was evaluated twice a week by quantifying fecal egg excretion 7–9 weeks post-infection by the Kato–Katz' thick smear method. Mice were euthanized the day after the last fecal examination was performed. Adult worms were recovered from the portal system and mesenteric veins, whereas liver and intestine were removed for enumeration of egg load. No differences in worm length or in measurements of the reproductive organs, tegument, and suckers were detected. Also oviposition was unaffected as the total number of eggs per female worm from the liver, the small and the large intestine was the same in both groups. An oogram evaluation revealed a lower percentage of mature (23.0% vs. 40.7%) and a higher percentage of immature (69.1% vs. 51.7%) eggs in the small intestine of the diabetic mice. We suggest that principally a hampered egg passage through the intestine tissue caused this reduction and that probably both the eggs and the impaired host response play a role.
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1. Introduction

Diabetes mellitus is a metabolic disorder, which is caused by the absence of, or an impaired response to insulin and is characterized by high blood glucose and is associated with immune dysfunction (Mandel and Mahmoud, 1978; Bessman and Sapico, 1992). The current diabetes epidemic is attributable predominantly to rising cases of type-2 diabetes, whose rate of prevalence is increasing and exerts a significant negative impact on the health and economies in developing countries (Dagogo-Jack, 2006). Epidemiological studies in a Brazilian rural population have shown that prevalence of obesity and associated diseases, such as diabetes also have increased, whereas physical activity has decreased (Velasquez-Melendez et al., 2007).

It is known that people suffering from diabetes mellitus are related to higher incidence of bacterial and fungal infections (Robertson and Polk, 1974), but clinical data on the association with parasites is very limited and not clear cut (Coovadia et al., 1993; Abaza et al., 1995; Nazligul et al., 2001). Human studies provided evidence supporting the hypothesis of a possible association between positive *Stongyloides stercoralis* serology and diabetes in patients from Brazil (Mendonça et al., 2006). Experimental studies though have shown that chemically-induced diabetic mice had higher *Trichinella spiralis* larval counts (Antonios et al., 1989) and also higher parasitemia and shorter survival times were noted in mice infected with *Trypanosoma brucei* (Amole et al., 1985) or *Trypanosoma cruzi* (Nagajyothi et al., 2009a,b; Tanowitz et al., 2009).

Diabetes mellitus has also been studied in relation with experimental schistosomiasis. Several experimental studies by Mahmoud et al. (1975,1976) and Mahmoud (1979), using chemically induced and genetically determined mice have shown a suppression of the granulomatous response around schistosoma eggs in

* Corresponding author.
E-mail address: machado@uerj.br (J.R. Machado-Silva).

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both the liver and lung, which was caused by an impaired cell mediated immunity. The infection of NOD mice with *Schistosoma mansoni* confers permanent protection against type 1 diabetes (Zaccone et al., 2003), with demonstrations that the mechanism of diabetes prevention involved soluble egg antigens (SEA). Such products can act direct on CD4 + T cells increasing expression of TGF-β, intregin β8 and galectins (Zaccone et al., 2009).

The egg of the flatworm *S. mansoni* is the main cause of pathological complications following the infection with this parasite. Oviposition by the adult worms and the subsequent passage of the eggs to the intestinal lumen depends largely on the host and its cellular immune response. Several host-derived hormones, like IL-7, thyroid and TNF have been described to influence worm growth and egg deposition (reviewed by Mendonça et al. (2000), Salzet et al. (2000)). In addition, T-cell deprived mice and animals treated with anti-inflammatory drugs have fewer eggs in their feces (Doenhoff et al., 1978; Doenhoff, 1998) and eosinophils and other inflammatory cells have been described to favor egg passage (Lenzi et al., 1987). Changes of the normal host metabolism due to other illnesses, or physiological complications could therefore disrupt oviposition or worm development. Malnutrition for instance, which is common in many areas where schistosomiasis is endemic has been described to affect egg viability and output, worm size and worm morphology in experimental animals (Akpom and Warren, 1975; Magalhães et al., 1986; Neves et al., 2001; Simões et al., 2002; Akpom, 1978, 1981).

In order to get a better understanding of the host–parasite interactions, we have studied schistosomiasis and diabetes mellitus in a murine model using streptozotocin (STZ), a drug that destroys pancreatic-cells resulting in hyperglaecemic and hypoinsulemic state of the host. Recently, studies with this host–parasite model do not found difference between non-diabetic and diabetic mice regarding tissue egg count and oogram pattern (Thabet et al., 2008). In this experiment, mice were first infected and treated with streptozotocin at 90 days post-infection.

In earlier experiments we have shown that streptozotocin, given at 45 days post-infection (dpi) affected the morphology of the reproductive organs of male and female worms and lowered the number of viable eggs in the intestine and the amount of eggs in the feces (Hulstijn et al., 2001, 2003). However, the morphological changes were caused directly by the drug. Here, we compared the fecal egg excretion, oviposition, worm recovery and morphology of diabetic and non-diabetic mice, infected after STZ injection, so no direct contact existed between the drug and the parasite.

2. Material and methods

2.1. Diabetes mellitus and Infection

Diabetes mellitus was induced by the administration of the drug streptozotocin (Sigma, St. Louis, USA) freshly dissolved in 40 mM citrate buffer (pH 4.5). Eleven, 45-days-old Swiss Webster mice were injected intraperitoneally with a single dose of STZ (180 mg per kg bodyweight), while 11 mice did not received the drug but were given citrate buffer only. Seven days after STZ injection, all animals were infected by tail exposure with 70 cercariae of *S. mansoni* of the Belo Horizonte (BH, Brazil) strain.

2.2. Blood glucose concentrations

Sixty-one days post-infection (dpi) blood samples were taken from the tail and glucose concentrations were measured using Glucometer Elite test strips (Bayer, Australia), a method based on the reaction of glucose with glucose oxidase and potassium ferricyanide.

Diabetics and their controls were weighed 45 days after birth and for the last time prior to killing. All mice were allowed to consume water and pellet chow *ad libitum*.

2.3. Worm recovery and morphometry

At 63 dpi all mice were sacrificed by dislocation of the cervical bone and worms were recovered from the portal and mesenteric veins and washed in saline. Male and female worms were then separated, fixated in AFA (2% acetic acid, 10% formaldehyde and 48% alcohol), stained with hydrochloric carmine (Neves et al., 1998) and whole-mounted on glass slides (Hulstijn et al., 2003). For morphometric analysis images were captured by either an analog camera (Sony, 640 × 480 pixels, RGB) using brightfield microscopy (Olympus BX50) or, in order to measure worm length by a digital camera (Olympus C2500L) with macro lens. Images were transferred to a computer and software for image analysis (Image Pro Plus, Media Cybernetics, USA) was used for the morphometric analysis of male and female worms. The following characters were measured in worms of both sexes: worm length, area of the oral and ventral suckers, and the shortest distance between them. The thickness of the tegument was analyzed in the posterior part at the height of the genital pore. In male worms the area and the number of the testicular lobes and in female worms the area of the ovary were measured.

2.4. Fecal and tissue egg counts

Stools of each mouse were collected separately 43, 47, 50, 54, 57 and 61 dpi and processed by the method of Kato–Katz (Katz et al., 1972). Two slides per mice were counted and averaged. An oogram was made from the first centimeter of the distal part of the small intestine and eggs were qualified according to our previous study (Machado e Silva et al., 1991). The remainder small intestine, the colon plus caecum and part of the liver were dissolved in 4% KOH and the number of eggs was determined (Cheever, 1968).

2.5. Animal use

The use of animals and the here presented experimental procedures were approved by the Ethical Commission for the care and use of experimental Animals (CEA), Biological Institute Roberto Alcantara Gomes, State University of Rio de Janeiro under protocol CEA/05/2001.

2.6. Statistical analysis

The statistical program SPSS 9.0 for Windows was used to facilitate calculations. The diabetic and non-diabetic groups were compared by Mann–Whitney *U*-test and values of $P < 0.05$ were considered significant. To see if measurements were associated the Pearson's correlation coefficient r was calculated and tested for significance (two-tailed).

3. Results

3.1. Blood glucose and weight

STZ-injected animals had blood glucose levels above 400 mg/DL with an average of 530.1 ± 74.3, while this was 103.3 ± 17.7 mg/DL in animals that had not received STZ ($P < 0.001$, Mann–Whitney *U*-test). The average weight of male and female mice 45 days after birth was 31.4 ± 5.3 g and 25.3 ± 2.4 g, respectively when diabetes was induced. Seventy days after STZ injection, at 63 days p.i., male diabetic mice weighed 31.1 ± 7.2 g against 39.8 ± 5.7 g of

Table 1
The recovery of adult *Schistosoma mansoni* worms from the mesenteric and portal veins of non-diabetic and diabetic mice.

Recovery	Group		P[a]
	Non-diabetic ($n = 11$)	Diabetic ($n = 11$)	
	Mean ± SD	Mean ± SD	
Males	8.2 ± 4.8	5.1 ± 2.2	0.126
Females	7.3 ± 3.5	4.2 ± 2.0	**0.030**
Total	15.5 ± 8.2	9.3 ± 4.0	0.064
Ratio m/f	1.08 ± 0.15	1.30 ± 0.40	0.292

Bold values means significant differences.
[a] *P*-values calculated with Mann–Whitney test.

non-diabetic males ($P = 0.076$). These numbers for the female animals were 24.3 ± 5.1 g vs. 34.1 ± 4.1 g ($P = 0.016$).

3.2. Worm recovery

The recovery of worms in male mice was lower than in female mice (10.0 vs. 14.3) and also the ratio male per female worm was slightly lower in male animals (1.08 vs. 1.28), but not significantly different ($P = 0.129$ and $P = 0.126$, respectively). The average amount of male and female worms was lower in diabetic mice although only the latter significantly (see Table 1). The ratio of male per female mice was slightly higher, but not significantly different in diabetic mice. The overall recuperation, i.e. the percentage of cercariae that grew into adult worms, was 22.1% in non-diabetic and 13.2% in diabetic mice ($P = 0.064$).

3.3. Fecal egg count

Evaluation of the egg excretion by the method of Kato–Katz revealed that diabetic mice had much lower amount of eggs in the feces (see Table 2). Since fewer females were recovered from diabetic mice, the egg counts were also calculated per female worm. Also these counts were about 3 times lower in diabetic mice at 61 dpi. Fecundity though was not affected because the total amount of eggs from both the liver and the intestine was not significantly different between the groups after the correcting the counts per female worm. Only the counts from the large intestine were significantly lower in diabetic mice. To see if percent wise a larger amount of eggs in diabetic mice was dislocated to the liver, the distribution of the eggs among the three locations (liver, small and large intestine) was calculated per mice and averaged. The percentage of eggs in swept to the liver was 34.1 ± 6.1% in non-diabetic and 39.1% ± 9.0 in diabetic mice; 59.5 ± 6.7 vs. 56.9 ± 8.7 in the small intestine and consequently the percentage in the large intestine were 6.4 ± 2.8 vs. 4.0 ± 3.3. None of these numbers was significantly different between the groups. Egg maturation was evaluated in an oogram from the small intestine where the several stages of immature, mature, and dead eggs were counted. The results showed no differences between the groups in the amount of dead eggs or eggshells but did reveal a lower percentage of mature eggs and consequently a higher percentage of immature eggs in the intestine of diabetic mice (see Table 3). No differences were observed in between the different stages of development of immature eggs.

Table 3
Egg maturation and viability in non-diabetic and diabetic mice evaluated by an oogram of the small intestine.

Eggs	Group		P[a]
	Non-diabetic ($n = 11$) Mean ± SD	Diabetic ($n = 11$) Mean ± SD	
Imature %	51.7 ± 12.0	69.1 ± 9.4	**0.008**
Mature %	40.7 ± 10.5	23.0 ± 10.4	**0.002**
Dead %	6.0 ± 3.1	6.8 ± 4.5	0.622
Eggshell %	1.6 ± 0.8	1.1 ± 0.9	0.264

[a] *P*-values calculated with Mann–Whitney test.

3.4. Morphology and morphometry

Male and female worms stained with hydrochloric carmine and whole-mounted on slides were analyzed by brighfield microscopy. No morphological divergences in either tegument or reproductive organs were observed between the two groups. Notable is one female worm recovered from a diabetic mouse of which the ovary seemed to be divided in two parts. However, other females recovered from the same mouse had normal ovaries, so the observation was not host-induced per se. Morphometric analysis also did not show differences between worms recovered from diabetic or non-diabetic mice. Oral and ventral suckers, tegument thickness, length and the size of the ovaries and testicular lobes were measured in each worm and averages were calculated per mouse (see Table 4).

Table 2
Fecal and tissue egg counts from non-diabetic and diabetic mice and the counts calculated per female worm. Fecal egg counts (eggs/g feces) were obtained from 43 to 61 days post-infection by the method of Kato–Katz. The amount of eggs in the tissue was counted after dissolving the liver, small and large intestine in 4% KOH.

	Counts		P[a]	Counts per female worm		P[a]
	Non-diabetic ($n = 11$)	Diabetic ($n = 11$)		Non-diabetic ($n = 11$)	Diabetic ($n = 11$)	
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Fecal egg counts	(eggs/g)			(eggs/g/F)		
43 dpi	4 ± 11	0 ± 0	0.148	1 ± 4	0 ± 0	0.148
47 dpi	235 ± 201	15 ± 28	**<0.001**	32 ± 18	2 ± 4	**<0.001**
50 dpi	572 ± 443	35 ± 32	**<0.001**	73 ± 31	7 ± 6	**<0.001**
54 dpi	384 ± 260	110 ± 66	**<0.001**	53 ± 18	29 ± 24	**0.005**
57 dpi	742 ± 503	172 ± 115	**0.004**	97 ± 51	39 ± 28	**0.003**
61 dpi	747 ± 525	163 ± 151	**0.001**	100 ± 43	34 ± 26	**0.001**
Tissue egg counts	(eggs)			(eggs/F)		
Liver	15731 ± 6368	11668 ± 5978	0.178	2318 ± 609	2840 ± 972	0.200
Small intestine	29673 ± 15257	17268 ± 9760	0.071	4077 ± 1020	4016 ± 699	0.768
Rectum and caecum	3176 ± 2285	1566 ± 2015	**0.045**	405 ± 127	299 ± 279	**0.045**
Total	48580 ± 22762	30503 ± 16721	0.061	6799 ± 1307	7155 ± 1392	0.577

Bold values means significant differences.
[a] *P* values calculated with Mann–Whitney test.

Table 4
Morphometric analysis (worm length, tegument thickness, area of the oral and ventral sucker; the distance between them and the area of the reproductive organs (testicular lobes or ovary) of male and female worms recovered from non-diabetic and mice diabetic.

	Male worms		P^a	Female worms		P^a
	Non-diabetic (n = 11) Mean ± SD	Diabetic (n = 11) Mean ± SD		Non-diabetic (n = 11) Mean ± SD	Diabetic (n = 11) Mean ± SD	
Length (mm)	8.7 ± 0.6	9.0 ± 1.1	0.718	11.4 ± 1.1	11.5 ± 1.4	0.818
Tegument (μm)	10.9 ± 1.5	10.8 ± 1.6	0.974	4.8 ± 0.5	5.0 ± 1.0	0.725
Suckers						
Oral (area) (μm^2)	25837 ± 8751	24429 ± 1700	0.818	3147 ± 404	2909 ± 734	0.409
Ventral (area) (μm^2)	35641 ± 9097	29451 ± 5205	0.071	1737 ± 550	1781 ± 347	0.637
Distance (μm)	206 ± 51	200 ± 41	0.818	150 ± 29	181 ± 53	0.099
Reproductive organs (μm^2)	27390 ± 2988	26442 ± 2685	0.670	38950 ± 4656	33325 ± 7292	0.140

[a] *P*-values calculated with Mann–Whitney test.

To see if measurements from male and female worms were associated Pearson's correlation coefficients were calculated. The areas of the oral and ventral sucker were strongly associated in male worms ($r = 0.931$, $P < 0.001$, $n = 96$), while no correlation was found between the sucker areas of female worms ($r = 0.219$, $P = 0.153$, $n = 44$) or between the sucker area and worm length in either sex. In male and female worms the length was slightly correlated with the distance between the suckers ($r = 0.321$, $P = 0.033$ in females and $r = 0.220$, $P = 0.035$ in males) and with the size of the reproductive organs (ovary: $r = 0.531$, $p < 0.001$, $n = 104$ and testicular lobes: $r = 0.386$, $P < 0.001$, $n = 117$). In male worms a positive correlation was found between the length and the tegument thickness ($r = 0.282$, $P = 0.001$, $n = 127$), while no such association exists in females ($r = -0.054$, $P = 0.600$, $n = 97$). Correlation was also calculated for the data obtained from the mice, to see if the average morphometric features of the worms from each mouse were associated with egg counts. Nor the size of either the ovary or the testicular lobes, nor the length of the worms was associated with the total amount of eggs counted in the liver and intestine.

4. Discussion

Most experimental studies on schistosomiasis have shown that the host–parasite relationship may be modified by the physiological status of the host (Kanuft and Warren, 1969; Coutinho et al., 2003). This study investigated whether streptozotocin-induced diabetes has an effect on acute schistosomiasis outcome compared to non-diabetic mice. Paired schistosomes migrate towards mesenteric veins, where still immature eggs are released, preventing them to transverse the gut tissue to reach the lumen and be voided with feces (Neves et al., 2007a).

In this study, instead of elevated numbers, our results present an almost fivefold diminution of *S. mansoni* eggs in the feces of diabetic mice compared to non-diabetic animals at 61 dpi. Part of this decrease could be explained by the lower recuperation of adult worms from the portal and mesenteric veins. However, other studies did not document differences regarding worm count between infected diabetic mice compared to control animals (Thabet et al., 2008). Although earlier reports have not revealed a reduction (Mahmoud et al., 1975; Mahmoud, 1979), high blood glucose or low insulin might diminish worm development or cercarial penetration. Schistosomes metabolize copious amounts of glucose (Bueding, 1950), but insulin does not influence its uptake and only non-specific binding occurs, suggesting that the worms do not have specific receptors for insulin or insulin like growth factor (Clemens and Basch, 1989). IL-7 and T4 modulate schistosome glucose metabolism through an alteration of the glucose metabolism of the infected host, namely through modulations of glucose and insulin circulating levels (Saule et al., 2005).

In an earlier experiment, we have shown STZ injected in mice already infected with *S. mansoni* (45 dpi) caused morphological alteration in the reproductive organs of male and female worms (Hulstijn et al., 2003). No differences of the male/female ratio were seen and also no morphological or morphometric differences were observed between the worms recovered from the diabetic and control group. Studies demonstrate that overexpression of genes and enzymes that may increases the glucose uptake could account for phenotypic alteration of schistosome development (Saule et al., 2005). In addition to this molecular and biochemical approaches, considering limitations of optical microscopy, we could deeper investigations of any variations of tissue organization in adult worms through confocal laser scanning microscopy (Machado-Silva et al., 1998). Previously, we evidenced that schistosomes from undernourished mice display quantitative and qualitative alterations in the reproductive organs and tegument (Neves et al., 2001).

Oviposition was not affected, as tissue egg counts per female worm were similar in the animals of both groups. These results were in accordance with the observations of Mahmoud et al. (1975,1976), Mahmoud (1979) and Thabet et al. (2008) who did not observe significant differences regarding tissue egg load. In this study, we showed lower liver and small intestine egg count, although this was not significantly different. This finding is not consistent with previous data showing a reduced tissue egg count in alloxan-induced diabetes in mice (Magalhães et al., 1978). Also, no differences were detected regarding tissue egg count in streptozotocin-induced diabetes compared to non-diabetic mice (Thabet et al., 2008).

Having been release in the mesenteric vasculature, immature eggs need about 5–6 days to the embryo reach maturity and starts eliminating lytic and antigenic secretions through micro pores present in the eggshell (Andrade, 2009). The current data suggest that eggs displayed lower conditions to mature in diabetic mice than controls. In a previous study, it was hypothesized that lipid-rich diets may be environmental modifiers which lead to exchanges between miracidium inside eggs, and nutrients primarily in the small intestine (Neves et al., 2007b).

Schistosome eggs across endothelial and mucosal barriers towards to the intestinal lumen, so that viable mature eggs can reach the outside environment with the host's feces. Molecules secreted from eggs (Ashton et al., 2001) induce cytokine production (Brindley, 2005) and local inflammation, where inflammatory cells sustain egg passage (Doenhoff et al., 1978, 1986; Damian, 1987; Lenzi et al., 1987; Doenhoff, 1998; Brindley, 2005; Andrade, 2009). The lack of insulin in the diabetic mice might therefore have caused a lowering in the Th2 response, which is necessary for the peri-ovular granulomatous reactions (Kaplan et al., 1998) facilitating the passage of eggs through the intestine tissue. Furthermore, lower fecal egg excretion has been described to occur due to changes in schistosomal egg adhesion to the endothelium and

several host adhesion molecules and egg carbohydrates are necessary for the adherence (Ngaiza and Doenhoff, 1990; File, 1995; Lejoly-Boisseau et al., 1999).

In an earlier experiment we have also shown a lower fecal egg excretion and a hampered egg maturation expressed by a high numbers of dead eggs (Hulstijn et al., 2001). Most probably, STZ-induced morphological alterations in the reproductive organs of male and female worms resulted in the high number of unviable eggs (Hulstijn et al., 2003). In the here presented experiment no contact existed between the drug and parasite, given that STZ was injected seven days before infection, however dead eggs were seen.

It is known that the architecture of the small intestine is changed due to the marked inflammation (De Man et al., 2001). The lack of insulin in the diabetic mice might therefore have caused a lowering in the Th2 response, which is necessary for the peri-ovular granulomatous reactions (Kaplan et al., 1998) facilitating the passage of eggs through the intestine tissue. Other possibility can be put forward to explain the lowering fecal egg output. Eggs may have lower capacity to across endothelial and mucosal barriers towards to the intestinal lumen. Interestingly, streptozotocin-induced diabetes in the rat model provoked changes in the quantitative architecture of the small intestine, which appears due to greater amounts mucosa, submucosa and muscularis externa than non-diabetic rats (Mayhew and Carson, 1989). However, diabetic small intestine shows changes in glucose transport due to numerical changes of villous enterocytes (Mayhew, 1996). Experiments are on going in our lab, aiming to determine a quantitative assessment of the mucosal architecture of streptozotocin-induced diabetes mice with concomitant schistosomiais infection. In conclusion, our data suggest that principally a hampered egg passage through the intestine tissue caused reduced egg excretion that probably both the eggs and the impaired host response play a role.

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