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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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1 **Molecular characterisation of immunological memory following homologous or**  
2 **heterologous challenges in the Schistosomiasis vector snail, *Biomphalaria***  
3 ***glabrata*.**

4  
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31 **Abstract**

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

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

47

48

49 **Keywords**

50 Immunological memory, specificity, Schistosoma, Biomphalaria, RNAseq, Proteomic,  
51 Vaccination

52

53

## 54 **1. Introduction**

55 Living organisms have evolved complex immune systems to confront  
56 pathogenicbiological-traits and diversity, from prokaryotes(Marraffini and Sontheimer,  
57 2008) to eukaryotes,from plants (Calil and Fontes, 2016; Dangl et al., 2013) to  
58 animals, and from invertebrates (Cirimotich et al., 2011; Haine et al., 2008; Moret and  
59 Schmid-Hempel, 2000, 2001; Portela et al., 2013) to vertebrates (Cooper and Alder,  
60 2006; Du Pasquier, 2004; Eason et al., 2004). “Benefiting from experience” or more  
61 specifically “remembering pathogens” has been a pressure that has led many  
62 immune systems to develop rapid and efficient protections against pathogens  
63 encountered earlier in life. This is due to a chain of events referred to as  
64 “immunological memory”. For innate immune systems, the memory processes are  
65 derived from evolutionary convergences and could have very different features that  
66 have led to different terminologies, i.e.trained immunity for vertebrates or immune  
67 priming/innate immune memory for invertebrates (Gourbal et al., 2018; Milutinovic  
68 and Kurtz, 2016; Netea et al., 2011; Pradeu and Du Pasquier, 2018).

69 In invertebrate species, despite the lack of a canonical adaptive immune system,  
70 immunological memory and specificity of immunological memoryhave been  
71 demonstrated using different strains of pathogens even though in most cases,a  
72 short-term memory is difficult to distinguish from an ongoing response(Jorge  
73 Contreras-Garduno et al., 2016; Kurtz and Franz, 2003; Lafont et al., 2017;  
74 Milutinovic and Kurtz, 2016; Pham et al., 2007; Pinaud et al., 2016; Witteveldt et al.,  
75 2004).

76 However, observations of memory specificity in invertebrate innate immune systems  
77 are largely phenomenological; and the underlying molecular and/or cellular  
78 mechanisms remain unknown. In vertebrates, trained immunity shows low or even no  
79 specificity(Netea et al., 2011; Netea and van der Meer, 2017). Thus, it may not be  
80 possible to explain the observations in invertebrate systems in terms of what we  
81 know about non-self-recognition and trained immunity in vertebrates(Litman,  
82 2006).Thus, if invertebrates do show specificity in their memory responses, what are  
83 the molecular and cellular determinants of such a specific immunological memory?

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

87 most widespread human parasitic disease after malaria, causing a substantial public-  
88 health burden with about 200 million humans infected in 74 countries and more than  
89 200,000 deaths annually (King et al., 2005).

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

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

111

## 112 **2. Materials and methods**

### 113 **2.1. *Biomphalaria* snail and *Schistosoma* parasites**

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

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

123

## 124 **2.2. Immunological memory experimental protocols**

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

138

## 139 **2.3. High throughput approach protocols**

140 For the RNAseq, pools of 20 BgBRE snails were recovered as previously described  
141 (Pinaud et al., 2016) at 1, 4 and 15 days after the challenges and equimolar amounts  
142 of each biological material collected at these time points were pooled together into 3  
143 separate samples named BRE, VEN and ROD corresponding to the 3 strains. Two  
144 pools of 20 naïve snails (naïve1 and naïve2) were sampled and used as controls.  
145 Statistical analyses were performed as already described (Pinaud et al., 2016)  
146 following the pipeline on our Galaxy Project server. Briefly, best quality reads were  
147 filtered (phred >29) and then reads were aligned with Bowtie (v2.0.2, quality mapping  
148 255) and PCR duplicates were removed thanks to SAM tools. DESeq2 software was  
149 used to compare duplicate samples of uninfected snails (naïve1 and naïve2) with our  
150 three challenge conditions (BRE, VEN and ROD) and all statistically different results  
151 (P value <0.05) were annotated manually (Blast X and Interproscan against nr  
152 database, Bast2GoPro for all the other available databases, and data from the  
153 literature). A heatmap was constructed from a log<sub>2</sub> fold change for each of the  
154 transcripts using Hierarchical Ascending Clustering (HAC) with Pearson correlation,

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

157

#### 158 **2.4. Vaccination**

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

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

186

#### 187 **2.5. Expression of relevant immunity genes following vaccination**

188 To determine if vaccination activates a functional immune response similar to the one  
189 observed following natural primo-infection with *Schistosoma mansoni* miracidia, we  
190 conducted a quantitative Real-time RT-PCR on relevant immune genes following  
191 vaccination. The differential gene expressions from RT-PCR following vaccination  
192 were compared to RNAseq Log2fold change data. Down- and up-regulated genes  
193 were selected based on RNAseq data (see supplementary Table 1 for details on  
194 primers and genes selected). BgBRE snails were injected with parasite extracts as  
195 described above in the vaccination section, and then hemocytes were recovered and  
196 gathered in 4 biological replicates of 3 individuals each. mRNAs were extracted using  
197 TRIZOL® (Sigma Life Science, USA) and then reverse transcribed to first strand  
198 cDNA as described by the manufacturer and treated with DNase (Maxima First  
199 Strand cDNA Synthesis Kit with dsDNase, Thermo Scientific, USA). Real-time RT-  
200 PCR analysis was performed in the LightCycler 480 System (Roche) with a volume of  
201 10 µL comprised of 20ng cDNA diluted with MilliQ-H<sub>2</sub>O in 2 µL, 5 µL of No Rox  
202 SYBR Master Mix blue dTTP (Takyon) and 1.5 µL for each of the 2X primers. The  
203 cycling program was: 95°C for 4 minutes, followed by 45 cycles of 95°C for 10 sec,  
204 60°C for 20 sec and 72°C for 20 sec. Expression levels of targeted genes were  
205 normalised relative to S19 gene thanks to the Pfaffl method (Pfaffl, 2001).

206

## 207 **2.6. Cell-free hemolymph transfer and protection phenotype**

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

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



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

225

## 226 **2.7. Comparative label-free proteomic of cell-free hemolymph**

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

253

## 254 **2.8. *In vitro* cell-free hemolymph toxicity assay: a validation of specificity**

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

268

### 269 **3. Results**

#### 270 **3.1. Long-lasting immunological memory in *Biomphalaria glabrata***

271 To test for duration of the innate immune memory process, BgBRE snails were  
272 primo-infected with 10 SmBRE miracidia and challenged at 15, 25, 56, 72, and  
273 140DPI, again with 10 SmBRE miracidia. For all-time points, primo-infected snails  
274 were always resistant to challenge (Table 1). All snails died after 140 DPI due to  
275 parasite pathogenesis (Table 1) as parasite from primo-infection developed to  
276 patency in snail tissue. These results demonstrated that, (i) immunological memory  
277 was acquired soon after infection (from 15 DPI) and (ii) was maintained during the  
278 snail's entire life span (Table 1).

