Defenses of susceptible and resistant Chinook salmon (Onchorhynchus tshawytscha) against the myxozoan parasite Ceratomyxa shasta

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1. Introduction

Resistance of salmonids to the myxozoan parasite Ceratomyxa shasta is an inherited trait [1–3] conferred through unique genetic loci [4], but the mechanism is unknown. Although strains of native fish sympatric with the parasite are considered “resistant” [1,2,5–9], they may become infected and succumb to disease at high parasite dose [10–13] and high water temperatures [14,15]. Researchers investigating the mechanisms of the fish host response proposed three defense strategies against C. shasta: resistance against parasite entry and establishment, the mounting of an effective immune response that contains and/or eliminates the parasite, and parasite tolerance [8,9,16,17]. In the years since the suggestion of these strategies, researchers have solved the C. shasta life cycle and developed a laboratory model for infection [18,19], identified the route of parasite invasion and migration [19], determined threshold parasite doses [10,13,20,21], and uncovered relationships between parasite genotype and pathogenicity in the salmonid host [22,23]. These advances permit a re-examination of the above hypotheses to account for unknown parasite dose, course of infection and parasite genotype.

The complex life cycle of C. shasta involves two hosts; a salmonid and a freshwater polychaete, Manayunkia speciosa. The infectious stage of the parasite, the actinospore, is released from the polychaete and attaches to the gills of a fish. In the initial stages of infection, the parasite invades the gill epithelium and enters the bloodstream, a site of proliferation and means of transport to the intestine [19]. As infection progresses, parasites migrate through the serosa and lamina propria and proliferate between mucosal epithelial cells of the intestine, culminating in myxospore maturation. In severe infections, lymphocytes infiltrate the tissue and the intestine becomes grossly enlarged, inflamed and necrotic, resulting in enteronecrosis.

Keywords:
- Resistance
- Immune response
- Inflammation
- Cytokine
- Myxozoan
(ceratomyxosis). Parasites may occlude the intestinal lumen and pathological changes coinciding with parasite proliferation can occur in other organs [17]. Based on current knowledge of the route of actinospore invasion and migration, we modify the first defense strategy hypothesis to: resistance against parasite entry and establishment at the gills and/or intestine.

Conclusions made concerning defense strategies from previous studies are obscured by researchers’ reliance on field experiments for parasite infection. In these cases, parasite dose and genotype were unknown and/or could not be controlled. In lethal infections of resistant Chinook salmon (Oncorhynchus tshawytscha) there was no evidence of parasite exclusion or containment in the gills or in the intestine [19]. Increases in plasma lysozyme, complement and phagocytosis were demonstrated in lethal infections; however, these innate defenses failed to provide protection against disease when challenged with a high parasite dose [13]. In sub-lethal C. shasta infections of unknown genotype in resistant rainbow trout (Oncorhynchus mykiss), the parasite was contained within granulomata [8] and in resistant steelhead (O. mykiss), parasites were detected only in the lumen of the intestine [16,17]. In susceptible rainbow trout that survived a low dose of a presumably non-pathogenic parasite genotype (1 parasite per fish of some genotypes are lethal [20]), parasites were also found in the lumen and there was evidence of antibody production [24]. These studies suggest two very different host responses; one in which the parasite is contained prior to sporulation (as typically occurs in the lamina propria) and the other in which the parasite progresses through the lamina propria to the intestinal lumen without extensive inflammation or necrosis (tolerance). The variation in responses could be attributed to differing parasite doses or reflect unique responses to specific parasite genotypes. Thus, examination of defense strategies requires the ability to conduct sub-lethal challenges with controlled parasite genotypes.

The goal of this study was to identify the strategies resistant salmonids have evolved to survive in sympatry with C. shasta, gills were not collected for qPCR from control fish at 24 h, but these fish were assessed for the presence of C. shasta infection in the intestine by PCR [26,27] at the end of the experiment.

DNA was extracted from gill tissue using the Qiagen DNeasy Blood and Tissue kit® (Valencia, CA, USA), eluted in 60 μL of AE buffer, then re-applied to the column and eluted again to increase yield. The DNA was then assayed by qPCR [25]. Samples were run in duplicate and Cq (quantification cycle) values were averaged for each sample. Two positive controls (DNA from infected fish tissue and synthetic C. shasta DNA template) and a negative control of molecular grade water were included. If the standard deviation of the duplicates was greater than 1, the samples were re-run. Samples in which parasite DNA was not detected were assigned a Cq of 40 to facilitate data analysis. A Student’s t-test was performed to determine if there were significant differences in the Cq values between strains using SAS v. 9.3 (SAS Institute, Cary, NC, USA).

Histological sections were prepared by the Veterinary Diagnostic Laboratory, Oregon State University (OSU) Corvallis, OR, and stained with May–Grunwald Giemsa. The presence and location of the parasite within the gill tissue were compared between strains.

2. Materials and methods

2.1. Fish strains

Naïve fish of a susceptible (Salmon River (SR) Hatchery, OR, USA) and resistant (Iron Gate (IG) Hatchery, CA, USA) strain of Chinook salmon (SR average 7.0 ± 1.2 g; IG average 6.2 ± 1.2 g) were transferred to the John L. Fryer Salmon Disease Laboratory, Oregon State University, Corvallis, OR. Fish were fed a daily commercial diet (Bio-Oregon, Longview, WA, USA) and reared in 13 °C specific pathogen free (SPF) well water until initiation of the experiment.

2.2. Parasite source

As a source of actinospores, a culture of the invertebrate host Manayunkia speciosa was infected with myxospores obtained from Chinook salmon (genotype 1) using methods previously described [19]. Water from the culture tank flowed into an aquarium where fish were held in separate cages segregated by strain for the parasite challenge. Three 1 L water samples were collected at the beginning of the fish challenge and parasite numbers were determined by quantitative PCR (qPCR) [25]. Water flow rate through the tank and parasite density measured by qPCR were used to estimate the total parasite dose for each experiment. Parasite exposure varied between the two experiments and is specified for each.

2.3. Resistance at the portal of entry (gills)

To compare C. shasta actinospore invasion in the gills, 10 fish of each strain were challenged simultaneously for 24 h. Based on qPCR estimates and flow rate, these fish were exposed to $1.7 \times 10^4$ actinospores fish$^{-1}$, below the estimated lethal threshold of $3.8 \times 10^4$ actinospores [13,21]. After 24 h, 5 fish from each strain were euthanized with an overdose of MS-222 (tricaine methosulphonate, Argent, Redmond, WA) and the gills from one side of the head of each fish were fixed for histology in Davidson’s fixative for 24 h then transferred to 70% ethanol. To assess parasite density, the other gill set was frozen for assay by qPCR. The remaining 5 fish from each strain were transferred to 13 °C SPF flow-through 25 L tanks to monitor the development of infection over 60 days. As a control, 5 uninfected fish from each strain were maintained identically, but in separate tanks. Because of the limited availability of these fish, gills were not collected for qPCR from control fish at 24 h, but these fish were assessed for the presence of C. shasta infection in the intestine by PCR [26,27] at the end of the experiment.

Histological sections were prepared by the Veterinary Diagnostic Laboratory, Oregon State University (OSU) Corvallis, OR, and stained with May–Grunwald Giemsa. The presence and location of the parasite within the gill tissue were compared between strains.

2.4. Comparison of histopathology and cytokine expression

2.4.1. Sample collection

In a second experiment, IG and SR strains of Chinook salmon were challenged with a dose of $4.3 \times 10^4$ actinospores fish$^{-1}$ in the exposure tanks. Thirty fish of each strain were placed in separate 40 × 15 cm cylindrical cages and held in a 136 L flow-through aquarium receiving water from the outflow of the infected poly-chaete colony for 24 h. An equal number of fish from each strain were held in cages in UV treated Willamette River water at the same flow rate for 24 h as a negative control. After exposure each treatment (IG infected (IGI), IG control (IGC), SR infected (SRI), SR control (SRC)), was divided into 3, 25 L SPF flow-through tanks at 13 °C with 10 fish per tank. Nine fish from each treatment (3 per replicate) were sampled at 12, 25 and 90 days after exposure.

Fish were euthanized with an overdose of MS-222 and intestinal samples were collected to compare histopathology and cytokine expression. A section of the posterior intestine (30 mg, approximately
0.5 cm) was excised, preserved in RNA Later (Qiagen) and stored at −80 °C until RNA extraction. The remainder of the intestine from each fish was preserved for histology as described in Section 2.3.

2.4.2. Histopathology processing and analyses

One transverse section per fish was stained with Giemsa to determine infection intensity and another with H&E for inflammation. The entire length of the intestine was examined at 200 × magnification. Infection intensity, in terms of number of parasite foci (one or greater parasites clustered in a group), was scored on a scale of 0–5: 0 = no foci, 1 = 1–5 foci, 2 = 6–10 foci, 3 = 11–15 foci, 4 = 16–20 foci, 5 = >20 foci. Inflammation was scored similarly: 0 = no foci, 1 = 1–10 foci, 2 = 11–20 foci, 3 = 21–30 foci, 4 = >30 foci. In areas of confluent inflammation, each villi where inflammation was present was considered a single focus of inflammation. For statistical analyses, comparisons between infection intensity and inflammation between strain and treatment on day 12, and treatment and day for only the IG fish were conducted using two separate Kruskal–Wallis tests followed by Wilcoxon two-sample tests for pairwise comparisons. Comparisons were not conducted in SR fish after day 12 because all fish had succumbed to infection 24 days post exposure.

2.4.3. Immunohistochemistry

Identification of IgG cells was conducted by immunohistochemistry using a monoclonal antibody raised in mouse against IgG cells (undetermined IgG class) in rainbow trout [28] in a third set of sections. Slides were deparaffinized, rehydrated and blocked with 0.33% hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity. Slides were then washed twice in 1 × PBS for 5 min and autoclaved in 0.01 M citrate buffer (pH 6) for 15 min at 121 °C for antigenic retrieval. After washing as before in 1 × PBS, slides were blocked for non-specific binding using 1.5% goat serum (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) was added. The DAB reaction revealed no differences in infection intensity or parasite location in the gills between strains; numbers of visible parasites were one or zero. Despite the low parasite numbers detected in the gills, all of the SRI held after the exposure succumbed to infection, with a mean day to death of 32.0 ± 9.0. All of the IGI survived to 60 days and myxospores were not detected in wet mounts upon termination. All of the control fish were negative by PCR.

Cytokine expression was initially measured in infected and control treatments using 3 pooled samples comprised of 3 fish each (1 fish from each replicate tank per pool) for each strain on each sampling day. When differences were detected between treatment and control fish using t-tests on log transformed data, cytokine expression was quantified in individual fish. The log-fold change in cytokine expression between the control and treatments were evaluated using REST 2009 software [29,30] with 1000 randomizations. Differences between the control and treatments were considered significant at p < 0.05.

3. Results

3.1. Resistance at the portal of entry (gills)

*Ceratomyxa shasta* was detected by qPCR in 100% of the gills of both resistant and susceptible strains of Chinook salmon exposed in experiment 1. Cq values were not different between SRI (29.97 ± 1.7) and IGI (30.46 ± 0.77) treatments (p = 0.56). Histology revealed no differences in infection intensity or parasite location in the gills between strains; numbers of visible parasites were one or zero. Despite the low parasite numbers detected in the gills, all of the SRI held after the exposure succumbed to infection, with a mean day to death of 32.0 ± 9.0. All of the IGI survived to 60 days and myxospores were not detected in wet mounts upon termination. All of the control fish were negative by PCR.

In the second experiment, infection in the SRI fish was fatal (mean day to death 22.0 ± 0.6), thus they were sampled only on day 12, while IGI cohorts survived to day 90. Therefore, measures of infection intensity, inflammation and numbers of IgG cells were compared between strains only at day 12. These measures were compared within the IG strain at days 12, 25 and 90. Neither parasites nor inflammation were detected in any of the controls. At day 12, infection intensity in the intestine was higher in SRI than IGI (p = 0.001), but inflammation was higher in IGI than SRI (Fig. 1a–b). SRI exhibited mild to moderate, multi-focal and locally extensive inflammation in the lamina propria, dominated by mononuclear cells with a few neutrophils (Fig. 2a–b). C. shasta trophozoites were observed in all layers of the posterior intestine (mucosal epithelium, lamina propria, muscularis, and serosa) from all nine of the SRI, but predominately in the mucosal...
epithelium (Fig. 3a–c). IGI exhibited moderate to diffuse lymphocytic enteritis (Fig. 2c–e), with trophozoites observed in eight of the nine fish (Fig. 3c–d). In three IGI, the mononuclear cell response was transmural, and scattered macrophages were present. Two of these also had increased vascularization in the lamina propria, evidenced by dilated blood vessels. Numbers of Ig⁺ cells were higher in the SRI than the IGI at day 12 (Fig. 1c, \( p < 0.001 \)) and were also higher than the SRC (Figs. 1c and 4, \( p < 0.001 \)), but numbers of Ig⁺ cells in IGI were not significantly different from IGC on day 12 (Fig. 1c, \( p = 0.222 \)).

Infection intensity and inflammation in IGI, as determined by histology, changed over time (Fig. 1a–b, \( p = 0.02; p < 0.001 \)), with both measures higher on day 12 and day 25 than on day 90. On day 25, *C. shasta* trophozoites were detected in five of nine IGI: one fish had an infection intensity score of five, the other four had a score of one. Inflammation on day 25 differed from day 12 not in severity, but in the location and distribution of host inflammatory cells (Fig. 2f–g). Host mononuclear cells were largely limited to the lamina propria and some fused villi in at least two of the fish on day 25 and trophozoites were present among the infiltrated cells. Inflammation in another two fish from this group was more diffuse, extending through the muscularis and serosal surface into the mesenteric adipose tissue. In one of these two fish, parasites were present within the adipose tissue, but numerous trophozoites were also present in the intestinal epithelium layer and destruction of villi was evident. In one fish, the steatitis extended from the serosal surface and surrounded a blood vessel. The number of Ig⁺ cells increased significantly on day 25 compared to day 12 (\( p < 0.001 \)) in the IGI and was also significantly higher than the IGC (Fig. 1c, \( p < 0.001 \)). By day 90, parasites were observed in only one fish and inflammation in the lamina propria was absent to minimal with lymphocytes scattered in the mesenteric adipose tissue (Fig. 2h–i). The number of Ig⁺ cells in the day 90 IGI was not significantly different from the IGC (\( p < 0.462 \)) and the Ig⁺ cells were also significantly lower at day 90 than day 25 (\( p < 0.001 \)).

### 3.3. Cytokine expression

Intestinal expression of IL-6, IL-10 and IFN-γ was upregulated (\( p < 0.001 \)) in SR fish 12 days post exposure. IFN-γ was the only cytokine significantly upregulated (\( p = 0.007 \)) in IG fish 12 days post exposure, but expression was seven times lower than that of SR fish. Expression of IFN-γ on day 25 in IG fish increased compared to day 12, but was no longer upregulated by day 90 (\( p = 0.54 \)). TNFα was upregulated in IG fish on day 90 (Table 2, \( p = 0.003 \)). Differences in expression were not detected for TGFβ and IL-2.

### Table 1

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<th>Primer concentration and efficiency</th>
<th>Product size (bp)</th>
<th>GenBank#</th>
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4. Discussion

In this study, the responses of resistant (IG) and susceptible (SR) Chinook salmon to *C. shasta* were followed from the site of parasite invasion to the intestine. Disease was fatal in SR fish but there was clearance and resolution of the infection in IG fish through an effective inflammatory response. The relatively equal numbers of parasites penetrating the gills of IG and SR Chinook suggests that resistance to invasion by the parasite does not occur. However, as proposed in the literature [8,9,16,17], resistant fish did show evidence of preventing parasite establishment in the intestine. This appears to have occurred by a twofold process; 1) limiting the number of parasites that invade/establish in the intestine, as evidenced by lower infection intensity and 2) an effective inflammatory response limiting parasite proliferation in that tissue. By 90 days resistant fish had recovered from the infection, as evidenced by elimination of parasites from the intestine coinciding with the reduction of inflammation, pro-inflammatory cytokine expression and Ig⁺ cells.

Differences between the timing and magnitude of the responses to parasite infection were evident between fish strains. Although both fish strains recruited Ig⁺ cells to the infection site, Ig⁺ cell recruitment occurred later in the resistant strain (day 25 as opposed to day 12 in the susceptible strain). We observed upregulation of the pro-inflammatory cytokines IFNγ and IL-6 in both fish strains, although the magnitude of expression was higher for SR than IG fish. In cold-water fish such as Chinook salmon, adaptive immunity typically occurs between six to twelve weeks post...

**Fig. 2.** Inflammation in Salmon River (SR) and Iron Gate (IG) Chinook salmon stained with Giemsa. Arrows indicate parasite foci. Areas of inflammation in the lamina propria of SR intestine at day 12 post infection adjacent to tissue with no inflammation (a, b). Inflammation in IG intestine in serosa, lamina propria, muscularis and stratum granulsum at day 12; although lymphocytes are numerous, no parasites are present (c–e). Inflammation in IG intestine at day 25 in lamina propria, surrounding blood vessels and resulting distortion of villi (f, g). Inflammation in IG intestine at day 90 largely limited to the lamina propria; no parasites are present (h, i).
tion intensity measured in the intestine of SR on day 12 was dose and the number of infecting parasites was similar, the infection may have suppressed both IL-6 and IFN-γ. This early cytokine response may also have aided in a better understanding of in vivo events. Earlier sampling may have continued to increase with disease progression. Earlier sampling may not have been sufficient to elicit the extent of inflammation observed in IG Chinook.

Reduced inflammation at day 12 in SR as compared to IG fish was unexpected given that cytokine expression and numbers of Ig+ cells were higher in SR fish and previous studies with susceptible fish have shown severe inflammation in moribund fish [19,20]. Inflammation in SR fish on day 12 occurred only in the lamina propria and did not arrest parasite proliferation or prevent parasites from reaching the epithelium. Because fish were not sampled at a time when they were clinically diseased, it is unknown if inflammation, cytokine expression and numbers of Ig+ cells would have continued to increase with disease progression. Earlier sampling may also have aided in a better understanding of inflammation patterns. For example, increased expression of the pro-inflammatory cytokines that respond quickly to infection, such as TNFα and IL-1β, may have occurred prior to day 12. If upregulation occurred prior to our sampling in IG fish, this could explain the increased histological inflammation in IG fish compared to SR fish. An alternate hypothesis for the lower inflammation observed in SR fish on day 12 is that IL-10, a potent anti-inflammatory cytokine, may have suppressed both IL-6 and IFN-γ [35,36]. In addition, although cytokine expression was upregulated in SR on day 12, this expression may not have been sufficient to elicit the extent of inflammation observed in IG Chinook.

While both Chinook strains were exposed to the same infectious dose and the number of infecting parasites was similar, the infection intensity measured in the intestine of SR on day 12 was approximately four times higher than that of IG fish. We offer two explanations for the reduced number of parasites in the intestine of the IG strain by day 12. First, parasites could be eliminated prior to arrival in the intestine (i.e. in the blood) and/or second, elimination could occur upon arrival in the intestine before our first sampling time. Although not examined in this study, we hypothesize that the immune response in resistant Chinook reduces the numbers of proliferating parasites in the blood during migration from the gills to the intestine between the time of initial infection and our first measurement on day 12. We have some support for this hypothesis; blood collected daily from IG fish after exposure to the parasite for two weeks indicated a reduction in parasite numbers by day 12 [37]. These additional defenses may limit the number of parasites invading the intestine and account for the difference in the timing of the response in the intestine between the Chinook strains.

Over the course of the infection, the numbers of parasites in the intestine of resistant fish remained low, likely because proliferation was controlled by the inflammatory response. Additionally, the high survival of infected IG fish in the presence of these extensive inflammatory lesions and resolution of the infection in cohorts by day 90 supports the successful regulation of this response. By day 90, there were no significant differences in IFN-γ expression, inflammation and the number of Ig+ cells from controls, indicating a return to basal levels. Therefore, although both strains of fish elicited an inflammatory response and recruited Ig+ cells to the intestine, the response was only effective for IG Chinook and was likely aided by the reduction of parasites prior to entering the intestinal tissues.

As a continued inflammatory response in the intestine would lead to immunopathology, downregulation of inflammatory cytokines prevent host tissue damage [38]. Although our study was limited to a single exposure to the parasite, decreased expression of IFNγ along with increased numbers of Ig+ cells suggests a switch from a TH1 helper (1 TH1) to a TH2 helper (2 TH2) response in resistant fish. For fish living in a river, exposure to C. shasta actinospores would be continuous, potentially leading to chronic inflammation. Thus, a switch to TH2 would prevent chronic inflammation and may stimulate a protective antibody response [39]. In susceptible rainbow trout infected with a presumably non-pathogenic strain of C. shasta, parasites were identified in the presence of increased mucosal IgT antibody [24]. Although it is not known if this response is protective against C. shasta, protective antibody responses have been demonstrated in fish surviving other myxozoan infections [40–43].

Previous studies examined the host response to C. shasta in susceptible rainbow trout infected with a different parasite genotype than used in this study [22,23]. Although this complicates comparisons between resistance strategies, we did see some similarities between infections among fish species. Ibarra et al. [8] noted formation of granulomata around the parasite and hypothesized that C. shasta resistance results from the ability of the fish to
mount an effective immune response. Bartholomew et al. [16] found parasites in the lumen without triggering inflammation and low mortality in resistant trout, suggestive of tolerance. In our study, although we did not observe granulomata or parasites in the lumen, resistant IG fish exhibited greater inflammation and had lower infection intensities compared to susceptible fish. Over time, infection intensities decreased, suggesting that the inflammatory response and/or the increase in numbers of Ig^+^ cells was capable of eliminating parasites prior to sporulation or movement into the intestinal lumen. Although the details of our observations were different than those observed in rainbow trout and steelhead, the hypotheses regarding resisting parasite establishment (in the intestine) as well as the development of an effective immune response in the form of an inflammatory response and/or antibody response are supported in this study.

Similarities exist in the host response against C. shasta and other myxozoans with tropism for the intestine of its fish host. In sharpsnout sea bream (Diplodus puntazzo) infected with Enteromyxum leei, the immune response is decreased compared with resistant gilthead sea bream (Sparus aurata), although infection severity is higher and disease progression is faster in the sharpsnout sea bream [44,45]. This finding is comparable to the response of the susceptible SR strain to C. shasta infection on day 12 of our study, where parasite intensity was higher in the resistant IG strain, but inflammation was lower. Gilthead sea bream also demonstrated recruitment of lymphocytes to the intestine and upregulation of pro-inflammatory cytokines early in infection [45,46]. This response is similar to the inflammatory response in the resistant IG fish in our study. Gilthead sea bream also displayed upregulation of anti-inflammatory cytokines later in the study, which may have correlated with a decrease in lymphocyte infiltration into intestinal tissues [45,46]. This trend was not detected in our study, although a sample point between 25 and 90 days may have provided further resolution of the response. Thus, an increased immune response in the intestine appears to be a common response to invasion by myxozoan parasites with intestinal tropism, but resistant fish appear to be able to mount a more effective response than susceptible fish.

5. Conclusions

Results of this study demonstrated that fish with increased resistance to enteronecrosis elicit an effective immune response capable of eliminating parasites. The effectiveness of the response is dependent on the parasite dose, as resistant fish can succumb to disease at high doses. The trends in cytokine expression and inflammatory cell recruitment to the intestine differed in timing and magnitude between susceptible and resistant fish. Expression of IFN^γ^, IL-6 and IL-10 was higher in susceptible fish at day 12, but expression continued to increase in resistant fish by day 25. Despite the increased expression of cytokines later in resistant fish, the histological inflammatory response was more intense in resistant fish at day 12. This may be due to upregulation of pro-inflammatory cytokines in resistant fish earlier in the infection or the expression of IL-10, a potent anti-inflammatory in susceptible fish. We also observed differences in the numbers of parasites detected in the intestine, with fewer parasites in the intestine of resistant fish. Because resistance is a genetically controlled trait [4], these fish may elicit an earlier immune response en route or upon arrival to

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<th>SE</th>
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<td>IFN^γ^</td>
<td>28.97</td>
<td></td>
<td>13.01–66.55</td>
<td>3.65</td>
<td></td>
<td>1.32–11.67</td>
<td>5.43</td>
<td></td>
<td>−1.16 to 41.23</td>
<td>−1.19</td>
<td></td>
<td>−2.78 to 1.85</td>
</tr>
</tbody>
</table>
the intestine. One mechanism for this may be pathogen recognition receptors. To better understand the role of innate immunity in prevention of clinical disease, future studies should determine the parasite dose range in which an effective immune response can be initiated but not overwhelmed. Additionally, new insights on the infectious dose, route of infection and timing of host response to the parasite in the intestine create new opportunities to investigate the possibility of a protective antibody response to C. shasta.

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