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# Molecular characterisation of immunological memory following homologous or heterologous challenges in the schistosomiasis vector snail, *Biomphalaria glabrata*

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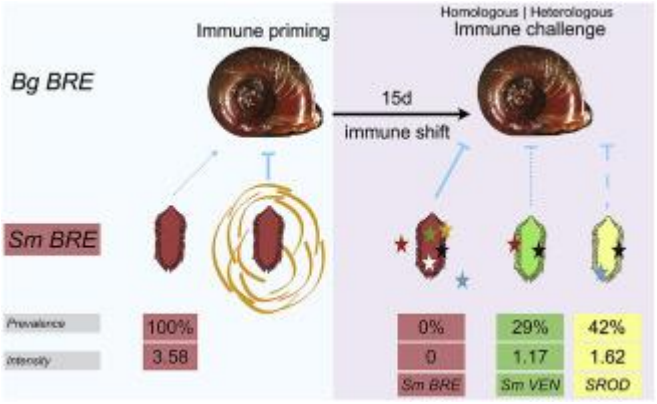
**Abstract :**

Invertebrate immune response may be primed by a current infection in a sustained manner, leading to the failure of a secondary infection with the same pathogen. The present study focuses on the Schistosomiasis vector snail Biomphalaria glabrata, in which a specific genotype-dependent immunological memory was demonstrated as a shift from a cellular to a humoral immune response.

Herein, we investigate the complex molecular bases associated with this genotype-dependant immunological memory response. We demonstrate that Biomphalaria regulates a polymorphic set of immune recognition molecules and immune effector repertoires to respond to different strains of Schistosoma parasites. These results suggest a combinatorial usage of pathogen recognition receptors (PRRs) that distinguish different strains of parasites during the acquisition of immunological memory. Immunizations also show that snails become resistant after exposure to parasite extracts. Hemolymph transfer and a label-free proteomic analysis proved that circulating hemolymph compounds can be produced and released to more efficiently kill the newly encountered parasite of the same genetic lineage.

## Graphical abstract




## Highlights

► Immunological memory (IM) has been now demonstrated in numerous invertebrate models. ► A high level of specificity of IM has been demonstrated in *Biomphalaria glabrata* snails. ► Efficiency of IM seems to be supported by a potential combinatorial usage of PRRs. ► PRRs and cytolytic/cytotoxic compounds were mandatory for parasite killing.

**Keywords** : Immunological memory, Specificity, Schistosoma, Biomphalaria, RNAseq, Proteomic, Vaccination

## 1. Introduction

Living organisms have evolved complex immune systems to confront pathogenicbiological-traits and diversity, from prokaryotes(Marraffini and Sontheimer, 2008) to eukaryotes,from plants (Calil and Fontes, 2016; Dangl et al., 2013) to animals, and from invertebrates (Cirimotich et al., 2011; Haine et al., 2008; Moret and Schmid-Hempel, 2000, 2001; Portela et al., 2013) to vertebrates (Cooper and Alder, 2006; Du Pasquier, 2004; Eason et al., 2004). “Benefiting from experience” or more specifically “remembering pathogens” has been a pressure that has led many immune systems to develop rapid and efficient protections against pathogens encountered earlier in life. This is due to a chain of events referred to as “immunological memory”. For innate immune systems, the memory processes are derived from evolutionary convergences and could have very different features that have led to different terminologies, i.e.trained immunity for vertebrates or immune priming/innate immune memory for invertebrates (Gourbal et al., 2018; Milutinovic and Kurtz, 2016; Netea et al., 2011; Pradeu and Du Pasquier, 2018).

In invertebrate species, despite the lack of a canonical adaptive immune system, immunological memory and specificity of immunological memoryhave been demonstrated using different strains of pathogens even though in most cases,a short-term memory is difficult to distinguish from an ongoing response(Jorge Contreras-Garduno et al., 2016; Kurtz and Franz, 2003; Lafont et al., 2017; Milutinovic and Kurtz, 2016; Pham et al., 2007; Pinaud et al., 2016; Witteveldt et al., 2004).

However, observations of memory specificity in invertebrate innate immune systems are largely phenomenological; and the underlying molecular and/or cellular mechanisms remain unknown. In vertebrates, trained immunity shows low or even no specificity(Netea et al., 2011; Netea and van der Meer, 2017). Thus, it may not be possible to explain the observations in invertebrate systems in terms of what we know about non-self-recognition and trained immunity in vertebrates(Litman, 2006).Thus, if invertebrates do show specificity in their memory responses, what are the molecular and cellular determinants of such a specific immunological memory?

In the present paper, we investigate this question in the Lophotrochozoan snail, *Biomphalaria glabrata,* and its metazoan parasite, the trematode *Schistosoma mansoni*. *Biomphalaria* snails are the vectorfor Schistosomiasis, which is the second

most widespread human parasitic disease after malaria,causing a substantial public-health burden with about 200 million humans infected in 74 countries and more than 200,000 deaths annually (King et al., 2005).

We previouslyreported that the immunological memory response of *Biomphalaria* to various strains of *Schistosoma* is specific (better protection observed in homologous challenges compared to heterologous challenges), although cross-reaction was certainly encountered (Portela et al., 2013).In the case of one Brazilian *Biomphalaria* / sympatric *Schistosoma* interaction, a transcriptomic and comparative proteomic approach revealed an apparent shift from a cellular immune response (where the hemocyte encapsulates the parasite) following primo-infection, toward a humoral immune response (soluble plasma factors will clear the parasite) following challenge(Pinaud et al., 2016). Following primo-infection, 2/3 of the parasites were encapsulated and 1/3 succeeded in infecting the snails that then harbour a viable, developing and productive parasite infection (Portela et al., 2013).At the molecular level, we identified a shift in transcript expression only associated with the challenge(Pinaud et al., 2016).

In the present paper, we further explore the specificity of the immunological memory response of the snail *Biomphalaria* particularly within the context of a specific genotype-dependant immune response. Using experiments at different scales, as well as transcriptomic responses, snail immunisation,*in-vitro* toxicity assays and a comparative proteomic approach of plasma compartment, we compared host response followinghomologous versus heterologous parasite challenges (figure 1) and we bring new insightsto help better understand the specificity of the immunological memory response in *Biomphalaria glabrata* snails.

## 2. Materials and methods

### *2.1. Biomphalaria* snail and *Schistosoma* parasites

One strain of *Biomphalaria glabrata* snail originating from Brazil (BgBRE) has been used for all experiments. Its sympatric *Schistosoma mansoni* parasite strain from Brazil (SmBRE) was used for homologous interactions while heterologous interactions were conducted with two different geographic isolates, with the first coming from the same species: *Schistosoma mansoni* from Venezuela (SmVEN); and the second from a different species: *Schistosoma Rodhaini* from Burundi (SROD). Infectivity phenotypes for all interactions were previously described as

highly compatible, with prevalence values from 80% to 100% (Portela et al., 2013; Theron et al., 1997).

### 2.2. Immunological memory experimental protocols

Duration of the immune memory was tested by primo-infecting BgBRE snails (n=100) with 10 SmBRE miracidia. Then, at 15, 25, 56, 72, and 140 days after primo-infection (DPI), snails were challenged with 10 SmBRE miracidia and 15 days after challenge snails were fixed in Raillet-Henri solution, and parasite prevalence was quantified as previously described (Portela et al., 2013). For RNAseq, BgBRE snails were primed with 10 SmBRE miracidia and then,25 days later, challenged with 10 miracidia from each strain (SmBRE, SmVEN, SROD). For vaccination, *in-vitro* toxicity and label-free proteomic approachesfrom cell-free hemolymph,BgBRE snails (n=150) were primo-infectedor experimentally injected with parasite extracts and challenged with 10 SmBRE miracidia, 15 days later. Parasite prevalence was quantified throughdissection of Raillet-Henry fixed snails 15 days after the immune challenge as previously described (Portela et al., 2013). For all of these experimental approaches, uninfected naïve snails were used as negative controls.

### 2.3. High throughput approach protocols

For the RNAseq, pools of 20 BgBRE snails were recovered as previously described (Pinaud et al., 2016) at 1, 4 and 15 days after the challenges and equimolar amounts of each biological material collected at these time points were pooled together into 3 separate samples named BRE, VEN and ROD corresponding to the 3 strains. Two pools of 20 naïvesnails (naïve1 and naïve2) were sampled and used as controls. Statistical analyses were performed as already described (Pinaud et al., 2016) following the pipeline on our Galaxy Project server. Briefly, best quality reads were filtered (phred >29) and then reads were aligned with Bowtie (v2.0.2, quality mapping 255) and PCR duplicates were removed thanks to SAM tools. DESeq2 software was used to compare duplicate samples of uninfected snails (naïve1 and naïve2) with our three challenge conditions (BRE, VEN and ROD) and all statistically different results (P value <0.05) were annotated manually (Blast X and Interproscan against nr database, Bast2GoPro for all the other available databases, and data from the literature). A heatmap was constructed from a log2 fold change for each of the transcripts using Hierarchical Ascending Clustering (HAC) with Pearson correlation,

as applied by the Cluster 3.0 (Eisen et al., 1998) and JavaTreeView software packages.

### 2.4. Vaccination

All developmental stages of *Schistosoma* parasites were recovered and whole extracts were prepared. Miracidia and cercaria (free-living swimming stages) were recovered in water, washed in PBS and pelleted by centrifugation. Primary sporocysts (Sp1) were recovered by *in-vitro* transformation from miracidia as previously described (Roger et al., 2008b) in CBSS for 24 hours. Secondary sporocysts (Sp2) were recovered in the hepato-pancreas of 30-day infected snails (Sp2 representing 70% of the hepato-pancreas at this time point). Hepato-pancreas from uninfected naïve snails were also recovered and used as a control (named: Sp2 control). All of the samples were natively extracted in 150 µL TBS-Tween 0.05% buffer. Three cycles of freeze-thaw (-80°c to 4°C) were followed by four pulses of sonication (Vibra-cell 75185) at 20% max amplitude for 20 seconds at 4°C; then proteins containing samples were titrated (2D Quant kit, GE Healthcare life sciences) and parasite stage extracts were stored at -80°C. A pool combining an equimolar amount of whole extracts from the 4 parasite stages (sample named “pool”) was constituted and used as a test for antigen synergistic effect.

For the homologous vaccination, 500 uninfected BgBRE snails were anaesthetised for 2h in 0.05% Pentobarbital sodium (200mg/ml Vetoquinol Dolethal) in 26°C drilling water, then injected in the head-foot region with 1µg in 10µL TBS-Tween 0.05% buffer of each SmBRE intra-molluscal stage protein extract (Miracidia, Sp1, Sp2, Sp2Control, Cercaria or Pool). Then, individual snails wereexposed to 10 SmBRE miracidia 15 days after vaccination. An injection of TBS-Tween buffer alone was used as a negative control. For heterologous vaccinations, 200 BgBRE naïve snails were individually injected under the conditions previously described with Miracidia and Sp1 extracts from SmVEN and SROD, then individually exposed to 10 SmBRE miracidia 15 days after vaccination. Finally, fifteen days after infection, snails were fixed in Raillet-Henry solution to quantify parasite prevalence as previously described (Portela et al., 2013). Experiments were done in triplicate.

### 2.5. Expression of relevant immunity genes following vaccination

To determine if vaccination activates a functional immune response similar to the one observed following natural primo-infection with *Schistosoma mansoni* miracidia, we conducted a quantitative Real-time RT-PCR on relevant immune genes following vaccination. The differential gene expressions from RT-PCR following vaccination were compared to RNAseq Log2fold change data. Down- and up-regulated genes were selected based on RNAseq data (see supplementary Table 1 for details on primers and genes selected). BgBRE snails were injected with parasite extracts as described above in the vaccination section, and then hemocytes were recovered and gathered in 4 biological replicates of 3 individuals each. mRNAs were extracted using TRIZOL® (Sigma Life Science, USA) and thenreverse transcribed to first strand cDNA as described by the manufacturer and treated with DNase (Maxima First Strand cDNA Synthesis Kit with dsDNase, Thermo Scientific, USA). Real-time RT-PCR analysiswas performed in the LightCycler 480 System (Roche) with a volume of 10 µL comprised of 20ng cDNA diluted with MilliQ-H2O in 2 µL, 5 µL of No Rox SYBR Master Mix blue dTTP (Takyon) and 1.5 µL foreach of the 2X primers.The cycling program was: 95°C for 4 minutes, followed by 45 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 20 sec. Expression levels of targeted genes were normalised relative to S19 gene thanks to the Pfaffl method (Pfaffl, 2001).