279

#### 280 **3.2. Transcriptomic results provide clues for specificity of immunological 281 memory**

282 A RNAseq approach was used to identify differentially expressed (DE) transcripts  
283 following challenges with homologous (SmBRE) or heterologous (SmVEN or  
284 SROD) parasites. Complete RNAseq results are available in supplementary Table 2.  
285 Venn Diagram (Fig. 2A) shows that among 4,202 DE transcripts, 22% (representing  
286 924 different transcripts) appeared as common regardless of the strain used for  
287 immune challenges. Strain-specific transcripts were identified for the homologous  
288 SmBRE challenge (45.8% representing 2,013 transcripts), for the heterologous

289 challenge with SmVEN (9.6% and 405 transcripts) and for the challenge with SROD  
290 (5% and 210 transcripts). The number of DE transcripts is decreasing with an increase  
291 in the genetic distance between the parasite used for the primo-infection and for the  
292 one used in the challenge. The heat map of these DE transcripts provides more  
293 precise information by highlighting 14 different transcript clusters (Fig. 2B). Clusters 1  
294 to 7 corresponded to up-regulated transcripts and clusters 8 to 14 to down-  
295 regulated ones. Both clusters 1 and 14 corresponded to transcripts shared between  
296 all challenge conditions. With 3,339 DE transcripts, the homologous challenge with  
297 SmBRE induced the largest response while the SmVEN strain induced the regulation  
298 of 1,838 transcripts, and SROD only 1,525 transcripts (Fig. 2B). Interestingly, many  
299 immunity-related transcripts can be identified in these clusters. Within them, three  
300 main families can be defined, (i) the receptors that potentially bind pathogen factors,  
301 (ii) effectors of immunity, capable of having a direct deleterious effect on the  
302 pathogens or activation effect on host immune response and (iii) other transcripts  
303 related to immunity triggering maturation, activation of immune response. In this way,  
304 the putative immuno-receptors are widely distributed across clusters but with  
305 differential distribution between the parasite strains. Immune effectors are  
306 narrowly distributed on the heatmap. For example biomphalysin, a beta-Pore Forming  
307 Toxin ( $\beta$ -PFT) secreted in snail plasma, capable of directly killing the parasite (Galinier  
308 et al., 2013) is notably present in cluster 1 (Fig. 2B). Cluster 1 groups the up-  
309 regulated transcripts differentially expressed regardless of the parasite strain. Thus,  
310 biomphalysin could participate in the humoral memory phenotype observed following  
311 the challenge in *B. glabrata* snails (Pinaud et al., 2016; Portela et al., 2013).  
312 Biomphalysin DE transcripts suggest that numerous variants of biomphalysin family  
313 members were involved in this immunological memory response (Supp-Table 2).  
314 Variants of biomphalysin are specifically up-regulated following SmBRE, SmVEN  
315 or SROD challenges while others are down-regulated following the SmVEN challenge  
316 (Fig. 2B, and Supp-Table 2).

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

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

327 For the third group of transcripts related to immunity, several immune regulators were  
328 identified. Collagen, Hemagglutinin, Haemoglobin and SCO-spondin are components  
329 of, or proteins associated with, the extra-cellular matrix (ECM), they showed a mosaic  
330 of transcript expression depending on the parasite strain used for the challenge.  
331 Collagen and hemagglutinin variants were associated with clusters 1 and 14.  
332 Haemoglobin 11 variants was down-regulated following SmBRE challenge. These  
333 results confirmed the complex restructuring of the ECM during the immune shift  
334 from a cellular to a humoral immune response associated to immunological  
335 memory. The serpin, a family of serine protease inhibitors, implicated mainly in  
336 inflammatory processes, displayed a complex pattern of expression. A total of 26  
337 variants were down-regulated in cluster 14 and 4 isoforms of serpin b6-b were up-  
338 regulated following the homologous challenge (cluster 7). Cadherin and  
339 protocadherin which belong to a superfamily of transmembrane proteins implicated in  
340 cell-to-cell binding or adhesion, were also identified. Following the SmBRE immune  
341 challenge, 40 different transcripts of this family were down-regulated including  
342 protocadherin fats 1, 3, 4 and 20. Similar patterns of regulation were also observed  
343 for 14 transcripts of hemocytin, a highly-glycosylated adhesive protein related to  
344 haemostasis or the encapsulation of foreign substances for self-defence.

345 In order to investigate the specificity of immunological memory towards the complex  
346 mixture of antigenic epitopes presented by *Schistosoma*, we focussed on putative  
347 immuno-receptors and we tried to decipher how a specific repertoire is  
348 activated following homologous or heterologous parasite challenges (Fig.  
349 3). Cumulative log<sub>2</sub> fold-change of each DE immuno-receptor transcript was used to  
350 highlight quantitative differences while the number of transcripts was used to highlight  
351 qualitative differences (Fig. 3). Homologous systems led to +70.59 and -70.70 log<sub>2</sub>  
352 fold-changes for 82 DE transcripts (Fig. 3A-B). The heterologous SmVEN and SROD  
353 challenges led to the modulation of 62 and 48 DE transcripts, respectively (Fig. 3A-  
354 B). The level of expression stands between +22.77 and -80.05 cumulative log<sub>2</sub> fold-  
355 change for SmVEN and +35.52 and -41.37 log<sub>2</sub> fold-change for SROD (Fig. 3A).  
356 Expression patterns of these transcripts differed strongly between parasites of

357 different genetic backgrounds (Fig. 3B). Only one fourth of the transcripts shared a  
358 similar modulation irrespective of the parasite strain used for the challenge (N=29,  
359 24.4%) (Fig. 3B). SmBRE-challenged snails expressed the most specific pattern of  
360 DE transcripts (N=42, 35.3%) while snails expressed less and less transcripts  
361 when challenged with SmVEN (N=19, 16.0%) or SROD (N=14, 11.8%) (Fig. 3B).  
362 Annotations provide important information concerning the families of immune  
363 recognition receptors selected by the host to cope with the diversity of the pathogens  
364 used for the challenges. In cluster 1 (shared up-regulated transcripts), selectin, c-type  
365 lectin, FREP 3.3, tenascin, CD205 protein, lymphocyte antigen 75-like and  
366 macrophage mannose receptor 1-like were identified (Fig. 3C). In cluster 12 (shared  
367 down-regulated transcripts), we identified: 3 transcripts of Pathogenesis Related  
368 Proteins (PathoRP), 2 variants of C1q-like 4 (C1q complement-like), 3 transcripts of  
369 c-type lectin (calcium-dependant lectin), 1 transcript of collectin-10, 2 transcripts of c-  
370 type mannose receptor-2, one CD209, a dermatopontin-3 and one transcript of  
371 Thioester-containing protein (TEP) (Fig. 3C). For the strain specific response,  
372 homologous challenge led to up-regulation of macrophage mannose receptor 1-like, 3  
373 transcripts of selectin, 5 Peptidoglycan recognition proteins (PGRP), 8 Fibrinogen  
374 related proteins (FREPs), one galectin, 4 transcripts of c-type lectin, 2 of alpha-2-  
375 macroglobulin and 2 of dermatopontin (cluster 6) (Fig. 3C). For down-regulation, one  
376 transcript of macrophage mannose receptor-1 like, BgFREP1, a Toll-like receptor, a  
377 mucin-5AC, a Galectin-3 and one transcript of TEP were identified (cluster 7). For the  
378 SmVEN heterologous response, most of the transcripts were down-regulated  
379 (clusters 5-8) including 3 transcripts of C1q-like, 4 transcripts of c-type lectin, and  
380 5 transcripts of macrophage-mannose receptor. For the SROD heterologous  
381 challenge, 2 transcripts of FREPs were down-regulated together with a Toll-like  
382 receptor and one C-type lectin (cluster 9) while 2 transcripts of macrophage mannose  
383 receptor 1-like were up-regulated with 8 transcripts of peptidoglycan binding domain-  
384 containing protein (cluster 4).

