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Clearance of schistosome parasites by resistant genotypes at a single genomic region in *Biomphalaria glabrata* snails involves cellular components of the hemolymph

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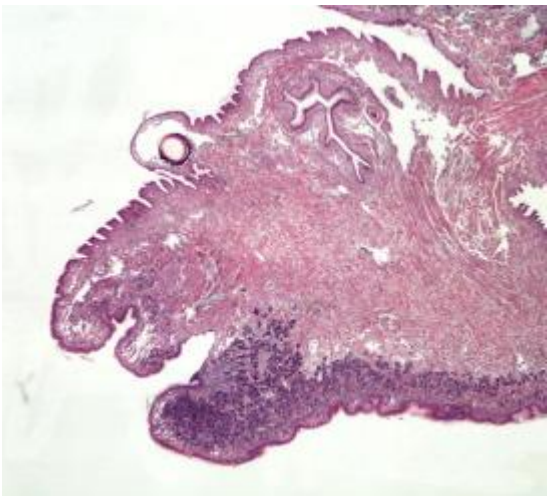
Abstract :

Schistosomiasis is one of the most detrimental neglected tropical diseases. Controlling the spread of this parasitic illness requires effective sanitation, access to chemotherapeutic drugs, and control over populations of the freshwater snails, such as Biomphalaria glabrata, that are essential intermediate hosts for schistosomes. Effectively controlling this disease, while minimising ecological implications of such control, will require an extensive understanding of the immunological interactions between schistosomes and their molluscan intermediate hosts. Here we histologically characterise the clearance of schistosome larvae by snails that exhibit allelic variation at a single genomic region, the Guadeloupe resistance complex. We show that snails with a resistant Guadeloupe resistance complex genotype clear schistosomes within the first 24-48 h, and that this resistance can be transferred to susceptible snails via whole hemolymph but not cell-free plasma. These findings imply that Guadeloupe resistance complex-coded proteins help to coordinate hemocyte-mediated immune responses to schistosome infections in Guadeloupean snails.

Graphical abstract



Highlights

► The Guadeloupe resistance complex (GRC) does not alter snail integument or miracidial penetration. ► The GRC can reduce the number of free parasites in snails. ► The GRC confers schistosome resistance via the hemolymph.

Keywords : Schistosomiasis, Biomphalaria glabrata, Histology, Hemocyte, Resistance, Hemolymph transfer, Plasma transfer

1. Introduction

Schistosomiasis is a devastating and neglected tropical illness that is responsible for hundreds of thousands of deaths each year, and afflicts over 250 million people in the developing world (WHO, 2012, 2016). This disease continues to persist despite mass chemotherapeutic drug administration by the World Health Organization (WHO), primarily because there are no effective vaccines targeting schistosomes, sanitation is lacking in some developing regions, and other mammalians can act as reservoir hosts and perpetuate the disease. Schistosomes require an intermediate snail host for their larval miracidial stage to develop, and regions that are unable to control these snails exhibit exacerbations in schistosomiasis cases (Sokolow et al., 2016). Presently, the only areas where schistosomes have been completely eliminated are regions where these intermediate snail hosts are extirpated, which makes them an essential target for disease control (Sokolow et al., 2016).

In the New World, miracidia from *Schistosoma mansoni* infect the aquatic snail *Biomphalaria glabrata*, which goes on to shed cercariae capable of causing human disease. This snail species has innate immune defenses that can both specifically and non-specifically target schistosomes for destruction (Coustau et al., 2015). The first line of defense against miracidial penetration is the integument of the snail. The integument provides a physical barrier of connective tissue and ciliated microvillus cells that protect from invading pathogens (Adlard, 2003). Miracidia must break through this barrier before they can infect any given individual. Successful miracidia, and the sporocysts that consequently develop, are then exposed to potentially degenerating humoral factors and attacked by motile hemocytes in the hemolymph and other tissues (Hanington et al., 2010; Loker, 2010). When schistosomes are recognized by hemocytes in resistant snails, they are encapsulated and exposed to anti-microbial effectors

including reactive oxygen species which destroy the schistosome (Bender et al., 2005; Loker, 2010). Miracidia and sporocysts have numerous ways of evading destruction by humoral factors and hemocytes. Notable evasion tactics involve obscuring sporocyst detection post-integument penetration (Peterson et al., 2009), and the production of oxidative scavengers to protect them from hemocyte-mediated damage if they are detected (Mourao Mde et al., 2009; Mone et al., 2011). Exposed snails that are able to recognise and efficiently degrade invading schistosomes can escape infection, and subsequently avoid transmitting the disease to humans (Portela et al., 2013; Pinaud et al., 2016).