**2.6. Cell-free hemolymph transfer and protection phenotype**

Individual naïve BgBRE snails were primed with 10 SmBRE miracidia and hemolymph was recovered from 30 snails 15 days later (sample was named 15DPI). Next,the snails were challenged with 10 SmBRE miracidia, and hemolymph from poolsof 30 individuals, sampled at 1, 4 and 15 days after the challenge (samples were named 1DC, 4DC and 15DC for “days post challenge”). Finally, a pool of hemolymph from 30 naïve snails served as the control (termednaïve). All samples were centrifuged (2,500 rpm for 15 min at 4°C) to pellet hemocytes and the supernatant, referred to as “cell-free hemolymph”, was kept at -80°C until used.

Three hundred naïve snails (10-12 mm) were injected with 10 µL of cell-free hemolymph of either naïve, 15DPI, 1DC, 4DC or 15DC samples or saline solution (Chernin’s balanced salt solution, CBSS (used as negative control of injection)), where 10 µl represented around 1/8 of the total hemolymph volume for the recipient snail. Fifteen days after injection, all snails were individually exposed to 10 SmBRE miracidia, and 15 days later, were fixed in Raillet-Henry solution to estimate parasite

prevalence. At the same time, 30 untreated snails were exposed to 10 SmBRE miracidia to quantify control prevalence used for protection calculation.All experimental approaches were conducted in triplicate.

### 2.7. Comparative label-free proteomic of cell-free hemolymph

Three hundred BgBRE snails were individually primo-infected with 10 SmBRE miracidia; and 15 days later, 150 snails were exposed to a homologous challenged with 10 SmBRE miracidia. For label-free proteomic sequencing, pools of hemolymph were recovered from either naïve, 15 days after primo-infection (15DPI) or 1 day after the challenge (1DC) snails. Twenty five µL of hemolymph was recovered from each snail for a total of 3,500 µL for each of the three samples. Each sample was then processed in the same way. Hemocytes were removed from hemolymph by centrifugation at 2,000 g, 5 min at 26°C. Then, hemoglobin was depleted from cell-free hemolymph by ultra-centrifugation at 40,000 g, 2.5 hours at 4°C. Cell-free hemolymphs (hemoglobin depleted) were extracted in Laemmli buffer 4X (Biorad, Hercules California, USA), boiled (95°C-100°C) for 5 min and stored at -80°C until used for label-free sequencing (EDyP service facilities, CEA Grenoble, France). Protein preparation and mass spectrometry-based proteomic analyses were conducted as described in (Milbradt et al., 2014). Briefly, extracted proteins were stacked in the top of an SDS-PAGE gel (NuPAGE 4 to 12%; Invitrogen) before in-gel digestion was performed using trypsin (sequencing grade; Promega). Resulting peptides were analysed in duplicate by online nanoscale liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) (UltiMate 3000 and LTQ-Orbitrap Velos Pro; Thermo Scientific) using a 120-min gradient. Peptides and proteins were identified using Mascot software (Matrix Science) and confronted against either, Uniprot database and translated transcriptome of *Biomphalaria glabrata* snail (available at: http://ihpe.univ-perp.fr/acces-aux-donnees/)or translated genome of *Schistosoma mansoni* (available at: http://schistodb.net/schisto/).A differential analysis was performed using extracted specific spectral counts (SSCs) where only the qualitative differences between naïve, 15DPI and 1DC samples, were considered.

### 2.8. *In vitro* cell-free hemolymph toxicity assay: a validation of specificity

Naïve BgBRE snails were primed either with 10 SmBRE miracidia or 10 SmVEN miracidia, and then challenged by 10 homologous SmBRE or SmVEN miracidia. Cell-free hemolymph was recovered from either thenaïve snails or 1 day after the challenge (1DC) as described above and then kept at -80°C until used. *In vitro* primary sporocysts (Sp1) of SmBRE, SmVEN and SROD were transformed *in-vitro* from miracidia as previously described (Roger et al., 2008b). *In vitro* toxicity assays were done by putting Sp1 in contact, at 26°C, with CBSS culture medium (control), or with 500µL of cell-free hemolymph from naïve snails or from 1DC snails challenged with SmBRE or SmVEN. Mortality was assessed after 4 hours of contact under a light microscope, with the Sp1 considered “dead” when we failed to observe motility and/or beating of the flame-cell flagella as previously described (Mone et al., 2011). All experiments were done in triplicate with different preparations of sporocysts and hemolymphs.

## 3. Results

### 3.1. Long-lasting immunological memory in *Biomphalaria glabrata*

To test for duration of the innate immune memory process, BgBRE snails were primo-infected with10 SmBRE miracidia and challenged at 15, 25, 56, 72, and 140DPI, again with 10 SmBRE miracidia. Forall-time points, primo-infected snails were always resistant to challenge (Table 1).All snails diedafter 140 DPI due to parasite pathogenesis (Table 1) as parasite from primo-infection developed to patency in snail tissue.These results demonstrated that, (i)immunological memory was acquired soon afterinfection (from 15 DPI) and (ii) was maintained during the snail’s entire life span(Table 1).

### 3.2. Transcriptomic results provide clues for specificity of immunological memory

A RNAseq approach was used to identify differentially expressed (DE) transcripts following challenges with homologous (SmBRE) or heterologous(SmVEN or SROD)parasites. Complete RNAseq results are available in supplementary Table 2. Venn Diagram (Fig. 2A) showsthat among 4,202 DE transcripts, 22% (representing 924 different transcripts) appeared as common regardless of the strain used for immune challenges. Strain-specific transcripts were identified for the homologous SmBRE challenge (45.8% representing 2,013 transcripts), for the heterologous

challenge with SmVEN (9.6% and 405 transcripts) and for the challenge with SROD (5% and 210 transcripts). The number of DE transcriptsisdecreasing with an increase in the genetic distance between the parasite used for the primo-infection and for the one used in the challenge. The heat map of these DE transcripts provides more precise information by highlighting 14 different transcript clusters (Fig. 2B). Clusters 1 to 7 corresponded to up-regulated transcripts and clusters 8 to 14 to down-regulatedones. Both clusters 1 and 14 corresponded to transcripts shared between all challenge conditions. With 3,339 DE transcripts, the homologouschallenge with SmBRE induced the largest response while the SmVEN strain induced the regulation of 1,838 transcripts, and SROD only 1,525 transcripts (Fig. 2B). Interestingly, many immunity-related transcripts can be identified in these clusters. Within them, three main families can be defined, (i) the receptorsthatpotentially bind pathogen factors, (ii) effectors of immunity, capable of havinga direct deleterious effect on the pathogens or activation effect on host immune response and (iii) other transcripts related to immunitytriggering maturation,activation of immune response. In this way, the putative immuno-receptors are widely distributed across clusters but with differential distribution between the parasite strains. Immune effectors are narrowlydistributed on the heatmap. For example biomphalysin, a beta-Pore Forming Toxin (β-PFT) secreted in snail plasma,capable of directly killing the parasite(Galinier et al., 2013) is notably present in cluster 1 (Fig. 2B). Cluster 1 groups the up-regulated transcripts differentially expressed regardless of the parasite strain. Thus, biomphalysin could participate inthe humoral memory phenotype observed following the challenge in *B. glabrata* snails (Pinaud et al., 2016; Portela et al., 2013). Biomphalysin DE transcripts suggest that numerous variantsofbiomphalysin family members were involved in this immunological memory response (Supp-Table 2). Variants of biomphalysin are specifically up-regulated following SmBRE, SmVEN orSRODchallengeswhile others aredown-regulated following the SmVENchallenge (Fig. 2B, and Supp-Table 2).

Anti-microbial peptides (AMPs) and the functionally related antimicrobial proteins werealso part of the immune effector family. Members of the *B. glabrata*lbp/bpi (lipopolysaccharide binding protein/bactericidal permeability increasing protein) family (22 transcript variants) are mainly down-regulated in response to the three parasite strains (cluster 14). Two members wereup-regulated in response tothe SmBRE strain (cluster 1) while one wasup-regulated in the response shared by

SmBRE and SROD strains (cluster 4). One form of mytimacin, a cysteine-rich antimicrobial peptide,previously described in the mussel *Mytilus* is up-regulatedinthe SmBRE specific cluster 7, while a variant is up-regulated in the shared response between SmBRE and SROD (Cluster 4).

For the third group of transcripts related to immunity, several immune regulatorswere identified.Collagen, Hemagglutinin, Haemoglobin and SCO-spondin arecomponents of, or proteins associated with, the extra-cellular matrix (ECM), theyshowed a mosaic of transcript expression depending on the parasite strain used for the challenge. Collagen and hemagglutinin variantswere associated with clusters 1 and 14. Haemoglobin11 variants was down-regulated following SmBRE challenge. These results confirmed the complex restructuration of the ECM during the immuneshift from a cellular toa humoralimmune responseassociated to immunological memory.The serpin, a family of serine protease inhibitors, implicated mainly in inflammatory processes,displayed a complex pattern of expression.A total of26 variantswere down-regulated in cluster 14 and4 isoforms of serpin b6-b were up-regulated followingthe homologous challenge (cluster 7). Cadherin and protocadherinwhich belong to a superfamily of transmembrane proteins implicated in cell-to-cell binding or adhesion, were also identified. Following the SmBRE immune challenge, 40 different transcripts of this family were down-regulated including protocadherin fats 1, 3, 4 and 20. Similar patterns of regulation werealso observed for 14 transcripts of hemocytin, a highly-glycosylated adhesive protein related to haemostasis or the encapsulation of foreign substances for self-defence.

In order to investigatethe specificity of immunological memory towards the complex mixture of antigenic epitopes presented by *Schistosoma*, we focussed onputative immuno-receptorsand we tried todecipherhow a specific repertoire is activatedfollowing homologous or heterologousparasite challenges (Fig. 3).Cumulative log2 fold-change of each DEimmuno-receptor transcript was used to highlight quantitative differences while the number of transcript was used to highlight qualitative differences (Fig. 3). Homologuoussystemsled to +70.59 and -70.70 log2 fold-changes for 82 DE transcripts (Fig. 3A-B). The heterologous SmVEN and SROD challenges led to the modulation of 62 and 48 DE transcripts, respectively (Fig. 3A-B). The level of expressionstandsbetween+22.77 and -80.05 cumulative log2 fold-change for SmVEN and+35.52 and -41.37log2 fold-change for SROD (Fig. 3A). Expression patterns of these transcriptsdifferedstrongly between parasites of

different genetic backgrounds (Fig. 3B).Only one fourth of the transcripts shared a similar modulationirrespective of the parasite strainused for the challenge(N=29, 24.4%) (Fig. 3B). SmBRE-challenged snails expressed the most specific pattern of DE transcripts (N=42, 35.3%) while snails expressed less and lesstranscripts whenchallenged with SmVEN (N=19, 16.0%) or SROD (N=14, 11.8%) (Fig. 3B). Annotations provide important information concerning thefamilies of immune recognition receptors selected by the host to cope with the diversity of the pathogens used for the challenges.In cluster 1 (shared up-regulated transcripts), selectin, c-type lectin, FREP 3.3, tenascin, CD205 protein, lymphocyte antigen 75-like and macrophage mannose receptor 1-like were identified (Fig. 3C).In cluster 12 (shared down-regulated transcripts), we identified: 3 transcripts of Pathogenesis Related Proteins (PathoRP), 2 variants of C1q-like 4 (C1q complement-like), 3 transcripts of c-type lectin (calcium-dependant lectin), 1 transcript of collectin-10, 2 transcripts of c-type mannose receptor-2, one CD209, a dermatopontin-3 and one transcript of Thioester-containing protein (TEP) (Fig.3C). For the strain specific response, homologous challenge led to up-regulationof macrophage mannose receptor 1-like, 3 transcripts of selectin, 5 Peptidoglycan recognition proteins (PGRP), 8 Fibrinogen related proteins (FREPs), one galectin, 4 transcripts of c-type lectin, 2 of alpha-2-macroglobulin and 2 of dermatopontin(cluster 6) (Fig.3C). For down-regulation, one transcript of macrophage mannose receptor-1 like, BgFREP1, a Toll-like receptor, a mucin-5AC, a Galectin-3 and one transcript of TEP were identified(cluster 7).For the SmVEN heterologous response,most of the transcripts were down-regulated (clusters 5-8) including 3 transcripts of C1q-like, 4transcripts of c-type lectin, and 5transcripts of macrophage-mannose receptor.For the SROD heterologous challenge, 2 transcripts of FREPs were down-regulated together with a Toll-like receptor and one C-type lectin (cluster 9) while 2 transcripts of macrophage mannose receptor 1-like were up-regulated with 8 transcripts of peptidoglycan binding domain-containing protein (cluster 4).