385

### 386 **3.3. Vaccination with parasite stage extracts**

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

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

396

### 397 **3.4. Vaccination:a tool to validate immunological memory specificity**

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

405

### 406 **3.5. Immune relevant gene-expression following vaccination**

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

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

426

### 427 **3.6. Cell-free hemolymph transfer and acquisition of protection**

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

444

### 445 **3.7. Comparative label-free proteomic of cell-free hemolymph**

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

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

462

### 463 **3.8. Specificity validation by *in-vitro* cell-free hemolymph toxicity assay**

464 The transcriptomic reprogramming is associated with a higher difference in the set of  
465 recognition proteins and effector molecules depending on the interaction with  
466 homologous or heterologous parasites. Moreover, the massive proteomic-sequencing  
467 approach demonstrated that most of these molecules were released into the  
468 hemolymph (Table 2). To confirm that cell-free hemolymph contains factors that  
469 support a specific activity against *in-vitro* cultured Sp1 of SmBRE, SmVEN or SROD, a  
470 cell-free hemolymph toxicity assay was conducted. SROD sporocysts were difficult to  
471 culture *in-vitro* (*i.e.*, 89% of mortality for SROD cultured in buffer saline, Table 3) thus  
472 the effects of naïve, SmBRE/SmBRE or SmVEN/SmVEN hemolymphs on SROD  
473 Sp1 survival have not been tested due to this high mortality rate (Table 3).

474 However, two conditions were tested, including hemolymph that was either recovered  
475 from BgBRE snails primed and challenged by SmBRE miracidia, or hemolymph that  
476 was primed and challenged by SmVEN miracidia. A significant difference in  
477 toxicity was observed when comparing mortalities induced by challenged snail  
478 hemolymph vs naïve snail hemolymph or CBSS culture medium. Hemolymph  
479 recovered from challenged snails increased the mortality of sporocysts compared to  
480 controls (Fisher exact tests: control vs SmBRE/SmBRE hemolymph for SmBRE  
481 sporocyst  $p = 0.0262$  and controls vs SmVEN/SmVEN hemolymph for SmBRE  
482 sporocyst  $p = 0.00001$  or for SmVEN sporocysts  $p = 0.00001$ ). However, one  
483 exception to this finding occurred for the controls vs SmBRE/SmBRE hemolymph for  
484 SmVEN sporocysts (Fisher exact test  $p = 0.8633$ ) (Table 3). Interestingly, the  
485 hemolymph recovered from challenged snails always induced greater mortality for  
486 homologous sporocysts compared to heterologous ones (Fisher exact tests:  
487 SmBRE/SmBRE hemolymph on SmBRE (47.48%) vs SmVEN (23.07%) sporocysts:  
488  $p = 0.0000252$  and SmVEN/SmVEN hemolymph on SmBRE (84.19%) vs SmVEN  
489 (95.20%) sporocysts:  $p = 0.000258$ ) (Table 3). These results confirmed the specificity  
490 in the humoral factors that are released in the hemolymph after the challenge that can  
491 participate in the binding, recognition and killing of primary sporocysts.



492

#### 493 **4. DISCUSSION**

494 An acquired resistance following a primary antigenic stimulation or encounter with a  
495 pathogen has been demonstrated for numerous animal models in various  
496 invertebrate phyla. Arthropods have been the most investigated group (Brown and  
497 Rodriguez-Lanetty, 2015; Kurtz and Franz, 2003; Rodrigues et al., 2010; Roth and  
498 Kurtz, 2009) but Cephalochordata, Echinodermata, Nematoda, Cnidaria, Ctenophora  
499 and Mollusca have also been investigated (Jorge Contreras-Garduno et al., 2016;  
500 Milutinovic and Kurtz, 2016). Some of these studies demonstrated the existence of a  
501 specific immunological memory against viruses, yeasts, and bacteria. However, these  
502 pathogens express a limited set of potential antigens or pathogen associated  
503 molecular patterns (PAMPs) like peptidoglycans, beta-glucans or Lipo-poly-  
504 saccharides (LPS), which can be easily recognised by the host immune  
505 system (Jorge Contreras-Garduno et al., 2016).

506 In the *Macrocyclops albidus*/*Schistocephalus solidus* model (Kurtz and Franz,  
507 2003) and *Biomphalaria glabrata*/*Schistosoma mansoni* model (Pinaud et al., 2016;  
508 Portela et al., 2013), both partners are phylogenetically related as they belong to the  
509 metazoa, bilateria lineage. This relative phylogenetic proximity is particularly  
510 interesting when studying the mechanisms involved in the specificity of immunological  
511 response. Here, both partners would be expected to share several antigenic  
512 determinants (Yoshino et al., 2012), and fine-scale host recognition processes would  
513 thus be expected to recognise specific pathogen determinants and to avoid auto-  
514 immunity. A genotype-dependant immunological memory has been demonstrated for  
515 *Biomphalaria glabrata* suggesting that snail immune response can distinguish  
516 between different parasite species and strains (Portela et al., 2013). Herein, we go  
517 further in the characterisation of the molecular bases for this specific immunological  
518 memory response.

519 An atypical transcriptomic profile was described following a natural primo-infection  
520 and challenge in *Biomphalaria glabrata* snails and was termed an 'immune  
521 shift' (Coustau et al., 2016; Pinaud et al., 2016). This response is characterised by an  
522 incomplete or absence of immune response following the primary encounter with  
523 *Schistosoma* worms (Portet et al., 2018b). This first interaction is considered compatible  
524 and thus the parasite can develop to patency in snail tissue. At the transcriptomic  
525 level, no strong immune response was observed in response to infection. However,

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

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

549 In snail/Schistosome interactions, similar processes have been demonstrated and  
550 host snail antigens shared with miracidia and sporocysts have been described on  
551 parasite larval surfaces and constitute another example of molecular  
552 mimicry (Yoshino and Bayne, 1983). In this context, our recent work on miracidium  
553 and sporocyst glycoproteins, the *Schistosoma mansoni* polymorphic mucins  
554 (SmPoMucs) (Galinier et al., 2017; Mone et al., 2010; Roger et al., 2008a), were  
555 identified as relevant carbohydrate or glycan candidates that share potential  
556 antigenic epitopes with snail antigens and support the "molecular mimicry" or "smoke  
557 screen" strategies developed by the parasite to avoid recognition (Mitta et al., 2012;  
558 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 response was observed, following the first encounter with  
562 the parasite. First, a cellular immune response, clears most of the parasites through  
563 hemocyte encapsulation. Then, during the secondary encounter with the same strain  
564 of parasite, circulating/plasma factors were involved in the elimination of the parasite.  
565 This shift in phenotype is supported by numerous differentially regulated immune  
566 transcripts identified 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 of the 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 resistant and 2,013 DE transcripts), the heterologous SmBRE/SmVEN system solely  
574 induced a partial protection of 71% with 405 DE transcripts and the SmBRE/SROD  
575 system led to 58% protection with only 210 DE transcripts (Fig. 2). However, it would  
576 be difficult to assume that the number of DE transcripts alone could explain both the  
577 improvement in protection observed and the specificity, even if they appeared to be  
578 correlated. The correlation between qualitative/quantitative transcript expression and  
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 and memory (Figs. 2 and 3).