Resistance of Guadeloupean *B. glabrata* (BgGUA) to Guadeloupean *S. mansoni* (SmGUA) has been shown to be strongly correlated with allelic variation in the Guadeloupe Resistance Complex (GRC), but not with mRNA expression levels of these genes (Tennessen et al., 2015b). The GRC is a gene region which contains novel genes with vast amino acid sequence differences and putative immune recognition functions. *Grctm6* is a novel GRC-encoded protein that has been shown to affect the number of cercariae shed per infected snail and is expressed in hemocytes (Allan et al., 2017b). Understanding the kinetics and biology of this new class of genes in schistosome defense is essential before they might be used in alternative schistosome control. Although these studies have highlighted the importance of the GRC during schistosome infection, little is known about the actual mechanisms by which snails bearing resistant genotypes differ from those bearing susceptible genotypes. Determining when resistant genotypes clear parasitic larvae, and whether cellular or humoral factors are involved in clearance, is essential to understanding the mechanistic role of the genes in the GRC.

Histological differences between highly resistant and highly susceptible strains of snails have been characterised extensively, but these studies did not describe the genes that may cause

93 the differences between those strains (Richards and Minchella, 1987; Borges et al., 1998; Loker,
94 2010; Nacif-Pimenta et al., 2012). Similarly, both cellular and humoral factors have been shown
95 to transfer resistance from highly resistant to highly susceptible strains, but again no study has
96 linked this transfer to allelic variation in specific genes (Sullivan et al., 1995, 2004; Vasquez and
97 Sullivan, 2001a, b, c; Pinaud et al., 2016). In the present study, we report that snails bearing
98 resistant GRC haplotypes have fewer unencapsulated sporocysts than snails bearing susceptible
99 haplotypes as early as 24 h p.i., despite having equivalent integument structure and susceptibility
100 to penetration by miracidia. We also show that resistance can be transferred from resistant GRC
101 genotypes to susceptible GRC genotypes via injections of the resistant haplotype's hemolymph
102 but not cell-free plasma. These findings demonstrate that GRC-mediated resistance to
103 schistosome exposure occurs during the early stages of infection and is likely cell-mediated.
104

105 ## 2. Materials and methods

106 *2.1. Maintenance, propagation, infection and inbred line derivation of B. glabrata and S.*
107 *mansoni*

108 *Biomphalaria glabrata* (BgGUA) and *S. mansoni* (SmGUA) were collected in 2005 from
109 Dans Fond (Theron et al., 2008) on the island of Guadeloupe, and maintained as previously
110 described (Theron et al., 2014; Tennessen et al., 2015b). The SmGUA strain of *S. mansoni* was
111 cycled through BgGUA and hamsters, and parasites were isolated from hamster livers or from
112 shedding snails. BgGUA snails were segregated based on their genotype at the GRC locus, and
113 separated into nine independently derived inbred lines as previously described: three *RR*
114 (resistant lines R^1, R^2, R^3), three *S1S1* (susceptible lines $S1^1$, $S1^2$, $S1^3$), and three *S2S2*
115 (susceptible lines $S2^1$, $S2^2$, $S2^3$) (Tennessen et al., 2015b; Allan et al., 2017b). To produce these

lines, independent founder snails, homozygous at the GRC locus, were allowed to self-fertilize for three generations. For all additional experiments, equal numbers of snails pooled from each of the three lines within a GRC genotype were used (*RR, S1S1, S2S2* or *RR, SS*; where *SS* is pooled *S1S1* and *S2S2*). Experiments were performed on size/age matched (~7 mm, ~7 weeks old) cohorts of snails which were housed identically. This research adheres to Public Health Service Domestic Assurance for humane care and use of laboratory animals (PHS Animal Welfare Assurance Number A3229-01), as Animal Care and Use Proposal 4360, and was approved by the Oregon State University Institutional Animal Care and Use Committee, USA.

2.2. Confirmation of infection phenotypes in BgGUA lines

In order to verify that the inbred lines of BgGUA that were to be used for functional work behaved phenotypically like outbred snails of the same genotype, we examined the infection phenotype of all inbred lines using the same exposure protocol that was used on outbred BgGUA (Tennessen et al., 2015b). Parasite exposures of BgGUA with SmGUA were carried out as previously described (Tennessen et al., 2015b; Allan et al., 2017b). Snails were incubated in 2 ml of dechlorinated water in individual wells of a 24 well plate containing 20 miracidia for 24 h, and then transferred into tanks containing 10-15 snails each to be monitored for infection. Three independent lines each of *RR* (R^1, R^2, R^3), *S1S1* ($S1^1$, $S1^2$, $S1^3$), and *S2S2* ($S2^1$, $S2^2$, $S2^3$) snails were used to verify the susceptibility of these lines (Fig. 1). Infections were done on a minimum of two separate occasions with the total number of snails that were exposed exceeding 27 for any given line or treatment (n= 32 $S1^1$,32 $S1^2$,28 $S1^3$, 35 $S2^1$, 33 $S2^2$, 27 $S2^3$, 55 R^1, 31 R^2, 42 R^3). Exposed snails were scored for cercarial shedding by placing individual snails in 2 ml of dechlorinated water in a 24 well plate for 3 h, and scored as either infected or uninfected. This

was done once per week between weeks 5-10 post exposure as previously described (Hanington et al., 2010, 2012; Pila et al., 2016b; Allan et al., 2017b).