### 3.3. Vaccination with parasite stage extracts

The parasite developedinto 3 successive stages within snail tissue (Fig. 4A). Parasite stages were thus recovered independently and used for vaccination.Vaccination with miracidia (prevalence 82.6% (N=86)), Sp2 (66% (N=50)), snail tissues (9% (N=83)), cercaria (81.4% (N=43)) and pool extracts(72.4% (N=98)) did not resultin significant

protection(Fisher exact test, p>0.01)compared to TBS-Tween injections (80.3% (N=61)) (Fig. 4B). However, Sp1 extract induceda significant protection (Fisher exact test, p=0.000145) with a prevalence of 47.9%(N=73 individuals) (Fig. 4B). Thus, snails were partially protected solely by Sp1 extract immunization, as other parasite stage extracts appearednot enoughimmunogenic to activate a significant protection.

### 3.4. Vaccination:a tool to validate immunological memory specificity

We analysed immune specificity using vaccination with Sp1 parasite extracts. Vaccinations with Sp1 protein extract from SmBRE, SmVEN or SROD were compared to TBS-Tween injection (Fig. 5). Only homologous vaccination provided significant protection from parasite infection (prevalence 47.9% (N=73), (Fisher exact test, p=0.000145)) (Fig. 5). Heterologous vaccinations did not differ from the TBS-Tween control with a prevalence of84.8% (N=33) for SmVEN, and 83.8% (N=37) for SROD (Fig. 5),respectively.

### 3.5. Immune relevant gene-expression following vaccination

To demonstrate that vaccination mimics natural infection and results in the activation of snail immune response, qRT-PCR on relevant immune genes wasperformed on hemocytes following vaccination and then compared to natural infections (Supplementary Fig. 1). Six candidate genes were selected for their putative role in immunological memoryin *Biomphalaria glabrata* based on a whole body RNAseq experiment (Supplementary Table 2). These candidates were selected based on their differential regulation following challenge and for their presence in the shared clusters 1 and 14 (same pattern of expression irrespective of the parasite strain). Vaccination induced the same pattern of response for these immune recognition receptors asthe known response generated by natural infection (Supplementary Fig. 1). The transcripts of FREP 3.3, C-type lectin and Selectin are down-regulated following primo-infection in the qRT-PCR approach,similar to the regulation observed in the RNAseq experiment(Supplementary Fig. 1). Glutamate synthase was up-regulated in both natural infection and vaccination(Supplementary Fig. 1). However, some differences exist for TEP and biomphalysin, which displayed opposite expression patternscomparingvaccination and natural infections(Supplementary Fig. 1). Altogether, these results suggest that vaccination can partially mimica natural

primary infection and can prepare the snail immune system to respond more efficiently to a subsequent parasite infection.

### 3.6. Cell-free hemolymph transfer andacquisition of protection

RNAseq approach reveals that most of the molecules identified as differentially represented between homologous and heterologous infections, corresponded to circulating immune recognition and/or effectorfactors. We thus investigated how the transfer of cell-free hemolymph from donor snails to naïve recipient snails could transfer protection against infection. Naïve, 15DPI,4DC and 15DC hemolymph did not significantly transfer resistance to naïve snails, compared to saline injection (Fig.6). Parasite prevalences were as follows: 76% (N=29) for saline injection, 76.5% (N=34)for naïve, 64.4% (N=59) for 15DPI, 59.4% (N=35) for 4DC and 81.8% (N=33) for 15DC (Fig. 6).However, transfer of cell-free hemolymph recovered one-day after the challenge (1DC)led to the acquisition of a significant protection against SmBRE infection (Fisher exact test, p=0.000145), with a prevalence of 42.9% (N=35) (Fig. 6). These results demonstrate that1 day after the challenge, relevant immune recognition molecules, immune mediators or cytotoxic/cytolytic effectorswere present in the snail hemolymph and, when injected,were able to activate and maintain an efficient immune protection even 15 days after cell-free hemolymphtransfer to naïve recipient snails.

### 3.7. Comparative label-free proteomic of cell-free hemolymph

Cell-free hemolymph recovered 1 day after the challenge (1DC) was able to confer a significant protection when transferred to naïve snails. Thus the characterisation of cell-free hemolymph composition was crucial forthe understanding of immune protection following the challenge. Using a comparative label-free proteomic sequencing approach, we characterised cell-free hemolymph circulating protein from naïve, 15 DPI and 1DC snails (Table 2). Here, biomphalysin was identified as the main protein in the 1DC cell-free hemolymph compounds (Table 2). This confirms, at the protein level, the RNAseq results showing that biomphalysin was up-regulated following the challenge (Fig. 2, cluster 1). For all cell-free hemolymph samples, the other identified proteins belong to lectins, recognition proteins, immune cell response mediators, glycan hydrolase, or extracellular matrix components (Table 2).These molecules support the immune shift phenotype observed in *Biomphalaria* snails that

turns from a cellular immune response following the primo infection, towards a humoral immune response following the challenge. Moreover, the same functional categories of molecules were observed in the RNAseq results (Fig.2), resulting in a reciprocal confirmation of transcriptomic and proteomic data.

### 3.8. Specificity validation by *in-vitro* cell-free hemolymph toxicity assay

The transcriptomic reprogramming is associated witha higher difference in theset of recognition proteins and effector molecules depending on the interaction with homologous or heterologous parasites. Moreover,the massive proteomic-sequencing approach demonstrated that most of these moleculeswere released into the hemolymph (Table 2). Toconfirm that cell-free hemolymphcontains factors that support a specificactivity against*in-vitro* cultured Sp1 of SmBRE, SmVEN or SROD, a cell-free hemolymph toxicity assay was conducted. SROD sporocysts were difficult to culture *in-vitro* (*i.e.,* 89% of mortality for SROD cultured in buffer saline, Table 3) thus the effects of naïve, SmBRE/SmBRE or SmVEN/SmVEN hemolymphs on SROD Sp1survival havenot beentested due to this high mortality rate (Table 3).

However, twoconditions were tested,including hemolymphthat was eitherrecovered from BgBRE snailsprimed and challenged by SmBRE miracidia, or hemolymph that was primed and challenged by SmVEN miracidia.A significant difference in toxicitywas observed whencomparing mortalities induced by challenged snail hemolymph *vs* naïve snail hemolymph or CBSS culture medium. Hemolymph recovered from challenged snails increasedthe mortality of sporocysts compared to controls (Fisher exact tests: control vs SmBRE/SmBRE hemolymph for SmBRE sporocyst $p = 0.0262$ and controls vs SmVEN/SmVEN hemolymph for SmBRE sporocyst $p = 0.00001$ or for SmVEN sporocysts $p = 0.00001$).However, one exception to this finding occurred for the controls vs SmBRE/SmBRE hemolymph for SmVEN sporocysts (Fisher exact test $p = 0.8633$) (Table 3).Interestingly, the hemolymph recovered from challenged snails always induced greater mortality for homologous sporocysts compared to heterologous ones (Fisher exact tests: SmBRE/SmBRE hemolymph on SmBRE (47.48%) vs SmVEN (23.07%) sporocysts: $p = 0.0000252$ and SmVEN/SmVEN hemolymph on SmBRE (84.19%) vs SmVEN (95.20%) sporocysts: $p = 0.000258$) (Table 3). These results confirmed the specificity in the humoral factors that are released in the hemolymph after the challengethat can participate in the binding, recognition and killing of primary sporocysts.

## 4. DISCUSSION

An acquired resistance following a primary antigenic stimulation or encounter with a pathogen has been demonstrated for numerous animal models in various invertebrate phyla. Arthropods have been the most investigatedgroup (Brown and Rodriguez-Lanetty, 2015; Kurtz and Franz, 2003; Rodrigues et al., 2010; Roth and Kurtz, 2009)butCephalochordata, Echinodermata, Nematoda, Cnidaria, Ctenophora and Mollusca have also been investigated(Jorge Contreras-Garduno et al., 2016; Milutinovic and Kurtz, 2016). Some of these studies demonstrated the existence of a specific immunological memory againstviruses, yeasts,andbacteria. However, these pathogens expressa limited set of potential antigensor pathogen associated molecular patterns (PAMPs)likepeptidoglycans, beta-glucansor Lipo-poly-saccharides (LPS), which can be easily recognised by the host immune system(Jorge Contreras-Garduno et al., 2016).

In the*Macrocyclops albidus*/*Schistocephalus solidus* model (Kurtz and Franz, 2003)and *Biomphalaria glabrata/Schistosoma mansoni* model(Pinaud et al., 2016; Portela et al., 2013), both partners are phylogenetically relatedas they belong to the metazoa, bilateria lineage. This relative phylogenetic proximity is particularly interesting when studyingthe mechanisms involved in the specificity of immunological response. Here, both partners would be expected to shareseveral antigenic determinants (Yoshino et al., 2012), andfine-scale host recognition processes would thus be expected to recognise specific pathogen determinants and to avoid auto-immunity. A genotype-dependant immunological memory has been demonstratedfor *Biomphalaria glabrata*suggesting that snail immune response can distinguish between different parasite speciesand strains(Portela et al., 2013). Herein, we go further in the characterisation of the molecular bases for thisspecific immunological memory response.

An atypical transcriptomic profile was described following a natural primo-infection and challenge in *Biomphalaria glabrata* snails andwas termed an‘immune shift’(Coustau et al., 2016; Pinaud et al., 2016).This response is characterised by an incomplete or absence ofimmune responsefollowingthe primary encounter with *Schistosoma*worms(Portet et al., 2018b).This firstinteractionisconsidered compatible and thus the parasite can developto patencyin snail tissue. At the transcriptomic level, no strong immune response was observedin response to infection.However,

followingthe secondary encounter with the same pathogen, the parasite was unable todevelop into snail tissue as the snails became totally resistant to infection. Only parasites from the challenge were killed,when parasites from the primo-infection persisted in snail tissue producing Sp2 and cercariae that lead to snail death 140 days after infection (Table 1).

Interestingly an analogous situation was demonstrated in experimental schistosomiasis infections in mice and monkeys (Smithers and Terry, 1967). The term ‘concomitant immunity’ has been used to describe the ability of the vertebrate host to mount an effective immune memory response against larval stages of the parasite whilst being unable to clear the persistent adult worms. The adult schistosomes are capable of evading the immune response which they provoke(Smithers and Terry, 1969). As the worms developed, a gradual acquisition of host antigens were detected and provide a possible explanation of concomitant immunity, and demonstrate the ability of adult worms to evade the immune response which acts against the unprotected schistosomula in a challenge infection(Clegg et al., 1971). These host antigens may be molecules of host origin incorporated by the parasite into its surface membrane or molecules synthesised by the parasite that mimic a host antigenic determinant. This refers to Raymond Damian's concept of 'molecular mimicry' or 'antigen sharing' between host and parasite which proposed that one of the mechanisms by which parasites could avoid the host immune response was by mimicking host molecules (Damian, 1997). Carbohydrate epitopes or protein glycosylation were more likely to be mimicked by the parasite and appeared as the best candidate to explain concomitant immunity(Damian, 1987).