582

#### 583 **4.1. Immunological memory against *Schistosoma* sp.**

584 Transcripts in the cluster 1 are up-regulated and correspond to the basal  
585 challenge response observed for all the parasite strains, symmetrically, those in  
586 cluster 14 correspond to down-regulated transcripts (Fig. 2). Within these clusters, we  
587 identified numerous factors already implicated in immune response pathways. Within  
588 immune recognition molecules or effectors, the lectin families are considered  
589 recognition factors in *Biomphalaria glabrata* snails. Lectins are defined as molecules  
590 that contain a lectin domain, previously described 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

593 lectin and selectin in cluster 1, highlights the importance of pathogen sensing  
594 in response to the secondary challenge. Lectin families have been shown to be highly  
595 diversified and polymorphic to cover an important range of pathogen diversity (Dheilly  
596 et al., 2015). This diversity should be enough to face the potential parasite  
597 strains/species antigenic diversity and to support the specific immune response  
598 observed herein. Numerous immune response-associated genes are frequently found  
599 in deep-sequencing analyses but their role within the context of immunity was seldom  
600 discussed. For example, snail mucins and collagens are often described as  
601 components of the extra-cellular matrix, but they must also be involved in cell motility  
602 or migration (Hunt et al., 1996). An up-regulation of these variants within these families  
603 may induce several pathways implicated in the recruitment of competent cells in order  
604 to block incompetent cells or pathogens as previously described for *Streptococcus*  
605 infection of macrophages (Dhar et al., 2017).

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

627 2013), can also participate in the regulation of the immune response (Hu et al.,  
628 2017), displaying cytokine functions, attracting haemocytes towards sites of injuries  
629 (Destoumieux-Garzon et al., 2016; Li and Song, 2010).

630 Finally, thioester-containing proteins (TEP) are complement-like proteins that  
631 act as opsonin, promoting phagocytosis, or lysis and sometimes even melanisation,  
632 in invertebrates (Le Clec'h et al., 2016), and arthropods (Blandin and Levashina,  
633 2004; Buresova et al., 2011; Volohonsky et al., 2017) and appear to also participate  
634 in primary cellular immune response and encapsulation in *Biomphalaria glabrata*  
635 snails (Mitta et al., 2012; Mone et al., 2010; Portet et al., 2018a). Following  
636 immunological memory acquisition in *Biomphalaria*, the cellular immune  
637 encapsulation response shifts to a humoral response (Pinaud et al., 2016) and is  
638 associated with the down-regulation of TEP in the homologous challenge RNAseq  
639 data 15 days after the primo-infection (Figs. 2 and 3) as well as the absence of TEP  
640 in 1DC proteome (Table 2).

641

#### 642 **4.2. Total protection in homologous challenge**

643 Following the homologous challenge, we observed a total protection. Immune  
644 recognition molecules and effectors were particularly up-regulated with an increase in  
645 both, the expression and diversity of different lectins (Fibrinogen-related proteins  
646 (FREPs), c-type lectin, and galectin-4) (Fig. 2 and 3, Supp Fig. 2).

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

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

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

677

#### 678 **4.3. Vaccination as proof of immune memory**

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

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

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

708 Finally, snails are better protected when vaccinated with homologous parasite  
709 extracts than with heterologous ones. These results revealed specificity of  
710 immunological memory in *Biomphalaria glabrata*. However, vaccination with Sp1  
711 extracts from SmBRE, SmVEN or SROD neither activated nor induced a total  
712 protection against reinfection, as it can be observed following natural infection.  
713 Vaccination with whole extracts of SmBRE Sp1 induced a partial protection against  
714 the homologous SmBRE challenge while heterologous Sp1 vaccination (SmVEN,  
715 SROD) did not result in any protection (fig. 5). Even immunizations with sympatric  
716 Sp1 or pools of whole parasite extracts cannot induce a total protection (Fig. 4B).  
717 This suggests that other immune pathways or mechanisms have to be activated to  
718 reach a 100% of protection. In this context, the parasite's development within snail  
719 tissues may be the source of a permanent snail immune stimulation through the  
720 release of proteins, nucleic acids, or excreted/secreted products by the parasite  
721 (Guillou et al., 2007; Lodes and Yoshino, 1990; Nowacki et al., 2015; Yoshino et al.,  
722 2014).

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

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

738

#### 739 **4.4. Cell-free hemolymph transfer**

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

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

762



#### 763 **4.5. *In-vitro* toxicity and specificity of primed plasma**

764 Toxicity assays show for the first time, the implication of humoral factors in the  
765 specificity of parasite clearance in an invertebrate. Homologous system displays a  
766 higher *in-vitro* toxicity despite the origin of the strain used (Fig. 6 and Table  
767 3). Immune factors are produced and secreted in the hemolymph compartment to  
768 specifically bind and kill the homologous parasite strains more efficiently than the  
769 heterologous ones (Table 3). These results suggest that the genotype-dependent  
770 specific memory response in *Biomphalaria* is supported by complex repertoires of  
771 circulating recognition molecules or effectors secreted in the hemolymph early after  
772 the challenge.

773 Cytotoxic activities may be due to some toxic molecules like the  
774 biomphalysin (Galinier et al., 2013; Pinaud et al., 2016) (Table 2). This beta-pore  
775 forming toxin may be transferred during vaccination and may remain toxic 15 days  
776 after injection to protect more than 50% of the tested snails (Fig. 6). Interestingly,  
777 biomphalysin is the main protein released in the hemolymph 1 day after the challenge  
778 (Table 2, Fig. 2 and 3) that can participate in parasite killing after challenge (Galinier  
779 et al., 2013; Pinaud et al., 2016). Moreover, a recent interactome approach revealed  
780 that different biomphalysin variants were found to interact with different pathogen  
781 species (bacteria, yeast, trematodes) (Tetreau et al., 2017) (Supp-Table 2), arguing  
782 for the potential role of biomphalysin in both immunological recognition/binding and  
783 pathogen clearance, leading to a higher level of specificity of immunological memory  
784 response in *Biomphalaria* snails.

785

#### 786 **4.6. Combinatorial repertoire of host recognition molecules and effectors to** 787 **face the very large number of parasite epitopes**

788 The peptidoglycan recognition protein family, FREPs, C-type lectins, macrophage  
789 mannose receptors, TLRs, selectin and galectin are all members of different putative  
790 immune recognition superfamilies (Adema et al., 2017; Dheilly et al., 2015; Ferrandon  
791 et al., 2007) (Figs. 2, 3). They contain domains capable of directly binding to pathogens  
792 as carbohydrate recognition domain (CRD), fibrinogen (FBG) or immunoglobulin (Ig)  
793 for the FREPs, the leucine-rich repeats (LRR) for TLR or carbohydrate-binding  
794 protein domain for C-type lectins (Ferrandon et al., 2004; Pasare and Medzhitov,  
795 2004). FREPs or C-type lectin, for example, were previously described in the  
796 interaction between *Biomphalaria* and *Schistosoma* in immune protein

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

822

823

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### 833 *Author contributions*