2.3. Histological analysis

We histologically examined exposed snails of each genotype to determine the extent of early sporocyst development at different time points. Additionally, we examined the histological structure of the integument to ascertain if any difference in this outer barrier could explain differences in the infection phenotype of the GRC genotypes. For histological analysis of infected snails, BgGUA that were exposed to 20 miracidia were collected after 24 h, 48 h, or 10 days, and removed from their shells and fixed as previously described, with some modifications (Pinaud et al., 2016). In brief, two each of R^1, R^2, $S1^1$, $S1^2$, $S2^1$, $S2^2$ BgGUA (n=4 *RR* and 8 *SS*) were dissected out of their shells and fixed in Bouin's solution (75% saturated aqueous picric acid, 20% formaldehyde, 5% acetic acid; pH 1.2-1.6). After a 48 h fixation, the snails underwent consecutive 12 h washes in 95% ethanol for 36 h, and were dehydrated using consecutive 12 h washes in butanol for 36 h prior to sectioning. Fixed whole BgGUA were embedded in paraffin, underwent transverse (3 µm thick) sectioning (RHEM platform, Montpellier, France), and were stained with azocarmine G and Heidenhain's azan (Pinaud et al., 2016). Approximately 300 sections were stained and analysed per snail, and all sporocysts in these sections were counted. Sections underwent the following: (i) re-hydration (toluene 95, 70, 30% ethanol and distilled water); (ii) coloration (azocarmine G, 70% ethanol/1% aniline, 1% acetic alcohol, distilled water, 5% phosphotungstic acid, distilled water, Heidenhain's azan) and (iii) dehydration (95% ethanol, absolute ethanol, toluene). The preparations were then mounted with Entellan (Sigma Life Science, St. Louis Missouri, USA) and examined. All embedding, staining and histological

analysis of exposed snails were completed by the group of Dr. Gourbal at the University of Perpignan, France.

For analysis of the integument of BgGUA, snails were removed from their shells and fixed in Bouin's solution as described above. Fixed snails were placed in histology cassettes and sections underwent routine diagnostic H&E staining as previouly described (Allan et al., 2014, 2017a). Slides were evaluated microscopically using a Nikon Eclipse 50i, and measurements were performed using the Nikon NIS-Elements Microscope Imaging Software. Integument thickness was measured on three distinct regions of the pedal sole from each snail. Four snails of each *RR*, *S1S1*, and *S2S2* were evaluated by a veterinary pathologist (Dr. Dores) at the Veterinary Diagnostic Laboratory at Oregon State University.

2.4. Enumeration of miracidia during exposure

We wanted to know whether miracidia penetrate the three genotypes with equal frequency. Therefore, we quantified the minimum number of miracidia that did not penetrate during exposure by counting the number of miracidia remaining in an exposure well after snails were removed following 2 h exposures (Theron et al., 1997). Miracidia were enumerated under a dissecting scope (n= 12 *RR*, 12 *S1S1*, 12 *S2S2*).

2.5. Hemolymph and cell-free hemolymph (plasma) transfer

To examine if resistance could be transferred via the direct addition of cells or endogenous molecules, *S2S2* or *RR* hemolymph was transferred to *S1S1* snails (thus avoiding injection of any genotype's own hemolymph into itself) 24 h before exposure to 20 miracidia. Two independent experimental exposures were conducted. Exposures were performed as