In snail/Schistosome interactions, similar processes have been demonstrated and host snail antigens shared with miracidia and sporocysts have been described on parasite larval surfaces and constitute another example of molecular mimicry(Yoshino and Bayne, 1983). In this context, our recent work on miracidium and sporocyst glycoproteins, the *Schistosoma mansoni* polymorphic mucins (SmPoMucs) (Galinier et al., 2017; Mone et al., 2010; Roger et al., 2008a), were identified as relevant carbohydrate or glycan candidates that share potential antigenic epitopes with snail antigensand support the “molecular mimicry” or “smoke screen” strategies developed by the parasite to avoid recognition(Mitta et al., 2012; Mitta et al., 2017; Portet et al., 2017).Thus concomitant immunity may exist solely if

559 efficient immune memory response exists, and this is exactly what we have observed
560 in the present paper.
561 Here, a shift in the immune responsewas observed, followingthe first encounter with
562 the parasite. First, a cellular immune response,clears most of the parasitesthrough
563 hemocyte encapsulation. Then, during the secondary encounter with the same strain
564 of parasite,circulating/plasma factors were involved in the eliminationof the parasite.
565 This shift in phenotype is supported bynumerous differentially regulated immune
566 transcriptsidentified in the RNAseq approach only following the challenge(Pinaud et
567 al., 2016)(Fig.2). This pattern of specific massive transcriptomic response appeared
568 as a characteristic of immunological memory in invertebrates(Greenwood et al.,
569 2017).
570 This pattern was also confirmed in the present study,irrespective ofthe strain of
571 parasite used for the secondary challenge. However, if homologous SmBRE/SmBRE
572 infection and challenge resulted in a total immune protection (100% of snail
573 resistants and 2,013 DE transcripts),the heterologous SmBRE/SmVEN systemsolely
574 induced a partial protection of 71% with 405 DE transcriptsand the SmBRE/SROD
575 system led to 58% protectionwithonly 210 DE transcripts (Fig. 2). However,it would
576 be difficult to assume that thenumber of DE transcriptsalone could explain both the
577 improvement in protectionobserved and the specificity, even if they appeared to be
578 correlated. The correlation between qualitative/quantitative transcript expressionand
579 protection in homologous or heterologous challenges has to be considered in
580 association with their potential involvement in pathogen recognition, pathogen killing,
581 specificity andmemory(Figs. 2 and 3).
582
583 **4.1. Immunological memory against *Schistosoma sp*.**
584 Transcripts in the cluster 1 areup-regulated and correspond to the basal
585 challenge response observed for all the parasite strains, symmetrically, those in
586 cluster 14correspond todown-regulatedtranscripts (Fig. 2). Within these clusters, we
587 identifiednumerousfactors alreadyimplicated in immune response pathways. Within
588 immune recognition molecules or effectors, the lectin familiesare considered
589 recognition factors in *Biomphalaria glabrata* snails. Lectins are defined as molecules
590 that containa lectin domain, previouslydescribed as carbohydrate-binding
591 proteins(Dheilly et al., 2015; Portet et al., 2017; Tetreau et al., 2017; Wu et al., 2017).
592 The presence of macrophage mannose receptor family members, FREP3.3, C-type

lectin and selectin in cluster 1,highlights the importance of pathogen sensing inresponse to the secondary challenge. Lectin families have been shown to be highly diversified and polymorphic to cover an important range of pathogen diversity (Dheilly et al., 2015). This diversity should be enoughto face the potential parasite strains/speciesantigenic diversity and to support the specific immune response observed herein. Numerous immune response-associatedgenes are frequently found in deep-sequencing analyses but their role within the context of immunity was seldom discussed.For example, snail mucins and collagens are often described as components of the extra-cellular matrix, but they must also be involved in cell motility or migration(Hunt et al., 1996). An up-regulation ofthese variantswithin these families may induce several pathways implicated in the recruitment of competent cellsin order to block incompetent cellsor pathogens as previouslydescribed for *Streptococcus* infection of macrophages(Dhar et al., 2017).

The biomphalysinappeared as a particularly relevant candidate.As a beta-pore forming toxin (Galinier et al., 2013),it remains the sole candidateable to directly kill schistosomes(Galinier et al., 2013). This highly toxic molecule has already been described in the immune memory protection observed after challenge in *Biomphalaria* snails(Pinaud et al., 2016). In the present work,its presence and its expression increase against all homologous or heterologous tested strains and species of parasites (Figs.2 and 3) and isidentified in cell-free hemolymph immediately following the challenge (Table 2). This circulating toxin, may be potentially released by competent immune cells that have been trained following the primary infection, and couldbe considered as the primaryfactor supporting immune shift in *Biomphalaria*immunological memory.Different biomphalysin transcripts are identified in massive transcriptomic approaches and these transcripts are difficult to align withreference biomphalysin genes,leavingthe possibility that several isoforms of this toxin could exist in *Biomphalaria* snails (Fig. 2B and Supp-Table 2). Label free proteomic (Table 3) and comparative interactome experiments between living pathogens and snail hemolymph demonstrate that it is often difficult to assign the identified peptides to a specific biomphalysin gene(Galinier et al., 2013; Tetreau et al., 2017). Altogether, these results provide clues for the potential role of this toxin in the humoral immunological memory responseobservedin *Biomphalariaglabrata* snails.Other immune effectors like lbp/bpi or mytimacin, described as antimicrobial peptides (AMPs) or proteins (Adema et al., 2017; Baron et al., 2016; Baron et al.,

2013), can also participate in the regulation of the immune response (Hu et al., 2017), displaying cytokine functions, attracting haemocytes towards sites of injuries (Destoumieux-Garzon et al., 2016; Li and Song, 2010).

Finally, thioester-containing proteins (TEP) are complement-like proteins that act as opsonin, promoting phagocytosis, or lysis and sometimes even melanisation, in invertebrates (Le Clec'h et al., 2016), and arthropods (Blandin and Levashina, 2004; Buresova et al., 2011; Volohonsky et al., 2017) and appear to also participate in primary cellular immune response and encapsulation in *Biomphalaria glabrata* snails (Mitta et al., 2012; Mone et al., 2010; Portet et al., 2018a). Following immunological memory acquisition in *Biomphalaria,* the cellular immune encapsulation response shifts to a humoral response (Pinaud et al., 2016) and is associated with the down-regulation of TEP in the homologous challenge RNAseq data 15 days after the primo-infection (Figs. 2 and 3) as well as the absence of TEP in 1DC proteome (Table 2).

### 4.2. Total protection in homologous challenge

Followingthe homologouschallenge, we observed a total protection. Immune recognition molecules and effectors were particularly up-regulated with an increase in both, theexpressionand diversity of different lectins (Fibrinogen-related proteins (FREPs), c-type lectin, and galectin-4) (Fig. 2 and 3, Supp Fig. 2).

In this context, FREPs wereof particular interest. These molecules have been highlighted in severaldifferent contexts in the study of *Biomphalaria* snails, (i) pathogen recognition (Mone et al., 2010; Roger et al., 2008b), (ii) resistance against parasites(Hanington et al., 2010; Hanington et al., 2012) and (iii) innate immune memory(Pinaud et al., 2016).RNAseq results showed that following a homologous challenge,the diversity and expression of FREP transcripts increase (Fig. 2, 3, and Supp Fig. 2) compared to heterologous challenges, leading to a potential increase in the ability of the host to recognise/bind to the encountered parasite following the challenge.Certain BgFREPs have been shown to form multimers, viewed using native PAGE or SDS-PAGE gels as noncovalent or covalent complexes(Adema et al., 1997; Zhang et al., 2008). For example, BgFREP4 is commonly found as a tetramer in snail plasma(Gordy et al., 2015; Zhang et al., 2008). Moreover, even if not demonstrated for *Biomphalaria* snails, other lectinslike the galectin are known to form multimers and their association leads totheactivation of bactericidal activity in

mammals and molluscs(Blazevits et al., 2016; Kawsar et al., 2011). Thus, we can hypothesizea putative dual-role of theselectin families, with both single-member pathogen recognition/binding and association with different members in dimer/multimer, as well as homo or hetero-multimers,providing additional functions to proteins that participate in an immunological response. Multimerization could be an additional process to increase the mollusc pathogen recognition repertoire, by increasing binding avidity or enhancing pathogen recognition specificityand making the invertebrate immune response more efficient(Schulenburg et al., 2007).

In the present study, we observe a complex expression pattern of diverse immune recognition molecules and/or effectors that drives a total immune protection following a homologous challenge. This complexity of immune recognitioneffectorsresults from the complexity of the antigenic landscape offered by *Schistosoma* parasite and may explained why previous RNAi studies targeting solely the FREPs or the BgTLR, resulted only in the partial invalidation of snail resistance or immunological memory(Hanington et al., 2010; Hanington et al., 2012; Pila et al., 2016; Pinaud et al., 2016).

### 4.3. Vaccination as proof of immune memory

Vaccination is the process whereby an individual is made immune to an infectious agent. This immunization with pathogen extracts, stimulates the immune system and yields a state of protection (i.e., immune response is stored and recalled) against subsequent infection. Thus, immunization is a tool that has the capacity to demonstrate the existence of an enhanced immune activity or immune memory in invertebrates. Here, vaccinations were developedusing native parasite extracts that correspondto a complex molecular extract containing proteins, sugars, salts, ions, hormones, nucleic acids, or other potential viral or bacterial associated organisms fromthe parasite. Thus it can be difficult to conclude which specific compounds (proteins, sugars, nucleic acids)were responsible for immunization and for the acquisition of resistance in immunized snails.However inflammatory response associated with needle injection of saline buffer (used as control of injection) was notenoughto activate protection(Fig.4). An absence of protection was also observed following immunization with hepato-pancreas extracts isolated from healthy individuals (used as Sp2 control injection) (Fig. 4). A tissue extract fromindividual snailswasnot enough to immunize recipient snails against*Schistosoma*infection.

However , vaccination with primary sporocyst extract (Sp1) was able toinduce a significant protection (47.9% of snails not infected following exposure), vaccination with Sp2 extract seemedto havea slight effect (not significant) and vaccination with cercariae extract did not protect snail at all (Fig. 4).While the snail immune system seemed able to recognise foreign pathogenic antigens and could activate an adapted and efficient immune response against them, it seems that it was unable to recognise antigens belonging to snails with same genetic background.

Efficient recognition of Sp1 suggests that evolutionary pressurestargeted recognition to the parasite's first larval stage,one could thusexpect that the Sp1 was the most immunogenic in comparison with those that developed later (Sp2 and cercariae).This observation fits well with the "molecular mimicry" hypothesis (Damian, 1987, 1997) and the gradual acquisition of host antigens by parasites during their development within host tissues.

Finally, snails are better protected when vaccinated with homologous parasite extracts than with heterologous ones. These results revealed specificity of immunological memory in*Biomphalaria glabrata*. However, vaccination with Sp1 extracts from SmBRE, SmVEN or SROD neitheractivatednor induced a total protection against reinfection, as it can be observed following natural infection. Vaccination with whole extracts of SmBRE Sp1 induceda partial protection against the homologous SmBRE challenge while heterologous Sp1 vaccination (SmVEN, SROD)did not results inany protection (fig. 5).Even immunizations with sympatric Sp1 or pools of whole parasite extracts cannot induce a total protection (Fig. 4B). This suggests that other immune pathways or mechanisms haveto be activated to reach a 100% of protection. In this context, the parasite's development within snail tissues may be the source of a permanent snail immune stimulation through the release of proteins,nucleic acids, or excreted/secreted productsby the parasite (Guillou et al., 2007; Lodes and Yoshino, 1990; Nowacki et al., 2015; Yoshino et al., 2014).

So, if vaccinationreveals sucha high degree of efficiency and potential specificity in the reaction of the *Biomphalaria glabrata* immune system, itdid not succeed in inducinga complete protection for all individuals tested within a heterogeneous population.The RT-q-PCR experiment that was based on a few candidate genes selected because they were differentially regulated in the massive transcriptomic approach, confirms that vaccination did not perfectly mimic natural

infection (Supp Fig. 1).Together, vaccination experiments demonstrate unequivocally the acquisition of immunological memory in *Biomphalaria* snails. Vaccination in invertebrates with economicimportance remains mainly focused on viral disease where successful vaccinations have been demonstrated(Chang et al., 2017; Green et al., 2016; Lafont et al., 2017; Syed Musthaq and Kwang, 2014; Taengchaiyaphum et al., 2017).However, our results onvaccination in the schistosomiasis vector snail *Biomphalariaglabrata* presents the first anti-metazoan vaccination in an invertebrate buthighlighted also the necessity to conduct additional investigations on the process of vaccination in molluscs and more generally in invertebrates.

### 4.4. Cell-free hemolymph transfer

When hemolymph is recovered 1 day after the immune challenge and injected into naïve snails, a high protection against infection (51% of snails protected following exposure) is observed(Fig. 5).Other plasma samples tested did not transfer any significant protection(Fig. 5). This result suggests that secretion of relevant immune factors into the plasma occurs early after the challenge and participates immediately in the defence of the organism against newly infecting parasites, while those from the primo-infection remained unharmed. These active molecules may decrease in the hemolymph as they are consumed in the anti-parasitic response. This may explain why the 4DC and 15DC cell-free hemolymph no longer provide protection when transferred to naïvesnails(Fig. 5).These factors seem important in support of the immune humoral response observed following secondary challenge infection and in the killing of the parasite. The label-free proteomic approach (Table 2) helped to identify some of these factors and in particular, the biomphalysin, whichmight act as the main humoral factor responsible for this immunological memory response.