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

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

842

### 843 *Conflict of interest*

844 The authors declare that no conflict of interest exists.

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### 847 **References**

- 848 Adema, C.M., Hertel, L.A., Loker, E.S., 1997. Infection with *Echinostoma paraensei* (Digenea)  
849 induces parasite-reactive polypeptides in the hemolymph of the gastropod host. in: N.E.  
850 Beckage (Ed.), *Parasites Pathog. Eff. Host Horm. Behav*,  
851 International Thomson Publishing, 76-98.
- 852 Adema, C.M., Hillier, L.W., Jones, C.S., Loker, E.S., Knight, M., Minx, P., Oliveira, G.,  
853 Raghavan, N., Shedlock, A., do Amaral, L.R., Arican-Goktas, H.D., Assis, J.G., Baba, E.H.,  
854 Baron, O.L., Bayne, C.J., Bickham-Wright, U., Biggar, K.K., Blouin, M., Bonning, B.C., Botka, C.,  
855 Bridger, J.M., Buckley, K.M., Buddenborg, S.K., Lima Caldeira, R., Carleton, J., Carvalho, O.S.,  
856 Castillo, M.G., Chalmers, I.W., Christensens, M., Clifton, S., Cosseau, C., Coustau, C., Cripps,  
857 R.M., Cuesta-Astroz, Y., Cummins, S.F., di Stephano, L., Dinguirard, N., Duval, D., Emrich, S.,  
858 Feschotte, C., Feyereisen, R., FitzGerald, P., Fronick, C., Fulton, L., Galinier, R., Gava, S.G.,  
859 Geusz, M., Geyer, K.K., Giraldo-Calderon, G.I., de Souza Gomes, M., Gordy, M.A., Gourbal, B.,  
860 Grunau, C., Hanington, P.C., Hoffmann, K.F., Hughes, D., Humphries, J., Jackson, D.J.,  
861 Jannotti-Passos, L.K., de Jesus Jeremias, W., Jobling, S., Kamel, B., Kapusta, A., Kaur, S.,  
862 Koene, J.M., Kohn, A.B., Lawson, D., Lawton, S.P., Liang, D., Limpanont, Y., Liu, S., Lockyer,  
863 A.E., Lovato, T.L., Ludolf, F., Magrini, V., McManus, D.P., Medina, M., Misra, M., Mitta, G.,  
864 Mkoji, G.M., Montague, M.J., Montelongo, C., Moroz, L.L., Munoz-Torres, M.C., Niazi, U.,  
865 Noble, L.R., Oliveira, F.S., Pais, F.S., Papenfuss, A.T., Peace, R., Pena, J.J., Pila, E.A., Quelais, T.,  
866 Raney, B.J., Rast, J.P., Rollinson, D., Rosse, I.C., Rotgans, B., Routledge, E.J., Ryan, K.M.,  
867 Scholte, L.L.S., Storey, K.B., Swain, M., Tennessen, J.A., Tomlinson, C., Trujillo, D.L., Volpi,  
868 E.V., Walker, A.J., Wang, T., Wannaporn, I., Warren, W.C., Wu, X.J., Yoshino, T.P., Yusuf, M.,

869 Zhang, S.M., Zhao, M., Wilson, R.K., 2017. Whole genome analysis of a schistosomiasis-  
870 transmitting freshwater snail. *Nature communications* 8, 15451.

871 Baron, O.L., Deleury, E., Reichhart, J.M., Coustau, C., 2016. The LBP/BPI multigenic family in  
872 invertebrates: Evolutionary history and evidences of specialization in mollusks. *Dev Comp*  
873 *Immunol* 57, 20-30.

874 Baron, O.L., van West, P., Industri, B., Ponchet, M., Dubreuil, G., Gourbal, B., Reichhart, J.M.,  
875 Coustau, C., 2013. Parental transfer of the antimicrobial protein LBP/BPI protects  
876 *Biomphalaria glabrata* eggs against oomycete infections. *PLoS Pathog* 9, e1003792.

877 Blandin, S., Levashina, E.A., 2004. Thioester-containing proteins and insect immunity. *Mol*  
878 *Immunol* 40, 903-908.

879 Blazevits, O., Mideksa, Y.G., Solman, M., Ligabue, A., Ariotti, N., Nakhaeizadeh, H., Fansa,  
880 E.K., Papageorgiou, A.C., Wittinghofer, A., Ahmadian, M.R., Abankwa, D., 2016. Galectin-1  
881 dimers can scaffold Raf-effectors to increase H-ras nanoclustering. *Sci Rep* 6, 24165.

882 Brewer, C.F., Miceli, M.C., Baum, L.G., 2002. Clusters, bundles, arrays and lattices: novel  
883 mechanisms for lectin-saccharide-mediated cellular interactions. *Curr Opin Struct Biol* 12,  
884 616-623.

885 Brown, T., Rodriguez-Lanetty, M., 2015. Defending against pathogens - immunological  
886 priming and its molecular basis in a sea anemone, cnidarian. *Sci Rep* 5, 17425.

887 Buresova, V., Hajdusek, O., Franta, Z., Loosova, G., Grunclova, L., Levashina, E.A., Kopacek,  
888 P., 2011. Functional genomics of tick thioester-containing proteins reveal the ancient origin  
889 of the complement system. *J Innate Immun* 3, 623-630.

890 Calil, I.P., Fontes, E.P., 2016. Plant immunity against viruses: antiviral immune receptors in  
891 focus. *Ann Bot*.

892 Chang, Y.H., Kumar, R., Ng, T.H., Wang, H.C., 2017. What vaccination studies tell us about  
893 immunological memory within the innate immune system of cultured shrimp and crayfish.  
894 *Dev Comp Immunol*.

895 Choe, K.M., Lee, H., Anderson, K.V., 2005. *Drosophila* peptidoglycan recognition protein LC  
896 (PGRP-LC) acts as a signal-transducing innate immune receptor. *Proc Natl Acad Sci U S A* 102,  
897 1122-1126.

898 Cirimotich, C.M., Dong, Y., Clayton, A.M., Sandiford, S.L., Souza-Neto, J.A., Mulenga, M.,  
899 Dimopoulos, G., 2011. Natural microbe-mediated refractoriness to *Plasmodium* infection in  
900 *Anopheles gambiae*. *Science* 332, 855-858.

901 Clegg, J.A., Smithers, S.R., Terry, R.J., 1971. Concomitant immunity and host antigens  
902 associated with schistosomiasis. *Int J Parasitol* 1, 43-49.

903 Cooper, M.D., Alder, M.N., 2006. The evolution of adaptive immune systems. *Cell* 124, 815-  
904 822.

905 Coustau, C., Kurtz, J., Moret, Y., 2016. A Novel Mechanism of Immune Memory Unveiled at  
906 the Invertebrate-Parasite Interface. *Trends Parasitol* 32, 353-355.

907 Damian, R.T., 1987. Molecular Mimicry Revisited. *Parasitology Today* 3, 263-266.

908 Damian, R.T., 1997. Parasite immune evasion and exploitation: reflections and projections.  
909 *Parasitology* 115 Suppl, S169-175.

910 Dangl, J.L., Horvath, D.M., Staskawicz, B.J., 2013. Pivoting the plant immune system from  
911 dissection to deployment. *Science* 341, 746-751.

912 De Marzi, M.C., Todone, M., Ganem, M.B., Wang, Q., Mariuzza, R.A., Fernandez, M.M.,  
913 Malchiodi, E.L., 2015. Peptidoglycan recognition protein-peptidoglycan complexes increase  
914 monocyte/macrophage activation and enhance the inflammatory response. *Immunology*  
915 145, 429-442.

916 Destoumieux-Garzon, D., Rosa, R.D., Schmitt, P., Barreto, C., Vidal-Dupiol, J., Mitta, G.,  
917 Gueguen, Y., Bachere, E., 2016. Antimicrobial peptides in marine invertebrate health and  
918 disease. *Philosophical transactions of the Royal Society of London. Series B, Biological*  
919 *sciences* 371.

920 Dhar, P., Ng, G.Z., Dunne, E.M., Sutton, P., 2017. Mucin 1 protects against severe  
921 *Streptococcus pneumoniae* infection. *Virulence*, 1-12.