185 described above with the following modifications. Hemolymph was collected from R^1, R^2, R^3
186 $S2^1$, $S2^2$ and $S2^3$ BgGUA snails (~10 snails per line) and pooled by genotype prior to injection
187 (providing pooled *RR* hemolymph batches consisting of hemolymph from R^1, *R,* and R^3; and
188 pooled *S2S2* batches of hemolymph consisting of hemolymph from $S2^1$, $S2^2$ and $S2^3$). The
189 number of cells in the hemolymph of the three genotypes of BgGUA was determined using a
190 haemocytometer and did not differ between groups. A small volume of sterile snail saline (SSS:
191 5 mM HEPES, 3.7 mM NaOH, 36 mM NaC1, 2 mM KC1, 2 mM MgCl2, 4 mM CaC12, pH 7.8)
192 was added to ensure equal cell numbers if any discrepancy was observed on a given
193 experimental day. Pooled *RR* or *S2S2* hemolymph was immediately injected into *S1S1* ($S1^1$, $S1^2$,
194 $S1^3$) snails (~15 snails/line, 20 µl/snail). Concurrently, *S1S1* ($S1^1$, $S1^2$, $S1^3$) snails (~15
195 snails/line, 20 µl/snail) were also injected with filter sterilized SSS as sham controls (Adema et
196 al., 1993; Pila et al., 2016a). As a third treatment, *RR* hemolymph was also left for 15 min and
197 centrifuged for 15 min at 2000 *g* (for cell-free plasma collection) and injected into *S1S1* ($S1^1$,
198 $S1^2$, $S1^3$) snails (~15 snails/line, 20 µl/snail on one occasion) (Pila et al., 2016b; Pinaud et al.,
199 2016; Allan et al., 2017b). Snails that survived past week 2 for cercarial scoring exceeded 31 per
200 group and were scored on week 5 (n=69 *S1S1* with SSS sham, n = 67 *S1S1* with *S2S2*
201 hemolymph, n = 31 *S1S1* with *RR* plasma, n= 74 *S1S1* with *RR* hemolymph). Hemocyte only
202 preparations were attempted, but cell viability and morphology were unacceptable for transfer
203 after isolation. These observations are consistent with previous findings that isolation and
204 resuspension of hemocytes can negatively affect their viability (Dr. Christopher Bayne, personal
205 observation).
206
207 *2.6. Statistical analysis*

Statistical analyses were carried using GraphPad Prism software (La Jolla, CA, USA) (Allan and Yates, 2015). A one-way ANOVA (or unpaired Student's *t*-test) with a Tukey post-test was used for comparing means ($P < 0.05$). Before reanalysis, a natural log transformation (ln) was performed if data did not have equal variances (Barlett's or F-test) (Allan and Habibi, 2012). Proportions were compared by calculating the Z score (standard score) of the population (Allan et al., 2017b).

3. Results

3.1. Resistance phenotypes are conserved among inbred lines of BgGUA

In the outbred BgGUA population the *R* allele confers an eight-fold decrease in the odds of infection (Tennessen et al., 2015b). Here we verified that inbred lines derived from that outbred population behaved similarly. All three lines homozygous for the resistant allele (*RR*) became infected ~20% of the time post-exposure, while all six families homozygous for one of the two susceptible GRC alleles (*S1S1, S2S2*) were infected at a rate of ~80% (Fig. 1) (Tennessen et al., 2015b; Allan et al., 2017b). These lines were then used for histological and mechanistic interrogation of these genotypes.

3.2. Despite equivalent integument anatomy and miracidial penetration, resistant BgGUA have fewer free sporocysts after exposure

The integument is one of the first lines of defense during pathogenic exposure. Therefore, we considered the possibility that GRC-encoded products could influence integument structure such that SmGUA larvae were less able to penetrate the integument of *RR* lines than *SS* lines. For this reason we compared, at the histological level, the integuments of *SS* and *RR* snails.

231 Additionally, by counting miracidia left behind when snails were removed after 2 h of exposure,
232 we collected independent data indicative of miracidial penetration of snail skin. At a histological
233 level, the structure, cellular composition and thickness (all determined by a veterinary
234 pathologist) of the integument did not vary among GRC genotypes (Fig. 2A, B). Additionally,
235 the number of miracidia unable to gain access (<1 on average) to the snail after 2 h did not differ
236 between resistant and susceptible snails, suggesting that equal numbers of miracidia penetrate
237 these snails during an exposure (Fig. 2C). Therefore, we have no reason to believe that miracidia
238 differentially penetrate the three genotypes.
239 We histologically examined snails 24 h, 48 h, and 10 days post-exposure. We found no
240 sporocysts in *RR* snails 10 days after exposure, so accordingly no additional analysis was
241 conducted. After as little as 24 h, sporocysts in *RR* snails were clearly in the late stages of
242 destruction, while the majority of sporocysts in *SS* snails were not evidently under immune
243 attack (Fig. 3A). Indeed, the number of 'free' (not degenerating, encapsulated or under any
244 identifiable suppression by the immune system) sporocysts was four-fold higher in *SS* snails
245 (Fig. 3C). However, the total number of sporocysts identified at 24 and 48 h did not differ
246 between *RR* and *SS* snails (Fig. 3B), an observation that is consistent with the conclusion that
247 miracidia penetrate all three genotypes equally well (Figs. 2, 3).
248
249 *3.3. Schistosome resistance is conferred to SS snails after transfer of RR hemolymph*
250 The integument is anatomically similar in *RR* and *SS* snails and equal numbers of
251 parasites gain access to each genotype; yet *RR* snails rapidly clear schistosomes whereas *SS*
252 snails generally fail to do so. Therefore, we sought to determine whether the protective factor is
253 carried in the hemolymph and, if so, whether it is humoral or cellular. We therefore attempted to