Moreover, while natural infection induces a 100% protection to secondary infection, vaccination and cell-free hemolymph transfer only reach around 50% of protection. This difference would be resolved by the sequence composition of plasma and parasite protein extract, but this must bestudied independently. Immunization means that the parasite -exogenous- molecule can activate the host’s immune system and can lead to anacquired immune resistance while the plasma transfer experiments confirm that circulating host molecules from infected individualscan activate the immune system of naïve individuals to reach a comparable immune protection.

### *4.5. In-vitro* toxicity and specificity of primed plasma

Toxicity assays show for the first time, the implication of humoral factors in the specificity of parasite clearance in an invertebrate. Homologous system displaysa higher *in-vitro* toxicity despite the origin of the strain used (Fig. 6 and Table 3).Immune factors are produced and secreted in the hemolymphcompartment to specifically bind and killthe homologous parasite strainsmore efficiently than the heterologous ones (Table 3). These results suggest that the genotype-dependent specific memory response in *Biomphalaria* is supported by complex repertoires of circulating recognition molecules or effectors secreted in the hemolymphearly after the challenge.

Cytotoxic activities may be due to some toxic molecules like the biomphalysin(Galinier et al., 2013; Pinaud et al., 2016) (Table 2). This beta-pore forming toxin may be transferred during vaccination and may remain toxic 15 days after injection to protect more than 50% of the tested snails (Fig. 6). Interestingly, biomphalysin isthe mainprotein released in the hemolymph1 day after the challenge (Table 2, Fig. 2 and 3)that can participate in parasite killing after challenge (Galinier et al., 2013; Pinaud et al., 2016).Moreover,a recent interactome approach revealed that different biomphalysin variants were found to interact with different pathogen species (bacteria, yeast, trematodes)(Tetreau et al., 2017) (Supp-Table 2), arguing for the potential role of biomphalysin in both immunological recognition/binding and pathogen clearance,leading to a higher level ofspecificityofimmunological memory responsein *Biomphalaria* snails.

### 4.6. Combinatorial repertoire of host recognition molecules and effectors to face the very large number of parasite epitopes

The peptidoglycan recognition protein family, FREPs, C-type lectins, macrophage mannose receptors, TLRs, selectin andgalectin are all members of different putative immune recognition superfamilies(Adema et al., 2017; Dheilly et al., 2015; Ferrandon et al., 2007) (Figs. 2, 3). They contain domainscapable of directly bind topathogens ascarbohydrate recognition domain (CRD), fibrinogen (FBG) or immunoglobulin (Ig) for the FREPs, the leucine-rich repeats (LRR) for TLR or carbohydrate-binding protein domain for C-type lectins(Ferrandon et al., 2004; Pasare and Medzhitov, 2004). FREPs or C-type lectin, for example,were previously described in the interaction between *Biomphalaria* and *Schistosoma* in immune protein

complexes(Mone et al., 2010; Tetreau et al., 2017; Wu et al., 2017) as well as in transcriptomic response(Hanington et al., 2012; Pinaud et al., 2016). Herein, we reveal for the first time, the complex repertoire of recognition and effectorfactors requiredto express an efficientimmunological memory response (Fig. 3 and Supp Fig. 2).These putative recognition molecules oreffectorsneed to be produced as extracellular or humoral factorsin order to cooperate altogether(Hargreaves and Medzhitov, 2005; Schulenburg et al., 2007). Such moleculesmayby successively expressed orassociated with each other to work in coherent multimers to increase recognition capabilities or to enhance substrate affinity(Brewer et al., 2002; Choe et al., 2005; De Marzi et al., 2015; Hargreaves and Medzhitov, 2005; Kang et al., 1998; Lee and Lee, 2000; Sharma et al., 2011; Wang et al., 2008).Together, the complex regulation and/or selection of different types of recognition molecules and effectors from the innate immune system certainly reflects the great antigenic complexity of immunogens facing the host. This complex response involving a multitude of often polymorphic genotype-dependent elements that, independently of each other or perhaps in combination with each other, contributes to provide the *Biomphalaria* snail with an efficient and specific immunological memory response. Recently we hypothesised that a molecular or epigenetic reprogramming of hemocyte sub-populationsmightallow for the expression of a higher quantity and diversity of immune recognition molecules and/or effectors, and support theimmunological memoryspecificity in*Biomphalaria*snails (Gourbal et al., 2018).We are convinced that investigating themolecular mechanisms supporting hemocyte reprogrammingwould now be mandatory to better understand the innate immunity, specificity and memory in invertebrates, and to decipher the overall complexity, originand evolution of metazoan innate and adaptive immune systems.

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*Author contributions*

SP, AP, JFA, RG, DD, BG design and performed the experiments and analysis. CSB, NA participated in animal breeding and parasite experimental infections. LB conducted the Label free proteomic experiments. SP, LdP, BG drafted the manuscript. JFA, DD, RG, LdP participated in the reviewing process. All authors read and approved the final version of the manuscript.

All data generated or analysed during this study are included in this published article [and its supplementary information files]. Transcriptome of *Biomphalaria* snail is available at: http://ihpe.univ-perp.fr/acces-aux-donnees/).

*Conflict of interest*

The authors declare that no conflict of interest exists.

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**Figure 1. Overview of experimental protocols and results**.

**Figure 2. Transcriptomic response exploration of innate immune memory specificity**.

(A) Venn diagram of differentially expressed (DE) transcripts following homologous or heterologous challenges. Snails were primo-infected by SmBRE and then challenged by SmBRE, SmVEN or SROD parasites. A correlation is observed between the level of protection following challenge and the number of DE transcripts. The homologous interaction SmBRE/SmBRE: 100% of protection and 2,013 specific DE transcripts;heterologous SmBRE/SmVEN: 71% of protection and 405 specific DE transcripts; heterologous SmBRE/SROD: 58% of protection and 210 specific DE transcripts. (B) Heat mapof differentially expressed transcripts following homologous and heterologous challenges. Each line represents a DE transcript (padj value <0.05), each row represents the strain of parasite used for the challenge. Colours represent the level of expression, green for down-regulated transcriptsand blue for up-regulated transcripts. Last column of the heat map highlighted the immune related transcripts identified based on Blast2Go annotation, domain search and known immune genes of the literature. Names in bold correspond to transcripts that have been yet described as involved in *Biomphalaria* / *Schistosoma* interaction in the literature.

**Figure 3. Immune recognition-related transcripts following homologous or heterologous challenge.**

(A) Graph showing cumulative Log2FC of DE immune recognition-related transcripts identifiedin BgBRE snail primo-infected by SmBRE and challenged with homologous (SmBRE) or heterologous (SmVENor SROD) parasites. Each colour in the graph correspond to a different DE transcript. (B)Venn diagram that indicates a relationship between the number of DE transcripts related to immune recognition and the homologous/heterologous associated protection (C)Heatmap of DE transcripts related to immune recognition.Each line is a DE transcript (padj value<0.05), each row the strain of parasite used and the colours depend on the level of expression, green for down-regulated and blue for up-regulated transcripts.Last column of the heat map indicates the immune receptor orrecognition transcripts identified in each cluster. Transcript annotations were defined using Blast2Go annotation, Blastp against opened protein databases, domain searches and data from the literature.

**Figure 4. Vaccination with *Schistosoma* parasite extracts.**

(A) Representation of *Schistosoma* parasite stages development into snail tissues (B) Prevalence observed following *SmBRE* infection in *BgBRE* snails previously vaccinated with different parasite native extracts (Miracidia, Sp1: Primary sporocyst, Sp2: Secondary sporocyst, Cercaria and an equimolar pool of Miracidia, Sp1, Sp2 and Cercaria.)

**Figure 5. Homologous versus heterologous vaccination with sporocyst extracts**

Prevalence observed following *SmBRE* infection in *BgBRE* previously vaccinated with sporocyst (Sp1) native extracts from homologous (*SmBRE*) or heterologous (*SmVEN*, *SROD*) parasites.

**Figure 6. Protection acquired following hemolymph transfer**

Protection against SmBRE infection observed in naïve BgBRE snails following different hemolymph transfer treatments. Naïve BgBRE snails were subjected to injection with, (i) saline solution (CBSS); (ii) cell-free hemolymph (plasma) of naïve snails, (iii)plasma from BgBRE snails primed with SmBRE; or (iv) plasma from BgBRE snails challenged with SmBRE and recovered at 1 day, 4 days or 15 days after challenge. Protection has been estimated using the following formula: (control prevalence-experimental sample prevalence)/(control prevalence). Control prevalence correspond to prevalence calculated for untreated snail following infection with 10 SmBRE miracidia.

**Supplementary figure 1. Q-RT-PCR expression of innate immune memory candidate genes following vaccination.**

BgBRE snails were either vaccinated or primo-infected with SmBRE Sp1 and gene expression wasquantified 15 days after treatment. Q-RT-PCR was conducted after vaccination and gene expression was compared to RNAseq data following primo-infection. See supplementary table 1 for gene details.

**Supplementary Figure 2. RNAseq-based FREP expressions following homologous or heterologous challenges.**

Histogram displays cumulative RNAseq Log2FC of differentially expressed FREP-related transcripts identified in BgBRE primo-infected by SmBRE and challenged with homologous (SmBRE) or heterologous (SmVEn and SROD) parasites.

Table 1. **Long-lasting immunological memory in *Biomphalaria glabrata* snails.**

| | Prevalence primo-infection BgBRE/SmBRE | |
|---|---|---|
| **BgBRE** | N=15 | 100% |
| | **Prevalence challenge BgBRE/SmBRE/SmBRE** | |
| **15 DPI BgBRE** | N= 15 | 0% |
| **25 DPI BgBRE** | N=15 | 0% |
| **56 DPI BgBRE** | N= 15 | 0% |
| **72 DPI BgBRE** | N= 15 | 6% |
| **140 DPI BgBRE** | N= 15 | 0% |
| **162 DPI BgBRE** | N= 15 | NA |

BgBRE/SmBRE: BgBRE snails primo-infected by SmBRE.
BgBRE/SmBRE/SmBRE: BgBRE snails primo-infected by SmBRE and Challenged by SmBRE.
DPI: days post-primo-infection.
NA: not available.

**TABLE 2: Label free proteomic of cell-free hemolymph**

| 15DPI | | | | | 1DC | | | Accession # (Biomphalaria transcriptom |
|---|---|---|---|---|---|---|---|---|
| Pep | SC | SSC | WSC | Pep | SC | SSC | WSC | |
| | | | | 8 | 11 | 2 | 6.47 | BV_Locus_580_Transcript_5/11_Confidence_0.412_Len |
| | | | | 7 | 10 | 2 | 5.8 | BBVBR_Locus_10747_Transcript_2/13_Confidence_0.4 |
| | | | | 3 | 3 | 3 | | cdhitest_out_45823 |
| | | | | 3 | 3 | 3 | | Bre_Locus_19841_Transcript_1/1_Confidence_0.000_Le |
| | | | | 3 | 3 | 3 | 3 | BB_Locus_1030_Transcript_1/1_Confidence_0.000_Len |
| 2 | 3 | 3 | 3 | 2 | 3 | 3 | 3 | Locus_37965_Transcript_3/3_Confidence_0.000_Lenght |
| 5 | 7 | 2 | 4.95 | 6 | 12 | 1 | 12 | Locus_628_Transcript_1/1_Confidence_1.000_Length_ |
| 2 | 2 | 2 | 2 | 4 | 7 | 5 | 7 | cdhitest_out_91870 |
| 2 | 2 | 2 | 2 | 3 | 5 | 5 | 5 | cdhitest_out_9901 |
| 2 | 2 | 2 | 2 | 4 | 4 | 4 | 4 | BBVBR_Locus_10035_Transcript_8/8_Confidence_0.091 |
| 1 | 1 | 1 | 1 | 4 | 4 | 4 | 4 | Locus_36128_Transcript_7/21_Confidence_1.000_Leng |
| 1 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | BBVBR_Locus_5580_Transcript_1/1_Confidence_0.000_ |
| 1 | 1 | 1 | 1 | 2 | 3 | 3 | 3 | cdhitest_out_3201 |
| | | | | | | | | BB_Locus_1608_Transcript_4/7_Confidence_0.182_Len |
| | | | | | | | | Locus_900_Transcript_90/213_Confidence_0.000_Leng |
| | | | | | | | | BR_Locus_1488_Transcript_3/5_Confidence_0.300_Len |
| | | | | | | | | BR_Locus_4448_Transcript_2/6_Confidence_0.429_Len |
| | | | | | | | | BV_Locus_3738_Transcript_3/12_Confidence_0.667_Len |
| 3 | 3 | 1 | 3 | | | | | BB_Locus_2700_Transcript_1/1_Confidence_0.000_Len |
| 2 | 2 | 2 | 2 | | | | | BV_Locus_1834_Transcript_29/75_Confidence_0.241_L |
| 3 | 3 | 2 | 2.5 | | | | | Locus_455_Transcript_36/36_Confidence_0.000_Lenght |
| 2 | 2 | 2 | 2 | | | | | BBVBR_Locus_1451_Transcript_1/2_Confidence_0.333_ |
| 2 | 2 | 2 | 2 | | | | | BB_Locus_173_Transcript_4/8_Confidence_0.310_Leng |
| | | | | 3 | 3 | 3 | 3 | BR_Locus_2953_Transcript_2/2_Confidence_0.000_Len |
| | | | | 2 | 4 | 4 | 4 | BBVBR_Locus_7868_Transcript_1/1_Confidence_0.000_ |
| | | | | 2 | 2 | 2 | 2 | BB_Locus_5998_Transcript_1/1_Confidence_0.000_Len |
| | | | | 3 | 3 | 3 | 3 | BBVBR_Locus_230_Transcript_32/53_Confidence_0.081 |
| | | | | 2 | 2 | 2 | 2 | BB_Locus_118789_Transcript_1/1_Confidence_0.000_L |
| | | | | 2 | 2 | 2 | 2 | Locus_35590_Transcript_13/16_Confidence_1.000_Len |