922 Dheilily, N.M., Duval, D., Mouahid, G., Emans, R., Allienne, J.F., Galinier, R., Genthon, C.,  
923 Dubois, E., Du Pasquier, L., Adema, C.M., Grunau, C., Mitta, G., Gourbal, B., 2015. A family of  
924 variable immunoglobulin and lectin domain containing molecules in the snail *Biomphalaria*  
925 *glabrata*. *Dev Comp Immunol* 48, 234-243.

926 Du Pasquier, L., 2004. Speculations on the origin of the vertebrate immune system. *Immunol*  
927 *Lett* 92, 3-9.

928 Eason, D.D., Cannon, J.P., Haire, R.N., Rast, J.P., Ostrov, D.A., Litman, G.W., 2004.  
929 Mechanisms of antigen receptor evolution. *Semin Immunol* 16, 215-226.

930 Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of  
931 genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95, 14863-14868.

932 Ferrandon, D., Imler, J.L., Hetru, C., Hoffmann, J.A., 2007. The *Drosophila* systemic immune  
933 response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol* 7,  
934 862-874.

935 Ferrandon, D., Imler, J.L., Hoffmann, J.A., 2004. Sensing infection in *Drosophila*: Toll and  
936 beyond. *Semin Immunol* 16, 43-53.

937 Galinier, R., Portela, J., Mone, Y., Allienne, J.F., Henri, H., Delbecq, S., Mitta, G., Gourbal, B.,  
938 Duval, D., 2013. Biomphalysin, a New beta Pore-forming Toxin Involved in *Biomphalaria*  
939 *glabrata* Immune Defense against *Schistosoma mansoni*. *PLoS Pathog* 9, e1003216.

940 Galinier, R., Roger, E., Mone, Y., Duval, D., Portet, A., Pinaud, S., Chaparro, C., Grunau, C.,  
941 Genthon, C., Dubois, E., Rognon, A., Arancibia, N., Dejean, B., Theron, A., Gourbal, B., Mitta,  
942 G., 2017. A multistrain approach to studying the mechanisms underlying compatibility in the  
943 interaction between *Biomphalaria glabrata* and *Schistosoma mansoni*. *PLoS Negl Trop Dis*  
944 11, e0005398.

945 Gordy, M.A., Pila, E.A., Hanington, P.C., 2015. The role of fibrinogen-related proteins in the  
946 gastropod immune response. *Fish & shellfish immunology* 46, 39-49.

947 Gourbal, B., Pinaud, S., Beckers, G.J.M., Van Der Meer, J.W.M., Conrath, U., Netea, M.G.,  
948 2018. Innate immune memory: An evolutionary perspective. *Immunol Rev* 283, 21-40.

949 Green, T.J., Helbig, K., Speck, P., Raftos, D.A., 2016. Primed for success: Oyster parents  
950 treated with poly(I:C) produce offspring with enhanced protection against *Ostreid*  
951 *herpesvirus type I* infection. *Mol Immunol* 78, 113-120.

952 Greenwood, J.M., Milutinovic, B., Peuss, R., Behrens, S., Esser, D., Rosenstiel, P.,  
953 Schulenburg, H., Kurtz, J., 2017. Oral immune priming with *Bacillus thuringiensis* induces a  
954 shift in the gene expression of *Tribolium castaneum* larvae. *BMC Genomics* 18, 329.

955 Guillou, F., Roger, E., Mone, Y., Rognon, A., Grunau, C., Theron, A., Mitta, G., Coustau, C.,  
956 Gourbal, B.E., 2007. Excretory-secretory proteome of larval *Schistosoma mansoni* and  
957 *Echinostoma caproni*, two parasites of *Biomphalaria glabrata*. *Mol Biochem Parasitol* 155,  
958 45-56.

959 Haine, E.R., Moret, Y., Siva-Jothy, M.T., Rolff, J., 2008. Antimicrobial defense and persistent  
960 infection in insects. *Science* 322, 1257-1259.

961 Hanington, P.C., Forys, M.A., Dragoo, J.W., Zhang, S.M., Adema, C.M., Loker, E.S., 2010. Role  
962 for a somatically diversified lectin in resistance of an invertebrate to parasite infection. *Proc*  
963 *Natl Acad Sci U S A* 107, 21087-21092.

964 Hanington, P.C., Forys, M.A., Loker, E.S., 2012. A somatically diversified defense factor,  
965 FREP3, is a determinant of snail resistance to schistosome infection. *PLoS Negl Trop Dis* 6,  
966 e1591.

967 Hargreaves, D.C., Medzhitov, R., 2005. Innate sensors of microbial infection. *Journal of*  
968 *clinical immunology* 25, 503-510.

969 Hu, B., Wen, C., Zhang, M., Jian, S., Yang, G., 2017. Identification and characterization of two  
970 LBP/BPI genes involved in innate immunity from *Hyriopsis cumingii*. *Fish & shellfish*  
971 *immunology* 60, 436-446.

972 Hunt, S.W., 3rd, Harris, E.S., Kellermann, S.A., Shimizu, Y., 1996. T-lymphocyte interactions  
973 with endothelium and extracellular matrix. *Crit Rev Oral Biol Med* 7, 59-86.

974 Jorge Contreras-Garduno, Humberto Lanz-Mendoza, Bernardo Franco, Adriana Nava, Mario  
975 Pedraza-Reyes, Canales-Lazcano, J., 2016. Insect immune priming: ecology and experimental  
976 evidences. *Ecological Entomology*, 351-366.

977 Kang, D., Liu, G., Lundstrom, A., Gelius, E., Steiner, H., 1998. A peptidoglycan recognition  
978 protein in innate immunity conserved from insects to humans. *Proc Natl Acad Sci U S A* 95,  
979 10078-10082.

980 Kawsar, S.M., Matsumoto, R., Fujii, Y., Matsuoka, H., Masuda, N., Chihiro, I., Yasumitsu, H.,  
981 Kanaly, R.A., Sugawara, S., Hosono, M., Nitta, K., Ishizaki, N., Dogasaki, C., Hamako, J.,  
982 Matsui, T., Ozeki, Y., 2011. Cytotoxicity and glycan-binding profile of a D-galactose-binding  
983 lectin from the eggs of a Japanese sea hare (*Aplysia kurodai*). *Protein J* 30, 509-519.

984 King, C.H., Dickman, K., Tisch, D.J., 2005. Reassessment of the cost of chronic helminthic  
985 infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. *Lancet*  
986 365, 1561-1569.

987 Kurtz, J., Franz, K., 2003. Innate defence: evidence for memory in invertebrate immunity.  
988 *Nature* 425, 37-38.

989 Lafont, M., Petton, B., Vergnes, A., Pauletto, M., Segarra, A., Gourbal, B., Montagnani, C.,  
990 2017. Long-lasting antiviral innate immune priming in the Lophotrochozoan Pacific oyster,  
991 *Crassostrea gigas*. *Scientific reports* 7, 13143.

992 Le Clec'h, W., Anderson, T.J., Chevalier, F.D., 2016. Characterization of hemolymph  
993 phenoloxidase activity in two *Biomphalaria* snail species and impact of *Schistosoma mansoni*  
994 infection. *Parasit Vectors* 9, 32.

995 Lee, R.T., Lee, Y.C., 2000. Affinity enhancement by multivalent lectin-carbohydrate  
996 interaction. *Glycoconj J* 17, 543-551.