transfer resistance to *SS* snails via *RR* hemolymph as it likely contains mobile hemocytes and humoral factors that may recognize and coordinate the initial innate internal defense response against schistosomes. *RR* hemolymph conferred a statistically significant ~10% increase in resistance of *S1S1* snails, while *RR* cell-free plasma or *S2S2* hemolymph had no effect on susceptibility (Fig. 4). Therefore, cells in *RR* hemolymph were able to significantly improve the resistance of susceptible snails. Interestingly, the susceptibility of *SS* snails increased to 100% during the transfer treatment, probably owing to stress, mechanical damage or the immunological consequences of preforming injections just 24 h before exposure. Attempts to transfer isolated hemocytes were unsuccessful because the majority of isolated hemocytes did not survive isolation, resuspension and passage through a transfer needle.

4. Discussion

Understanding the immunological interactions between *B. glabrata* and schistosomes is believed to be essential to the future control of schistosomiasis (Pearce and MacDonald, 2002; Mourao Mde et al., 2009; Stefanic et al., 2010; Bhardwaj et al., 2011; Valentim et al., 2013; Theron et al., 2014; Coustau et al., 2015; Guidi et al., 2015; Tennessen et al., 2015a, b; Pila et al., 2016b; Pinaud et al., 2016; Sokolow et al., 2016; Allan et al., 2017b). Compatible snail species evade schistosome infection via complex physical and immunological mechanisms often involving parasite recognition (Bender et al., 2005; Goodall et al., 2006; Hanington et al., 2010; Loker, 2010; Moremen et al., 2012; Theron et al., 2014; Coustau et al., 2015; Tennessen et al., 2015b; Pinaud et al., 2016; Allan et al., 2017b). The mechanistic bases of these interactions, and their importance to the spread of schistosomiasis by snails, are still under intensive scrutiny.

The GRC region has been shown to have a strong allelic correlation with the resistance of BgGUA to SmGUA (Tennessen et al., 2015b). Proteins in the GRC have been suggested to have an immune recognition function due to their putative structure (Tennessen et al., 2015b; Allan et al., 2017b). Furthermore, at least one protein in this region has been shown to be integral to the suppression of schistosomes in these snails and is expressed in the hemolymph (Allan et al., 2017b). Determining how the GRC influences the progression of schistosome infection is an integral part of understanding how these genes may allow some snails to be highly resistant to infection. To facilitate the functional study of resistant and susceptible individuals, homozygous BgGUA lines were developed based on their genotype at the GRC. These lines maintained the resistance phenotypes that were previously observed in the outbred population, allowing for more extensive functional assessments of infection (Tennessen et al., 2015b; Allan et al., 2017b).

In the present study, we determined that individual snails with resistant GRC genotypes destroy more compatible sporocysts in the initial days following infection than susceptible individuals, despite apparently having the same initial schistosome burden. We went on to show that some of this resistance can be transferred to susceptible snails by injecting them with resistant hemolymph, and that hemocytes are probably essential for this defensive priming. Therefore, GRC proteins are likely involved in the initial response of hemocytes to schistosome infection.

After the shell, the molluscan integument is the first line of defense against most pathogens and parasites. This structure consists of an epithelium and subepithelium, which predominantly consist of ciliated cuboidal cells and secretory cells, respectively (Adlard, 2003). These cells comprise a physical barrier to invaders, as well as a motile and secretary network that can damage or remove pathogens. Although the integument has never been implicated in

differences among snail strains in susceptibility to schistosomes, we decided to check for variation among genotypes in this structure to rule out the possibility that differences between GRC genotypes in thickness or structure could be causing fewer miracidia to penetrate *RR* snails. Additionally, given that there were few developing sporocysts (~1-4 sporocysts in 300 sections/snail) found per snail, this examination also allowed us to demine if the first physical barrier the miracidia encounter differed between genotypes and could have been partially responsible for so few sporocysts. The integuments of *RR* and *SS* BgGUA were indistinguishable with regards to cell structure, cell composition and thickness at a histological level. Accordingly, the parasitic burden was equivalent across *RR* and *SS* snails, suggesting that schistosome resistance is probably due to an immune response to sporocysts after the miracidia have penetrated the snail. It is possible that more subtle differences in the integument, or in the kinetics of schistosome penetration between genotypes, could be differentiated by electron microscopy or immunohistochemistry. However, we found similar numbers of total schistosome larvae in *RR* and *SS* snails at 24 and 48 h post-exposure and similar numbers of miracidia left behind after 2 h exposures. So, it is unlikely that such subtle differences in the integument influenced the overall ability of SmGUA to penetrate the integument of BgGUA. The number of parasites found in the snails and remaining in the wells does not account for all of the parasites from the exposure. It is possible that some of these parasites were attached to the shell, or caught in the mucus of the snail and lost from our analysis. Additional microscopic methods could be used in future studies to ascertain where some of these parasites end up, and the roles that the mucus and shell may play in infection.

