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Previous hookworm infection attenuates the immune response to a subsequent *Schistosoma mansoni* infection in an experimental model

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Research Paper

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Abstract

This study investigated the co-infection dynamics of *Schistosoma mansoni* and *Ancylostoma ceylanicum* in an experimental model. Two experiments were conducted to investigate varying infection orders. In experimental group 1, subjects were initially infected with *A. ceylanicum*, followed by *S. mansoni* infection. In group 2, subjects were first infected with *S. mansoni*, followed by *A. ceylanicum* infection. The co-infected groups were compared to mono-infected groups for further analysis. Parameters assessed included weight, fecal egg elimination, blood cell counts, IgG response, histopathological analysis, and granuloma morphometry. Results indicated that the timing of infections influenced weight loss. Co-infected animals lost weight similarly to *A. ceylanicum*-infected animals when *A. ceylanicum* preceded *S. mansoni.* The co-infected groups did not experience worsened anemia, despite both parasites being hematophagous. No linear correlation was found between *S. mansoni* egg counts and anti-SEA IgG production. The response to the *S. mansoni* adult worm antigen was significantly higher in the *S. mansoni* mono-infected group compared to the group previously infected with hookworm. Co-infection with *A. ceylanicum* after *S. mansoni* infection also interfered with the host response. It caused a delay in the peak of *S. mansoni* egg elimination compared to the *S. mansoni* monoinfected group. Additionally, fewer eggs were retained in the liver compared to single infections. These findings help elucidate the interactions between different parasites and their hosts in the same endemic area, providing valuable information to enhance strategies for disease control and management.

Introduction

Neglected tropical diseases currently impact over one-sixth of the global population, with helminth infections being the primary contributors to this burden (Gandhi *et al.* 2022). These parasitic infections pose significant public health challenges, affecting individuals worldwide, although they are more prevalent in rural communities of tropical and subtropical regions (Perera and Ndao 2021).

Notable among the helminths capable of infecting humans are hookworms (*Necator americanus*, *Ancylostoma duodenale*, and *Ancylostoma ceylanicum*) and the trematode *Schistosoma mansoni*, known for their high prevalence and associated physiological disorders (Anderson and Enabulele 2021; Loukas *et al.* 2016). Hookworms, soil-transmitted helminths, reside in the small intestine of hosts, causing an estimated loss of 2.1 million disability-adjusted life years (DALYs) (Bartsch *et al.* 2016). Meanwhile, the adult stages of *S. mansoni* inhabit the mesenteric veins of definitive hosts.

Due to limited funding research, drug production, and control efforts, schistosomiasis mansoni and hookworm infections remain classified as neglected tropical diseases (Engels and Zhou 2020). Given shared epidemiological factors, such as inadequate sanitation and low socioeconomic and educational levels, these infections may coexist in the same region, leading to co-infection. Literature reports indicate polyparasitism involving these species globally (Gordon *et al.* 2020; Hodges *et al.* 2012). Studies by Fetene *et al.* (2021) suggest that hookworm and *S. mansoni* infections contribute to anemia in children, impacting their health status, although the full extent of this issue remains uncertain.

Recent studies have focused on understanding the interactions between different parasites and their hosts during co-infection (Che-Kamaruddin and Isa 2023; Szabo *et al.* 2024). Such

interactions can result in synergistic or antagonistic effects on immune responses, influencing clinical manifestations (Brito *et al.* 2006). While several co-infection studies exist among helminths (Beiromvand *et al.* 2018; Yoshida *et al.* 1999), among microparasites (viruses, bacteria, and protozoa) (Gümüşer *et al.* 2018; Kwenti *et al.* 2018), and between helminths and microparasites (Ahmed *et al.* 2017; Salazar-Castañón *et al.* 2018), to our knowledge, no study has explored the interaction between hookworms and *S. mansoni*.

This study aims to investigate co-infection between these two parasites using the hamster (*Mesocricetus auratus*) as an experimental model. This rodent replicates infections by *S. mansoni* and the hookworm *A. ceylanicum*, exhibiting weight loss, decreased growth, anemia, and chronic infection akin to human infections. Through this work, we aim to shed light on the co-infection dynamics and its impact on the host's immune response and health status.

Two hypotheses were formulated for this study. The first suggested that a prior infection by one helminth, inducing a similar type 2 immune response, might confer parcialprotection against subsequent infection by a second helminth. The second hypothesis suggests that simultaneous co-infection by two hematophagous worms could intensify host damage, exacerbating anemia and other pathological parameters. Notably, our findings revealed that co-infection did not exacerbate the health status of the hamsters, suggesting modulation of the host's immune response.

Methods

Ethical considerations

The authors confirm that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals (Animal Care Ethics Committee at the Universidade Federal de Minas Gerais - Protocol number 52/2011).

Experimental design

To assess the effects of co-infection by *A. ceylanicum* and *S. mansoni*, two experiments were conducted, with the sequence of infection alternated. The experimental design aimed to evaluate the impact of infection, including the immune response, at different stages of infection, including the acute and chronic phases, and to investigate whether the order of infection would affect the parameters under evaluation. A schematic diagram providing an overview of infection timings and monitoring periods is presented in Figure 1.

In Experiment 1, hamsters were divided into four groups: uninfected (NI-1) (n=14), infected with 50 filarid larvae (L3) of *A. ceylanicum* (Acey-1) (n=14), infected with 30 cercariae of *S. mansoni* (Sm-1) (n=14), and initially infected with 50 L3 of *A. ceylanicum* and subsequently co-infected with 30 cercariae of *S. mansoni* after 20 days (Acey + Sm) (n=14). The groups in Experiment 1 were monitored for 75 days.

In Experiment 2, hamsters were divided into another four groups: uninfected (NI-2) (n=16), infected with 50 L3 of *A. ceylanicum* (Acey-2) (n=21), infected with 30 cercariae of *S. mansoni* (Sm-2) (n=21), and initially infected with 30 cercariae of *S. mansoni* and subsequently co-infected with 50 L3 of *A. ceylanicum* after 65 days (Sm + Acey) (n=21). The groups in Experiment 2 were monitored for 90 days. Discrepancies in the number of animals among groups resulted from anticipated mortality during the extended experimental periods, which occurred in some instances.

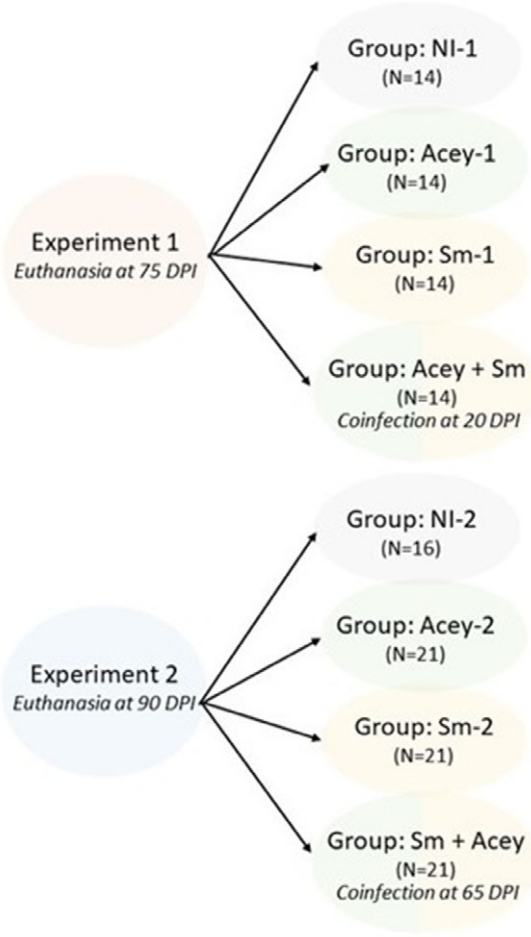

Figure 1. Experimental design of co-infections. Experiment 1: Hamsters were divided into four groups: uninfected (NI-1), infected with *Ancylostoma ceylanicum* (Acey-1), infected with *Schistosoma mansoni* (Sm-1), and initially infected with *A. ceylanicum* and subsequently co-infected with *S. mansoni* (Acey + Sm). Experiment 2: Hamsters were divided into another four groups: uninfected (NI-2), infected with *A. ceylanicum* (Acey-2), infected with *S. mansoni* (Sm-2), and initially infected with *S. mansoni* and subsequently co-infected with *A. ceylanicum* (Sm + Acey) (n=21). DPI: Days post-infection.

Infection

Hamsters were infected with cercariae of the LE strain of *S. mansoni*, which has been maintained through successive passages in *Biomphalaria glabrata* and *M. auratus*. Cercariae isolated from *S. mansoni*-infected snails underwent washing, concentration, and quantification, followed by subcutaneous injection of 30 cercariae per hamster, following the protocol outlined by Peters and Warren (1969).

Hamsters were infected with filarioid larvae (L3) of *A. ceylanicum*. Coprocultures were prepared according to the method described by Roberts and O'Sullivan (1950), using fecal samples from infected hamsters. Larvae were recovered using the Baermann-Moraes method. The animals were orally infected with an average of 50 L3 per hamster using a gavage needle.

Comparison of blood spoliation

Blood samples were obtained sublingually and homogenized with 0.5 M EDTA anticoagulant. In Experiment 1, samples were collected on days 0, 30, 60, and 75 post-infection (DPI) (euthanasia).

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In Experiment 2, collection times were on days 0, 30, 60, and 90 DPI (euthanasia). The blood samples were used to evaluate erythrogram using volumetric impedance hematology analysis equipment (BC-2800 Vet Mindray). Additionally, a differential leukocyte count was conducted using an optical microscope (Olympus BH2, Japan), and immunological assays were performed using the blood samples.

Comparison of animal weight

Each animal's weight was measured using a semi-analytical scale on the day of infection and every seven days thereafter until the day of euthanasia.

Parasitological examination of feces

Fecal samples from *S. mansoni*-infected animals were collected starting from 30 DPI and analyzed following the method described by Ritchie (1948) every two days. The samples were processed by resuspending the pellet in 1 ml of 3.7% buffered formaldehyde solution. Fifty µl aliquots were then taken, and readings were conducted in quadruplicate after staining with lugol. The total number of eggs was estimated based on the mean of eggs obtained in the readings and the estimated value for the 1 ml volume. Additionally, the number of eggs per gram of feces (EPG) was calculated for each group infected by *A. ceylanicum*. This quantification was performed from 10 DPI, every two days, using a McMaster chamber to estimate the parasite load.

Recovery of adult worms

The hamsters were euthanized via intraperitoneal administration of an anesthetic overdose (45 mg/kg xylazine hydrochloride solution and 240 mg/kg ketamine). Subsequently, the abdominal and thoracic cavities of the hamsters infected with *S. mansoni* were opened, exposing the viscera for visualization of the portal system. After ligating the rectum, the portal vein was dissected at the fusion region of its mesenteric afferents. A needle connected to an infusion pump (Automatic Pippeting Brewer Machine, model 60453, B.D.) was inserted into the thoracic aorta to perform perfusion with saline solution containing EDTA (0.85% NaCl and 0.05% EDTA 5M). The liquid containing the leaked worms through the ruptured portal vein of each animal was collected in individual cups. The infusion was repeated by inserting the needle into the hepatic sinus. The material collected from each animal was decanted for 30 minutes, with the supernatant being aspirated and discarded. More saline solution was added, and the process was repeated until the supernatant was clear. The worms present in each cup were transferred to Petri dishes and observed under a stereoscopic microscope, where they were separated by sex and counted.

For hamsters infected with *A. ceylanicum*, each animal was placed in a dorsal decubitus position, and the abdominal cavity was opened. The small intestine was removed and opened longitudinally, followed by scraping of the mucosa to detach the adult worms into a Petri dish containing a PBS (Phosphate Buffered Saline) solution with a pH of 7.4. The recovered worms were then quantified, separated by sex, and counted.

Quantification of S. mansoni eggs retained in the liver

The quantification of eggs retained in the liver tissue was determined according to Cheever's method. Briefly, after perfusion of animals infected with *S. mansoni*, the greater lobe of the liver was removed, weighed, and digested with a 5% potassium hydroxide (KOH) solution. The digested liver tissue was then centrifuged to sediment it. Egg counting was performed in duplicate, and the total egg count was estimated from the results.

Histological processing and evaluation

The small intestine was fixed on filter paper using a 3.7% buffered formaldehyde solution, and after 24 hours, it was rolled from the back end onto itself. After another 24 hours, the samples were transferred to identified histological cassettes and immersed in fresh buffered 3.7% formaldehyde solution. The cassettes containing the samples were then placed in stainless steel baskets and processed in an automated equipment (Easypath-Leica). Briefly, the tissue was immersed in water for 10 minutes to remove excess formaldehyde, followed by dehydration in baths of 70%, 80%, 90% alcohol, and absolute alcohol for 30 minutes each. Subsequently, the samples were cleared in xylene baths for two periods of 15 minutes each. Finally, the samples were processed in a histological paraffin bath for 15 minutes. The dehydrated and cleared samples were then transferred to embedding cassettes, and the cassettes were filled with a histological paraffin wax solution containing 2% beeswax.

The slides were examined under a light microscope at a magnification of 400x and scanned using the Leica DFC340FX microchamber associated with the Leica DM5000B microscope. The images obtained were analyzed using ImageJ software. The small intestine sections were qualitatively evaluated for mucosal alterations, including hypercellularity of the lamina propria, hypersecretion of Paneth cells, goblet cell hyperplasia, and erosion, as well as submucosal and muscle alterations. Morphometric analysis was performed to obtain measurements of villi (height and thickness) and crypts (height). Ten hamster villi per group were analyzed.

Granulomas present in the sections were evaluated qualitatively and morphometrically for the inflammatory process, average granuloma area, and evolutionary stage based on the predominant component: necrotico-exudative, productive, fibrosis cure, and fibrosis cure according to the classification by Raso *et al.* (2012).

Antigen preparation and Enzyme-linked immunosorbent assay (ELISA)

The preparation of antigens from the excretory and secretory (ES) products of adult worms of *A. ceylanicum* and total protein extract followed the method described by Serafim *et al.* (2014). Soluble adult worm antigen (SWAP) and *S. mansoni* egg antigen (SEA) were obtained according to Ali *et al.* (2012). The concentrations of antigens were estimated according to Bradford (1976).

Polystyrene microtiter plates (BD Falcon) were sensitized with 100 µl/well of the total protein extract solution of *A. ceylanicum* (1 µg/ml) or ES (5 µg/ml) or SWAP (1 µg/ml) or SEA (3 µg/ml) in carbonate/bicarbonate buffer (0.05M NaHCO3 pH 9.6). For blocking, the plates were incubated for 90 min at 25°C in a PBS-0.05% Tween 20 (PBST) solution containing 3% casein. Next, plasma samples were analyzed individually (diluted 1:100), beginning with an incubation for 18 h at 4°C.

A secondary antibody (biotinylated anti-Armenian and Syrian hamster IgG, BD Pharmingen), diluted 1:5,000, was subsequently added for 2 h at 4°C. Streptavidin (Sigma-Aldrich, USA), diluted 1:3,000 in 0.05% PBST, was then added, and the plate was incubated for 20 min. The assay was developed with TMB® (BD OptEIA, USA)

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for 10 min in the dark, and the reaction was stopped by adding 100 µl of 2 M H2SO4 to each well of the plate. The plate was read at 450 nm on a spectrophotometer (VersaMax Microplate Reader, Molecular Devices Inc., USA), using SoftMax Pro software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. The Kolmogorov-Smirnov test was used to assess the distribution of the data. For analyses between two groups, paired or unpaired T-tests were used for parametric data, and Wilcoxon Matched Pairs or Mann-Whitney tests were used for non-parametric data. For analyses involving three or more groups, parametric data were analyzed using ANOVA or Repeated Measures ANOVA, followed by Tukey tests. Non-parametric data were analyzed using Kruskal-Wallis or Friedman tests, followed by Dunn's tests. The Grubbs test was used to identify any outliers. A significance level of $p \leq 0.05$ was considered for all tests.

Results

Ancylostoma ceylanicum infection is responsible for weight loss in the co-infected or monoinfected group

In Experiment 1, the animals were weighed at 0 DPI and then every seven days until the end of the experiments. The NI-1 and Sm-1 groups demonstrated steady weight gain across all assessment dates. In contrast, those in the Acey-1 and Acey + Sm groups experienced periodic fluctuations, alternating between weight gain and loss throughout the experiment. A statistically significant difference in weight loss was observed among groups infected with *A. ceylanicum*, whether monoinfected or co-infected with *S. mansoni*, from 28 DPI onwards compared to the NI-1 group: Acey-1 ($p < 0.05$), Acey + Sm ($p < 0.05$), Sm and Acey-1 ($p < 0.05$), and Sm-1 and Acey + Sm ($p < 0.05$). This difference persisted throughout the study period. No statistically significant differences were observed in the comparison of NI-1 with Sm-1 or Acey-1 with Acey + Sm groups during the experiment (Figure 2A).

In Experiment 2, the animals were weighed weekly until the end of the experiment at 90 DPI. Comparisons between NI-2 and the infected groups (Acey-2, Sm-2, and Sm + Acey) and among those infected with each individual species showed no statistically significant differences among the groups (Figure 2B).

Delayed *S. mansoni* oviposition due to co-infection

The output kinetics of *A. ceylanicum* eggs in the feces of the Acey-1 group and Acey + Sm are shown in Figure 3A. Patency was observed at 16 DPI for both groups. The oviposition peak occurred at 32 DPI for the Acey-1 group and 26 DPI for Acey + Sm. *Schistosoma mansoni* eggs were observed in the feces of the Sm-1 and Acey + Sm groups starting from 42 DPI. The oviposition peak occurred at 46 DPI for both groups (Figure 3B).

In Experiment 2, *S. mansoni* eggs were observed in the Sm-2 and Sm + Acey groups from 43 DPI. The oviposition peak occurred at 71 DPI in the Sm-2 group and at 82 DPI in the Sm + Acey group (Figure 3C), demonstrating that *A. ceylanicum* co-infection, even after *S. mansoni* infection, altered the oviposition peak. The patency of *A. ceylanicum* for the Acey-2 and Sm + Acey groups occurred at 17 DPI.

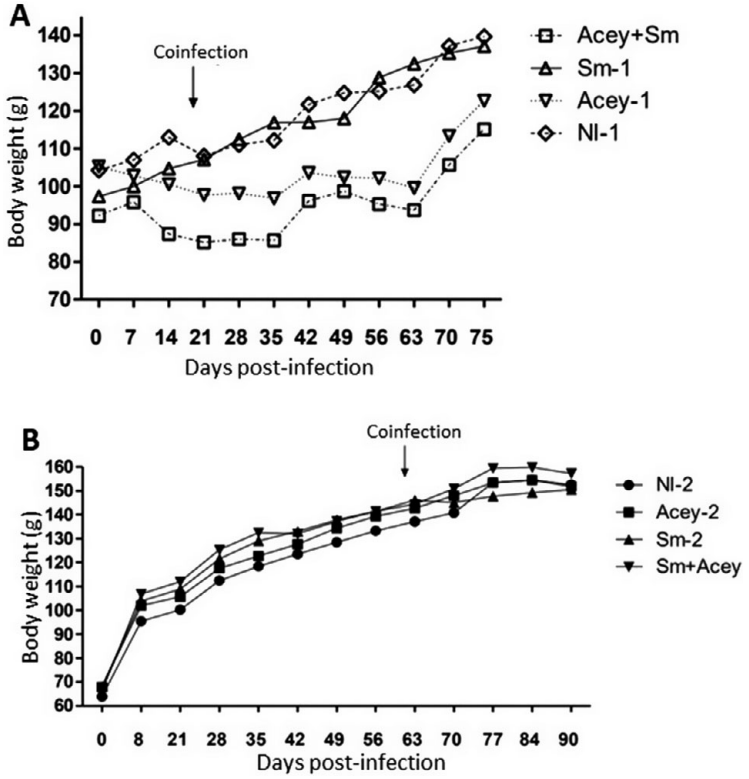

Figure 2. Average body weight of the groups throughout the experiment. A) Experiment 1: Non-infected (NI-1), infected with 50 L3 of *A. ceylanicum* (Acey-1), infected with 30 *S. mansoni* cercariae (Sm-1), and infected with *A. ceylanicum* followed by *S. mansoni* infection (Acey+Sm). B) Experiment 2: Non-infected (NI-2), infected with 50 L3 of *A. ceylanicum* (Acey-2), infected with 30 *S. mansoni* cercariae (Sm-2), and infected with 30 *S. mansoni* cercariae followed by *A. ceylanicum* infection after 60 days (Sm+Acey).

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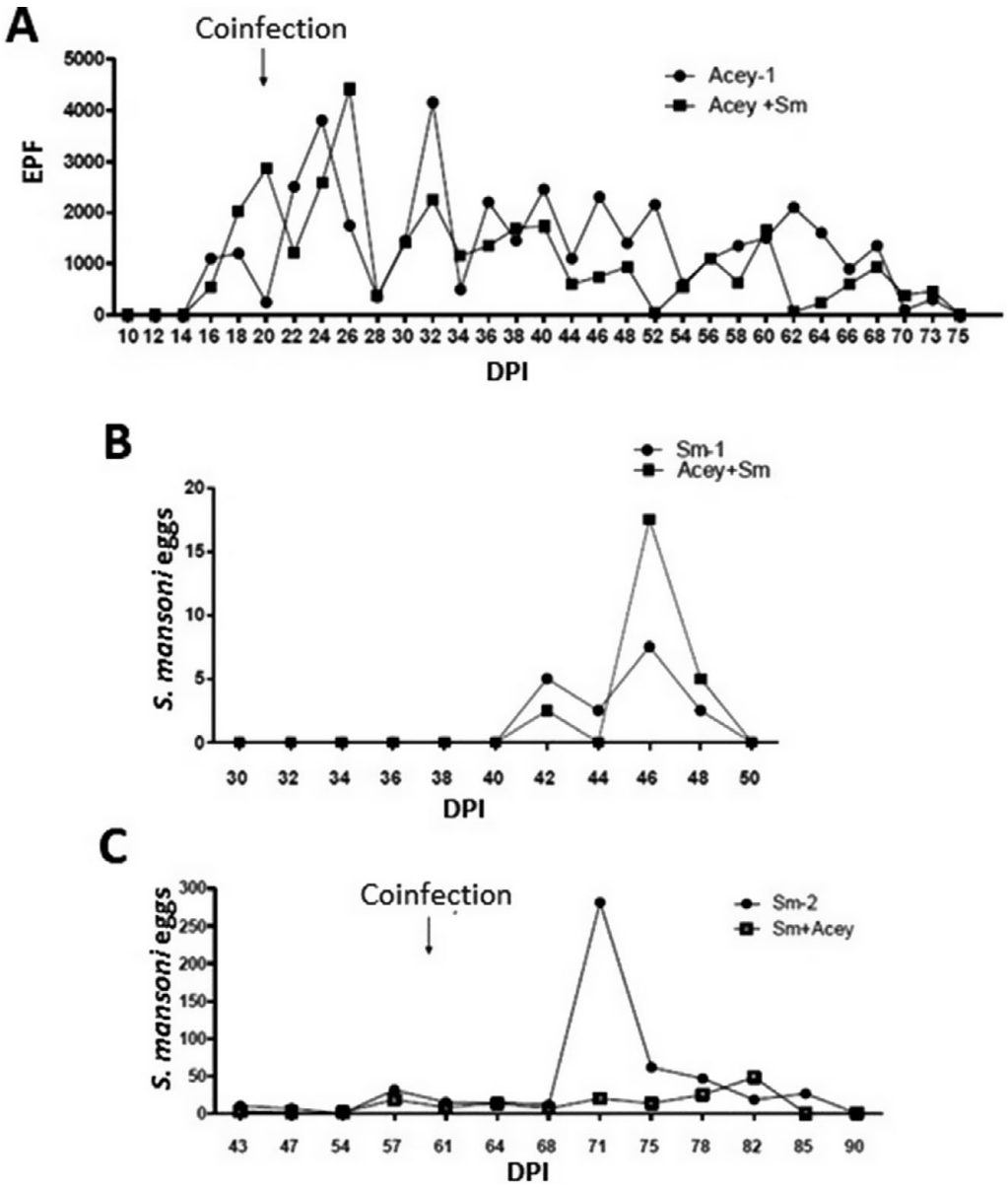

Figure 3. Oviposition of *Ancylostoma ceylanicum* and *Schistosoma mansoni* during single infection or co-infection. (A) *A. ceylanicum* oviposition from Experiment 1, *A. ceylanicum* infection followed by *S. mansoni* infection. (B) *S. mansoni* oviposition from Experiment 1. (C) *S. mansoni* oviposition from Experiment 2, *S. mansoni* infection followed by *A. ceylanicum* infection. Abbreviations: Acey-1: group infected with 50 L3 of *A. ceylanicum*. Acey + Sm: group infected with 50 L3 of *A. ceylanicum* and co-infected with 30 cercariae of *S. mansoni* at 20 DPI. Sm-1: group infected with 30 cercariae of *S. mansoni.* Sm-2: group infected with 30 cercariae of *S. mansoni.* Sm + Acey: group infected with 30 cercariae of *S. mansoni* and co-infected with 50 L3 of *A. ceylanicum* at 60 DPI. DPI = Days post-infection.

Co-infection interferes with the successful establishment of the second inoculated parasite

Figure 4 illustrates the mean of recovered worms in infected animals from Experiments 1 and 2. In Experiment 1, animals were euthanized for adult worm recovery after 75 DPI. Statistical differences were not observed in the recovery of *S. mansoni* between single infection (Sm-1) and co-infection (Acey + Sm) (Figure 4A). Similarly, no difference in the recovery of *A. ceylanicum* between single infection (Acey-1) and co-infection (Acey + Sm) was observed (Figure 4B).

In Experiment 2, animals were euthanized for adult worm recovery after 90 DPI. A statistical difference was observed in the recovery of *A. ceylanicum* between the single infection group (Acey-2) and the co-infected group (Sm + Acey), with a smaller number of worms recovered from the co-infected group (Figure 4C). However, no difference in the recovery of *S. mansoni* between single infection (Sm-2) and co-infection (Sm + Acey) was observed (Figure 4D).

Co-infection with *A. ceylanicum* in hamsters previously infected with *S. mansoni* caused a decrease in the number of *S. mansoni* eggs retained in the liver

In Experiment 1, no statistical difference was observed in the number of eggs retained in the liver between groups. However, in Experiment 2, the Sm-2 group had a significantly higher number of eggs retained in the liver compared to the Sm + Acey co-infected group ($p < 0.05$).

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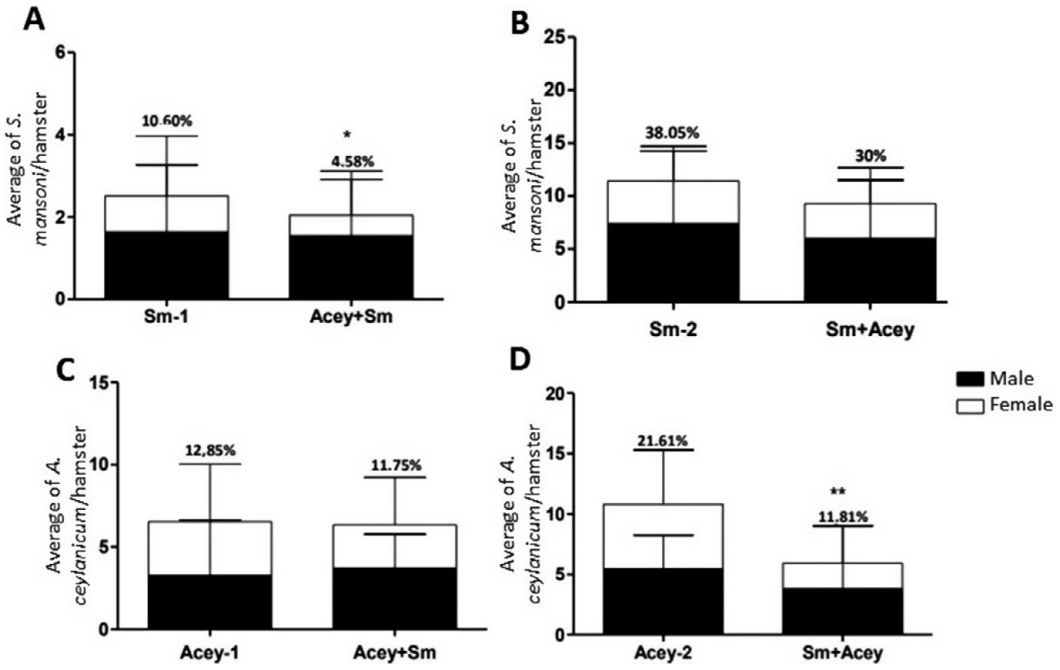

Figure 4. Worm Recovery. Average recovery of adult *S. mansoni* worms from the mesenteric vessels of hamsters in Experiment 1 (infection of *A. ceylanicum* followed by *S. mansoni* infection) (A) and Experiment 2 (infection of *S. mansoni* followed by *A. ceylanicum* infection) (B). Average recovery of adult *A. ceylanicum* worms from the small intestine of hamsters in Experiment 1 (C) and Experiment 2 (D). Abbreviations: Acey-1: group infected with 50 L3 of *A. ceylanicum.* Acey + Sm: group infected with 50 L3 of *A. ceylanicum* and co-infected with 30 cercariae of *S. mansoni* at 20 DPI. Sm-2: group infected with 30 cercariae of *S. mansoni.* Sm + Acey: group infected with 30 cercariae of *S. mansoni* and co-infected with 50 L3 of *A. ceylanicum* at 60 DPI.

Infection with *A. ceylanicum* resulted in a reduction in the number of red blood cells, hemoglobin levels, and hematocrit levels over the course of infection in both co-infected and mono-infected groups

In Experiment 1, the hemogram performed at 30 DPI revealed a significant reduction in the number of red blood cells for the Acey-1 ($p < 0.01$) and Acey + Sm ($p < 0.05$) groups compared to the NI-1 group. Similarly, hemoglobin and hematocrit levels were also reduced significantly in the Acey-1 ($p < 0.001$) and Acey + Sm ($p < 0.01$) groups compared to the NI-1 group. No statistically significant difference was observed between the Acey-1 and Acey + Sm groups. The Sm-1 group did not show significant changes in any of the evaluated parameters compared to the NI-1 group.

At 60 DPI, the number of circulating erythrocytes was reduced for the Acey-1 ($p < 0.05$) and Acey + Sm ($p < 0.05$) groups compared to NI-1. However, the Sm-1 group did not exhibit alterations in red blood cell, hemoglobin, or hematocrit levels compared to the NI-1 group. Hemoglobin levels showed a significant change for the Acey-1 ($p < 0.05$) and Acey + Sm ($p < 0.05$) groups compared to NI-1. In all groups, hemoglobin levels were below the physiological level. No statistically significant difference in hematocrit was observed between the NI-1 and infected groups.

At 75 DPI, increased levels of red blood cells, hemoglobin, and hematocrit were observed in all groups compared to blood counts at 30 and 60 DPI. Regarding the number of erythrocytes, the Acey-1 group showed lower values compared to the other groups, with a statistically significant difference in relation to NI-1 ($p < 0.01$). Additionally, a significant difference was observed between the Acey + Sm group and NI-1 ($p < 0.05$). Regarding hemoglobin levels, a significant difference was observed when comparing the NI-1 group with the Acey-1 ($p < 0.001$) and Sm-1 ($p < 0.01$) groups. For hematocrit values, differences were observed between the groups in the comparison of NI-1 and Acey-1 groups ($p < 0.01$), as well as between the Acey-1 and Acey + Sm groups ($p < 0.05$).

In Experiment 2, the hemogram performed at 90 DPI revealed alterations in red blood cell, hemoglobin, and hematocrit levels. A significant reduction in the number of red cells was observed for the Acey-2 ($p < 0.001$) and Sm + Acey ($p < 0.05$) groups compared to the NI-2 group. This reduction was also observed in hemoglobin levels and hematocrit values. There was no statistically significant difference in the comparison of the Acey-2 and Sm + Acey infected groups in the aforementioned parameters. Additionally, no difference was observed in the comparison between the Sm-2 group and the NI-2 group.

In Experiment 1, the leukogram performed at 30 DPI and 60 DPI showed no changes in total leukocytes, lymphocytes, total neutrophils, or eosinophils in the comparison between the infected and NI-1 groups. At 75 DPI, the leukogram revealed a change in lymphocyte counts, with a statistically significant difference observed in the comparison between the NI-1 and Sm-1 groups ($p < 0.01$) and between the Sm-1 and Acey + Sm infected groups ($p < 0.01$), where the Sm-1 group presented higher values. However, the other parameters evaluated did not show any significant alterations. In Experiment 2, at 90 DPI, the leukogram also showed a change in lymphocyte counts, with a statistically significant difference observed in the comparison between the NI-2 and Sm + Acey groups ($p < 0.01$). Similarly, no significant alterations were observed in the other parameters evaluated.

Total IgG levels in hamster plasma in response to antigens from *S. mansoni* and *A. ceylanicum*

In Experiment 1, hamster plasma collected at 30, 60, and 75 DPI was assessed for total IgG levels against Soluble Egg Antigens

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(SEA), Soluble Worm Antigen Preparation (SWAP), crude extract (CE), and excreted/secreted products (ES) of *A. ceylanicum*. In Experiment 2, these analyses were conducted at 90 dpi. Figures 5 and 6 present the results of these assays for various blood collection time points.

At 30 and 65 dpi, the Sm-1 and Acey+Sm groups exhibited an average absorption below the cutoff value against the SWAP antigen, with no statistical difference compared to the NI-1 group (Figures 5A and 5E). No reaction against the SEA antigen was observed in the Sm-1 and Acey+Sm groups, except for one animal in the 65 DPI group (Figure 5B and 5F). However, at 75 DPI, all plasmas from the Sm-1 and Acey+Sm groups showed absorbance above the cutoff value for the SWAP antigen (Figure 5I), with a statistical difference compared to the infected groups, Sm-1 ($p < 0.01$), Acey+Sm ($p < 0.05$), and the NI-1 group. Additionally, a significant reduction in anti-SWAP IgG levels was observed in the Acey+Sm group compared to the Sm-1 group at 75 DPI ($p < 0.05$) (Figure 5I). Although all animals infected with *S. mansoni* recognized SWAP, the same did not occur for SEA (Figure 5J). A statistical difference was observed when comparing the NI-1 group with the infected Sm-1 ($p < 0.01$) and Acey+Sm ($p < 0.05$), with no significant difference between Sm-1 and Acey+Sm in response to SEA antigens.

The Acey-1 and Acey+Sm groups displayed absorption values above the cutoff against the CE and ES antigens of *A. ceylanicum*, with statistical differences compared to the NI-1 group ($p < 0.01$ for 30 DPI) (Figure 4C and 4D), $p < 0.01$ and $p < 0.001$ for 65 DPI, Figure 4G and H, respectively, to CE and ES antigens; and ($p < 0.01$ for 75 DPI (Figure 4K and 4L). No significant difference was observed in the comparison between the Acey-1 and Acey+Sm groups at 30, 65, and 75 DPI, indicating that co-infection with *S. mansoni* did not interfere with the humoral immune response against *A. ceylanicum* infection. Conversely, animals initially infected with *A. ceylanicum* and co-infected with *S. mansoni* showed a significant reduction in anti-SWAP IgG levels, suggesting that *A. ceylanicum* may have modulated the immune response against *S. mansoni* (Figure 4I).

Regarding Experiment 2, in which *S. mansoni* infection preceded *A. ceylanicum* infection, and the animals were euthanized at 90 DPI, the groups infected with *S. mansoni* exhibited a distinct response to SWAP and SEA antigens. Conversely, groups infected with *A. ceylanicum* (Acey) demonstrated reactivity to the CE and ES antigens of *A. ceylanicum*. No cross-reactivity was observed between different groups and their respective specific antigens.

The Sm-2 group demonstrated reactivity to SEA and SWAP antigens of *S. mansoni*, with a statistical difference observed compared to the non-infected (NI)-2 group ($p < 0.01$) and the Acey-2 group ($p < 0.001$) (Figure 6A and 6B). No significant difference was noted between the Sm-2 group and the Sm+Acey group concerning SEA and SWAP antigens of *S. mansoni*. The Acey-2 group exhibited significant reactivity to CE and ES antigens of *A. ceylanicum* compared to the NI-2 group ($p < 0.001$) and the Sm-2 group ($p < 0.001$) (Figure 6C and 6D). However, there was no statistically significant difference between the Acey-2 and Sm+Acey groups regarding the CE and ES antigens of *A. ceylanicum*. The Sm+Acey group exhibited reactivity to all tested antigens, with a statistically significant difference observed only between the Sm+Acey and NI-2 groups ($p < 0.01$) (Figure 6A, 6B, 6C, and 6D). No statistical differences were observed between the monoinfected groups (Acey-2 or Sm-2) and the co-infected group (Sm+Acey) concerning SEA, SWAP, CE, and ES antigens of *A. ceylanicum*.

Histological analysis

In Experiment 1, the mucosa of the hamsters of the NI-1 group presented normal histological appearance, with intact structures, and mean villus-crypt ratio of 3:1. Paneth cells and goblets were present in normal amounts. Changes in the architecture of the small intestine were observed in all infected groups, such as loss of intestinal epithelium, reduction of intestinal villi height, and hypertrophy of Lieberkün crypts. There were reductions in villus height in the Acey-1 ($p < 0.05$) and Sm-1 ($p < 0.05$) groups in comparison to the NI-1 group. No significant changes were observed in the comparison between the Acey + Sm group and the NI-1 group or the others infected. Regarding crypt hypertrophy, it was only significant in the Acey + Sm group. The villi / crypt ratio of infected groups showed a significant reduction when compared to NI-1.

The analysis of granulomas was performed only for the small intestine, as it is the location of *A. ceylanicum*. The number of granulomas formed in the small intestine was similar in the Sm-1 and Acey + Sm groups. Co-infection did not change the composition of the granuloma or size, and the result can be visualized in Table 1. No granulomas were observed in the fibrosis cure phase because of the time of infection.

In Experiment 2, the hamsters of the NI-2 group presented normal histological appearance of the mucosa, with intact structures and mean villus-crypt ratio of 3:1. Paneth cells and goblets were quantitatively and qualitatively normal. In the morphometric evaluations, no significant changes were observed in villus height in the comparison between groups infected with the NI-2 group or with each other. We observed significant Liberkün crypt hypertrophy only in the comparison between the co-infected Sm + Acey group ($P < 0.01$) and the NI-2 group. Changes in the architecture of the small intestine were observed in all infected groups, such as loss of intestinal epithelium, reduction of height of intestinal villi, and hypertrophy of Lieberkün crypts. No significant changes were observed in the comparison between the Sm + Acey group and the NI-2 group or the other infected groups. All infected groups showed alteration of villus-crypt ratio to 1: 1. The number of granulomas formed in the small intestine was similar in the Sm-2 and Sm + Acey groups. The result can be observed in Table 1. Co-infection did not alter the composition of the granuloma, evolution or size (Supplementary Material).

Discussion

Schistosomiasis mansoni and ancylostomiasis are parasitic infections caused by distinct helminth species, primarily impacting economically marginalized populations. This investigation arises from the coexistence of these parasites in specific geographical regions, highlighting the need fora comprehensive understanding of their interactions within a common host (Gray *et al.* 2010; Hotez *et al.* 2006). As parasitic infections continue to pose significant public health challenges, unraveling the underlying mechanisms governing the interplay between these helminths becomes paramount for devising more effective strategies in disease control and treatment (Furtado *et al.* 2019).

Unraveling the complex mechanisms that influence their interaction is crucial for formulating more effective disease control programs and treatments. Experimental co-infection models have revealed evidence of cross-immunity, immunosuppression, and intra- and interspecific competition during co-infections (Gümüşer *et al.* 2018; Kwenti *et al.* 2018). In this study, we meticulously examine the interaction between *A. ceylanicum* and *S. mansoni*,

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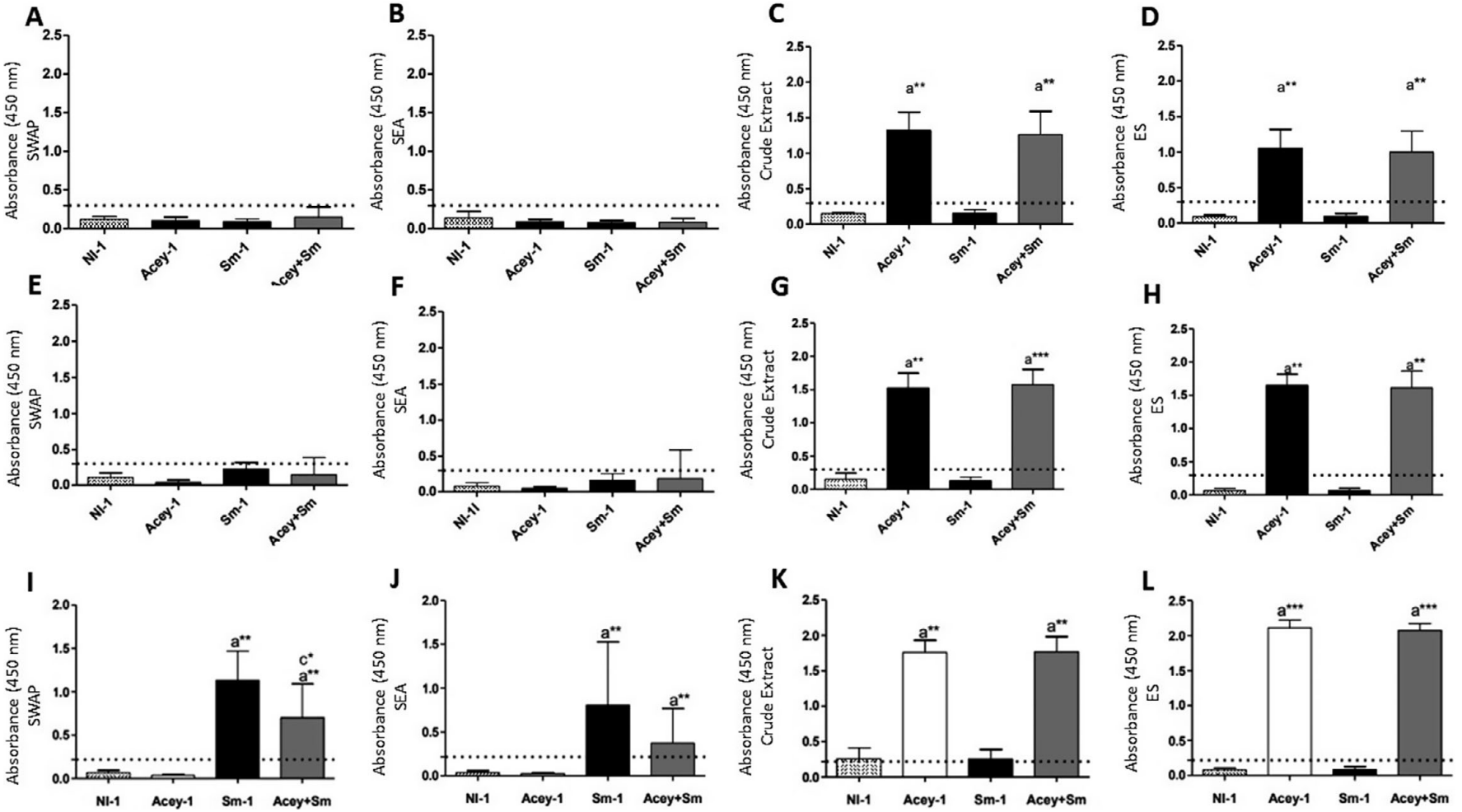

Figure 5. Levels of total IgG in hamster plasma collected at various time points post-infection (35, 60, and 75 DPI) against different antigens. The antigens tested include Soluble Worm Antigen Preparation (SWAP), Soluble Egg Antigens (SEA), crude extract of *Ancylostoma ceylanicum*, and excreted/secreted products (ES). The experimental groups consisted of hamsters categorized as follows: uninfected (NI-1), infected with 50 L3 of *A. ceylanicum* orally (Acey-1), infected with 50 L3 of *A. ceylanicum* and 30 cercariae of *Schistosoma mansoni* subcutaneously (Sm-1), and co-infected with 50 L3 of *A. ceylanicum* and 30 cercariae of *S. mansoni* at 20 DPI (Acey + Sm). Each group comprised 10 or 11 hamsters. The dotted line represents the cut-off value, and statistical significance compared to the NI-1 group is denoted by ** = $p < 0.01$. Panels A to D depict plasma obtained at 35 DPI, panels E to H represent plasma obtained at 60 DPI, and panels I to L show plasma obtained at 75 DPI.

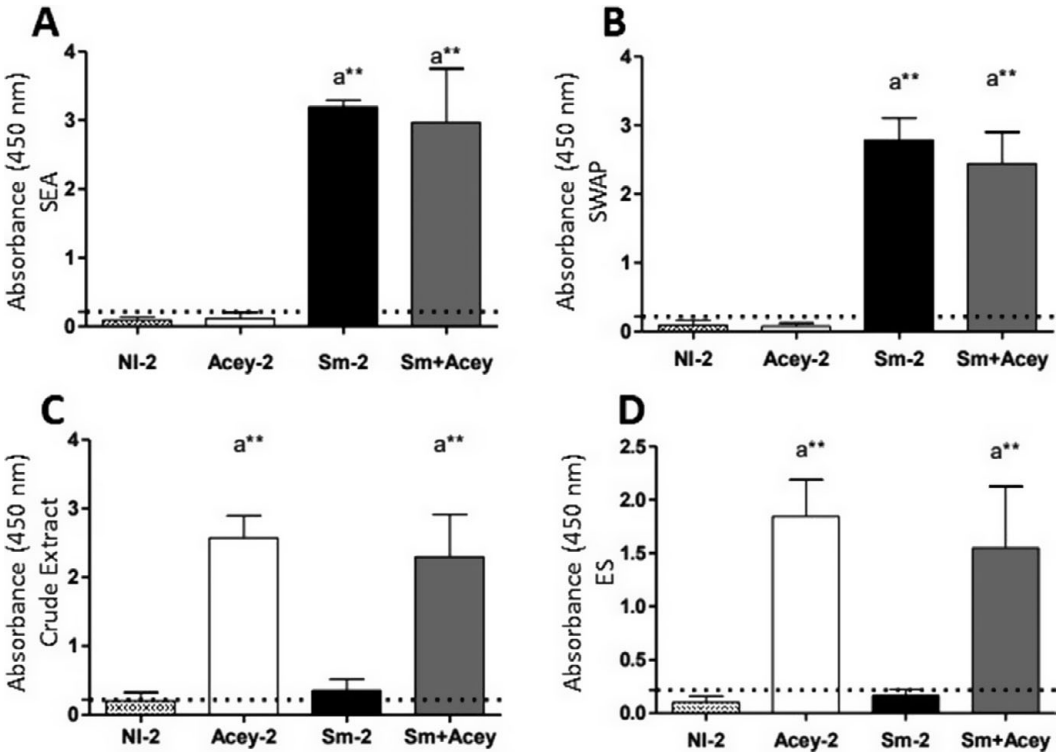

Figure 6. Levels of total IgG in hamster plasma against various antigens. Panel A represents SEA, Panel B represents SWAP, Panel C represents the crude extract of *Ancylostoma ceylanicum*, and Panel D represents ES. The experimental groups are as follows: Not infected (NI) (n = 6), Acey-2 (n = 20), Sm-2 (n = 7), and Sm + Acey (n = 11). A dotted line indicates the cut-off value. Statistical significance compared to the NI-2 group is indicated by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Plasma samples were collected at 90 DPI.

Table 1. Analysis of the evolutionary stage in granulomas of the small intestine

Group	Granuloma				Total
	Necrotic-exudative	Productive	Productive healing by fibrosis	Healing by fibrosis	
Sm–1	3	5	0	0	8
Acey+Sm	3	4	0	0	7
Sm2	22	26	5	3	56
Acey+Sm	20	29	13	3	65

assessing parasitological, morphological, and immunological parameters. Our hypotheses are based on the potential implications of simultaneous infections by *A. ceylanicum* and *S. mansoni.*

The first hypothesis suggests that the simultaneous presence of these two hematophagous worms could lead to synergistic effects, exacerbating the development of anemia in co-infected groups. The second hypothesis proposes that co-infection with *A. ceylanicum* and *S. mansoni* could influence the host's immune response, specifically by amplifying the Th2 immune pathway. This hypothesized immunomodulation aims to explore the potential mitigation of lesions and overall pathogenesis in the host, as Th2 responses are typically associated with anti-parasitic immune reactions.

Hamsters serve as a model in various studies (da Silva *et al.* 2019; Ferreira *et al.* 2020; Furtado *et al.* 2019; Serafim *et al.* 2014), and *A. ceylanicum* infection faithfully replicates the clinical situation observed in humans, including biochemical and hematological alterations (Furtado *et al.* 2024; Pacanaro *et al.* 2014; Silva Medeiros *et al.* 2022). Our results align with the findings in the literature, as infection with *A. ceylanicum* contributes to the weight loss and morbidity (Bundy *et al.* 1995; Hotez and Pritchard 1995; Stoltzfus *et al.* 1997). In our study, we observed that animals infected with *A. ceylanicum* exhibited decreased activity, increased prostration, and reduced feed intake, which is in agreement with clinical scenarios described in the literature (da Silva *et al.* 2019; Ferreira *et al.* 2020).

In Experiment 1, after *S. mansoni* reached sexual maturity (22 DPI), a significant decrease in *A. ceylanicum* oviposition was observed. This reduction in the number of female worms and their egg production parallels findings from co-infection studies involving *S. mansoni* and *Strongyloides venezuelensis*, where a decrease in both the infection duration and fertility of female *S. venezuelensis* was reported (Gazzinelli and Mello 2008). Similarly, co-infections with *Hymenolepis diminuta* and *H. microstoma* have demonstrated that *S. mansoni* can reduce both the longevity and reproductive success of co-infecting helminths (Andreassen *et al.* 1990). Our results support the notion that the order in which these helminths are introduced into the host is a key determinant in the outcome of co-infections. Specifically, the first helminth to infect the host

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seems to dominate the immune response, thereby compromising the success of subsequent infections. In our study, this effect was clearly evident: A previous infection with *A. ceylanicum* caused a significant reduction in the recovery rate of *S. mansoni* worms, while introducing *A. ceylanicum* 65 days after *S. mansoni* infection also resulted in a decreased recovery of *A. ceylanicum*. These findings suggest that the immune dynamics induced by the first helminth infection can limit the establishment and success of a subsequent infection, highlighting the complexity of host-parasite interactions in co-infection scenarios (Vanalli *et al.* 2020).

According to Lello *et al.* (2004), the interaction among different helminths within a single host can occur through various mechanisms: direct actions of the parasites, secretion of products into the medium, manipulation of intestinal physiology, competition, or crowding effects. Other interactions are likely related to the host's immune system. In our study, we observed interactions between the two species under investigation. The chronology and order of infections also had an impact on the results. In the first experiment, when hamsters were pre-infected with *A. ceylanicum*, it did not interfere with the establishment of the second infection by *S. mansoni*, as indicated by the simultaneous patency of the second infection with their respective controls. However, the number of adult *S. mansoni* worms recovered was lower compared to the group mono-infected with *S. mansoni.* Throughout the oviposition follow-up of *A. ceylanicum*, similar oscillations were observed in both the Acey-1 and Acey + Sm groups. The oviposition of *S. mansoni* was higher at 46 DPI for both groups in Experiment 1. The fecal exams were conducted using pooled samples from all the animals in each group, which means that the data reflected the collective results rather than individual variations. This approach limited our ability to perform statistically significant analyses to assess fertility differences at the individual level. Consequently, we were unable to analyze the fertility variations among individual female parasites within each group.

Hematological parameters indicated a reduction in erythrocytes count, hemoglobin levels, and hematocrit value associated with *A. ceylanicum* infection, and this decrease was not exacerbated by co-infection. *A. ceylanicum* infection is characterized by microcytic and hypochromic anemia, along with a malabsorption syndrome, both attributed to the hematophagous behavior of the parasite (Silva Medeiros *et al.* 2022). This parasitic activity leads to the destruction of microvilli, as demonstrated in the current study. At the initiation of infection, when blood loss begins, the bone marrow intensifies its activity, producing reticulocytes. However, this response decreases with a decline in iron levels and a reduction in erythropoiesis, symptoms not observed in individuals with an iron-rich diet (Furtado *et al.* 2024).

The occurrence of anemia during hookworm infection may be determined by factors such as parasitic load, the presence of other clinical factors, and iron reserves (Serafim *et al.* 2014). Studies suggest that anemia caused by infection with the main species of the genus *Schistosoma* (*S. mansoni*, *S. haematobium*, and *S. japonicum*) can result from four possible mechanisms: iron deficiency, retention of erythrocytes by the spleen, autoimmune hemolysis, and anemia as a result of inflammation (Friedman *et al.* 2005).

An epidemiological study conducted by Butler *et al.* (2012) in Kenya with 2,750 children aged 9 to 12 years concluded that the primary mechanism causing anemia in schistosomiasis is associated with the inflammation triggered by the infection. Hemoglobin levels, along with serum ferritin levels and its receptor in the cell, were analyzed in this study after exclusion of co-infections with *Plasmodium* sp. and hookworms (Butler *et al.* 2012).

Co-infection induced the production of IgG antibodies against both infections, and there was no cross-reactivity between the antigens used. However, IgG assessment does not differentiate between old and new infections. In the groups infected with *S. mansoni*, all hamsters presented IgG against SWAP, even in animals where adult worms of *S. mansoni* were not recovered. The reduction in the population of the second infection likely occurred after the worms reached the adult stage, not in the larval stage as proposed by Yoshida *et al.* (1999). A linear correlation between the amount of eggs retained in the liver and the production of anti-SEA IgG or the elimination of eggs in the feces was not observed. However, in the Sm + Acey group, there was lower IgG production against SWAP, indicating that co-infection decreased the response to *S. mansoni.*

Competition between parasite species or strains within hosts is a significant evolutionary force in infections, and parasites develop various strategies to enhance their chances of growth or reproduction over competitors. In the current study, no clear winner was identified between the two parasites, likely due to the insufficient infection rate to instigate robust competition between them.

In the histopathological analyses, animals infected with *A. ceylanicum* exhibited a reduction in intestinal villi size, consistent with previous literature, including studies by our group (Alkazmi and Behnke 2013; Dias et al. 2013). This reduction was also observed in our experiment with different parasitic loads (Serafim *et al.* 2014). Granulomas formed in the small intestine, where the two species exhibit greater interaction, were evaluated. However, no influence of *A. ceylanicum* infection on the formation, evolution, or development of granulomas in the small intestine was observed. Therefore, co-infection did not act as a protective or exacerbating factor, considering granuloma diameter and its evolutionary classification as parameters.

In a study on co-infection between *H. poligirus* and *S. mansoni*, Bazzone *et al.* (2008) observed a significant reduction in immunopathology triggered by egg deposition in the liver of mice. The reduction in disease severity was associated with a decrease in cytokines related to the Th1/Th17 response and an increase in Th2 response and regulatory cytokines. However, no changes were observed in the number of eggs or fecundity of adult worms. The reduction in granuloma severity in co-infection may be linked to the enhancement of the Th2 response and the production of regulatory cytokines such as IL-10, crucial for immunomodulation and the transition from acute to chronic granuloma (Zheng *et al.* 2020). Although granulomas serve to contain *S. mansoni* eggs, the immune response associated with fibrosis may lead to the development of portal hypertension, interruption of venous blood flow in the liver, and cirrhosis (Atia *et al.* 2023; Roderfeld *et al.* 2020).

The hypothesis of exacerbation of the Th2 response stems from the ability of both *A. ceylanicum* and *S. mansoni* to polarize a Th2 response, crucial for host protection and reducing infection lethality (Loukas and Prociv 2001). A study by Aribodor *et al.* (2019) detected double and triple co-infections in primary school students with schistosomiasis and soil-transmitted helminthiasis in Nigeria. However, our results showed that there was no decrease or increase in the pathogenesis of either infection, which is an important finding for populations living in areas endemic to both species of helminths.

The introduction of *S. mansoni* after *A. ceylanicum* infection did not lead to a decrease in the number of recovered *S. mansoni* worms. However, a notable reduction in the number of eggs retained in the liver for this group compared to the group solely infected with *S. mansoni* was observed, indicating a modulation of

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the host's response due to *A. ceylanicum* infection. Even though egg laying by worms typically begins around 40 days post-infection, the process of egg retention extends into the chronic phase of the disease. Given that egg laying is a crucial factor for the life cycles of both parasites, the decrease in retained eggs could have a negative impact on the persistence of the parasites. Particularly for *S. mansoni*, where the primary pathogenesis stems from granuloma formation in various host organs, the preceding *A. ceylanicum* infection causing a decrease in retained eggs would be advantageous for the host.

In conclusion, co-infection did not alter the establishment or patency of the infection, but the recovery of the second introduced helminth was lower in both experiments. Despite both helminths being hematophagous, an exacerbation of anemia was not observed based on parameters such as the number of erythrocytes, hemoglobin levels, and percentage of hematocrit. Therefore, the co-infection between *A. ceylanicum* and *S. mansoni* did not aggravate anemia. Furthermore, although the number of *S. mansoni* eggs retained in the liver was lower in the experiment where *A. ceylanicum* was inoculated after *S. mansoni*, the number of granulomas and their development remained unchanged during co-infection, contrary to the initial hypothesis. This unexpected outcome challenges the initial hypothesis and underscores the complexity of host-parasite interactions. It sheds light on intricate mechanisms that govern the outcomes of co-infections, holding significant importance in the broader context of developing effective strategies for controlling parasitic diseases worldwide.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X25000033.

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Competing interest. The author(s) declare none.

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