997 Li, C.Y., Song, Y.L., 2010. Proline-rich domain of penaeidin molecule exhibits autocrine  
998 feature by attracting penaeidin-positive granulocytes toward the wound-induced  
999 inflammatory site. *Fish & shellfish immunology* 29, 1044-1052.

1000 Litman, G.W., 2006. How *Botryllus* chooses to fuse. *Immunity* 25, 13-15.

1001 Lodes, M.J., Yoshino, T.P., 1990. The effect of schistosome excretory-secretory products on  
1002 *Biomphalaria glabrata* hemocyte motility. *J Invertebr Pathol* 56, 75-85.

1003 Marraffini, L.A., Sontheimer, E.J., 2008. CRISPR interference limits horizontal gene transfer in  
1004 staphylococci by targeting DNA. *Science* 322, 1843-1845.

1005 Milbradt, J., Kraut, A., Hutterer, C., Sonntag, E., Schmeiser, C., Ferro, M., Wagner, S., Lenac,  
1006 T., Claus, C., Pinkert, S., Hamilton, S.T., Rawlinson, W.D., Sticht, H., Coute, Y., Marschall, M.,

1007 2014. Proteomic analysis of the multimeric nuclear egress complex of human  
1008 cytomegalovirus. *Mol Cell Proteomics* 13, 2132-2146.

1009 Milutinovic, B., Kurtz, J., 2016. Immune memory in invertebrates. *Semin Immunol* 28, 328-  
1010 342.

1011 Mitta, G., Adema, C.M., Gourbal, B., Loker, E.S., Theron, A., 2012. Compatibility  
1012 polymorphism in snail/schistosome interactions: From field to theory to molecular  
1013 mechanisms. *Dev Comp Immunol* 37, 1-8.

1014 Mitta, G., Gourbal, B., Grunau, C., Knight, M., Bridger, J.M., Theron, A., 2017. The  
1015 Compatibility Between *Biomphalaria glabrata* Snails and *Schistosoma mansoni*: An  
1016 Increasingly Complex Puzzle. *Adv Parasitol* 97, 111-145.

1017 Mone, Y., Gourbal, B., Duval, D., Du Pasquier, L., Kieffer-Jaquinod, S., Mitta, G., 2010. A large  
1018 repertoire of parasite epitopes matched by a large repertoire of host immune receptors in  
1019 an invertebrate host/parasite model. *PLoS Negl Trop Dis* 4.

1020 Mone, Y., Ribou, A.C., Cosseau, C., Duval, D., Theron, A., Mitta, G., Gourbal, B., 2011. An  
1021 example of molecular co-evolution: reactive oxygen species (ROS) and ROS scavenger levels  
1022 in *Schistosoma mansoni*/*Biomphalaria glabrata* interactions. *Int J Parasitol* 41, 721-730.

1023 Moret, Y., Schmid-Hempel, P., 2000. Survival for immunity: the price of immune system  
1024 activation for bumblebee workers. *Science* 290, 1166-1168.

1025 Moret, Y., Schmid-Hempel, P., 2001. Immune defence in bumble-bee offspring. *Nature* 414,  
1026 506.

1027 Netea, M.G., Quintin, J., van der Meer, J.W., 2011. Trained immunity: a memory for innate  
1028 host defense. *Cell host & microbe* 9, 355-361.

1029 Netea, M.G., van der Meer, J.W., 2017. Trained Immunity: An Ancient Way of Remembering.  
1030 *Cell host & microbe* 21, 297-300.

1031 Nowacki, F.C., Swain, M.T., Klychnikov, O.I., Niazi, U., Ivens, A., Quintana, J.F., Hensbergen,  
1032 P.J., Hokke, C.H., Buck, A.H., Hoffmann, K.F., 2015. Protein and small non-coding RNA-  
1033 enriched extracellular vesicles are released by the pathogenic blood fluke *Schistosoma*  
1034 *mansoni*. *J Extracell Vesicles* 4, 28665.

1035 Pasare, C., Medzhitov, R., 2004. Toll-like receptors and acquired immunity. *Semin Immunol*  
1036 16, 23-26.

1037 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-  
1038 PCR. *Nucleic Acids Res* 29, e45.

1039 Pham, L.N., Dionne, M.S., Shirasu-Hiza, M., Schneider, D.S., 2007. A specific primed immune  
1040 response in *Drosophila* is dependent on phagocytes. *PLoS Pathog* 3, e26.

1041 Pila, E.A., Tarrabain, M., Kabore, A.L., Hanington, P.C., 2016. A Novel Toll-Like Receptor (TLR)  
1042 Influences Compatibility between the Gastropod *Biomphalaria glabrata*, and the Digenean  
1043 Trematode *Schistosoma mansoni*. *PLoS Pathog* 12, e1005513.

1044 Pinaud, S., Portela, J., Duval, D., Nowacki, F.C., Olive, M.A., Allienne, J.F., Galinier, R., Dheilly,  
1045 N.M., Kieffer-Jaquinod, S., Mitta, G., Theron, A., Gourbal, B., 2016. A Shift from Cellular to  
1046 Humoral Responses Contributes to Innate Immune Memory in the Vector Snail *Biomphalaria*  
1047 *glabrata*. *PLoS pathogens* 12, e1005361.

1048 Portela, J., Duval, D., Rognon, A., Galinier, R., Boissier, J., Coustau, C., Mitta, G., Theron, A.,  
1049 Gourbal, B., 2013. Evidence for specific genotype-dependent immune priming in the  
1050 lophotrochozoan *Biomphalaria glabrata* snail. *J Innate Immun* 5, 261-276.

1051 Portet, A., Galinier, R., Pinaud, S., Portela, J., Nowacki, F., Gourbal, B., Duval, D., 2018a.  
1052 BgTEP: An Antiprotease Involved in Innate Immune Sensing in *Biomphalaria glabrata*. *Front*  
1053 *Immunol* 9, 1206.

1054 Portet, A., Pinaud, S., Chaparro, C., Galinier, R., Charriere, G.M., Allienne, J.F., Duval, D.,  
1055 Gourbal, B., 2018b. Sympatric and allopatric evolutionary contexts shape differential  
1056 immune response in *Biomphalaria* / *Schistosoma* interaction. *BioRxiv* 378034.

1057 Portet, A., Pinaud, S., Tetreau, G., Galinier, R., Cosseau, C., Duval, D., Grunau, C., Mitta, G.,  
1058 Gourbal, B., 2017. Integrated multi-omic analyses in *Biomphalaria*-*Schistosoma* dialogue  
1059 reveal the immunobiological significance of FREP-SmPoMuc interaction. *Dev Comp Immunol*  
1060 75, 16-27.

1061 Pradeu, T., Du Pasquier, L., 2018. Immunological memory: What's in a name? *Immunol Rev*  
1062 283, 7-20.

1063 Rodrigues, J., Brayner, F.A., Alves, L.C., Dixit, R., Barillas-Mury, C., 2010. Hemocyte  
1064 differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. *Science*  
1065 329, 1353-1355.

1066 Roger, E., Grunau, C., Pierce, R.J., Hirai, H., Gourbal, B., Galinier, R., Emans, R., Cesari, I.M.,  
1067 Cosseau, C., Mitta, G., 2008a. Controlled Chaos of Polymorphic Mucins in a Metazoan  
1068 Parasite (*Schistosoma mansoni*) Interacting with Its Invertebrate Host (*Biomphalaria*  
1069 *glabrata*). *PLoS Negl Trop Dis* 2, e330.