Pep: Number of identified peptides
SC: Spectral Counts : mass spectra allow the identification of peptides belonging to the protein
SSC: Specific Spectral Counts : mass spectra allow the identification of peptides belonging specifically to the protein
WSC: Weighted Spectral Counts : SSC + the weight of unspecific SC (in the case of peptides shared between several proteins)
Biomphalaria transcriptome and proteome were availables at: http://ihpe.univ-perp.fr/acces-aux-donnees/
15DPI: 15 days post primo-infection
1DC: 1 day post challenge

Table 3. Cell-free hemolymph toxicity

| | | % of mortality | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sporocysts SmBRE | | Sporocysts SmVEN | | Sporocysts SROD | |
| **CBSS culture medium** | | N= 71 | 23.94% | N= 89 | 21.34% | N= 72 | 89.47% |
| **CBSS supplemented with** | **naive hemolymph** | N= 60 | 21.05% | N= 118 | 17.41% | NA | |
| | **SmBRE/SmBRE hemolymph** | N= 106 | 47.48%[a,b] | N= 143 | 23.07%[b] | NA | |
| | **SmVEN/SmVEN hemolymph** | N= 203 | 84.19%[a,c] | N= 162 | 95.20%[a,c] | NA | |

a, Fisher exact test < 0.05 compared to controls
b, Fisher exact test < 0.05 comparing SmBRE or SmVEN sporocysts with SmBRE/SmBRE hemolymphe
c, Fisher exact test < 0.05 comparing SmBRE or SmVEN sporocysts with SmVEN/SmVEN hemolymphe

NA: not available

Supplementary Table 1. Primers of Q-RT-PCR

| Transcript names | Annotations | Primer names and sequences [§] | |
|---|---|---|---|
| BBVBR_Locus_52081_Transcript_1/1_Confidence_0.000_Length_987 | fibrinogen-related protein 3.3 [Biomphalaria glabrata] | Frep3.3 | Forward: CCTCTGTCGAAGGCGAACTCTATT |
| | | | Reverse: AACGTCGGCCTTTCAACAGTTATT |
| BB_Locus_14654_Transcript_1/1_Confidence_0.333_Length_433 | PREDICTED: c-type lectin | C-type Lectin | Forward: TAATCGCGAGCTGTCACCACAA |
| | | | Reverse: AGCGTCCAGCCGTTACAATACA |
| BBVBR_Locus_2588_Transcript_1/2_Confidence_0.667_Length_595 | uncharacterized protein [Aplysia californica]_&_selectin partial | Selectin | Forward: GGGCGTTTGAATGGCGATGAAT |
| | | | Reverse: TGCATTTCGTCGAGGAGGTGTT |
| BB_Locus_44392_Transcript_1/3_Confidence_0.667_Length_1083 | putative glutamate synthase [NADPH]-like isoform X1 [Aplysia californica] | Glutamate synthase | Forward: TGCAGGTCCACTGCCAATGATA |
| | | | Reverse: CTCCTTGTGAGGGAGCATGTGT |
| cdhitest_out_225206 | thioester-containing protein [Euphaedusa tau] | TEP | Forward: GGCGTGGAATCAATCTGGTCTA |
| | | | Reverse: TGGAAGTGAGTCTGATGGTGTT |
| BB_Locus_4989_Transcript_4/4_Confidence_0.000_Length_1252 | biomphalysin [Biomphalaria glabrata] | Biomphalysin | Forward: CGTGGAGGAATGGTTTGGGTAGT |
| | | | Reverse: TCAACGACAAGCAGCTGGAAGA |

[§] Synthesized by ©Eurogentec, Belgium Sepop Desalt

Supplementary Table 2. Biomphalysin diversity

| Up-regulated Biomphalysins following challenge | | | | | | Log2FC vs control | | |
|---|---|---|---|---|---|---|---|---|
| Transcript iD in RNAseq | Sequence | Blast2GO Annotation | Locus iD [1] | Transcript iD [2] | protein sequence | SmBRE | SmVEN | SROD |
| BB_Locus_4989_Transcript_4/4_Confidence_0.000_Length_1252 | GTAGGGAATAGTTGTTCTGGGTCTTACCCACCATAACCGTATACTTTGC TGCTGATCGTCGAGCAATGGTTGGGCTAGTTGATATTTAAGTCGTATC TGACTTCATTTGGCTATTTTTGTTTGTTGTTGATCTCGAATACTCAAA AGCTGTTTTATAAGACAGCGATATTTTCAAAACATCACCCAAGTCAAA GTTGAGTGAAACTGAAACTTCGTGACTATTCTTCCAGCTGCTTGTCGT TGAATGCTCTATTGTTTCCGTTACTGTCTTTGAATATTCAAATAGTTT GGAAGAGCTTTCATCCAGATGGTTGAACTCTACGCCAGTATGTACAGT ATCTGGTTTCAATTCATCAACAACACTTTGCCCATATTTAATTTCTTT GACAGCTAAATTCCAATTAATATAATCAATGGCAAGTCTTTCGTAACA TTTGTAACCTTCACAATATATTAACGGCCAAAAAAGCCTCGAATCAGC CACCCATGAATCTCCGCTTCTGTAAAAATCTCTCCACAATTGACTTC TTTAGTACCCAAACCCCAGCCGTAGCCAAGTAGATGCGCCAGATTGGC CAGGTTCCCACAACGTTGTGTCCATAATGTTTGTCTCAGTTTATGTAT ATCATCAATTACCATTGGTTTGTCTGCCATCTTACAACATTGGAGACT ATCTAAGCAAATGCAAGTAGTGCAGTTGCTTTGTAGAGACGCGGTTAT ATAATAACCTGCTTTGCAAGAATATAAACCTTTCCTAATAAAGTTTAT GTTATAGACGTAGCAATCCCCATAATAGAAAGGGTGATTGCGCTGGCTT TGCGCACCTTGCATCTTCAATCTGCCCTAAATAGCCTTCGCTTATAGA CAAGGATCGATACAAACCATGAAGAAAGAAACTCCCGGCCAATTAAC CCAAACGTCAAATCTATCTAATTTGTACGTCCAGTCATCGTAGACAAC TTGAATCTCGCTGTTTGACCACTGGTGTGGGGCGCTGCAGCATTGTAC GCCTTCTAAAAGATACAGCTTGTCGTCGTCCATGTATGTGTTTCTGTC GAAGCCGTGGATATAGTATTGAGATTCTTTGCATACAGATAGTCCAGT GGAATCAAAGCTGTGCAACCAACTTTGTATGTACATAAGGGTTTGC GGTTTTTATTAGTGTCACACTGACACAGATGAATAAAAATAACTTCAT TACTGCCAAATAAAGTAGTTTATTCTGGAATTGTTTAAGCAGAACAAG CAGT | biomphalysin [Biomphalaria glabrata] | LGUN_random_Scaffold6076 | BGLB000076 | MKLFLFICVSVTLIKTANPLCTYTSWLHSFDS TGLSVCKESQVTIHGFDRNTYWDDDKLYLLEG VQCCSAPHQWSNSEIQVVYDDWTYKLDRFDVW VNCPAGFFLHGLYRSLSISEGYLGQIEDARCA KPANHPFYYGDCVVYNINFIRKGLYSCHAGYY ITGLVKSNCNYLRCLDSLQCCKMADKPMVIDD VHKLKTNIMDTTLWNLANLLGYGWCLGTK GINVGEDFYRSGDSWVADSRLFWHLTYCEGYK CYERLAIDYINWNLAVKEIKYQGSVVDELKPD AVHTGVEFWHLNESSSKLFEYSKTVTETIEHS TTSSWKNSHEVSVLNFPLGDVLKISLSYKTA FEYSRSTTNRNSQMKSDTLKISTTQTIPPRSA AKYTVMVGKTRTTIPYTAVIIAKFNVKFRGFL RWGKGYGSPITWYHYKHKGSGDRPTVPYTFGD QSEAFYTALKRESETQAHFWLHNEMIRNYFSA RQLINRLTNETQYEFTLNGKLEHVAGTKIDVI WEKARLNRRSIRDKIKSSTPQLDNSTFFATVG PNDKPVDVKYPEVVLVNSEPFQFESIPFDPDS N | 1.583538 | 1.13634 | 1.40829 |
| BB_Locus_121387_Transcript_1/1_Confidence_0.800_Length_221 | TTGATCGCCGATTCAATCTCGCTTTCTCCCAGATGACATCAATCTTTG TTCCCGCAACATGCTGCAGTTTCCATTCAGTGTAAATTCATATTGAG TCTCATTTGTGCAGTCTATTAATGAGTTGACGTGCTGAAGGGTAGTTCT TTATCATCTCGTTCCACAACCAAGGCATAGATTGAGTTTCGCTTTCTC GTTTAAGAGCTGTGTAGAAAGCTTCAGAT | biomphalysin [Biomphalaria glabrata] | LGUN_random_Scaffold579 | BGLB000125 | MRLFLFPCVSVTLIKTANPLCTNTSWHHSFDS AGQEACKESEYYIHGFDRNTYWDDDKLYLLEG VQCCSAPHQWSNSEIQVVYDDWTYKLDRFDVW VNCPAGFFLHGLYRSLSISEGYLEQIEYARCT KPANHPFYYGECYVQNINFITKGLYSCNAGYY ITGLYKGNCNNLRCLDSLQCCKMADKPMVIDD IHKLKTNIMDTTLWNLANLALLGYGWCLGTK GINVGEDFYRSGDSWVADSRLFWHLTKCEGDK CNERLAIDYIDWNLAVKETKYQGSVVDELKPD AVHTGVEFWHLNESSSKLFEYSKTVTETIEHS TTSSWKNSHEFSVSLNFNLFNILQTSLSYKTA FENSRSTTDTNSQMKSDTLKISTTQTIPPRSA AKFTVLVGKTRTTIPYTAVIIAKFNVKFRGFL RWGKGPGSPTTWYHHQHKGSGDRPTVPYTFGD KSEAFYTALKRESETQAHFWLHNEMIRNYFSA RQLINRLTNETQYEFTLNGKLEHVAGTKIDVI WEKARLNRRSIRDKIKSSTPQFDNSTFFATVG PNDKPVDVKYPEVVLVNSEPFQLESIPVDPNS I | 1.532214 | 0 | 1.37917 |
| BBVBR_Locus_58035_Transcript_3/3_Confidence_0.000_Length_1115 | TGATAATTAGTTGTTGGGCTACTGAAGCCTTGCCCAAATCTTAAGAAT CCCCTAAGTCCCACGCTGAACTTGGCAATGATGACAGCCGTATAAGGA ATTGTTGTTCTGGTTTTGCTCATCTTAATTGTGTACTCTGCAGCTGAG TGAGGAGGGATGGTTTGGGTAGAAGTCGTTTTTAAAGTATGTGACGTC GTTGGCTGTTTTCCTTGTTAGTCAATTGGGAATACTCAAAAGCTACT TTAAATGTCCTTGATGCTTTGCCTATATCTTTTAACCCTAACTCCAAT GAAAACGATAATTCTAGGCTAGTCTCCAACTGCTGGTCATTGAATGT TCAATTGTATCTATGACTGCATATGAACGCTCAACTGCTACCGTAGAG CTAGTATTCAGGTGGTTTCTTTTTGGCCACTTTCTATATTTTCTGTA TTTAATTTATCAATAACCTTTTGTCCATATTTAATTTCCTTTACAGCT AAATTCCAATTTCTGTAATCAATGGCAAGTCTTTTATCACACTTGTCC CCTTTACAATAGTACTCTGTGGTTAGCCACCCAGGAATCGTGCTCTCTC CTAAAATCTTCACCTACGTTGACGCCTTTGGTACCGTAGCAATAGCCG TAGCCCAGTTTGTCCGCCAGATTGGCCAAGTTCGTCAATGTTGTGTCC ATAACTTCGTCTTCAGTTTATCGACATCGTCTATTATCTCTGGTTTG TCCGCCATCTTACAACAATAGAGTTTATCTATACAATGAAGGTAGTCA CAGTTACCTTTATATAGGCCTTTTACATAGTAACCTTCGTTGCAAGAA AATAAACCGTTCTCGTTAAAATTTATGTCCTGACTATAACAGTCGCCA TAATAGAAAGGGTGTTCGGCTGGTTTAGTGCACCTTGCAGTTTCAATA TGATATAAGTAGCCTGCGTACCAGGGCCATCCTGAATCTGTTCGCAGT AAACCCTGAAGAAAGAATCCCGTAGGGCAATTAGCCCATGCGTTATAT TTATAATACAATGTAGGTACCCAGTCAGCGTACACAACTTCGAAGCTCA CCGTTGGACCACTGAGATGGGGCACTGCAGCACTGTACACCTTCTAAA TAAGTTAACTG | biomphalysin [Biomphalaria glabrata] | LGUN_random_Scaffold3364 | BGLB021232 | MFVLKFGVLLALIGKGSALCANTSWWDSFNSP GQSKCNEANFYINGLSRNEYKKDQLTYLEGV QCCSAPSQWSNGELQVVYADWVPTLYYKYNTW ANCPTGFFLQGLLKTDSGWFWAGYLYNIETA RCTKPAEHPFYYGDCIPQDINFNEWGLFSCNE GYYVKGLYKGNCDYLHCIDKLYCCKMADKPEI IDDVDKLKTKIMDTTLTNLANLADKLGYGYCY GTKGVNVGEDFRREHDSWVANTEYYCKGDKCD KKLAIDYFNWNLAVEEIKYQQEVIDKLNTENI ESGQKENHLNTSSTVAVERSYAVMDTIEHSMT SSWKTSLELSFSLEFGFKETGKASSTFKVAFE YSQSTTKKNSQTTSHTLKTTSTQTIPPHSAAE YTIKMSKTRTTIPYTAVIIAKFSVGLRGFLRF GQGFSSPTTNYHFHHKGSDEQSVPYTFGSQS EAFYTALKRESETQSKPWLWNDMFKNHPSVRH VINKLTNESQYEFTLNGKLEHVAGTKIDITWK KVELKDLNRRSVLDESKADTPSPNNITPATV GPKDKPVDVEYPEVHLGNADQVKLETGPVNQN SS | 0 | 0 | 1.25927 |

| Down-regulated Biomphalysins following challenge | | | | | | Log2FC vs control | | |
|---|---|---|---|---|---|---|---|---|
| Transcript iD in RNAseq | Sequence | Blast2GO Annotation | Locus iD [1] | Transcript iD [2] | protein sequence | SmBRE | SmVEN | SROD |
| BV_Locus_9409_Transcript_7/8_Confidence_0.471_Length_415 | CAACTGAAGGGAGGTAGGCTTATATTGCAGAAAATGTTTTTACAAAT CTTTGTGGCAGTCACGCTGGTGCAGTATGTCAGCTCTCAATGCACCTA TTCCTCATGGTGGTACAGTTTGATACTCCTGGACAACTCTAAATGTAA TGAGATCAATTCCTACATCAATGCACTGGACAGAAATGACGTGAACTG GGCAGCAACGCCTCTTAGTAACTAGAAGGTGTTCCAATGTTGTCGACC CCCCGCTCCATGGAATAACGTTGAACAGCAAGTGGTCTACGAAGATTG GACCGCCACTTTGGACTCTGATTACACCTGGGCTTTTGTCGTGATAGG CTACTTCCTTCAAGGCTTGTACAGGTCAGATACTGGATGGCCAAGATT CAAAGGCTATCTTTTCAATCTCGAGAGCGCA | biomphalysin [Biomphalaria glabrata] | LGUN_random_Scaffold10 | BGLB000137 | MFLQIFLAVTLVQTVFSSQCTYSSWWYSFDTPG QSKCNDINSYINALDRNDVNWADDALSNLEGV QCCRPPAPWNNVEQQVVYEDWTATLDSDYTWA FCRVGYFLQGLYRSDTGWFRFKGYLFNLESAR CTKPANHPLMYGTCQDIDVSSCWGRKGQCSCP GGYFLTGLYRADGDDLYFLKKIRCCTPAAKPL EMDEKSKIQTRIMDTTLWNMATLAHYMGYGWC YGCHGLAVGEDFTRNGFTWAADTRSFWGKWCE GDRNGERLNLVPGDWGFAVKEIYGKSYIEDL QAESVDSGVLYNRASSPVTESIERSKTIQETI THSTTSTFTNSHELGVELEFEIASVKGKASYK TRFEYSTSTTNSKSISETQGFTKQSSITLGPM EGARYEYIMSKSRTTVPYTAIITFKFSTEMKG FLRWEDGNGNFHQDYRTNSGRPTFNYRPGDSS VPFYKALKKQSDNNEGVWHWGMLFQKFPDARR VINRLTDETQYQPTLTGKLEKVEGTSVNVRWE RMKLNRRDVSGNDAPGSNITTYIAASGPADKP AVVEYPKVNLNNKEPFKPIEIVTEVKV | 0 | -1.261642 | 0 |
| BV_Locus_9409_Transcript_5/8_Confidence_0.412_Length_388 | ACGCTAGACAATCAGACGTAGTTTTCAATCGCAGAAATAAAACATCAA CCAATCATTAAAAAATGTTAGTGAAATTTTTGTTGCTGCTACGCTGG TGCAGTATGTCAGCTCTCAATGCACCTATTCCTCATGGTGGTACAGTT TTGATACTCCTGGACAATCTAAATGTAATGAGATCAATTCCTACATCA ATGCACTGGACAGAAATGACGTGAACTGGGCAGCAACGCCTCTTAGTA ACTTAGAAGGTGTTCAATGTTGTCGACCCCCCGCTCCATGGAATAACG TTGAACAGCAAGTGGTCTACGAAGATTGGACCGCCACTTTGGACTCTG ATTACACCTGGGCTTTTTGTCGTGATAGGCTACTTCCTTCAAGGCTTGT ACCG | biomphalysin [Biomphalaria glabrata] | LGUN_random_Scaffold10 | BGLB000033 | MLVQFLFAATLLQTVFSSQCTYSSWWYSFDTPG QSKCNEINSYINALDRNDVNWADDALSNLEGV QCCRPPAPWNNVEQQVVYEDWTATLDSDYTWA FCRVGYFLQGLYRSDTGWFRFKGYLFNLESAR CTKPANHPLMYGMCQDIDVSSCWGRKGQCSCP GGYFLTGLYRADGDDLYFLKKIRCCTPAAKPL EMDEKSKIQTRIMDTTLWNMATLAHYLGYGWC YGCRGVAVGEDFTRNGFTWAADTRSFWGKWCE GDRNGERLNLVPGDWGFAVKEIYGKSYIEDL QAESVDSGVLYNRASSPVTESIDRTKTIEETV THSTTSTFTNSHELGIELNFEIASVSGKASYT TKFEYSKATTNEKSISQTAGFTKKSSITLGPM EGARYEIMSKSRTTVPYTAIITFKFSTEMKG FLRWEDGNGNFHQDYRTNSGRPTYNYRPGDSS VPFYKALKKQSDNNEGVWHWGMLFQKFPDARR VINRLTDETQYQPTLTGKLEKVEGTSVNVRWE KIKLNRRDVSGNDAPGSNITTYIAASGPADKP AVVEYPKVNLNNKEPFKPIEIVTEVKV | 0 | -1.197732 | 0 |

1. Locus iD from the *Biomphalaria glabrata* genome annotation on VectorBase (https://www.vectorbase.org/)
2. Transcript iD from the *Biomphalaria glabrata* genome annotation on VectorBase (https://www.vectorbase.org/)

Figure 1

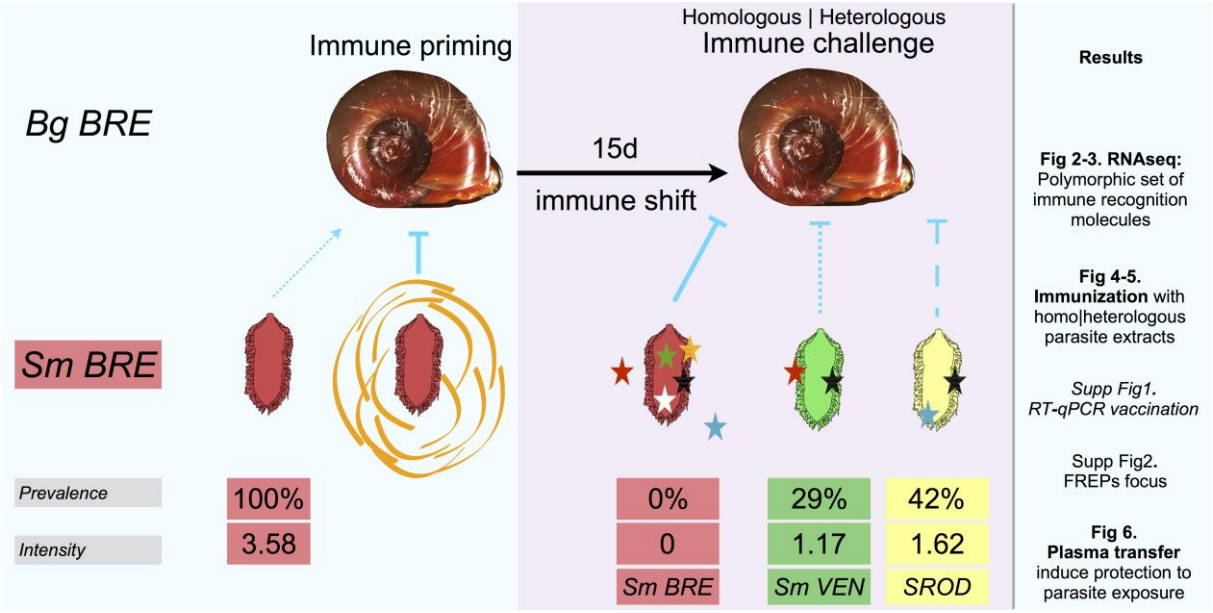



Figure 2

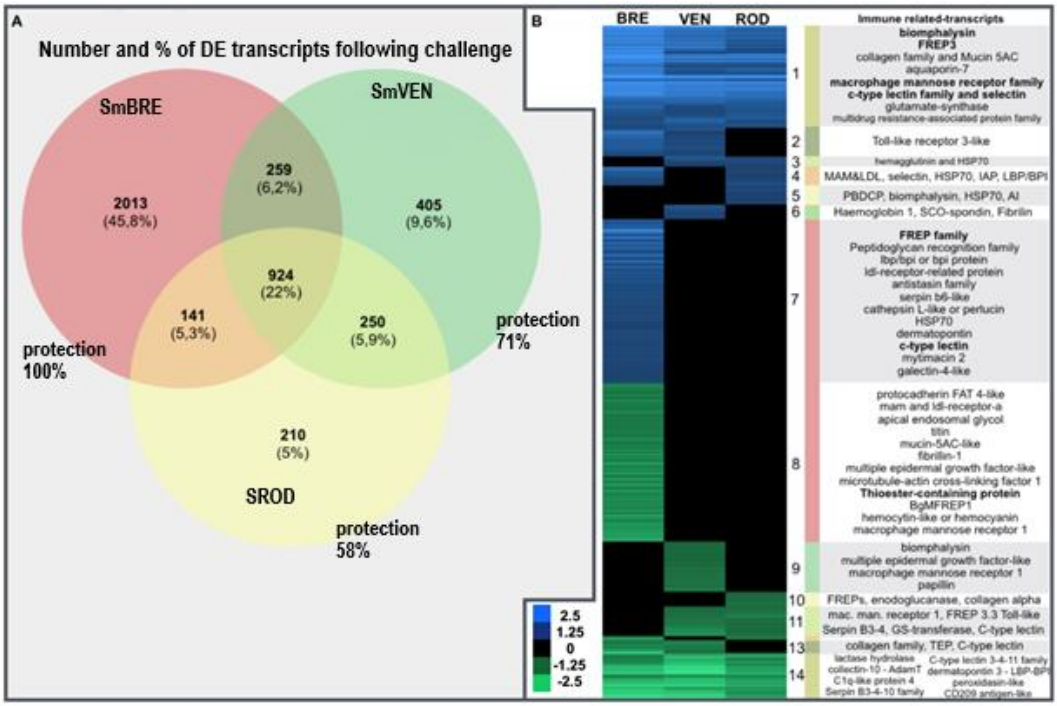


Figure 3

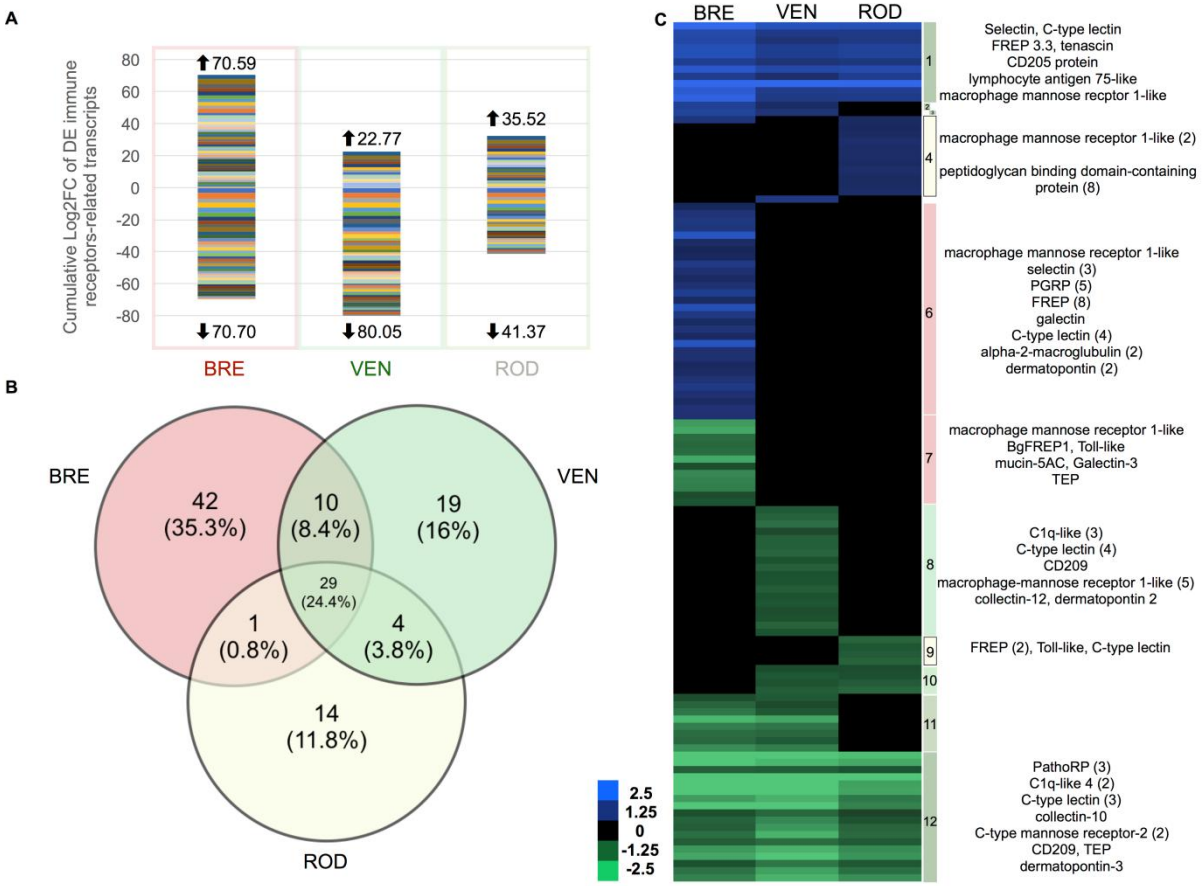



Figure 4

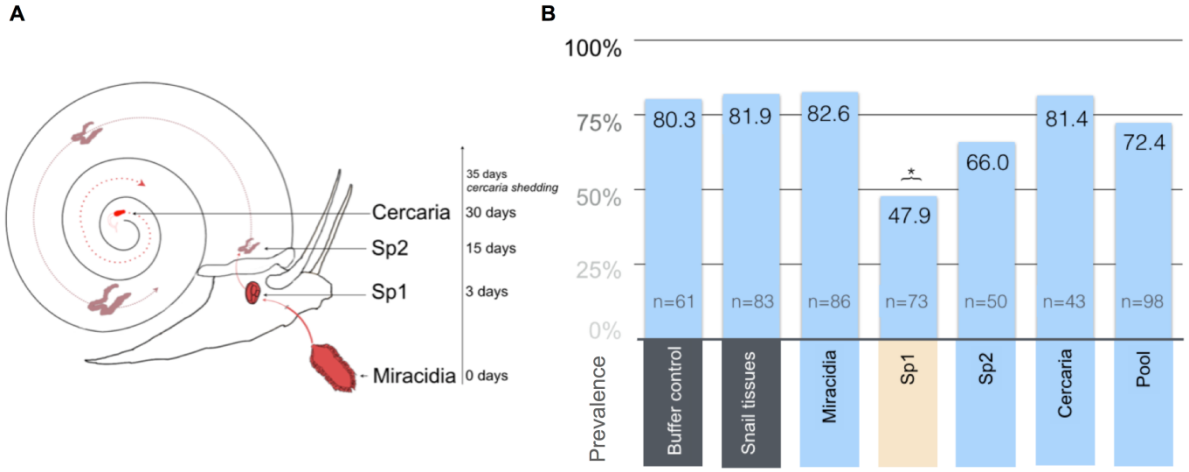


Figure 5

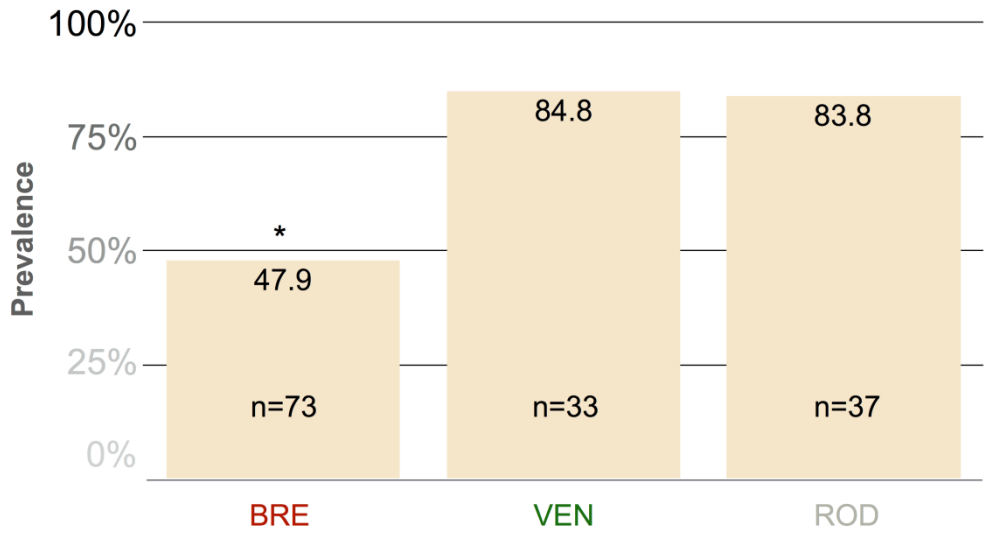
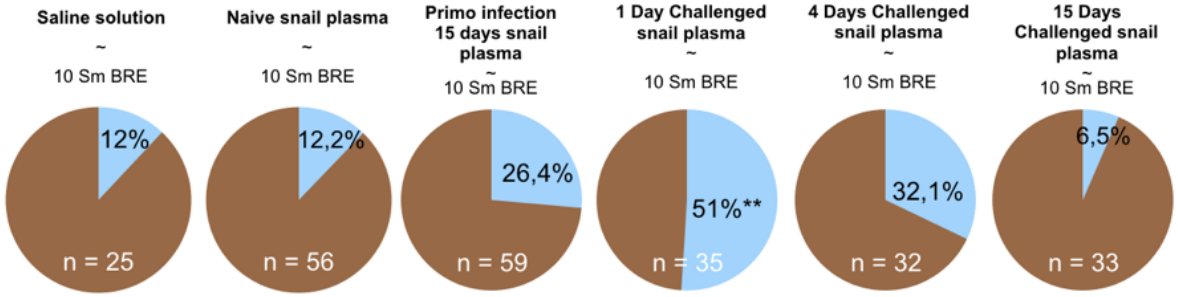
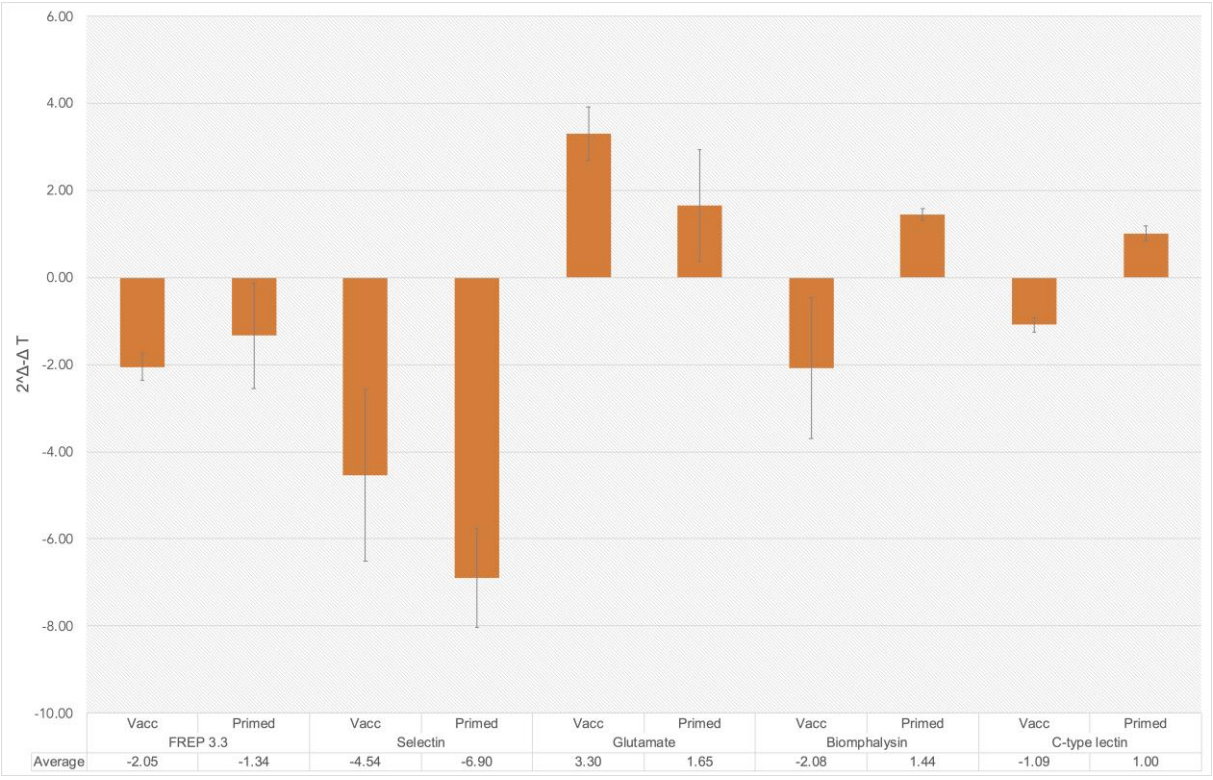



Figure 6




Supplementray Figure 1



Supplementray Figure 2