The developmental capacity of miracidia has been shown to be proportional to the number of miracidia that penetrate the host (Theron et al., 1997). In general, sporocysts that do

not survive have been shown to be encapsulated within the first 48 h, and the immune response to these sporocysts, mediated by hemocytes and humoral factors, has been shown to be faster and more extensive in more resistant strains of *B. glabrata* (Theron et al., 1997; Pereira et al., 2008). Parasite larvae which are not destroyed can be found developing weeks after an exposure in individuals that become infected, but prior to cercarial shedding. Indeed, we found that *RR* individuals had fewer 'free' (not encapsulated by hemocytes or degenerated by humoral factors) sporocysts despite having the same initial miracidial burden after exposure. *RR* snails were able to clear parasite larvae such that that no histological evidence of exposure was evident after 10 days in any of the *RR* snails that we examined.

Given that the transfer of cell-free hemolymph has been shown to confer resistance during the initial phases of infection in other strains of *B. glabrata* (Pereira et al., 2008; Pinaud et al., 2016), we aimed to determine if the transfer of different hemolymph treatments could improve the resistance of *SS* individuals. The transfer procedure itself increased the infection outcome to 100% in control *SS* snails, likely due to stress, mechanical damage, hemolymph dilution, induction of an unintended immune response, or a combination of these factors. Despite this, transferred *S2S2* hemolymph and *RR* plasma (cell-free hemolymph including humoral factors) did not alter the resistance of *S1S1* snails, but *RR* hemolymph conferred a 10% increase in resistance. This result implies that proteins encoded by genes in the GRC region are most likely present in *RR* hemocytes (as *RR* plasma alone did not confer resistance), and are partially responsible for the resistance phenotypes we observed and the efficient clearance of schistosome larvae by *RR* snails, as previously hypothesized (Tennessen et al., 2015b; Allan et al., 2017b). These results also confirm that humoral factors in *RR* BgGUA are probably not responsible for their greater resistance to schistosomes, but we cannot eliminate the possibility that they may

work synergistically with hemocytes because we were unable to transfer a pure hemocyte preparation.

In summary, we found that schistosomes are equally likely to penetrate the three BgGUA genotypes, but that *RR* snails clear schistosomes much more effectively and rapidly – most within 48 h. This difference between GUA region genotypes is similar to what has been observed in comparisons between highly differentiated lines of snails that differ in their susceptibility to particular strains of *S. mansoni* (Richards and Minchella, 1987; Theron et al., 1997; Borges et al., 1998; Loker, 2010; Nacif-Pimenta et al., 2012). However, we believe this is the first time such variation can be ascribed to variation in particular genes. We postulate that proteins encoded in the GRC region are acting via hemocytes in the hemolymph because hemolymph from *RR* snails was able to transfer some resistance to *SS* individuals, while cell-free plasma was not. Previous work showed that proteins coded for by the GRC regions have structures reminiscent of immune recognition proteins and are expressed in hemolymph (Tennessen et al., 2015b; Allan et al., 2017b). These previous observations and our results here are all consistent with the hypothesis that proteins encoded in the GRC region are involved in pathogen recognition by hemocytes.

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Figure Legends

Fig 1. Resistance phenotypes are conserved among inbred Guadeloupean *Biomphalaria glabrata* (BgGUA) lines. The susceptibility of nine independent homozygous BgGUA lines (3x *S1S1, S2S2*and *RR*) after exposure to 20 Guadeloupean *Schistosoma mansoni* (SmGUA) miracidia (*n*= 32 $S1^1$,32 $S1^2$,28 $S1^3$, 35 $S2^1$, 33 $S2^2$, 27 $S2^3$, 55 R^1, 31 R^2, 42 R^3). Data are presented as the proportion of infected snails (+/- the S.E. of proportions). Significant differences (P <0.05, Z score of proportion) are denoted by asterisks (*).

Fig 2. Resistant genotypes do not have modified integument anatomy or thickness, nor do they prevent *Schistosoma mansoni* miracidial penetration in Guadeloupean *Biomphalaria glabrata* (BgGUA). (A) The head-foot integument architecture of *S1S1, S2S2,* and *RR* BgGUA (scale bar = 100 µm). (B) The thickness of the integument of the head-foot of *S1S1, S2S2,* and *RR* BgGUA (*n*= 4). (C) The number of miracidia remaining in a well 2 h post-BgGUA exposure to 20 miracidia (*n*=12). Data presented as means (+/- S.D.). No significant differences (P >0.05, ANOVA) were observed.

Fig 3. Resistant Guadeloupean *Biomphalaria glabrata* (BgGUA) have fewer free sporocysts, and destroy schistosomes during the initial stages of infection. (A) Free and encapsulated sporocysts in BgGUA 24 h post-exposure (scale bar = 200 µm). (B) Total number and (C) number of free (unencapsulated/undegenerated) sporocysts found in individual *SS* and *RR* BgGUA 24 h and 48

516 h post-exposure. Data presented as means (+/- S.D.). Significant differences (P <0.05, student's
517 t-test) are denoted by asterisks (*).

518

519 **Fig 4.** Transfer of resistant (*RR*) hemolymph, but not cell-free plasma or sensitive (*SS*)
520 hemolymph, confers resistance to Guadeloupean *Schistosoma mansoni* (SmGUA). The
521 susceptibility of homozygous *S1S1* snails after exposure to 20 miracidia and transfer treatments.
522 Groups of snails were treated with sham (SSS), *S2S2* hemolymph (HL), *RR* plasma, or *RR*
523 hemolymph (HL) 24 h pre-exposure (n=69 *S1S1* + sham, n = 67 *S1S1* + *S2S2* HL, n = 31 *S1S1* +
524 *RR* plasma, n= 74 *S1S1* + *RR* HL). Data are presented as the proportion of infected snails (+/- the
525 S.E. of proportions), and significant differences (P <0.05, Z score of proportion) are denoted by
526 an asterisk (*).

527

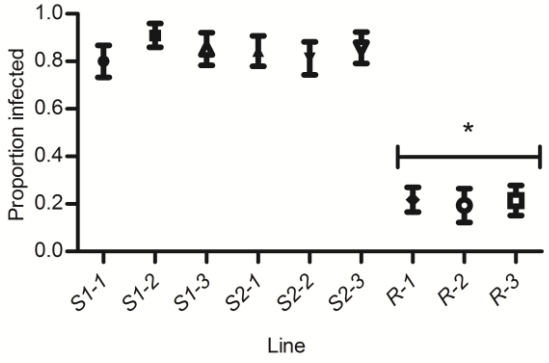

1.0
0.8
0.6
0.4
0.2
0.0
Proportion infected
*
S1-1
S1-2
S1-3
S2-1
S2-2
S2-3
R-1
R-2
R-3
Line

Figure 1

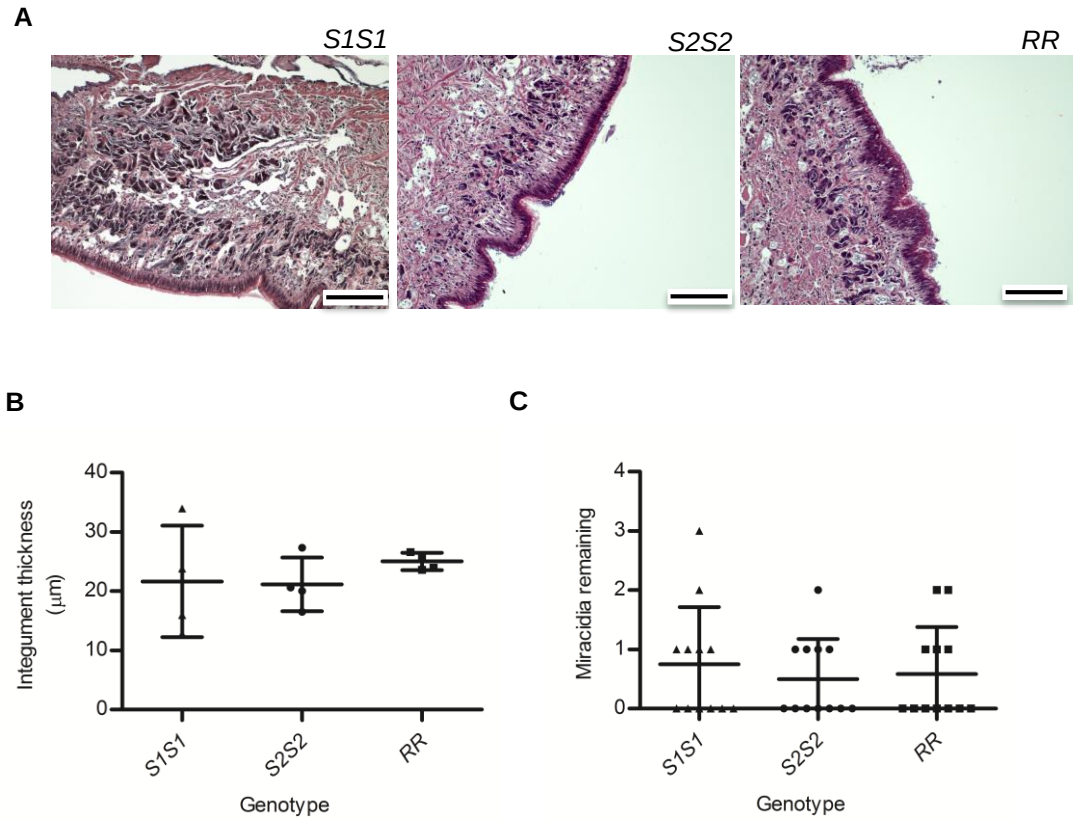
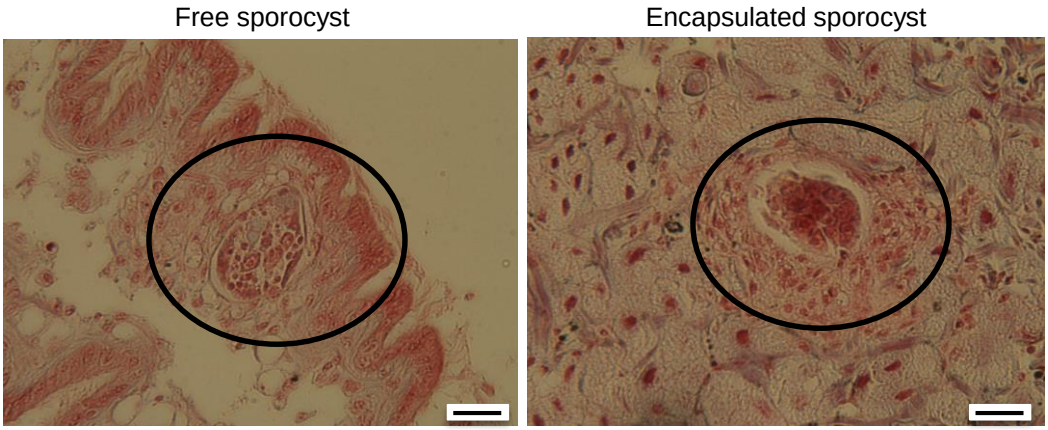
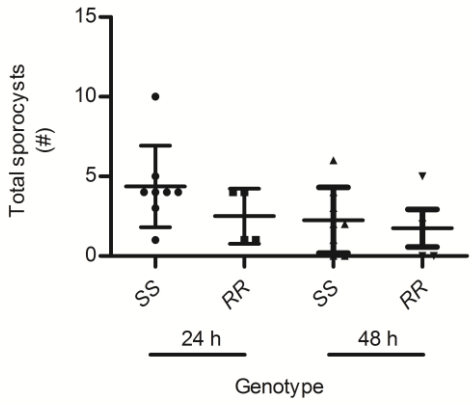

A
S1S1
S2S2
RR
B
Integument thickness (μm)
40
30
20
10
0
S1S1
S2S2
RR
Genotype
C
Miracidia remaining
4
3
2
1
0
S1S1
S2S2
RR
Genotype

Figure 2

A


Free sporocyst
Encapsulated sporocyst

B


Total sporocysts (#)
15
10
5
0
SS
RR
SS
RR
24 h
48 h
Genotype

C

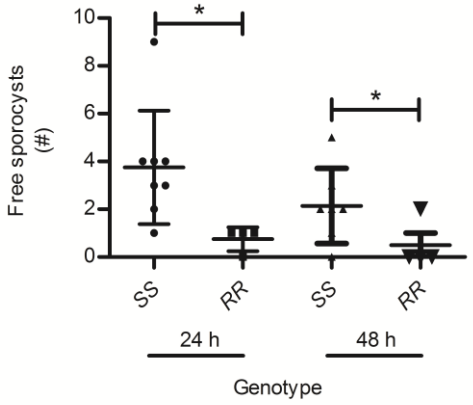

Free sporocysts (#)
10
8
6
4
2
0
*
*
SS
RR
SS
RR
24 h
48 h
Genotype

Figure 3

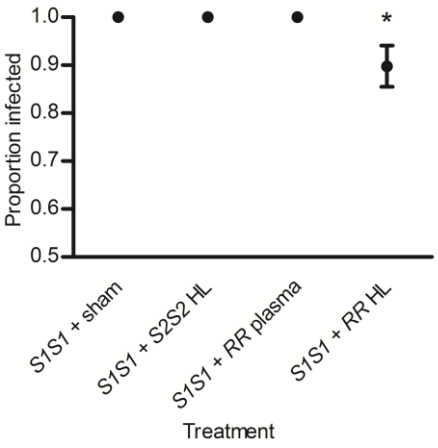

1.0
0.9
0.8
0.7
0.6
0.5
Proportion infected
*
S1S1 + sham
S1S1 + S2S2 HL
S1S1 + RR plasma
S1S1 + RR HL
Treatment

Figure 4