1070 Roger, E., Mitta, G., Mone, Y., Bouchut, A., Rognon, A., Grunau, C., Boissier, J., Theron, A.,  
1071 Gourbal, B.E., 2008b. Molecular determinants of compatibility polymorphism in the  
1072 *Biomphalaria glabrata*/*Schistosoma mansoni* model: New candidates identified by a global  
1073 comparative proteomics approach. *Mol Biochem Parasitol* 157, 205-216.

1074 Roth, O., Kurtz, J., 2009. Phagocytosis mediates specificity in the immune defence of an  
1075 invertebrate, the woodlouse *Porcellio scaber* (Crustacea: Isopoda). *Dev Comp Immunol* 33,  
1076 1151-1155.

1077 Schulenburg, H., Boehnisch, C., Michiels, N.K., 2007. How do invertebrates generate a highly  
1078 specific innate immune response? *Mol Immunol* 44, 3338-3344.

1079 Sharma, P., Dube, D., Sinha, M., Mishra, B., Dey, S., Mal, G., Pathak, K.M., Kaur, P., Sharma,  
1080 S., Singh, T.P., 2011. Multiligand specificity of pathogen-associated molecular pattern-  
1081 binding site in peptidoglycan recognition protein. *J Biol Chem* 286, 31723-31730.

1082 Smithers, S.R., Terry, R.J., 1967. Resistance to experimental infection with *Schistosoma*  
1083 *mansoni* in rhesus monkeys induced by the transfer of adult worms. *Transactions of the*  
1084 *Royal Society of Tropical Medicine and Hygiene* 61, 517-533.

1085 Smithers, S.R., Terry, R.J., 1969. Immunity in schistosomiasis. *Ann N Y Acad Sci* 160, 826-840.

1086 Syed Musthaq, S.K., Kwang, J., 2014. Evolution of specific immunity in shrimp - a vaccination  
1087 perspective against white spot syndrome virus. *Dev Comp Immunol* 46, 279-290.

1088 Taengchaiyaphum, S., Nakayama, H., Srisala, J., Khiev, R., Aldama-Cano, D.J., Thitamadee, S.,  
1089 Sritunyalucksana, K., 2017. Vaccination with multimeric recombinant VP28 induces high  
1090 protection against white spot syndrome virus in shrimp. *Dev Comp Immunol* 76, 56-64.

1091 Tetreau, G., Pinaud, S., Portet, A., Galinier, R., Gourbal, B., Duval, D., 2017. Specific Pathogen  
1092 Recognition by Multiple Innate Immune Sensors in an Invertebrate. *Front Immunol* 8, 1249.

1093 Theron, A., Pages, J.R., Rognon, A., 1997. *Schistosoma mansoni*: distribution patterns of  
1094 miracidia among *Biomphalaria glabrata* snail as related to host susceptibility and sporocyst  
1095 regulatory processes. *Exp Parasitol* 85, 1-9.

1096 Volohonsky, G., Hopp, A.K., Saenger, M., Soichot, J., Scholze, H., Boch, J., Blandin, S.A.,  
1097 Marois, E., 2017. Transgenic Expression of the Anti-parasitic Factor TEP1 in the Malaria  
1098 Mosquito *Anopheles gambiae*. *PLoS Pathog* 13, e1006113.



1099 Wang, L., Gilbert, R.J., Atilano, M.L., Filipe, S.R., Gay, N.J., Ligoxygakis, P., 2008.  
1100 Peptidoglycan recognition protein-SD provides versatility of receptor formation in  
1101 *Drosophila* immunity. *Proc Natl Acad Sci U S A* 105, 11881-11886.  
1102 Witteveldt, J., Cifuentes, C.C., Vlak, J.M., van Hulten, M.C., 2004. Protection of *Penaeus*  
1103 *monodon* against white spot syndrome virus by oral vaccination. *Journal of virology* 78,  
1104 2057-2061.  
1105 Wu, X.J., Dinguirard, N., Sabat, G., Lui, H.D., Gonzalez, L., Gehring, M., Bickham-Wright, U.,  
1106 Yoshino, T.P., 2017. Proteomic analysis of *Biomphalaria glabrata* plasma proteins with  
1107 binding affinity to those expressed by early developing larval *Schistosoma mansoni*. *PLoS*  
1108 *Pathog* 13, e1006081.  
1109 Yoshino, T.P., Bayne, C.J., 1983. Mimicry of snail host antigens by miracidia and primary  
1110 sporocysts of *Schistosoma mansoni*. *Parasite immunology* 5, 317-328.  
1111 Yoshino, T.P., Brown, M., Wu, X.J., Jackson, C.J., Ocadiz-Ruiz, R., Chalmers, I.W., Kolb, M.,  
1112 Hokke, C.H., Hoffmann, K.F., 2014. Excreted/secreted *Schistosoma mansoni* venom allergen-  
1113 like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression. *Int J*  
1114 *Parasitol* 44, 551-563.  
1115 Yoshino, T.P., Wu, X.J., Liu, H., Gonzalez, L.A., Deelder, A.M., Hokke, C.H., 2012. Glycotope  
1116 sharing between snail hemolymph and larval schistosomes: larval transformation products  
1117 alter shared glycan patterns of plasma proteins. *PLoS Negl Trop Dis* 6, e1569.  
1118 Zhang, S.M., Zeng, Y., Loker, E.S., 2008. Expression profiling and binding properties of  
1119 fibrinogen-related proteins (FREPs), plasma proteins from the schistosome snail host  
1120 *Biomphalaria glabrata*. *Innate immunity* 14, 175-189.  
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1123 **Figure 1. Overview of experimental protocols and results.**

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1125 **Figure 2. Transcriptomic response exploration of innate immune memory**  
1126 **specificity.**

1127 (A) Venn diagram of differentially expressed (DE) transcripts following homologous or  
1128 heterologous challenges. Snails were primo-infected by SmBRE and then challenged  
1129 by SmBRE, SmVEN or SROD parasites. A correlation is observed between the level  
1130 of protection following challenge and the number of DE transcripts. The homologous  
1131 interaction SmBRE/SmBRE: 100% of protection and 2,013 specific DE  
1132 transcripts; heterologous SmBRE/SmVEN: 71% of protection and 405 specific DE  
1133 transcripts; heterologous SmBRE/SROD: 58% of protection and 210 specific DE  
1134 transcripts. (B) Heat map of differentially expressed transcripts following homologous  
1135 and heterologous challenges. Each line represents a DE transcript (padj value  
1136 <0.05), each row represents the strain of parasite used for the challenge. Colours  
1137 represent the level of expression, green for down-regulated transcripts and blue for  
1138 up-regulated transcripts. Last column of the heat map highlighted the immune related  
1139 transcripts identified based on Blast2Go annotation, domain search and known  
1140 immune genes of the literature. Names in bold correspond to transcripts that have  
1141 been yet described as involved in *Biomphalaria* / *Schistosoma* interaction in the  
1142 literature.

1143

1144 **Figure 3. Immune recognition-related transcripts following homologous or**  
1145 **heterologous challenge.**

1146 (A) Graph showing cumulative Log<sub>2</sub>FC of DE immune recognition-related transcripts  
1147 identified in *BgBRE* snail primo-infected by SmBRE and challenged with homologous  
1148 (SmBRE) or heterologous (SmVEN or SROD) parasites. Each colour in the graph  
1149 correspond to a different DE transcript. (B) Venn diagram that indicates a relationship  
1150 between the number of DE transcripts related to immune recognition and the  
1151 homologous/heterologous associated protection (C) Heatmap of DE transcripts  
1152 related to immune recognition. