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Natalie Gooder
*Minnesota State University, Mankato*

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By

Natalie Gooder

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This thesis has been examined and approved by the following members of the student's committee.

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Advisor

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Committee Member

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Committee Member

# The Effects of Heat-Killed Echinostomatid Parasites on Mice Treated with Dextran Sodium Sulfate (DSS)

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**Abstract**

**Objective:** Autoimmune diseases are chronic, incurable, and affect approximately 50 million Americans. This is a strong need for better ways to treat autoimmune diseases. Parasites and parasite proteins have been observed to protect mice from symptoms of induced colitis in mice treated with dextran sodium sulfate (DSS), a model for inflammatory bowel disease (IBD). I hypothesize that non-living echinostomatid parasites, given during DSS treatment, can decrease intestinal inflammation and weight loss, providing a possible novel treatment for IBD.

**Methods:** A range of DSS concentrations (0.5%-3%) were delivered to female C57BL/6 mice in their drinking water in order to determine the concentration that induced colitis but not severe morbidity; 1% DSS was determined to be optimal. Next, two groups of mice were administered 1% DSS in their drinking water for the first six days of the nine-day experiment, while two groups received regular water throughout. The mice in one of the DSS treatment groups and one of the water control groups were orally administered heat-killed metacercariae in PBS on the third day of the experiment, while the remaining mice were administered just PBS as a control treatment. Weight of the mice and intestinal distress were monitored, as was water and food consumption. At the end of the experiment, colon lengths were measured and splenic cytokines (IL-4, IFN-γ, IL-10, and IL-2) were assessed by ELISA.

**Results:** Mice with DSS induced colitis that received the parasite treatment demonstrated more weight loss ($p=0.01$) and poorer intestinal distress ($p=0.02$) on the ninth day when compared to mice with DSS induced colitis that did not receive parasite treatment. The spleens from mice

receiving DSS and parasite treatment showed lower levels of regulatory cytokine IL-2 than mice receiving DSS but not parasite treatment ($p=0.013$). Additionally, mice treated with DSS consumed less food on average than untreated mice ($p<0.0001$).

**Conclusion:** In contrast to my hypothesis, I found that feeding mice heat-killed echinostomatid parasites during DSS treatment did not improve symptoms of colitis. Future studies could be performed to determine if non living parasites can counteract symptoms of colitis, a possible novel treatment for patients with IBD.

# 1. Introduction

## 1.1. *IBD and the Hygiene Hypothesis*

The 20th century brought forth better sanitation, high quality water, vaccines, and antibiotics within industrialized countries, leading to a decline in microbial and parasitic infections (1,2,3). However, the 20th century also ushered in asthma, allergies, and a host of autoimmune diseases that had been uncommon previously and remain so in less developed countries (2-4). While genetics and viruses are likely to be involved in autoimmunity (for example, if one identical twin has type 1 diabetes, the other twin has a 30% greater chance of developing the disease (5)), the increase in these diseases within the last 50 years has been so rapid that environmental changes are strongly suggested (5,6).

Epidemiologic data and animal experimentation suggest that the elimination of helminth parasites contributes to the increasing prevalence of some immune mediated diseases, including inflammatory bowel disease (IBD), type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), asthma, and food allergies (2). IBD, including Crohn's disease (CD), which affects both the distal ileum and colon, and ulcerative colitis (UC), which affects the colon only, is characterized by chronic intestinal inflammation and epithelial damage (1,2,5-9) and usually begins during the second or third decade of life (8). IBD has been steadily increasing since the mid-20th century, making it a significant health problem within developed countries (1,2). It is estimated that over 2 million people in the USA or Europe have IBD (2,8), which is a 3-4-fold increase since 1950 (5). In 2014, the CDC reported an incidence rate of anywhere between 3 and 20 cases of IBD per 100,000 individuals in the US (7).

The etiology of IBD is not certain and can vary between patients with UC or CD. Because the colon and terminal ileum contain most of the intestinal flora (2), one hypothesis is that IBD can result from an uncontrolled immune response to the normal gut flora (1). Another hypothesis is that IBD is caused by an increased production of autoantibodies towards goblet cells (10). No known bacterial or viral infections have been linked to IBD, although genetics can play a role in predisposing patients (1,2,6-9). Epidemiological studies show an inverse relationship between the frequency of helminth worm infections and IBD (1). IBD is common in industrialized countries where human helminth infections are relatively sparse. Likewise, IBD is uncommon in less developed areas of the world where helminth infections are endemic, such as tropical countries in Asia and Africa (Fig. 1) (8,9).

The rarity of IBD in tropical countries cannot be explained based on genetics alone, as descendants of immigrants from other countries acquire the higher risk of IBD of the adopted developed country (9). The hygiene hypothesis was initially proposed by Strachan for hay fever and is now proposed for several immunological disorders, including allergic diseases and asthma, cardiovascular diseases, T1D, MS, and IBD (1). Hay fever was less frequent in children from larger families where exchange of infections was common, as well as children exposed to a farming environment, or certain food born or oral-fecal infections (3). This led to the hypothesis that the stimulation of the immune system by microbes or microbial products protects from the development of inflammatory diseases, therefore a reduced exposure to infectious agents may explain the rise in allergic and autoimmune diseases (3)

The hygiene hypothesis for IBD is supported by geographical distribution, as IBD is seen more frequently among patients with a higher socioeconomic status, associated with better sanitation, high-quality water, and better medical standards (1). In contrast, countries with higher infant mortality rates have lower reported incidence of IBD (1).

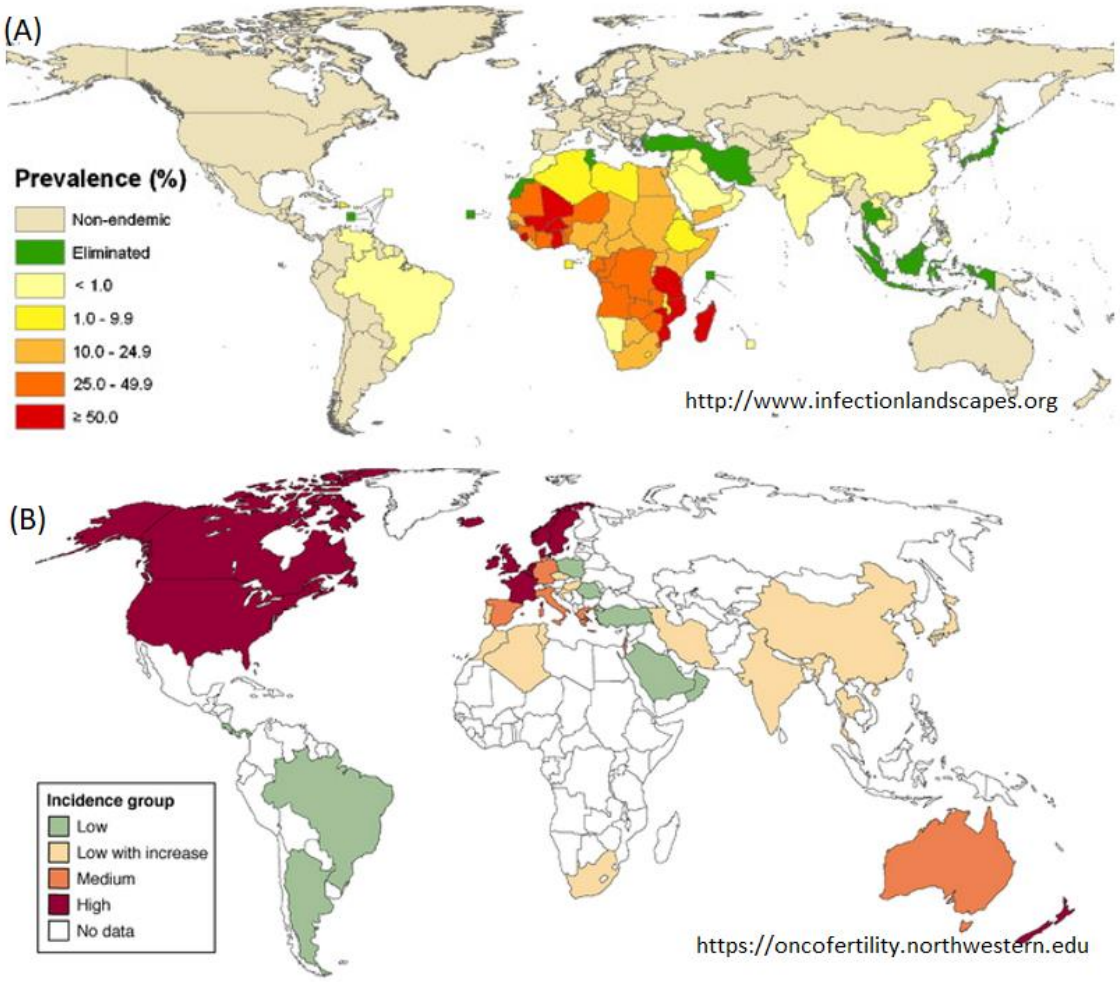


**Fig. 1.** Global data shows an inverse correlation of IBD and helminth infections. (A) 2012 prevalence of *Schistosoma* trematode infections (https://www.infectionlandscapes.org). (B) 2015 incidence of IBD (https://oncofertility.northwestern.edu).

## 1.2. *The Immune System*

The immune system is made up of both nonspecific (innate) and specific (adaptive) branches. The innate immune system includes phagocytes, such as neutrophils and macrophages, dendritic cells, and cytotoxic cells like natural killer (NK) cells, which work to rapidly control a pathogenic invasion (1,9). Adaptive immune cells, known as B and T lymphocytes, are antigen specific (1). Antigens are taken up by antigen presenting cells (APCs)

expressing MHC classes, such as dendritic cells and macrophages (1). APC's present fragments of the antigen to T cells, and function to release cytokines, or signaling proteins, and direct naïve T cells to differentiate into T helper cells ($T_H1$, $T_H2$) or regulatory T cells ($T_{REG}$) (1,4,9). B cells can recognize free antigens and can serve as APCs, leading to optimal antigen-specific CD4+ T cell expansion (1). They can also differentiate into plasma cells that produce antibodies (IgG, IgA, IgM, IgE, IgD), which fight infection by tagging pathogens for destruction by phagocytes and other effector cells (4,11). Adaptive immune responses are initially slow but improve on repeated exposure to a given antigen by the formation of B and T memory cells (1).

The balance between activating and inhibitory subsets of immune cells is important for the immune system to work optimally (11,12). This is most clearly understood for T cells, in which effector T cells and regulatory T cells have opposing roles in maintaining this balance (11,12). CD4+ $T_H1$ cells secrete proinflammatory cytokines IFN-γ and IL-12 (Fig. 2), which enhance macrophage activity and promote the production of IgG during a normal immune response (1,9,10,13). These cytokines are also associated with autoimmune diseases such IBD, T1D, RA, and MS (1-3,5-8,11-20). $T_H1$ cells additionally secrete IL-2, which is important for the proliferation of T cells, and research suggests that IL-2 specifically induces $T_{REG}$ cells and is an important regulator of autoimmunity (1,21). CD4+ $T_H2$ cells secrete IL-4, IL-5, IL-9, and IL-13 (Fig. 2) which promote B cell production of IgE and signal the release of granules within basophils and eosinophils (1,9,10,13). Basophils contain histamines within their granules and are associated with allergies, whereas eosinophils are often associated with helminth infections (1,20). Additionally, $T_H2$ cells can secrete IL-10, a cytokine with well-known anti-inflammatory and immunosuppressive properties (10). IL-10 blocks inflammatory cytokine production by T cells, NK cells, and macrophages, and can also induce T cell anergy (a state of immune unresponsiveness) in self-reactive T cells (10).

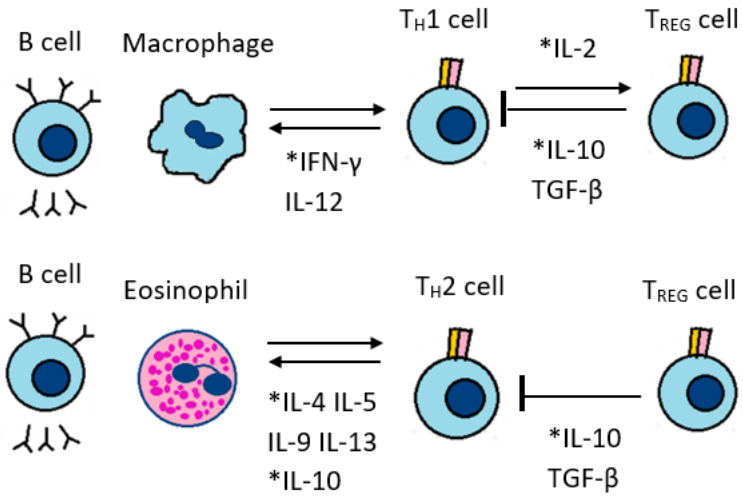


**Fig. 2.** Overview of T cell subsets and cytokine production. $T_H1$ specific cytokines IFN-γ and IL-12 cycle between immune cells and induce inflammatory responses. $T_H2$ specific cytokines IL-4, IL-5, IL-9, and IL-13 are triggered by allergic or parasitic infections and release granules in eosinophils or basophils. $T_H2$ cells can also be a source of regulatory cytokine IL-10. $T_{REG}$ cells function to regulate immune responses by producing IL-10 and TGF-β, which block cytokine production of other T cells subsets. Additionally, $T_H1$ cells produce IL-2 which stimulates $T_{REG}$ cell production. *Indicates cytokines analyzed in this study

$T_H1$ and $T_H2$ cells cross regulate one another (9). IFN-γ secreted by $T_H1$ cells directly suppresses IL-4 production and inhibits the differentiation of naïve T cells into $T_H2$ cells, whereas IL-4 and IL-10 inhibit the secretion of IL-12 and IFN-γ, blocking polarization into $T_H1$ cells (9). T cells isolated from the colons of patients suffering from Crohn's disease produce large amounts of IFN-γ and little IL-4 or IL-10 (9). Shifting the paradigm of a $T_H1$ cytokine-rich environment towards a $T_H2$ polarized environment was proposed as a mode of therapy for autoimmune diseases including IBD (21), since chronic inflammation within the GI tract is $T_H1$ mediated (2,5,6,8,9,15,16,22).

The murine gut also harbors large numbers of Foxp3 CD4+ $T_{REG}$ cells, which function to restrain the host immune response to the normal intestinal flora (2). $T_{REG}$ cells release IL-10 and TGF-β (Fig. 2) and regulate the immune system by inhibiting pro-inflammatory cytokine production (2,4,11). B regulatory cells ($B_{REG}$) are also a source of IL-10 and control intestinal inflammation by decreasing IFN-γ levels (11,12). Furthermore, once stimulated $B_{REG}$ cells continue to produce IL-10, leading to long term regulation of the immune system (11).

## 1.3. *Helminth Parasites and IBD*

Helminth parasites colonize more than one third of the world population and human infections are common within developing countries (1). For chronic infection to occur, the parasite must first avoid clearance by the host immune system by altering mucosal and systemic immunity and inhibiting inflammatory responses (8). The exact mechanism for this is unknown and can vary between different autoimmune diseases and different animal models. During the acute phase of a parasitic infection, a $T_H2$ response is initiated within the host (19), leading to IL-4, IL-10, and IgE production and the release of granules within eosinophils (9,10). A shift from $T_H1$ to $T_H2$ profiles in hosts was initially used to explain evidence that helminth parasites protect from autoimmune disease but does not explain the rise in allergies within developed countries, which are $T_H2$ dependent (1-3,5,7,14).

Helminths can also protect from allergic reactions within a host, suggesting that additional mechanisms of regulation must occur that are independent of $T_H2$ cytokines (2). It is suggested that during the chronic phase of infection, helminths produce several products with immune modulatory properties including molecules that induce $T_{REG}$ cells and inhibit the effector function of self-reactive T cells (2,19). However, other studies suggest that parasite induced protection of IBD can inhibit $T_H1$ proliferation without the need for IL-10 or TGF-β (2). In one study it was demonstrated that helminth infections in IL-10 deficient mice still prevent colitis (2). It has also been shown that regulatory dendritic cells and alternatively activated macrophages (M2) inhibit T effector cell function in a manner independent of $T_{REG}$ cells, IL-10 and TGF-β (2). Also, because intestinal helminths must breach the mucosal barrier to reach the immune system it has been suggested that direct interactions between intestinal helminths and the gut epithelium may play a role in regulating symptoms of IBD (2).

## 1.4. *Effect of Parasites on Colitis*

The effects of helminth parasites on autoimmune diseases have been tested in multiple studies within the last 20 years, confirming that parasites can protect from chronic inflammation (1-5,7,9,12,13,15-20,23). IBD is characterized by intestinal inflammation and epithelial damage, and includes symptoms of bloody diarrhea, pain, fatigue, and weight loss (6-9,14,16,20,22). Chemical-induced colitis models, including trinitrobenzene sulfonic acid (TNBS), and dextran sulfate sodium (DSS) are widely used in research studies and closely mimic the symptoms of human IBD (9,16,20,22-24). DSS induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon (24). The DSS-induced colitis model has been shown to induce changes in $T_H1/T_H2$ cytokine profiles, leading to an increase in pro-inflammatory cytokines (8,16,20).

Elliott et al. proposed the hypothesis that the loss of exposure to parasitic worms increased the risk of IBD and illustrated a protective response of *Schistosoma mansoni* infection on TNBS-induced colitis is mice (1). *Schistosoma mansoni* is a water-borne blood fluke that infects human blood vessels near the intestinal tract, causing intestinal schistosomiasis (14,20). Schistosomes undergo their intermediate life cycle by infecting snails, then adult larvae can penetrate through the skin of humans (20). Clinical symptoms develop when schistosome eggs migrate through intestinal tissue (20). Smith et al. demonstrated a protective response of *S. mansoni* worms on DSS-induced colitis in BALB/c mice, showing that mice subcutaneously infected with male cercariae and treated with 5% DSS 7-8 weeks post infection had significantly reduced intestinal distress indicated by less weight loss, lower intestinal scores, and longer colon lengths after dissection then uninfected mice (20). Infection of BALB/c mice with *S. mansoni* was shown to protect mice from symptoms of colitis through the induction of M2 macrophages, independent of $T_{REG}$ cells, IL-10, and TGF-β (2). Smith et al. further demonstrated that *S.*

*mansoni* eggs do not induce protection from DSS-induced colitis in BALB/c mice by subcutaneously infecting mice with both male and female cercariae and treating with DSS 4 weeks after egg laying had commenced (20). Mice developed more severe colitis when infected with *S. mansoni* eggs then uninfected mice, suggesting that protection from colitis is only achieved through the adult larvae cycle of *S. mansoni* (20).

In response to overwhelming evidence that helminth parasite infections protect from induced colitis in mice, Summers and Weinstock et al. performed a series of clinical studies, treating IBD patients with *Trichuris suis* eggs (1,2,8). *Trichuris suis* is an intestinal whipworm commonly found in pigs (8,14.). *T. suis* does not require an intermediate host; its eggs are passed in feces from infected animals and its juvenile larvae can directly penetrate the mucosa of the intestines (14). While *T. suis* is not a human parasite, its juvenile worms can colonize a human host for several weeks and are eliminated thereafter without any specific therapy (8). If clinical symptoms occur in humans, they are mild and treatable (8,14). In these studies, 29 patients with long standing Crohn's Disease, and 45 patients with Ulcerative Colitis were orally infected with *Trichuris suis* eggs (1,2,8). After 24 weeks, 21 patients with CD were in remission, and another two had improved symptoms (2,8). Additionally, about 50% of patients with UC reported improved symptoms (1,8).

The use of living infectious parasites may not be necessary for treating patients with autoimmune disease. It has been shown that excretory/secretory products of *Fasciola hepatica* parasites, collected after culturing them in vitro, exert a potent immune-modulatory effect in BALB/c and C57BL/6 mice by activated regulatory M2 macrophages, suppressing dendritic cell maturation, and inhibiting $T_H1$ cell differentiation (13,19). Another study delivered a single recombinant parasite protein, galectin, isolated from the *Toxascaris leonine* nematode to C57BL/6 mice prior to treatment with DSS (16). Galectins are highly conserved animal lectins

with an affinity for β-galactose containing oligosaccharides (16,25), and have been shown to affect different functions relevant to innate and adaptive immunity (16). Previous studies have suggested that galectin-8 may play a suppressive role in IBD and RA. Mice injected with galectin-8 (rTI-GAL) before treatment with DSS demonstrated less weight loss, lower intestinal scores, longer colons after dissection, lower levels of IFN-γ and IL-4, and higher levels of IL-10 and TGF-β compared to mice not injected with rTI-GAL before DSS treatment (16).

In previous studies of parasites on mice models of colitis, the subcutaneous infection with a parasite, or the injection of a parasite protein was delivered before treatment with DSS, as a prevention method of DSS-induced colitis (Fig. 3) (9,16,20). It is currently unknown whether administering parasites or parasite products during treatment with DSS can also decrease symptoms of induced colitis in mice. Also, because a parasite protein is enough to protect mice from DSS-induced colitis (16), we questioned whether feeding mice non-living whole parasites could also decrease symptoms of induced colitis. This study addresses whether feeding C57BL/6 mice whole, heat-killed parasites after several days of DSS treatment will decrease symptoms of DSS-induced intestinal damage, resulting in less weight loss, improved intestinal health, and longer colon lengths after dissection, and whether a decrease in symptoms is accompanied by a shift in $T_H1$ cytokines to $T_H2$ and $T_{REG}$ cytokines.

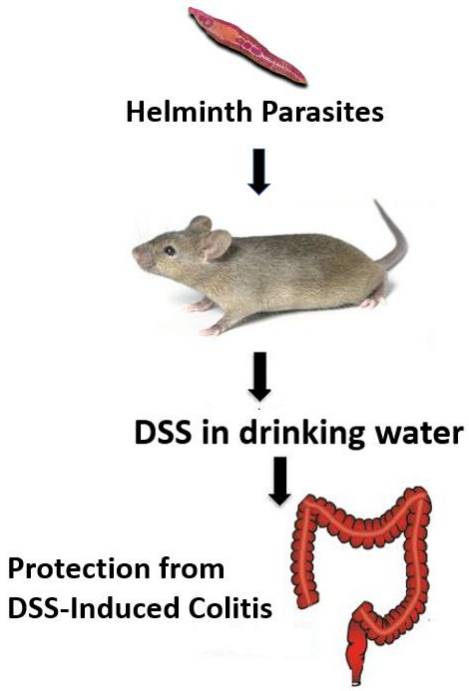


**Fig 3.** Parasites or recombinant parasite proteins (galectin) have been shown to protect from DSS-induced colitis in mice when mice are infected with cercariae or injected with galectin prior to treatment with DSS.

## 1.5. *Rationale and Hypothesis*

Echinostomatids are a family of intestinal parasites from the class *Trematoda* (flatworms) and can cause disease in mammals and birds (1,14,26). Typically, the life cycle of echinostomatids includes three different hosts: an invertebrate first intermediate host, typically a mollusk, a second intermediate host carrying the encysted metacercarial stage, and a vertebrate definitive host (26). Echinostome metacercariae can be obtained locally from infected snails. Here, we test the effects of feeding heat-killed echinostome metacercariae to C57BL/6 mice after three days of DSS treatment. We hypothesize that C57BL/6 mice treated with DSS for 6 days and administered heat-killed metacercariae during DSS treatment will show less weight loss, improved intestinal health, and longer colon lengths after dissection than mice treated with DSS but not administered metacercariae. Furthermore, we hypothesize that a decrease in symptoms can be linked to a decrease in inflammatory cytokines IFN-γ and IL-4, and/or an increase in regulatory cytokines IL-10 and IL-2.

## 1.6 *Summary*

Research described in this thesis demonstrates that 3%, 2.25%, or 1.5% DSS dissolved in drinking water for 6 days causes loose stools that are highly bloody and/or weight loss of over 25% of the initial body weight, whereas 1% DSS causes loose stools and weight loss of less than 25% of the initial body weight, and 0.5% DSS does not cause significant intestinal distress. Furthermore, mice treated with 3%, 2.25%, 1.5%, or 1% DSS drink less water on average than untreated mice. Mice treated with 1% DSS for 6 days and administered heat-killed echinostome metacercariae during DSS treatment do not show less weight loss, improved intestinal health, or longer colons after dissection when compared to mice treated with 1% DSS and administered

PBS. Inversely, mice administered metacercariae during DSS treatment show more weight loss and higher intestinal scores on the ninth day. However, mice administered PBS and treated with 1% DSS lose less weight than mice treated with 1% DSS in the initial trial, and mice administered PBS and given regular water gain less weight than control mice in the initial trial. Analysis of splenic cytokines show a decrease in regulatory cytokine IL-2 levels in mice treated with DSS and administered metacercariae when compared to mice in all other groups. Furthermore, mice treated with 1% DSS consume less food then untreated mice.

We conclude that heat-killed metacercariae fed orally to C57BL/6 mice after several days of DSS treatment do not decrease symptoms of DSS-induced colitis but may exacerbate symptoms. Because previous studies provided an ongoing parasite stimulus to protect from immune mediated symptoms (1,2,9,13,16,18,20), we question whether delivering multiple doses of metacercariae during DSS treatment could decrease symptoms of induced colitis. We also question whether gavaging mice or administering PBS orally affects water consumption and therefore weight loss. Further studies may be necessary to determine whether nonliving whole parasites can be used as a form of treatment for models of IBD or other autoimmune diseases.

## 2. Methods

### 2.1. *DSS-induced model of colitis*

It has been previously shown that mice models of colitis can be established in C57BL/6 mice by dissolving small amounts of dextran sodium sulfate (DSS) salt into drinking water (16,20,23,24). An optimal DSS dosage is needed to test the effects of heat-killed parasite treatment on mediating DSS-induced colitis to model the autoimmune disorder, inflammatory bowel disease (IBD). Many factors affect DSS-induced colitis, including mouse age, gender, strain, and environmental conditions of vivarium (23), therefore a range of DSS doses were tested. An optimal dosage will result in the slow and steady onset of symptoms, and the mice will remain healthy enough to last throughout the experimental period (23).

Since the effects of DSS can be variable, five mice were included per group. Three groups were given different DSS (36-50,000 M. Wt., MP Biomedicals) concentrations dissolved in drinking water with a fourth group used as a control. Initially, a range of 1.5%-3% DSS was tested, then the experiment was repeated using 0.5%-1.5% DSS. Prior to each 9-day experiment, mice were sedated using a mixture of 4% isoflurane gas with 1% oxygen and identified with ear tags, then assigned to random groups (randomlists.com/team-generator). On Day 0, the mice were weighed, and the groups adjusted so that each group had a similar average weight (Tables 1 and 2). Mice in each group were divided into two cages to prevent overcrowding. To minimize cage variance, each treatment group had one cage with two mice and one cage with three mice. DSS treatment was given on days 0-5, and regular water on days 6-9.

**Table 1.** Mice ID numbers and initial weight for Experiment 1.

| Amount of DSS | Age on Day 0 | Mice ID | Weight (g) |
|---|---|---|---|
| | 7 w, 1 d | 913 | 16.92 |
| | 7 w, 1 d | 915 | 15.87 |
| **1.5% DSS** | 6 w, 6 d | 919 | 17.64 |
| | 6 w, 6 d | 933 | 15.76 |
| | 6 w, 6 d | 921 | 18.17 |
| | | Average= | **16.87** |
| | 7 w, 7 d | 910 | 18.46 |
| | 6 w, 6 d | 925 | 16.4 |
| **2.25% DSS** | 7 w, 1 d | 912 | 15.75 |
| | 7 w, 1 d | 918 | 16.93 |
| | 6 w, 5 d | 928 | 16.92 |
| | | Average= | **16.89** |
| | 7 w, 1 d | 917 | 17.17 |
| | 7 w, 7 d | 911 | 18.58 |
| **3% DSS** | 7 w, 1 d | 916 | 15.5 |
| | 6 w, 6 d | 923 | 17.76 |
| | 6 w, 6 d | 924 | 17.42 |
| | | Average= | **17.29** |
| | 6 w, 6 d | 920 | 17.3 |
| | 6 w, 4 d | 929 | 18.04 |
| **0% DSS** | 6 w, 6 d | 927 | 16.7 |
| | 6 w | 930 | 17.4 |
| | 6 w | 932 | 15 |
| | | Average= | **16.89** |

**Table 2.** Mice ID numbers and initial weight for Experiment 2.

| Amount of DSS | Age on Day 0 | Mice ID | Weight (g) |
|---|---|---|---|
| | 7 w, 3 d | 32 | 17.69 |
| | 7 w, 3 d | 29 | 17.62 |
| **0.5% DSS** | 8 w | 14 | 17.85 |
| | 8 w | 17 | 17.44 |
| | 7 w, 3 d | 34 | 17.48 |
| | | Average= | **17.62** |
| | 7 w, 3 d | 33 | 18.85 |
| | 6 w, 6 d | 23 | 18.72 |
| **1% DSS** | 8 w | 15 | 18.57 |
| | 6 w, 6 d | 21 | 18.03 |
| | 6 w, 2 d | 31 | 13.84 |
| | | Average= | **17.6** |
| | 6 w, 6 d | 22 | 18.12 |
| | 6 w, 6 d | 25 | 18.14 |
| **1.5% DSS** | 8 w | 13 | 18.25 |
| | 6 w, 3 d | 47 | 16.86 |
| | 6 w, 5 d | 36 | 16.78 |
| | | Average= | **17.63** |
| | 6 w, 5 d | 35 | 17.11 |
| | 6 w, 6 d | 24 | 19.18 |
| **0% DSS** | 6 w, 3 d | 45 | 15.46 |
| | 8 w | 19 | 17.68 |
| | 8 w | 18 | 18.36 |
| | | Average= | **17.56** |

The control group was given regular water throughout the experiment. DSS solutions/water were changed every two days with the remaining volume recorded and divided by the number of mice per cage to determine average water consumption.

At approximately the same time each day, mice were individually weighed and placed in an empty cage to allow for stool collection. Stool consistency was recorded, and two different areas of the stool were smeared on a hema-screen™ card (Immunostics) to detect for fecal blood. Hema-screen cards contain the compound guaiac (alpha-guaiaconic acid), which is

oxidized by hemoglobin, resulting in a dark blue color within 30 seconds in the presence of blood. For the initial experiment, stools were rated on a scale of 0-4 for stool consistency and presence of fecal blood (0=Normal stool, -blood; 1=normal stool, +blood, or soft stool, -blood; 2=soft stool, +blood, or loose stool, -blood; 3=loose stool, + blood, or diarrhea, -blood; 4=diarrhea, +blood) (Table 3). For the second experiment, visible blood in stools was included as a factor (4=loose bloody stool, +blood; 5=bloody diarrhea, +blood) (Table 4).

At the beginning of each experiment, a humane handling weight (75% of weight on Day 0) was calculated for all mice and if any mice reached that body weight prior to the end of the experiment they were euthanized by $CO_2$ gas to minimize extreme suffering. At the end of each experiment, all remaining mice were euthanized by $CO_2$ gas.

**Table 3.** Intestinal Scores for Experiments 1 and 3

| Intestinal Score | Visible Stool Consistency | Presence of Blood as determined by hema-screen |
|---|---|---|
| 0 | Normal | - |
| 1 | Normal | + |
| 1 | Soft | - |
| 2 | Soft | + |
| 2 | Loose | - |
| 3 | Loose | + |
| 3 | Diarrhea | - |
| 4 | Diarrhea | + |

**Table 4.** Intestinal Scores for Experiment 2

| Intestinal Score | Visible Stool Consistency | Presence of Blood as determined by hema-screen |
|---|---|---|
| 0 | Normal | - |
| 1 | Normal | + |
| 1 | Soft | - |
| 2 | Soft | + |
| 2 | Loose | - |
| 3 | Loose | + |
| 3 | Diarrhea | - |
| 4 | Loose, bloody | + |
| 4 | Diarrhea | + |
| 5 | Diarrhea, bloody | + |

2.2. *Counteraction of DSS-induced intestinal distress with Echinostomatid parasites*

It has been previously shown that overexpression of the parasite protein galectin in mice models of IBD protects the mice from developing intestinal distress by inducing a large $T_{REG}$

population (16). The effects of heat-killed echinostome metacercariae were tested in an attempt to counteract the symptoms of DSS-induced intestinal distress (Fig. 4). As with the

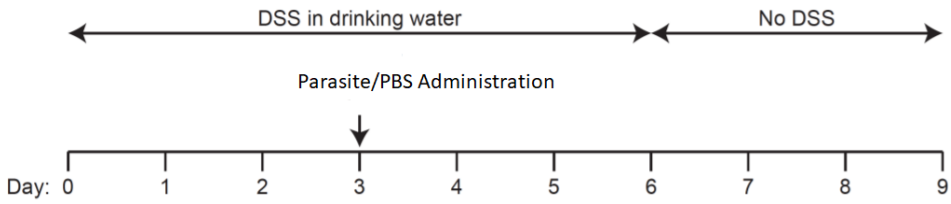


**Fig 4.** Timeline of Experiment 3: Administration of heat-killed Echinostomatid metacercariae to mice treated with 1% DSS. DSS was dissolved in drinking water for 6 days followed by regular water for 3 days. Metacercariae or PBS was orally delivered to mice on Day 3.

previous tests, female C57BL/6 mice were used, but with eight mice per group to enable detection of modest changes. Four groups were included to properly test all variables (DSS + Parasites, Water + Parasites, DSS + PBS, and Water + PBS). Mice were identified and assigned groups as described in section 2.1 (Table 5). 1% DSS treatment was given to mice in Groups 1 and 3 on days 0-5, and regular water on days 6-9. Regular water was given to mice in Groups 2 and 4 for all 9 days. Mice weight, stool consistency, and presence of fecal blood were recorded daily. DSS solutions/water were changed every two days with the remaining volume used to determine average water consumption. Food was also weighed every two days and used to determine average food consumption in the same method as water consumption.

Echinostome metacercariae were isolated from infected *Lymnaea* snails that were obtained through Dr. Sorensen (MSU, Mankato). The infected snails were dissected and metacercariae were isolated and counted using a dissecting microscope and divided between 16 tubes (approximately 30-40 metacercaria per tube). Metacercariae were stored at room temperature for 2-3 days in a salt solution (Lockes). On the day before administration to mice, metacercariae were washed twice with PBS (phosphate-buffered saline). PBS was then added to a total volume of 200 µL to all tubes placed in a thermocycler (Prime, TECHNE) at 72°C for 15

**Table 5.** Mice ID numbers and initial weight for Experiment 3.

| Treatment Group | Age on Day 0 | Mice ID | Weight (g) | Treatment Group | Age on Day 0 | Mice ID | Weight (g) |
|---|---|---|---|---|---|---|---|
| | 7 w, 2 d | 232 | 16.7 | | 7 w, 1 d | 222 | 16.9 |
| | 7 w, 2 d | 225 | 17 | | 7 w, 1 d | 224 | 17.9 |
| | 7 w, 4 d | 243 | 16.65 | | 7 w, 4 d | 229 | 17.18 |
| | 7 w, 4 d | 245 | 14.4 | | 7 w, 3 d | 235 | 16.7 |
| **DSS + Parasite** | 7 w, 4 d | 248 | 15.8 | **DSS + PBS** | 7 w, 3 d | 238 | 15.3 |
| | 7 w, 5 d | 252 | 16.68 | | 7 w, 4 d | 242 | 16.12 |
| | 7 w, 5 d | 254 | 18.16 | | 7 w, 4 d | 249 | 17.62 |
| | 7 w, 5 d | 256 | 18.97 | | 7 w, 4 d | 251 | 16.88 |
| | | Average= | **16.795** | | | Average= | **16.825** |
| | 7 w, 4 d | 233 | 15.92 | | 7 w, 2 d | 227 | 17.42 |
| | 7 w, 3 d | 226 | 17.58 | | 7 w, 2 d | 228 | 17.8 |
| | 7 w, 3 d | 236 | 17.75 | | 7 w, 3 d | 234 | 17.5 |
| | 7 w, 3 d | 237 | 16.5 | | 7 w, 4 d | 239 | 14.72 |
| **Water + Parasite** | 7 w, 4 d | 240 | 15.85 | **Water + PBS** | 7 w, 4 d | 250 | 17.93 |
| | 7 w, 4 d | 244 | 17.62 | | 7 w, 5 d | 253 | 16.16 |
| | 7 w, 4 d | 246 | 16.95 | | 7 w, 5 d | 255 | 16.52 |
| | 7 w, 4 d | 247 | 17.05 | | 7 w, 5 d | 257 | 17 |
| | | Average= | **16.9025** | | | Average= | **16.88125** |

minutes followed by 95°C for 15 minutes to kill the metacercariae, as previously demonstrated in Hafalla et al. (27), and stored at 4°C overnight. Before weight and stool measurements were collected on Day 3, metacercariae in PBS or 200 µL PBS was administered to mice using an 18-gauge feeding tube 1.5 inches in length with a rounded tip. Mice were held by the scruff of the neck and the gavage tube was placed in the diastema of the mouth and gently advanced along the upper palate until the esophagus was reached. Once proper placement was verified, metacercariae in PBS or PBS was administered by a 1 cc syringe attached to the end of the feeding tube. After dosing, the mice were returned to their cage and monitored for signs of labored breathing or distress for 5-10 minutes, and again after a few hours. Feeding tubes and gavages were examined under the dissecting microscope to confirm that the metacercariae were no longer present.

After data collection on Day 9, all mice were euthanized by $CO_2$ gas and dissected to harvest the spleens and intestines. Spleens were placed into wells containing 1 mL sterile cold PBS and disrupted with the flat plunger end of a sterile syringe. Cells suspensions were transferred to sterile microfuge tubes containing 1 ml PBS and 100 µL of 1.1% IGEPAL (Alfa Aesar) and kept on ice. Cell suspensions were vortexed for 1 minute, then centrifuged for 5 minutes at 8000 rpm. Supernatants were transferred to new sterile microfuge tubes and stored at -20°C until analyzed for pro-inflammatory cytokines IFN-γ, parasite specific cytokine IL-4, and regulatory cytokines IL-10 and IL-2 presence, using enzyme-linked immunosorbent assay (ELISA). Intestines were placed into tubes containing 10 mL of 10% formalin and kept at 4°C for analysis of colon length. To measure colon length, intestines were straightened and measured from the ileocecal junction to the rectum.

### 2.3. *Analysis of splenic cytokines IL-4, IFN-γ, IL-2, and IL-10*

Splenic supernatants were vortexed briefly and diluted twofold or tenfold with sterile PBS. A Mouse $T_H1/T_H2$ Uncoated ELISA kit (ThermoFisher Scientific) was purchased. The detection ranges given by the manufacturer for each cytokine were: IL-4, 4-500 pg/mL; IFN-γ, 15-2000 pg/mL; IL-2, 2-200 pg/mL; and IL-10, 30-4000 pg/mL. In all samples where the calculated cytokine concentration was at or below the given sensitivity, it was graphed as the lowest detectable level for that cytokine. To analyze cytokine concentrations, ELISA plates were coated with 100 µL/well of IL-4, IFN-γ, IL-2, or IL-10 capture antibody in Coating Buffer then sealed and incubated overnight. The following day wells were aspirated 3 times with Wash Buffer and blocked with 200 µL/well of ELISA/ELISASPOT diluent. The plates were incubated at room temperature for 1 hour, then the wells were aspirated once with Wash Buffer. Standards

were prepared according to the manufacturer's instructions and diluted to make a 6-point standard curve (Fig. 5). 100 µL/well of standards or samples were added to appropriate wells. Three or four specimens per treatment group were included with two or three replicates per sample. The plates were sealed and incubated overnight. The following day, the wells were

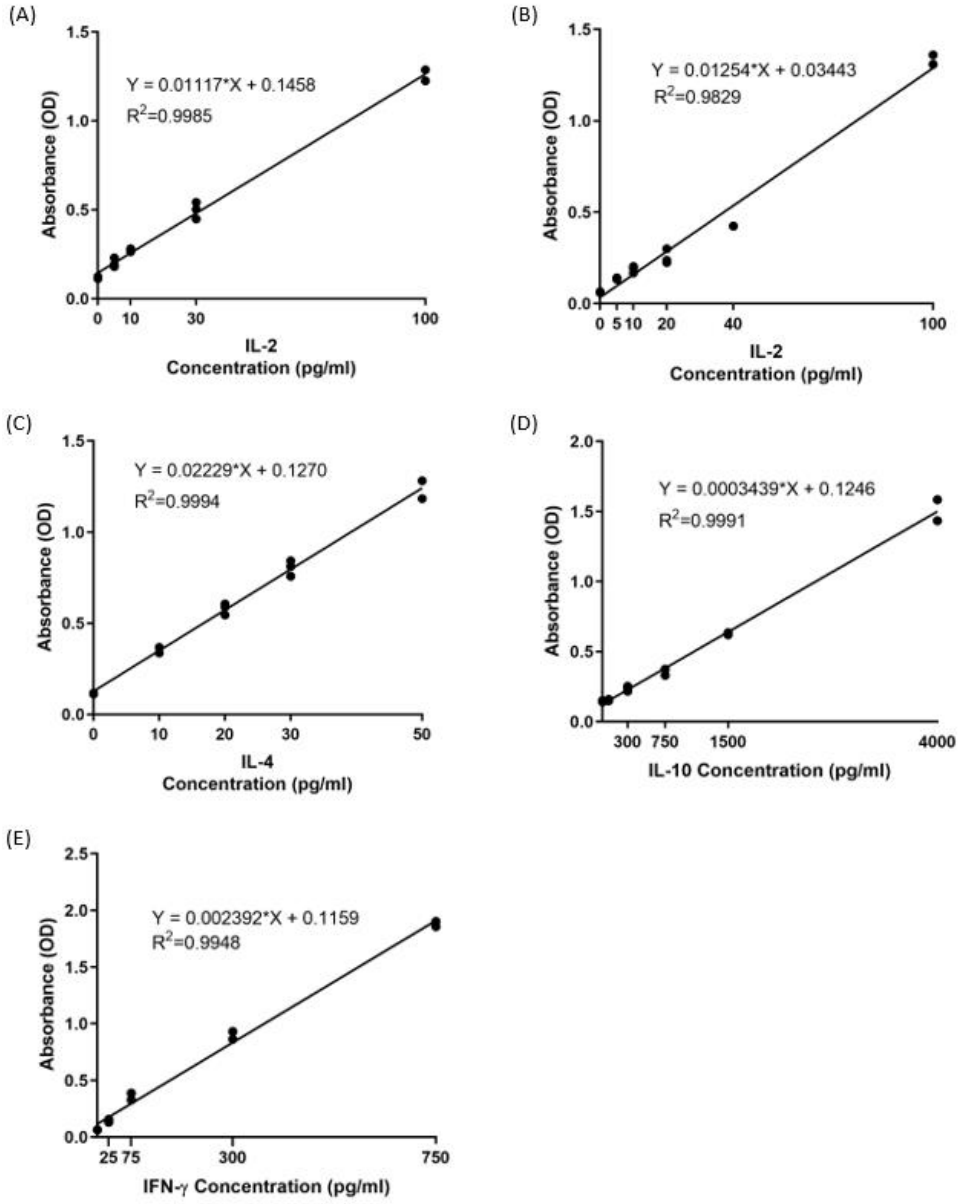


**Fig. 5.** ELISA mouse $T_H1/T_H2$ standard curves used for cytokines IL-2, IL-4, IL-10, and IFN-γ. Standards were diluted according to manufacturer's instructions and used to make a 6-point curve with two or three duplicates per sample. GraphPad Prism 7 was used to calculate standard curve equations and $R^2$ values. (A-B) $T_H1$; $T_{REG}$ cytokine IL-2. (C) $T_H2$; parasite specific cytokine IL-4. (D) $T_H2$; $T_{REG}$ cytokine IL-10. (E) $T_H1$, pro-inflammatory cytokine IFN-γ.

aspirated 5 times with Wash Buffer then 100 µL/well of IL-4, IFN-γ, IL-2, or IL-10 diluted detection antibody was added, and plates were sealed and incubated at room temperature for 1 hour. Wells were aspirated 5 times with Wash Buffer then 100 µL/well of diluted Avidin-HRP enzyme was added, and plates were sealed and incubated at room temperate for 30 minutes. Wells were aspirated 7 times with Wash Buffer then 100 µL/well of TMB (tetramethylbenzidine) substrate solution was added, and plates were incubated at room temperature for 15 minutes. 50 µL/well of Stop Solution was added and plates were read at 450 nm in a MultiSkan Spectrum plate reader (ThermoFisher). For IL-4, IFN-γ, and IL-10, one ELISA plate was analyzed (three or four specimens per treatment group), and for IL-2, two ELISA plates were analyzed (five or six specimens per treatment group). For all plates, GraphPad Prism 7 was used to calculate standard curve equations and interpolate x-values. X-values were multiplied by 2 or 10 depending on the dilution factor to give cytokine concentrations.

### 2.4. *Statistical analysis*

GraphPad Prism 7 software was used to perform all statistical testing. For comparison of groups for weight change, intestinal score, food consumption, and water consumption, repeated measures, 2-way ANOVA matching: stacked with Tukey's multiple comparisons test was performed. For data sets with missing values, mixed-affects model: matching: stacked with Tukey's multiple comparisons test was performed instead. For comparison of groups for colon length, 1-way ANOVA with Tukey's multiple comparisons test was performed. For comparison of groups for IL-2 concentrations, 1-way ANOVA with Tukey's multiple comparisons test, or unpaired t test with Welch's correction was performed. For all group comparisons, results were expressed as group means with standard deviations. For cytokine concentrations, linear

regression was used to calculate standard curve equations and interpolate x values. For comparison of individual data points, linear regression was used to calculate line equations, $R^2$ values, and p values. P values of <0.05 were considered statistically significant for all tests.

## 3. Results

### 3.1. *3%, 2.25%, and 1.5% DSS administered through drinking water induces acute intestinal inflammation in C57BL/6 mice and causes excessive weight loss.*

The overall goal of this study was to test the effects of non-living parasites on mice models of IBD, by orally administering heat-killed metacercariae to mice treated with DSS. Therefore, an optimal concentration of DSS which causes weight loss and loose stools while allowing mice to survive the entire experiment (mice that lose >25% of their body weight must be euthanized for ethical purposes), was necessary for this study.

An initial experiment was performed on 20 female, C57BL/6 mice, ages 6-8 weeks. Five mice per treatment group were included. Individual mice were given ear tags prior to the experiment and randomly assigned treatment groups. Individual mice were then weighed, and treatments groups were adjusted slightly so that each group weighed approximately the same (Table 1). DSS treatment cages were administered either 3%, 2.25%, or 1.5% DSS dissolved in drinking water for 6 days, followed by regular water for 3 days. Control cages were administered regular water for all 9 days. DSS solutions/water were replaced every two days and the remaining volume from each cage was recorded. Average water consumption per

mouse was calculated by dividing the remaining volume from each cage by the number of mice (two or three) per cage. Weight, stool consistency, and presence of fecal blood (hema-screen) were recorded daily for individual mice. Stool consistency and presence of fecal blood were used to assign individual mice an intestinal score from 0-4 (Table 3). If a mouse lost >25% of its body weight at any time during the experiment it was euthanized after data collection for that day. At the end of 9 days, all remaining mice were euthanized.

Over the course of treatment, the group receiving 3% DSS began testing positive for fecal blood on the first day and developed loose stools starting on the fourth day. When compared to the control group, mice receiving 3% DSS had higher intestinal scores from the first to the seventh day, ($p<0.0001$, 2- way ANOVA) (Fig. 6A). Mice receiving 3% DSS lost more weight than the control group from the fifth to the seventh day ($p\leq0.0002$, 2-way ANOVA) (Fig. 6B). Two mice receiving 3% DSS lost over 25% of their body weight on the seventh day, and the remaining three mice on the eighth day (Table 6). Mice receiving 3% DSS also consumed less water on the sixth day ($p=0.055$) and eighth day ($p=0.0001$, 2-way ANOVA) when compared to the control group (Fig. 6C).

Similarly, the group receiving 2.25% DSS began testing positive for fecal blood on the first day and developed loose stools starting on the fourth day. When compared to the control group, mice receiving 2.25% DSS had higher intestinal scores from the first to the seventh day ($p<0.0001$, 2-way ANOVA) (Fig. 6A). Mice receiving 2.25% DSS also lost more weight than the control group from the fifth to the seventh day ($p<0.001$, 2-way ANOVA) (Fig. 6B). Four mice receiving 2.25% DSS lost over 25% of their body weight on the eighth day, and the remaining

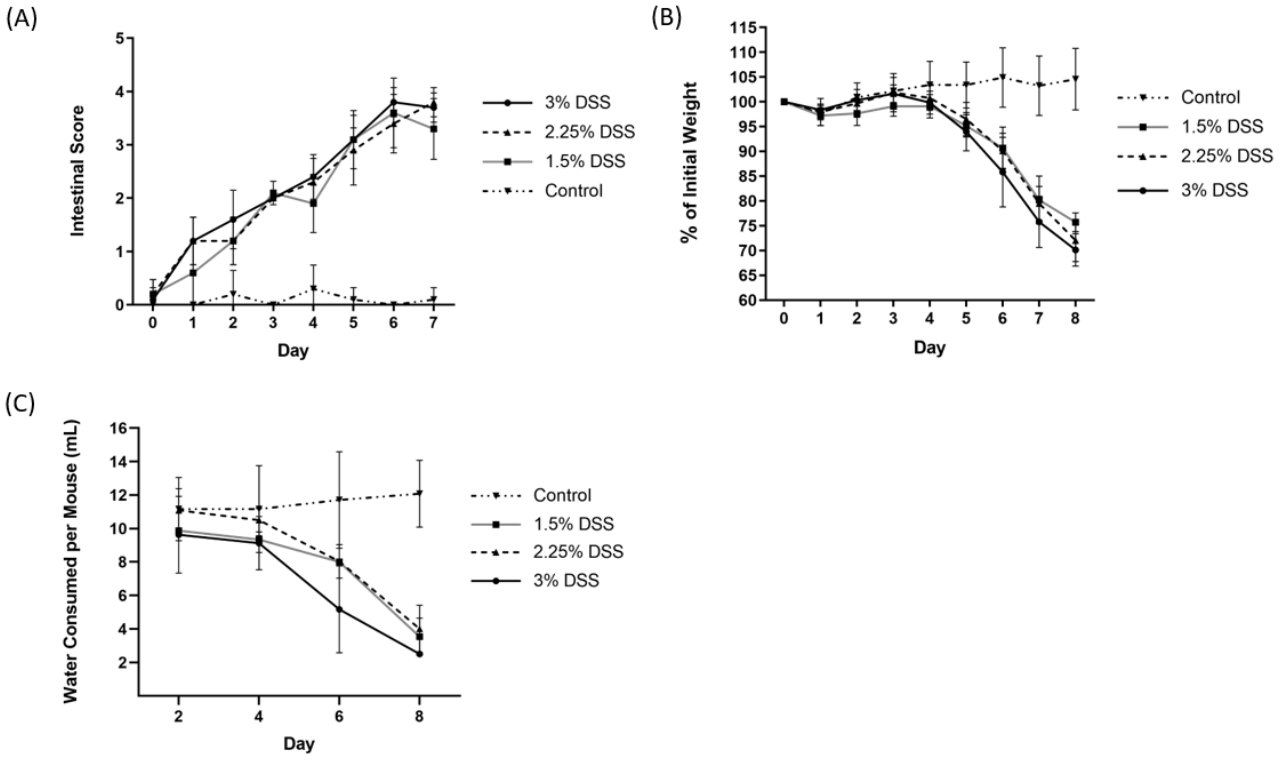


**Fig. 6**. 3%, 2.25%, or 1.5% DSS induces acute intestinal distress in C57BL/6 mice but causes excessive weight loss. 3%, 2.25%, or 1.5% DSS was dissolved in drinking water and administered to mice for 6 days, followed by regular water for 3 days. Control mice were administered regular water for all 9 days. Five mice per treatment group were included. For all panels, group means, and standard deviations are plotted. (A) Stool consistency and presence of fecal blood (hema-screen) were used to provide mice an intestinal score of 0-4. (B) Weight change during treatment, expressed as a percentage change from day 0. (C) Average water consumption per mouse was calculated by dividing the remaining volume in cages every two days by the number of mice per cage. Statistical differences of groups ($p<0.05$) was determined using RM 2-way ANOVA, matching: stacked with Tukey's multiple comparisons.

one mouse on the ninth day (Table 6). Mice receiving 2.25% DSS also consumed less water on the eighth day when compared to the control group ($p=0.0008$, 2-way ANOVA) (Fig. 6C).

The group receiving 1.5% DSS began testing positive for fecal blood on the second day and developed loose stools starting on the fifth day. When compared to the control group, mice receiving 1.5% DSS had higher intestinal scores from the second to the seventh day ($p<0.0005$, 2-way ANOVA) (Fig. 6A). A linear equation was calculated for intestinal score from the fourth to sixth day, when intestinal distress increased significantly ($y=0.85x-1.383$, linear regression) (Appendix 1A). Mice receiving 1.5% DSS also lost more weight than the control group from the

**Table 6.** Number of mice that lost over 25% of their initial body weight on each day during Experiment 1.

| Treatment Group (n=5) | # of mice that lost >25% body weight | | | |
|---|---|---|---|---|
| | Days 1-6 | Day 7 | Day 8 | Day 9 |
| Control | 0 | 0 | 0 | 0 |
| 1.5% DSS | 0 | 1 | 2 | 2 |
| 2.25% DSS | 0 | 0 | 4 | 1 |
| 3% DSS | 0 | 2 | 3 | N/A |

fifth to the seventh day ($p \leq 0.0012$, 2-way ANOVA) (Fig. 6B). One mouse receiving 1.5% DSS lost over 25% of their body weight on the seventh day, an additional one mouse on the eighth day, and the remaining two mice on the ninth day (Table 6). Mice receiving 1.5% DSS also consumed less water on the eighth day when compared to the control group ($p=0.0005$, 2-way ANOVA) (Fig. 6C).

3.2. *1% DSS administered through drinking water induces acute intestinal inflammation in C57BL/6 mice without causing excessive weight loss or highly bloody stools.*

Because the first tested range of 1.5%-3% DSS did not reveal an optimal model of IBD (no differences between DSS groups were demonstrated for weight loss or intestinal scores, and most mice in all DSS treatment groups lost >25% of their body weight before day 9), the experiment was repeated using a lower range of 0.5%-1.5% DSS. Twenty female C57BL/6 mice, ages 6-8 weeks were identified and assigned treatment groups using the same method as the first experiment. Five mice per treatment group were included (Table 2). DSS treatment cages were administered either 1.5%, 1%, or 0.5% DSS dissolved in drinking water for 6 days, followed by regular water for 3 days. Control cages were administered regular water for all 9 days. DSS solutions/water were replaced every two days and average water consumption per mouse was calculated as previously described. Weight, stool consistency, and presence of fecal blood (hema-screen) were recorded daily for individual mice. Mice were assigned an intestinal score

from 0-5 as previously described, but with the addition of visible blood in stools as a factor (Table 4). If a mouse lost >25% of its body weight at any time during the experiment, it was euthanized after data collection for that day. At the end of 9 days, all remaining mice were euthanized.

Similar to the first experiment, mice receiving 1.5% DSS began testing positive for fecal blood on the second day and developed loose stools starting on the fifth day. Mice receiving 1.5% DSS had higher intestinal scores than control mice from the second to the eighth day ($p \leq 0.05$), and higher scores than mice receiving 0.5% DSS from the fifth to the eighth day ($p < 0.0037$, mixed-affects model) (Fig. 7A). A linear equation was calculated for intestinal score from the fourth to sixth day, when intestinal distress increases significantly ($y=1.55x-4.483$, linear regression) (Appendix 1B). Mice receiving 1.5% DSS lost more weight than control mice from the fifth to the eighth day ($p \leq 0.0019$), and lost more weight than mice receiving 0.5% from the sixth to the eighth day ($p < 0.0001$, 2-way ANOVA) (Fig. 7B).

Additionally, mice receiving 1.5% DSS had higher intestinal scores than mice receiving 1% DSS on the sixth day ($p=0.0266$, mixed model analysis), and lost more weight than mice receiving 1% DSS on the seventh and eighth days ($p \leq 0.0026$, 2-way ANOVA). All five mice receiving 1.5% DSS lost over 25% of their body weight on the eighth day (Table 7). Also, mice receiving 1.5% DSS consumed less water than control mice and mice receiving 0.5% DSS on the eighth day ($p \leq 0.002$, 2-way ANOVA) (Fig. 7C).

Similarly, the group receiving 1% DSS began testing positive for fecal blood on the second day and developed loose stools starting on the fifth day. Mice receiving 1% DSS had higher intestinal scores than control mice on the fourth to the the eighth day ($p \leq 0.05$) and higher scores than mice receiving 0.5% DSS from the sixth to the eighth day ($p \leq 0.0056$, mixed-

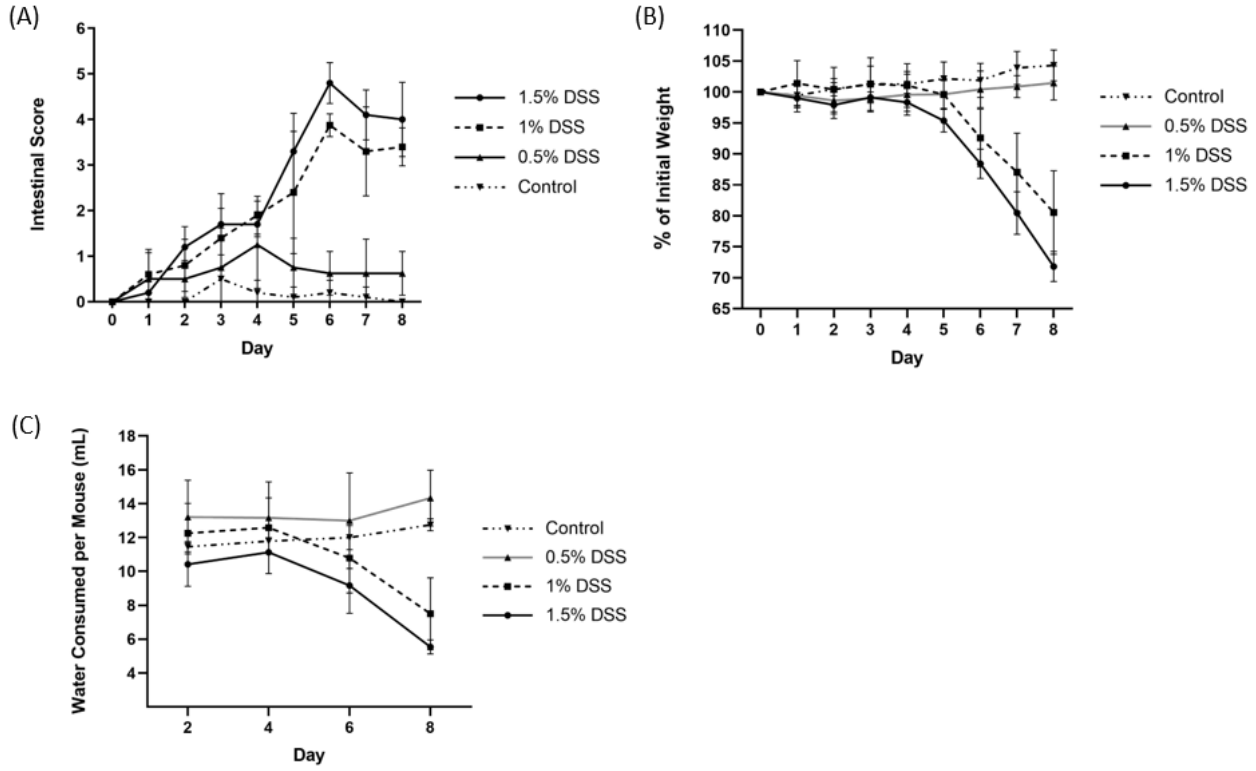


**Fig. 7**. 1% DSS induces acute intestinal distress in C57BL/6 mice without causing excessive weight loss or highly bloody stools. 1.5%, 1%, or 0.5% DSS was dissolved in drinking water and administered to mice for 6 days, followed by regular water for 3 days. Control mice were administered regular water for all 9 days. Five mice per treatment group were included. For all panels, group means, and standard deviations are plotted. (A) Stool consistency and presence of fecal blood (hema-screen) were used to provide mice an intestinal score of 0-5. (B) Weight change during treatment, expressed as a percentage change from day 0. (C) Average water consumption per mouse was calculated by dividing the remaining volume in cages every two days by the number of mice per cage. Statistical differences of groups ($p<0.05$) was determined using RM 2-way ANOVA or mixed-affects model, matching: stacked with Tukey's multiple comparisons.

model analysis) (Fig. 7A). A linear equation was calculated for intestinal score from the fourth to sixth day ($y=0.968x-2.128$, linear regression) (Appendix 1C). Mice receiving 1% DSS lost more weight than control mice and mice receiving 0.5% DSS from the sixth to the eighth day ($p<0.0001$, 2-way ANOVA) (Fig. 7B).

One mouse receiving 1% DSS lost over 25% of their body weight on the eighth day, and two additional mice on the ninth day (Table 7). Also, mice receiving 1% DSS consumed less water than control mice and mice receiving 0.5% DSS on the eighth day ($p\leq0.0235$, 2-way ANOVA) (Fig. 7C).

**Table 7.** Number of mice that lost over 25% of their initial body weight on each day during Experiment 2.

| Treatment Group (n=5) | # of mice that lost >25% body weight | | | |
|---|---|---|---|---|
| | Days 1-6 | Day 7 | Day 8 | Day 9 |
| Control | 0 | 0 | 0 | 0 |
| 0.5% DSS | 0 | 0 | 0 | 0 |
| 1% DSS | 0 | 0 | 1 | 2 |
| 1.5% DSS | 0 | 0 | 5 | N/A |

The group receiving 0.5% DSS began testing positive for fecal blood on the second day but did not develop loose stools throughout the experiment. Mice receiving 0.5% DSS did not had higher intestinal scores or lose more weight than control mice throughout the experiment. Mice receiving 0.5% DSS also did not consume less water than control mice (Fig. 7C).

3.3. *Administration of heat-killed echinostome metacercariae to mice treated with 1% DSS does not decrease induced intestinal distress but appears to exacerbate symptoms.*

The results of the initial trial revealed that 1% DSS, when given for 6 days, induces acute intestinal distress in C57BL/6 mice without causing excessive weight loss (four out of the five mice did not lose 25% of their body weight before 9 days), or highly bloody stools; therefore, 1% DSS was used in this phase of the project, which tested the effects of heat-killed echinostome metacercariae on mice treated with DSS.

Thirty-two female, C57BL/6 mice, ages 6-8 weeks were identified and assigned treatment groups in the same method as the first two experiments. Eight mice per treatment group were included to ensure proper controls were tested: Group 1 (DSS + Parasite), Group 2 (Water + Parasite), Group 3 (DSS + PBS), and Group 4 (Water + PBS). (Table 5). Mice in Groups 1 and 3 were given 1% DSS dissolved in drinking water for 6 days, followed by regular water for 3 days. Mice in Groups 2 and 4 were given regular water for all 9 days. DSS solutions/water were

replaced every two days and average water consumption per mouse was calculated as previously described. Average food consumption per mouse was calculated in the same method as water consumption.

Thirty to forty echinostome metacercariae, dissected from infected snails and heat-killed prior to the experiment, were orally administered on Day 3 to each mouse in Groups 1 and 2. PBS was orally administered on Day 3 to each mouse in Groups 3 and 4. Weight, stool consistency, and presence of fecal blood (hema-screen) were recorded daily. Mice were assigned an intestinal score from 0-4 as previously described in the first experiment (Table 3). On the ninth day, all mice were euthanized, and spleens and intestines were dissected. Intestines were stored in formalin for analysis of colon length. To measure colon length, intestines were straightened and measured from the ileocecal junction to the rectum. Spleens were disrupted and dissolved in a solution of 1 mL PBS with 0.1 mL 1.1% IPEGAL. Splenic supernatants were kept at -20°C for analysis of pro-inflammatory cytokines IL-4 and IFN-γ, and regulatory cytokines IL-2 and IL-10 by ELISA.

Group 1 (DSS + Parasite) began testing positive for fecal blood on the first day and developed loose stools starting on the seventh day. Mice in Group 1 had higher intestinal scores than mice in Group 2 (Water + Parasite) on the second day, and from the fourth to the ninth day ($p \leq 0.0379$) and higher scores than Group 4 (Water + PBS) from the second to the ninth day ($p \leq 0.0049$, 2-way ANOVA) (Fig. 8A). Mice in Group 1 (DSS + Parasite) also lost more weight than mice in Group 2 (Water + Parasite) and Group 4 (Water + PBS) from the seventh to the ninth day ($p \leq 0.046$, 2-way ANOVA) (Fig. 8B). Additionally, mice in Group 1 had higher intestinal scores and lost more weight than mice in Group 3 (DSS + PBS) on the ninth day ($p \leq 0.02$, 2-way ANOVA).

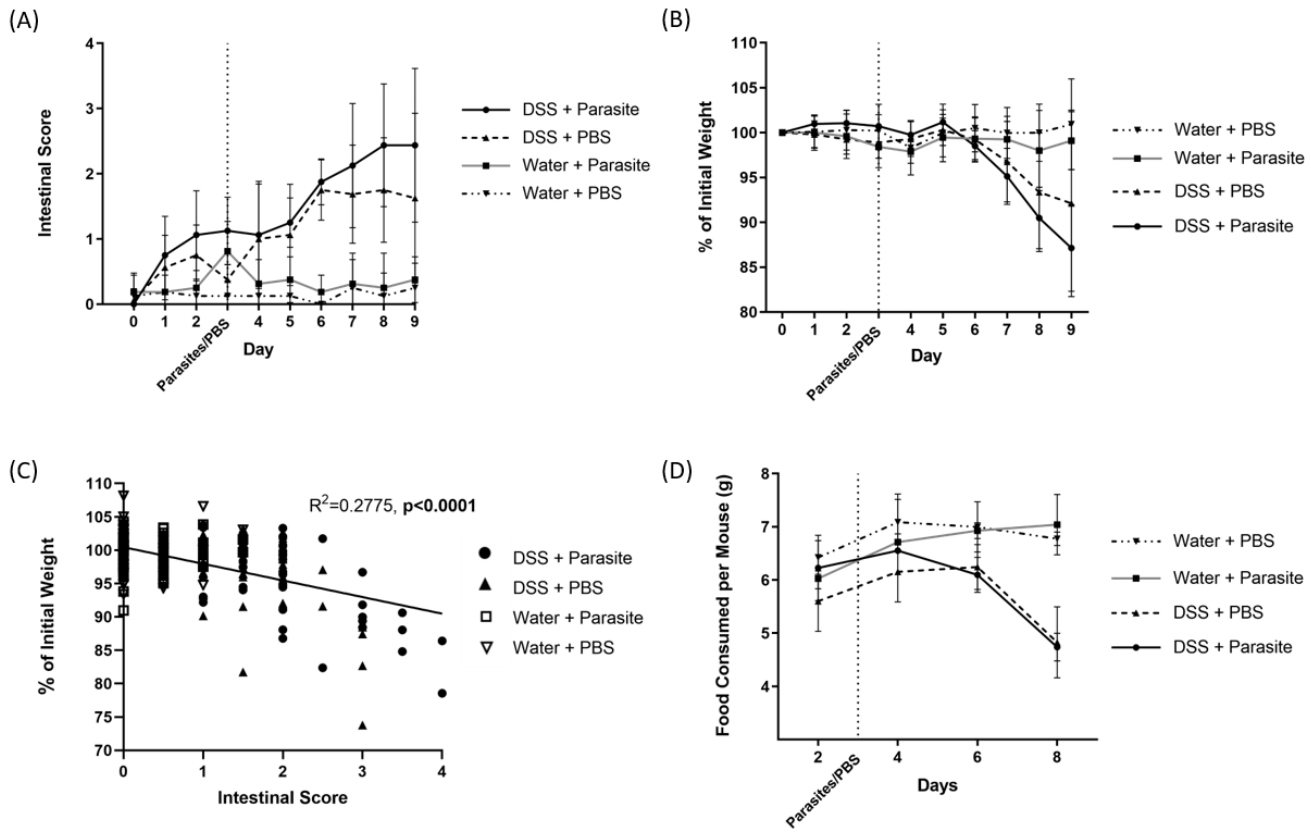


**Fig. 8**. The effects of heat-killed echinostome metacercariae on DSS-induced intestinal distress in C57BL/6 mice. Two groups of experimental mice were given 1% DSS dissolved in drinking water for 6 days, followed by regular water for 3 days. Two groups of control mice were given regular water for all 9 days. Eight mice per treatment group were included. On Day 3, mice in Groups 1 and 2 were orally administered metacercariae and mice in Groups 3 and 4 were orally administered PBS. For panels A, B, and D, group means, and standard deviations are plotted. For panel C, each symbol represents one mouse. (A) Stool consistency and presence of fecal blood (hema-screen) were used to provide an intestinal score of 0-4. (B) Weight change during treatment, expressed as a percentage change from day 0. (C) Correlation of intestinal score to weight change on each day for all mice. (D) Average food consumption per mouse was calculated by dividing the remaining volume in cages every two days by the number of mice per cage. Statistical differences of groups ($p<0.05$) was determined using RM 2-way ANOVA matching: stacked with Tukey's multiple comparisons. Linear regression was used to determine $R^2$ and p values for panel C.

It was also revealed that mice in Group 1 (DSS + Parasite) did not consume less water than mice in Group 2 (Water + Parasite) or Group 4 (Water + PBS) but did consume less food than mice in Groups 2 and 4 on the eighth day ($p<0.0001$, 2-way ANOVA) (Fig. 8D). Colon length analysis revealed that mice in Group 1 (DSS + Parasite) exhibited shorter colons after dissection then mice in Groups 2 and 4 ($p\leq0.0037$, 1-way ANOVA) (Fig 9B.).

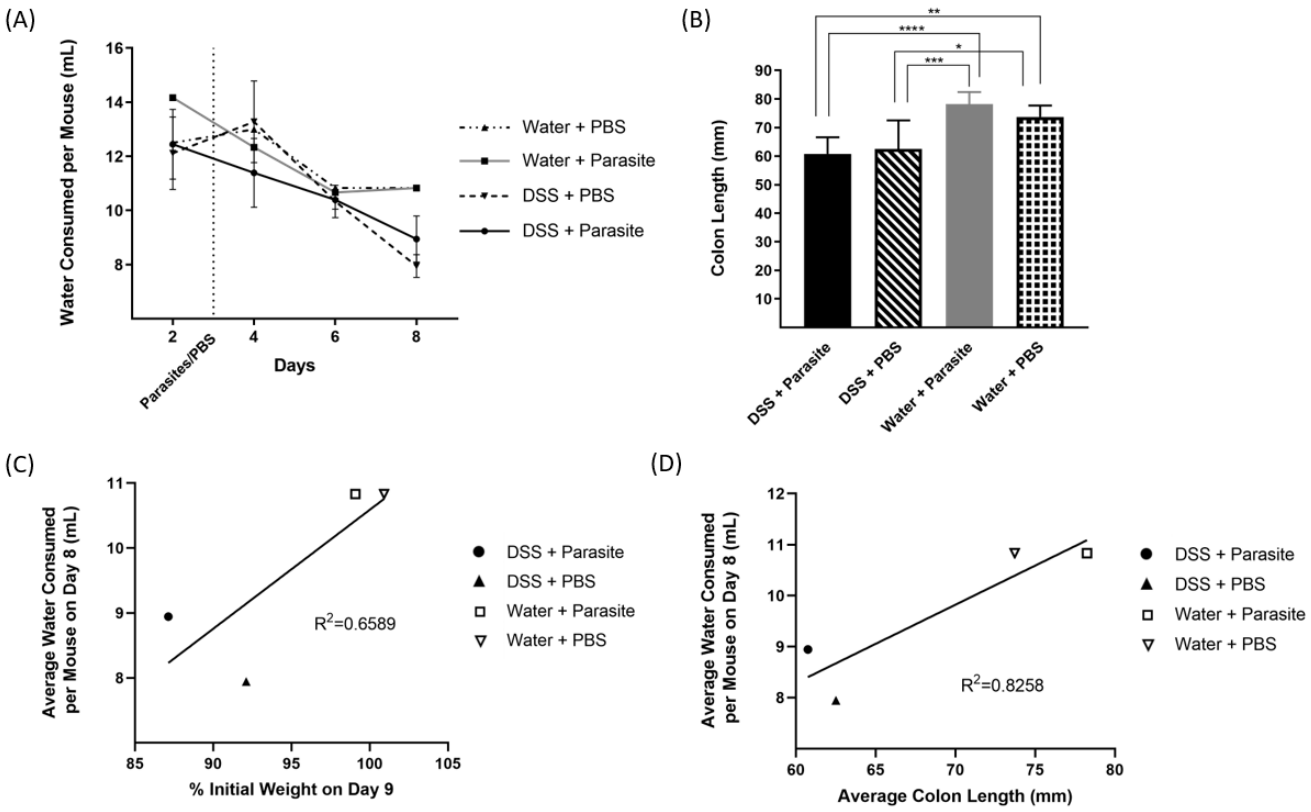


**Fig. 9.** Average water consumption and colon lengths of mice treated with 1% DSS for 6 days and administered heat-killed metacercariae or PBS on Day 3. For panels A-B, group means, and standard deviations are plotted. (A) Average water consumption per mouse was calculated by dividing the remaining volume in cages every two days by the number of mice per cage (RM 2-way ANOVA, matching stacked with Tukey's multiple comparisons test). (B) Colon length was determined by straightening intestines and measuring the length from the ileocecal junction to the rectum (1-way ANOVA with Tukey's multiple comparisons test). (C) Correlation of average water consumption on Day 8 to average weight change on Day 9 (linear regression). (D) Correlation of average water consumption on Day 8 to average colon length (linear regression).

Mice in Group 3 (DSS + PBS) began testing positive for fecal blood on the first day and developed loose stools starting on the seventh day. Mice in Group 3 (DSS + PBS) had higher intestinal scores than mice in Group 4 (Water + PBS) from the fourth to the ninth day ($p \leq 0.01$), and higher scores than mice in Group 2 (Water + Parasite) from the sixth to the ninth day ($p < 0.0001$, 2-way ANOVA) (Fig. 8A). A linear equation was calculated for intestinal score from the fourth to sixth day ($y = 0.3750x - 0.6042$, linear regression) (Appendix 1D). Mice in Group 3 (DSS + PBS) lost more weight than mice in Groups 2 (Water + Parasite) and 4 (Water + PBS) on the eighth and the ninth days ($p \leq 0.0187$, 2-way ANOVA) (Fig. 8B).

It was also revealed that mice in Group 3 (DSS + PBS) did not consume less water than mice in Groups 2 (Water + Parasite) and 4 (Water + PBS) (Fig. 9A) but did consume less food than mice in Groups 2 and 4 on the eighth day ($p<0.0001$, 2-way ANOVA) (Fig. 8D). Colon length analysis revealed that mice in Group 3 (DSS + PBS) exhibited shorter colons after dissection then mice in Group 2 (Water + Parasite) and Group 4 (Water + PBS) ($p\leq0.0133$, 1-way ANOVA) (Fig. 9B).

Mice in Group 2 (Water + Parasite) did not develop loose stools or progressive weight loss throughout the experiment (Fig. 8A, B). However, two out of eight mice in Group 2 (Water + Parasite) tested positive for fecal blood on the third day, one mouse on the fourth day, one mouse on the fifth day, and one mouse on the eighth day. Mice in Group 2 showed no significant differences between mice in Group 4 (Water + PBS) for weight change, intestinal scores, colon length, water consumption, or food consumption.

Mice in group 4 (Water + PBS) did not develop loose stools or progressive weight loss throughout the experiment (Fig. 8A, B).

An overall correlation between weight change and intestinal score was observed for all mice throughout the experiment ($p<0.0001$, linear regression) (Fig. 8C). This correlation was further revealed for mice in DSS treatment groups (Appendix 2A, B), but not for mice in untreated groups (Append. 2C, D). A correlation between average water consumption on the eighth day and average weight change on the ninth day was observed (Fig. 9C), as well as a correlation between average water consumption on the eighth day and average colon length (Fig. 9D).

The results of the initial trial showed no differences between identical groups in the first two experiments. Mice receiving 1.5% DSS in the first experiment showed no differences in

weight change or water consumption throughout the experiment (Fig. 10A, B). Furthermore, mice receiving water in the first experiment showed no difference in weight change or water consumption than mice receiving water in the second experiment (Fig. 10A, B). However, our results revealed that mice receiving 1% DSS and PBS in the third experiment lost less weight than mice receiving 1% DSS in the second experiment from the sixth to the eighth day ($p \leq 0.001$, 2-way ANOVA) (Fig. 10C). The data also suggested that mice receiving water and PBS in the

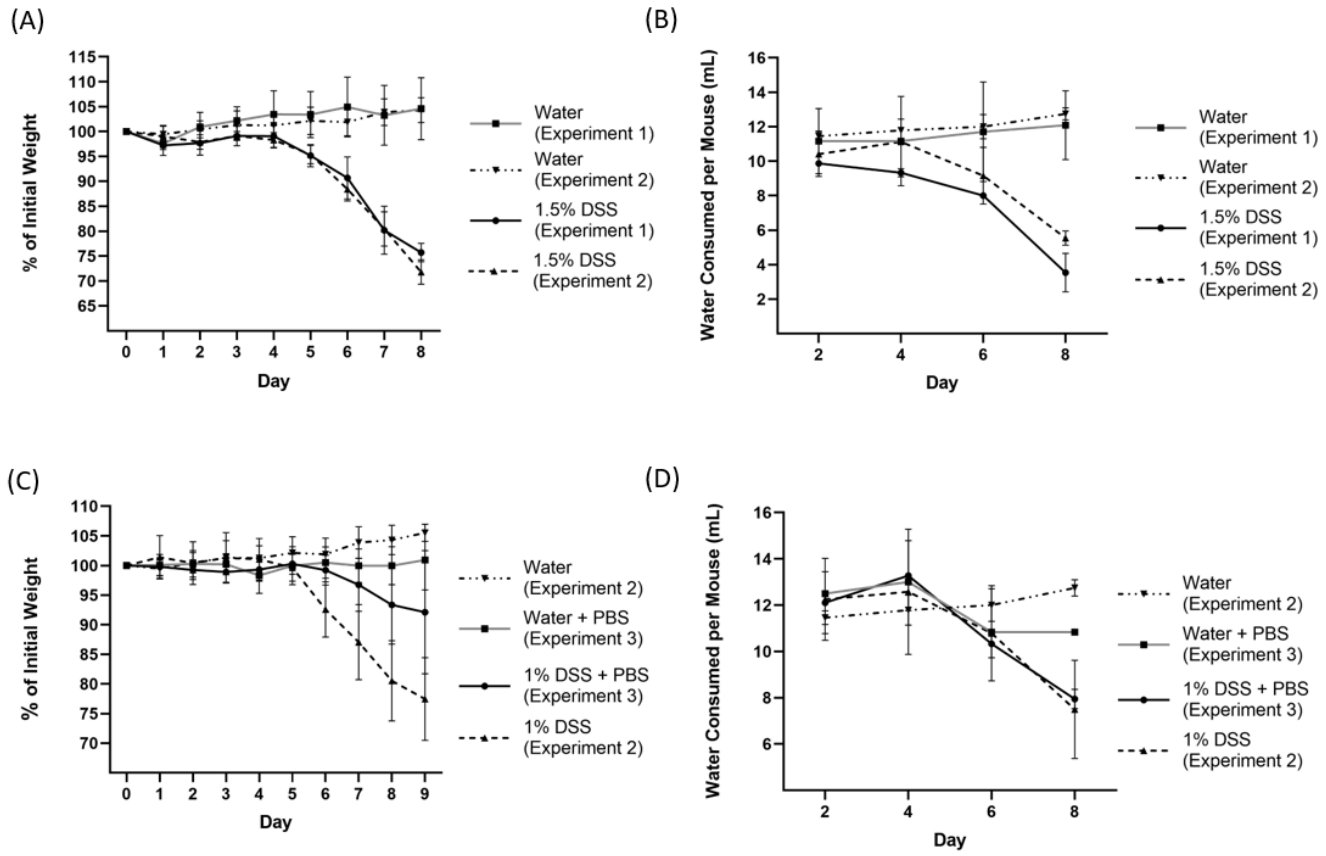


**Fig. 10**. Analysis of weight change and water consumption between groups for all three experiments. For all panels, group means, and standard deviations are plotted. (A) Weight change, expressed as a percentage change from day 0, for mice receiving 1.5% DSS or water in the first two experiments. (B) Average water consumption, measured by dividing the remaining volume every two days by the number of mice per cage, for mice receiving 1.5% DSS or water in the first two experiments. (C) Weight change for mice receiving 1% DSS or water in experiments two and three. (D) Average water consumption for mice receiving 1% DSS or water in experiments two and three. Statistical differences between groups ($p<0.05$) was determined using RM 2-way ANOVA matching: stacked with Tukey's multiple comparisons.

third experiment gained less weight than mice receiving water in the second experiment on the eighth day (p=0.065, 2-way ANOVA) (Fig. 10C).

The results of the second experiment revealed that mice receiving 1% DSS consumed less water on average than mice receiving water on the eighth day (p=0.0068, 2-way ANOVA) (Fig. 10D), however mice receiving 1% DSS and PBS in the third experiment did not consume significantly less water than mice receiving water and PBS in the third experiment (Fig. 10D). Furthermore, while mice receiving water in the second experiment consumed approximately the same amount of water on average throughout the experiment, mice receiving water and PBS in the third experiment consumed less water on the sixth and eight days (Fig. 10D).

3.4. *Mice treated with 1% DSS and administered heat-killed echinostome metacercariae showed decreased levels of regulatory cytokine IL-2 when compared to mice in all other groups.*

Spleens from individual mice were dissected on Day 9 and were disrupted and dissolved in a solution of 1 mL PBS and 0.1 mL 1.1% IPEGAL. Supernatants were analyzed using a Mouse $T_H1/T_H2$ ELISA kit (Thermofisher Scientific). The detection ranges for each cytokine were reported by the manufacturer to be: IL-4, 4-500 pg/mL; IFN-γ, 15-2000 pg/mL; IL-2, 2-200 pg/mL; and IL-10, 30-4000 pg/mL. In all samples where the calculated cytokine concentration was at or below the given sensitivity, it was plotted as the lowest detectable concentration for that cytokine (Table 8).

In order to detect levels of IL-2, a cytokine associated with $T_H1$ cells and $T_{REG}$ cell differentiation, five or six mice per treatment group were analyzed on two ELISA plates. We showed that detectable levels of IL-2 (≥2 pg/ml) were present in the spleens of four out of five tested mice in Group 1 (DSS + Parasite), all tested mice in Group 2 (Water + Parasite), all tested

**Table 8.** Splenic levels of regulatory cytokines IL-2 and IL-10, parasite specific cytokine IL-4, and pro-inflammatory cytokine IFN-γ in mice treated with 1% DSS for 6 days and administered metacercariae or PBS on Day 3. Splenic supernatants from each treatment group were analyzed by a mouse $T_H1/T_H2$ ELISA kit. Mice not tested were recorded as n/t.

| Treatment Group | Mice ID | IL-2 | IL-4 | IL-10 | IFN-γ |
|---|---|---|---|---|---|
| | 232 | n/t | n/t | *0 | *0 |
| | 225 | 5.547 | n/t | *0 | n/t |
| | 243 | *0 | n/t | *0 | n/t |
| **DSS + Parasite** | 245 | n/t | n/t | 431.695 | *0 |
| | 248 | 6.362 | *0 | n/t | *0 |
| | 252 | n/t | *0 | n/t | n/t |
| | 254 | 3.028 | *3.166 | n/t | n/t |
| | 256 | 2.729 | n/t | n/t | n/t |
| | 233 | 4.835 | n/t | *0 | n/t |
| | 226 | 4.812 | n/t | *0 | n/t |
| | 236 | 13.588 | n/t | *0 | *0 |
| **Water + Parasite** | 237 | 4.792 | n/t | n/t | n/t |
| | 240 | n/t | 7.44 | n/t | n/t |
| | 244 | 9.826 | *0 | n/t | *0 |
| | 246 | 8.768 | 9.566 | n/t | *0 |
| | 247 | 8.619 | *0 | n/t | *0 |
| | 222 | n/t | n/t | *0 | n/t |
| | 224 | 4.806 | n/t | *9.032 | n/t |
| | 229 | 9.491 | n/t | *0 | *0 |
| **DSS + PBS** | 235 | n/t | n/t | *0 | n/t |
| | 238 | 8.719 | *0 | n/t | *0 |
| | 242 | 8.954 | *0 | n/t | *0 |
| | 249 | n/t | *3.176 | n/t | n/t |
| | 251 | 6.907 | n/t | n/t | *0 |
| | 227 | 14.548 | n/t | 487.336 | *0 |
| | 228 | 8.164 | n/t | 105.517 | n/t |
| | 234 | 15.018 | n/t | 43.214 | n/t |
| **Water + PBS** | 239 | 21.767 | n/t | n/t | 34.921 |
| | 250 | 11.08 | *0 | n/t | *0 |
| | 253 | n/t | *0 | n/t | n/t |
| | 255 | 6.281 | *0 | n/t | *0 |
| | 257 | n/t | *0 | n/t | n/t |

* indicates concentration was below detectable level (IL-2=2 pg/ml, IL-4= 4 pg/ml, IL-10= 30 pg/ml, IFN-γ= 15 pg/ml)

mice in Group 3 (DSS + PBS), and all tested mice in Group 4 (Water + PBS) (Table 8, Fig. 11A).

Statistical analysis revealed that mice in Group 1 (DSS + Parasite) had significantly lower levels of

IL-2 than mice in Group 4 (Water + PBS) (p=0.0036, 1-way ANOVA) (Fig. 11A).

Statistical analysis further revealed that mice in Group 1 (DSS + Parasite) had lower levels of IL-2 than mice in Group 2 (Water + Parasite) (p=0.026) (Fig. 12C), and mice in Group 3 (DSS + Water) (p=0.0128, unpaired t test) (Fig. 12E).

A correlation between IL-2 concentration and weight change on the ninth day (p=0.0345) (Fig. 12A), intestinal score on the ninth day (p=0.03) (Fig. 12B), and weight on the

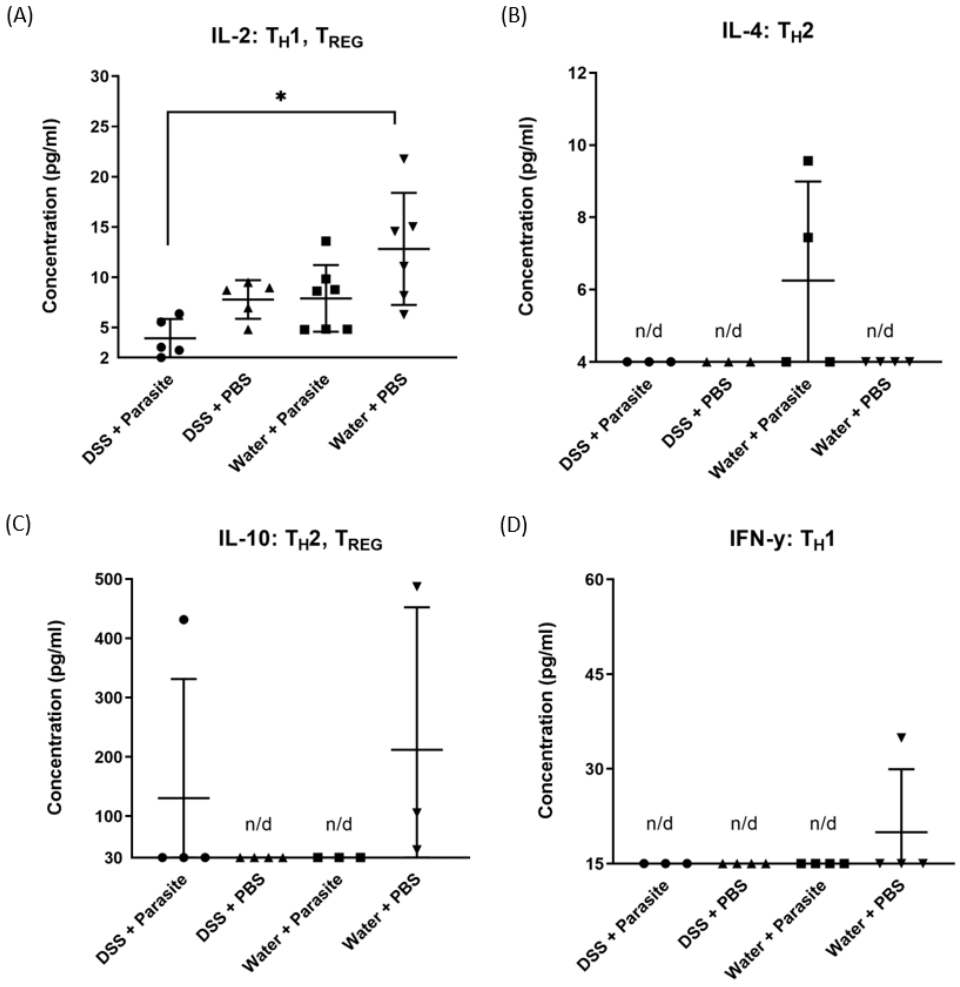


**Fig. 11**. Analysis of splenic cytokines in mice treated with 1% DSS for 6 days and administered metacercariae or PBS on Day 3. Spleens were harvested after 9 days and supernatants were analyzed using ELISA. For all panels, group means, and standard deviations are indicated, and each symbol represents one mouse. (A) $T_H1$, $T_{REG}$ cytokine IL-2. (B) $T_H2$ and parasite specific cytokine IL-4. (C) $T_H2$, $T_{REG}$ cytokine IL-10. (D) $T_H1$ and pro-inflammatory cytokine IFN-γ. Statistical differences of groups ($p<0.05$) was determined using 1-way ANOVA with Tukey's multiple comparisons test.

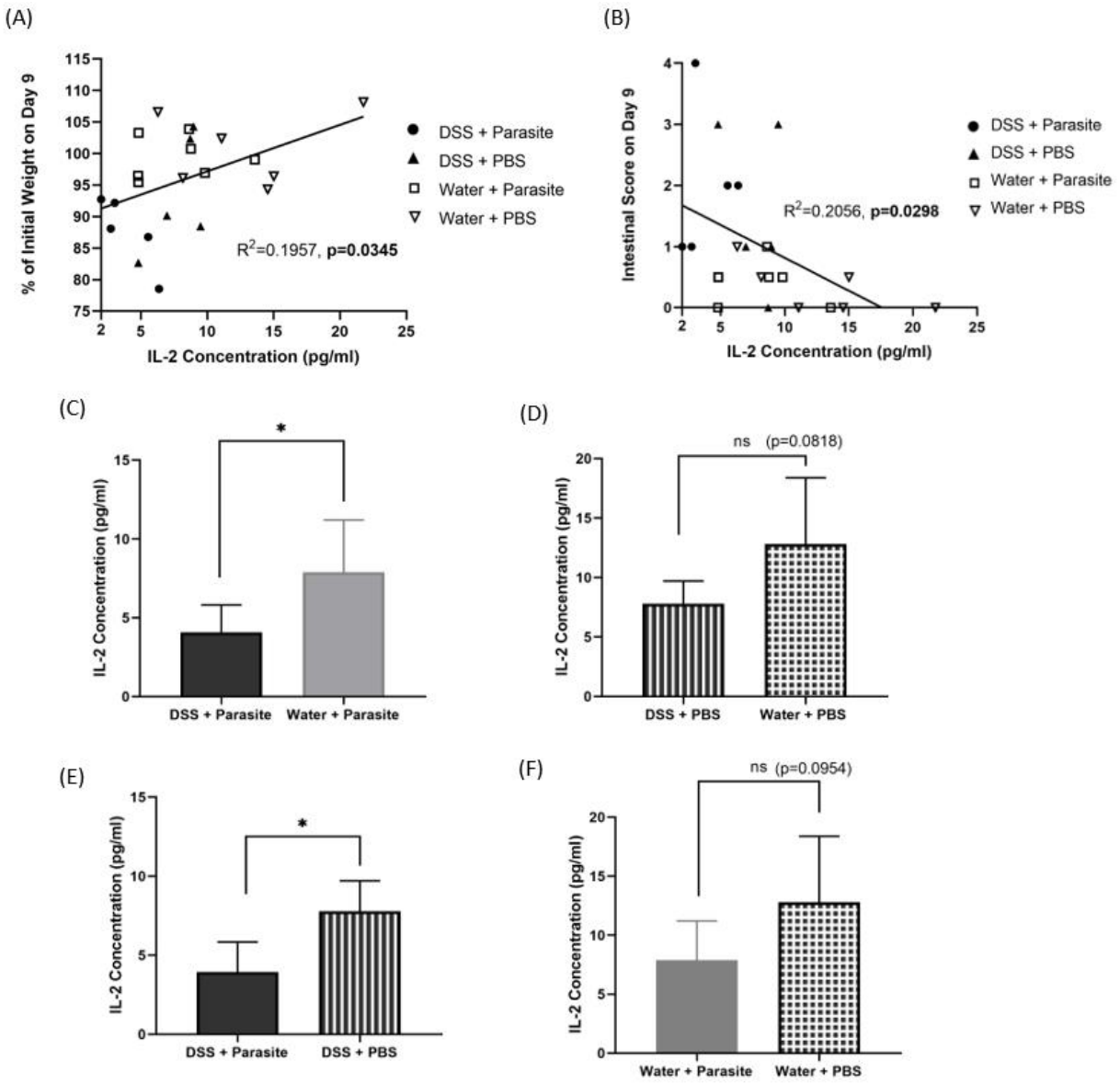


**Fig. 12.** Analysis of splenic cytokine IL-2 in mice treated with 1% DSS for 6 days and administered metacercariae or PBS on Day 3. Spleens were harvested after 9 days and supernatants were analyzed for regulatory cytokine IL-2 (n=5-6 per treatment group) using ELISA. For panels A and B, each symbol represents one mouse. For panels C-F, group means, and standard deviations are plotted. (A) Correlation of weight change on Day 9 and IL-2 concentration. (B) Correlation of intestinal score on Day 9 and IL-2 concentration. (C) IL-2 concentrations in groups receiving parasites. (D) IL-2 concentrations in groups receiving PBS. (E) IL-2 concentrations in groups receiving DSS. (F) IL-2 concentrations in groups receiving water. For panels A and B, linear regression was used to determine $R^2$ and p values. Statistical differences between groups ($p<0.05$) (Panels C-F) was determined using unpaired t test with Welch's correction.

ninth day (p=0.0232, linear regression) (Appendix 3A) was observed. No correlation between IL-2 concentration and initial weight was observed (Appendix 3B).

In order to detect levels of IL-4, a cytokine associated with $T_H2$ cells and parasite expulsion, three or four mice per treatment group were analyzed on one ELISA plate. We showed that detectable levels of IL-4 (≥4 pg/ml) were not present in the spleens of mice in Group 1 (DSS + Parasite), Group 3 (DSS + PBS), or Group 4 (Water + PBS), and were present in the spleens of two out of four tested mice in Group 2 (Water + Parasite) (Table 8, Fig. 11B).

In order to detect levels of IL-10, a cytokine associated with $T_H2$, $T_{REG}$, and $B_{REG}$ cells, three or four mice per treatment group were analyzed on one ELISA plate. We showed that detectable levels of IL-10 (≥30 pg/ml) were not present in the spleens of mice in Group 2 (Water + Parasite), or Group 3 (DSS + PBS), and were present in the spleen of one out of four tested mice in Group 1 (DSS + Parasite), and all tested mice in Group 4 (Water + PBS) (Table 8, Fig. 11C).

In order to detect levels of IFN-γ, a cytokine associated with $T_H1$ cells and autoimmunity, three or four mice per treatment group were analyzed on one ELISA plate. We showed that detectable levels of IFN-γ (≥15 pg/ml) were not present in the spleens of mice in Group 1 (DSS + Parasite), Group 2 (Water + Parasite), or Group 3 (DSS + PBS), and were present in the spleen of one out of four tested mice in Group 4 (Water + PBS) (Table 8, Fig. 11D).

## 4. Discussion

IBD is an autoimmune disease characterized by chronic intestinal inflammation and epithelial damage (1,2,5-9). Epidemiological studies show an inverse relationship between the frequency of helminth worm infections and IBD (1,2,5,7,8,15). IBD is common in industrialized

countries where human helminth parasite infections are relatively sparse, whereas helminth parasites, including species from Class *Trematoda* and Phylum *Nematoda*, are still common in tropical countries where IBD is scarce. (1,2,5,8,15). Various studies have been conducted on mice models of IBD in which mice were protected from chemically induced colitis (DSS or TNBS) by a helminth infection, or an isolated helminth protein (galectin) delivered prior to inducing symptoms of colitis (1,2,8,9,15,16,20). Parasite have been shown to release proteins that induce a shift in $T_H1$ to $T_H2$ profiles, inhibiting IFN-γ and effector T cells, and producing IL-4 and IL-10 (1-5,7-9,13,15,17-20). IFN-γ is a pro-inflammatory $T_H1$ cytokine associated with the chronic inflammation in autoimmune diseases such as IBD (1,6,9,15,16,20,22). IL-4 is a $T_H2$ cytokine associated with parasite expulsion in the initial phase of infection (1-3,5,7,15,17,18), and IL-10 is a $T_H2$ and regulatory cytokine often associated with the protective effects of helminth parasites (1-3,5,7,11-13,15-18). Furthermore, parasite proteins may induce regulatory mechanisms including the production of $T_{REG}$ cells, $B_{REG}$ cells, regulatory DCs and macrophages, and regulatory cytokines TGF-β, IL-10, and IL-2 (1-5,7-9,13,15-21). IL-2 is a $T_H1$ cytokine shown to induce $T_{REG}$ cells following an infection (21).

We questioned whether noninfectious helminth parasites (heat-killed echinostome metacercariae) delivered orally to C57BL/6 mice after several days of treatment with DSS would decrease symptoms of DSS-induced colitis in mice. We hypothesized that mice treated with DSS for 6 days and administered heat-killed metacercariae during DSS treatment would show less weight loss, improved intestinal health, longer colons after dissection, decreased levels of pro-inflammatory cytokines IFN-γ or IL-4, and/or increased levels of regulatory cytokines IL-2 and IL-10 when compared to mice treated with DSS and not administered metacercariae.

In order to test our hypothesis, we first established an optimal model of DSS-induced colitis in female C57BL/6 mice by dissolving different concentrations of DSS (3%, 2.25%, 1.5%,

1%, and 0.5%) in drinking water for 6 days, followed by regular water for 3 days. The goal of the initial trial was to find an "mid-point" concentration of DSS that induces moderate intestinal distress in female C57BL/6 mice living in the MSU, Mankato vivarium. Weight, stool consistency, and presence of fecal blood were recorded daily, and water consumption was recorded every two days. If mice lost over 25% of their body weight at any time they were euthanized after data collection that day. After 9 days all remaining mice were euthanized following data collection.

In the initial trial we demonstrated that mice given 3%, 2.25%, or 1.5% DSS for 6 days developed highly bloody stools and/or excessive weight loss of >25% of their body weight. Also, mice given 3%, 2.25%, 1.5%, and 1% DSS consumed less water on average than control mice. One possible reason that mice consumed less water is that DSS may be bitter tasting, which could condition mice to drink less. Another reason is that mice may eventually become too sick to drink as much, leading to dehydration and an increase in symptoms. We further demonstrated that mice given 1% DSS for 6 days developed loose stools that tested positive for fecal blood, and lost more weight than control mice, but <25% of their body weight, and that mice given 0.5% DSS developed soft stools which occasionally tested positive for fecal blood, but did not lose more weight than control mice. From the results of the initial trial, we conclude that 1% DSS is the optimal concentration that induces moderate intestinal distress; therefore 1% DSS was chosen for the next phase of the study.

During the next phase, mice were fed heat-killed echinostome metacercariae in an attempt to counteract symptoms of DSS-induced colitis. Two groups of experimental mice received 1% DSS dissolved in drinking water for 6 days followed by regular water for 3 days, and two groups of control mice received regular water for all 9 days. One group of experimental mice and one group of control mice were orally administered heat-killed metacercariae in PBS

on the third day of DSS treatment (DSS + Parasite, Water + Parasite). The other two groups were orally administered PBS on the third day of DSS treatment (DSS + PBS, Water + PBS). Weight, stool consistency, and presence of fecal blood were recorded daily, and water and food consumption were recorded every two days. After 9 days all mice were euthanized, and intestines and spleens were dissected. Colon lengths were recorded for individual mice and spleens were disrupted and tested for inflammatory cytokine IFN-γ, parasite specific cytokine IL-4, and regulatory cytokines IL-10 and IL-2, using ELISA.

In this phase we demonstrated that mice administered heat-killed echinostome metacercariae during treatment with 1% DSS did not show a decrease in weight loss, improved intestinal health, or longer colons after dissection than mice treated with 1% DSS but not administered metacercariae. Inversely, on the ninth day of the experiment, mice administered metacercariae during DSS treatment weighed significantly less and had poorer intestinal health than mice treated with DSS but not administered metacercariae, suggesting that heat-killed metacercariae may exacerbate symptoms of colitis in DSS treated mice. Analysis of splenic cytokines showed that mice treated with DSS and administered metacercariae had lower levels of regulatory cytokine IL-2 than mice treated with DSS but not administered metacercariae.

It was also noted that mice receiving 1% DSS in the second experiment lost more weight than mice receiving 1% DSS in their drinking water and gavaged with PBS in the third experiment, and that control mice receiving water in the second experiment gained more weight than mice drinking water and gavaged with PBS in the third experiment. The data also demonstrated that control mice receiving water in the second experiment drank more water than mice drinking water and gavaged with PBS in the third experiment.

Since there was little variation between the first and second experiments (mice receiving 1.5% DSS or control mice in the first experiment did not show statistical differences between weight change or water consumption compared to mice receiving 1.5% DSS or control mice in the second experiment), this was a surprising result. It is possible that gavaging mice affects the amount of water consumption and therefore weight change. If this experiment were to be repeated, mice should also be gavaged during the initial trial when determining the optimal level of DSS.

A significant correlation between weight change and intestinal score was observed in mice treated with DSS, demonstrating that increased weight loss is associated with poorer intestinal health. This was expected since DSS induces symptoms of both weight loss and intestinal distress in mice (16,20,22,23). We also demonstrated that mice treated with DSS exhibited shorter colons after dissection than mice that did not receive DSS, regardless of parasite treatment. This result was expected since mice treated with DSS have been previously shown to exhibit shorter colons (16,20). A correlation between average colon length and average water consumption on Day 8 was observed, demonstrating that less water consumption is associated with shorter colons. Since mice treated with DSS consume less water it is possible that dehydration is what causes the shortening of colons. A correlation between weight change on Day 9 and average water consumption on Day 8 was also observed, demonstrating that less water consumption contributes to more weight loss.

Previous literature describing the effects of parasites on mice models of colitis described a shift in T cell subsets leading to a decrease in inflammatory cytokines and an increase in regulatory cytokines (16). In this study we demonstrated that mice administered metacercariae during DSS treatment showed lower levels of the regulatory cytokine IL-2 than mice in all other groups. A correlation between IL-2 concentrations and weight change, intestinal score, and

weight on the ninth day was observed, demonstrating that lower IL-2 levels are associated with increased symptoms of colitis. We did not observe a correlation between IL-2 concentration and initial weight, confirming that IL-2 levels analyzed in this study are not dependent on mouse weight, but rather on intestinal health.

Since previous literature describing the effects of parasites on DSS-induced colitis in mice did not analyze IL-2, we questioned the role of IL-2 in mice treated with DSS and administered heat-killed metacercariae. We confirmed that a decrease in IL-2 levels was driven by both DSS treatment and parasite administration. The DSS model induces a shift in T cell subsets, leading to an increase in $T_H1$ cells and pro-inflammatory cytokines. Because of this we would expect to see lower levels of regulatory cytokines during symptoms of colitis. IL-2 is thought to induce $T_{REG}$ cells at the end of an infection. The data showed that mice treated with DSS but not administered parasites exhibited intestinal distress while treated with DSS but showed a decrease in symptoms once regular water was given, suggesting that IL-2 was produced once DSS was no longer administered, leading to remission in mice.

Mice treated with DSS and administered parasites continued to exhibit intestinal distress once regular water was given. The data also showed that mice not treated with DSS but administered parasites exhibited acute intestinal distress on the third day, several hours after metacercariae were orally administered. Cytokine analysis revealed that mice receiving parasite treatment but not DSS showed detectable levels of IL-4 (although it is noted that only a small sample size was tested for this cytokine.) In the initial stage of a parasite infection, a pro-inflammatory response and the production of cytokine IL-4 is induced, which allows the body to expel the parasites, while the long-term phase of a parasite infection induces a regulatory response. It is possible that a single dose of heat-killed metacercariae fed to mice initiates or

prolongs a pro-inflammatory immune response and may block the production of regulatory cytokine IL-2 in DSS-treated mice once DSS is no longer administered.

Detectable levels of the regulatory cytokine IL-10 were present in the spleens of all tested mice not treated with DSS or administered parasites, and in one tested mouse treated with DSS and administered parasites. Detectable levels of the pro-inflammatory cytokine IFN-γ were only present in tested mice not treated with DSS or administered parasites. It is noted that only a small sample size was tested for both IL-10 and IFN-γ. Because of faulty reagents or equipment, only one ELISA plate was conclusive for each cytokine. Because of this, three or four mice per treatment group were analyzed for IFN-γ, IL-4, and IL-10. If this experiment were to be repeated it may be necessary to measure fewer cytokines in order to have more replicates.

In this study we also demonstrated that mice treated with 1% DSS consumed less food on average then mice not treated with DSS, suggesting that as mice become sick, they are unable to eat as much. Because food consumption was not measured during the initial trial, it is possible that a lack of nutrients, as well as dehydration, contributes to weight loss in mice models of colitis.

Previous literature describing the effects of parasites in mice models of colitis reported inducing a parasite stimulus before treatment with DSS and demonstrated a protective response of parasites against DSS-induced colitis (1-3,5,7-9,15,16,20). Also, previous literature reported inducing a long-term parasite stimulus, via a live infection, or multiple injections with galectin. It is unknown if non-living, whole parasites can be used as a form of treatment in mice models of colitis. Because the parasites in this study were noninfectious and delivered while symptoms of colitis were present, they most likely passed through the digestive tract. During the clinical phase of colitis, nutrients and water are less likely to be absorbed through the intestinal tract. It

is possible that heat-killed metacercariae delivered during DSS treatment do not remain inside of the body long enough to induce a regulatory immune response and decrease symptoms of colitis.

If this experiment were to be continued, multiple doses of metacercariae may be needed to stimulate the immune system into forming a regulatory response. This could be done by obtaining more infected snails and feeding metacercariae to mice on multiple days. Also, the metacercariae may need to be administered during a period of remission, so that they remain inside of the body long enough to induce a regulatory immune response. This is could be done by expanding the experiment to 18 days and administering metacercariae in between treatment with DSS.

Based on the data we conclude that heat-killed metacercariae administered to female C57BL/6 mice during treatment with 1% DSS do not protect mice from DSS-induced symptoms of colitis but appear to exacerbate symptoms, possibly by blocking regulatory IL-2 production once DSS administration is stopped. Furthermore, a single dose of metacercariae may not be sufficient to alter the immune symptom to favor a regulatory response, and multiple doses given throughout DSS treatment or during periods of remission may be required. Further studies are necessary to determine whether delivering non-infectious whole parasites as a treatment method can stimulate the immune system to form a regulatory response, thus reducing chronic inflammation and relieving symptoms of autoimmunity.

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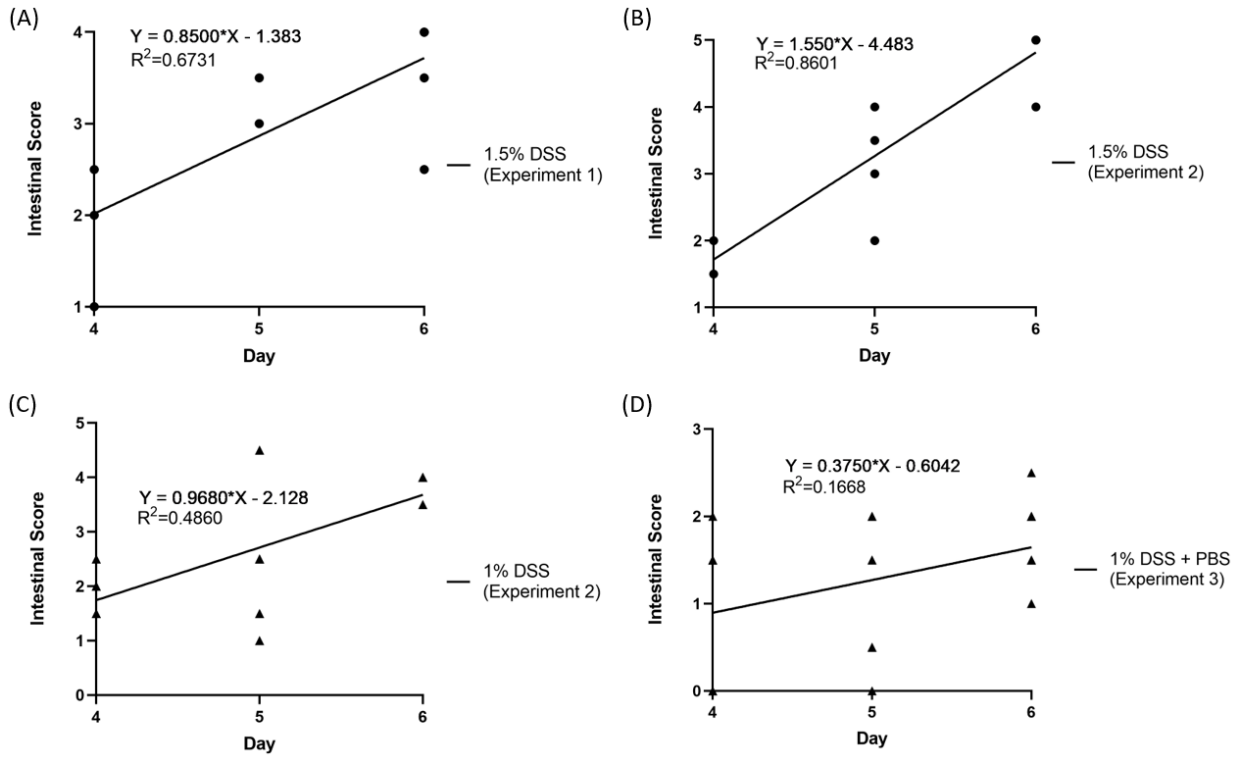
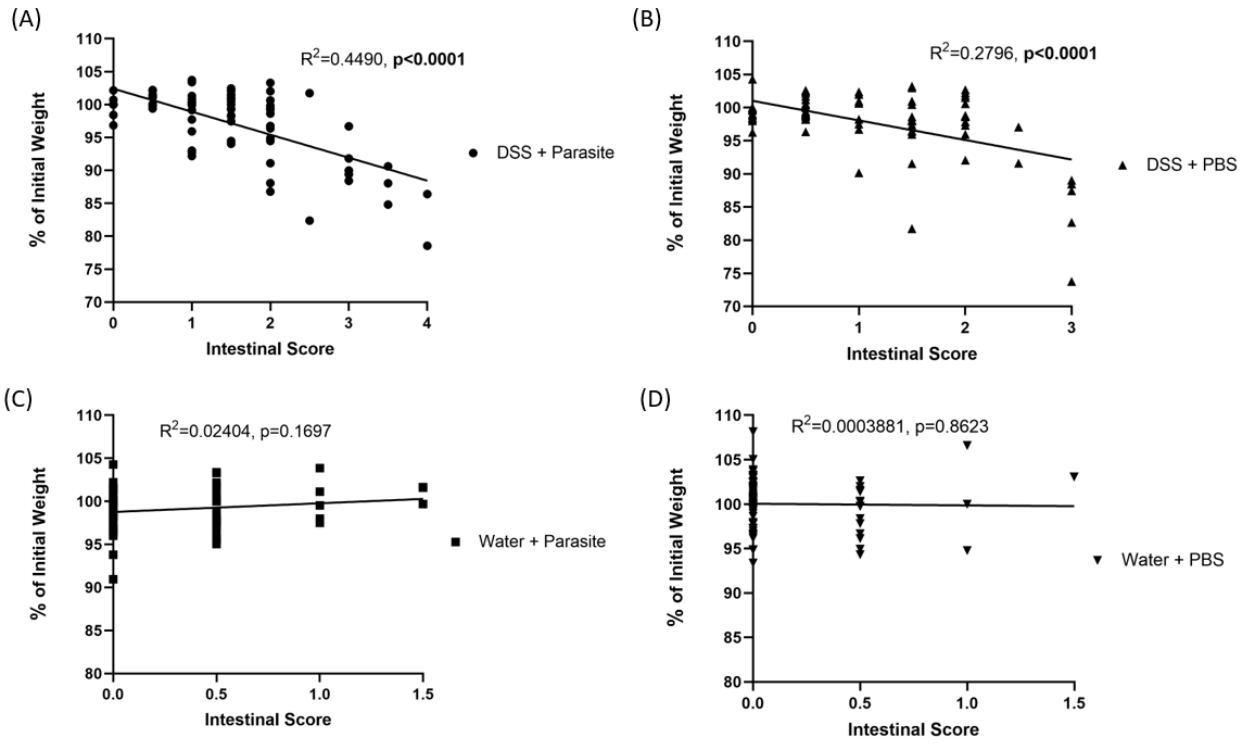
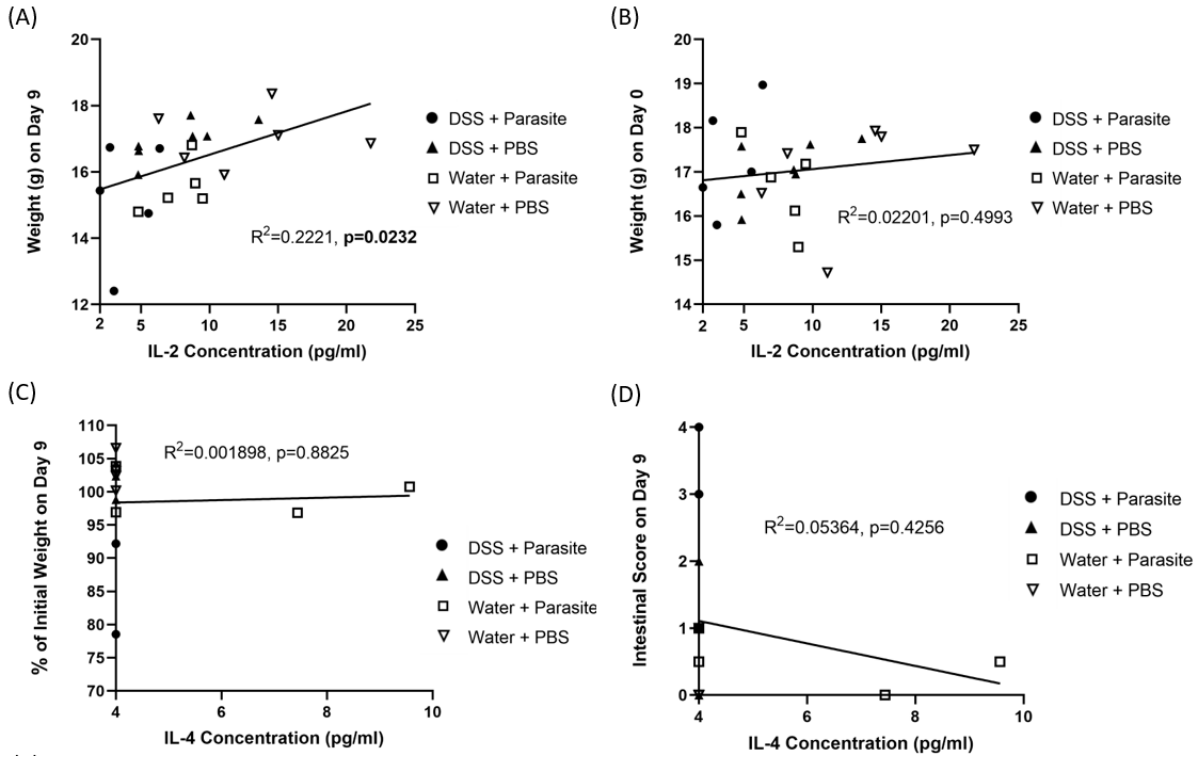


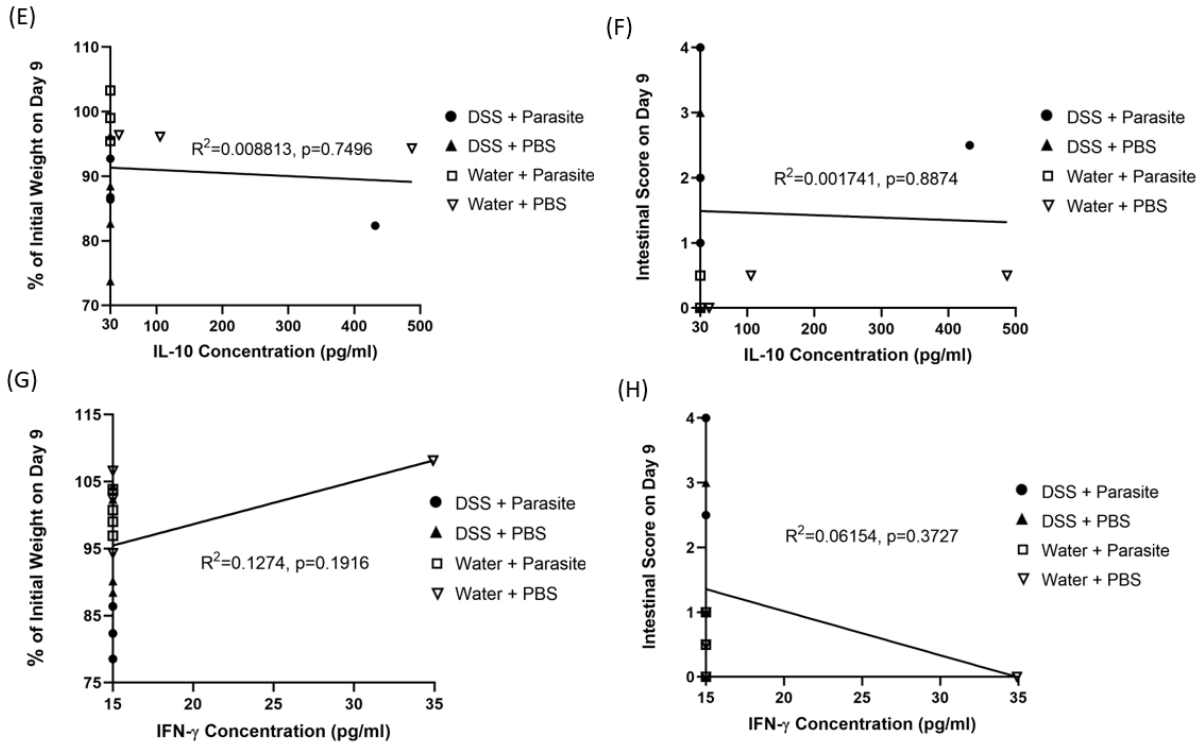

**Appendix 1.** Linear equations of intestinal distress for mice treated with 1.5% DSS or 1% DSS for all experiments. Intestinal scores increase at a significant rate from the fourth to sixth day when mice are treated with 1% DSS or 1.5% DSS for 6 days. For panels A-C, five mice were included, and for panel D, eight mice were included. (A) Mice receiving 1.5% DSS during experiment 1. (B) Mice receiving 1.5% DSS during experiment 2. (C) Mice receiving 1% DSS during experiment 2. (D) Mice receiving 1% DSS during experiment 3. Linear regression was used to determine line equations and $R^2$ values.



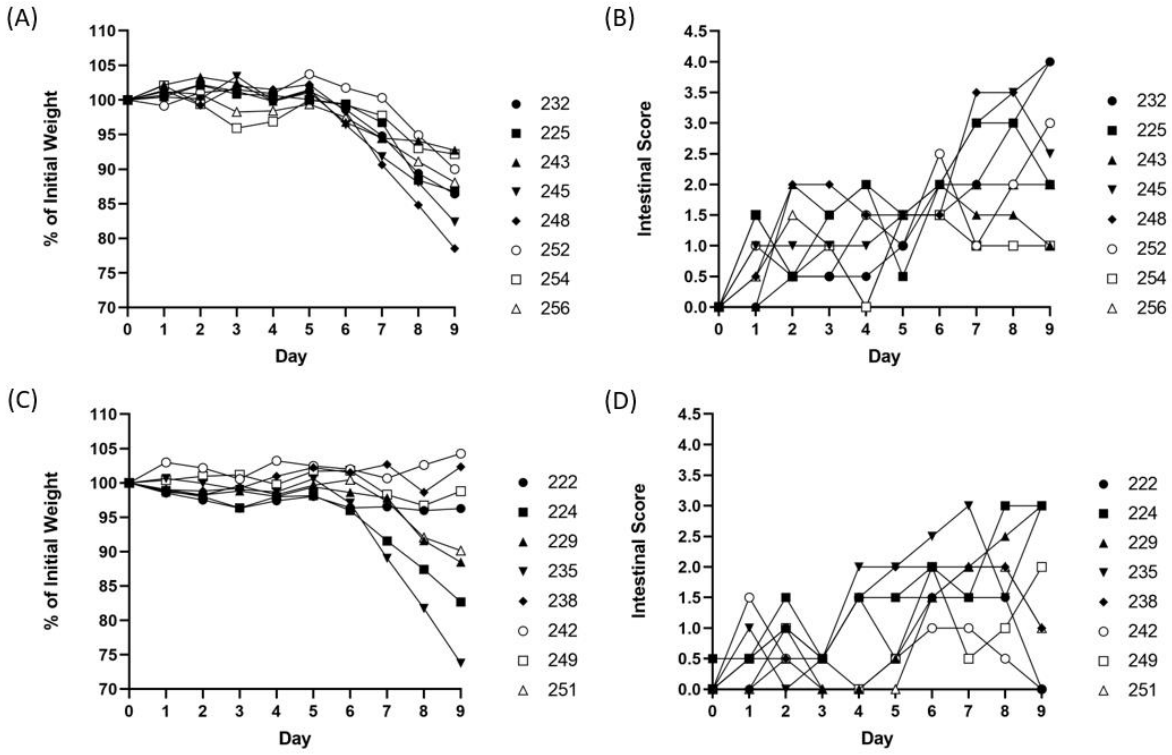

**Appendix 2.** Correlation of weight change and intestinal score for mice treated with 1% DSS for 6 days and administered metacercariae or PBS on day 3. For all panels, correlation of weight change, expressed as a percentage change from day 0, to intestinal score (0-4) are graphed for individual mice (n=8 per group). (A) DSS and parasite treatment. (B) DSS treatment and PBS. (C) Water and parasite treatment. (D) Water and PBS. Linear regression was used to determine $R^2$ and p values.



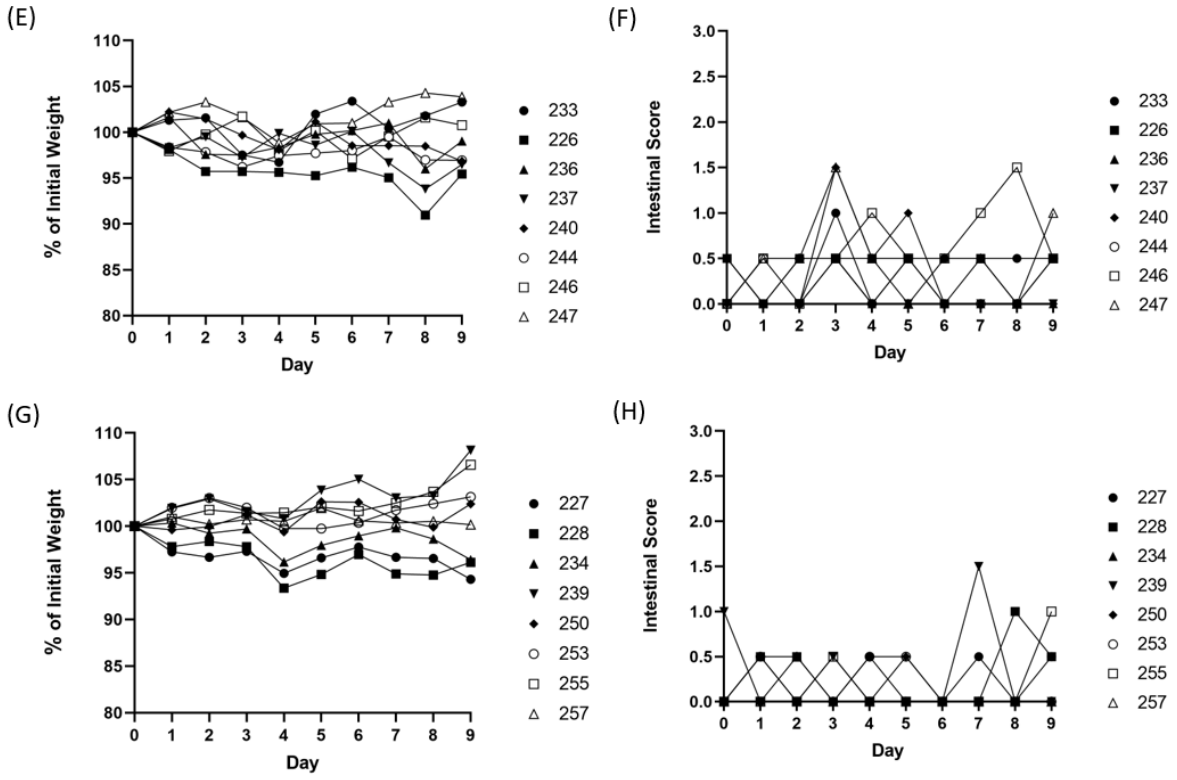

**Appendix 3.** Correlations of splenic cytokines and weight, weight change, or intestinal score on Day 9 in mice treated with 1% DSS and administered metacercariae or PBS on Day 3. Spleens were harvested after 9 days and supernatants were tested for regulatory cytokines IL-2 and IL-10, parasite specific cytokine IL-4, and pro-inflammatory cytokine IFN-γ using ELISA. For all panels, each symbol represents one mouse. (A) Weight on Day 9 and IL-2 concentration. (B) Weight on Day 0 and IL-2 concentration. (C) Weight change on Day 9, expressed as a percentage change from Day 0, and IL-4 concentration. (D) Intestinal score on Day 9 and IL-4 concentration. Linear regression was used to determine $R^2$ and p values.




**Appendix 3 (continued).** Correlations of splenic cytokines and weight change or intestinal score on Day 9 in mice treated with 1% DSS and administered metacercariae or PBS on Day 3. For all panels, each symbol represents one mouse. (E) Weight change on Day 9 and IL-10 concentration. (F) Intestinal score on Day 9 and IL-10 concentration. (G) Weight change on Day 9 and IFN-γ concentration. (H) Intestinal score on Day 9 and IFN-γ concentration. Linear regression was used to determine $R^2$ and p values.




**Appendix 4.** Weight change and intestinal scores for individual mice treated with 1% DSS for 6 days and administered heat-killed metacercariae or PBS on Day 3. For left panels, weight change, expressed as a percentage change from day 0, are graphed for individual mice (n=8 per group). For right panels, intestinal scores (0-4) are graphed for individual mice (n=8 per group). Panels A-B: DSS and parasite treatment. Panels C-D: DSS treatment and PBS.




**Appendix 4 (continued).** Weight change and intestinal scores for individual mice treated with 1% DSS for 6 days and administered heat-killed metacercariae or PBS on Day 3. For left panels, weight change, expressed as a percentage change from day 0, are graphed for individual mice (n=8 per group). For right panels, intestinal scores (0-4) are graphed for individual mice (n=8 per group). Panels E-F: Water and parasite treatment. Panels G-H: Water and PBS.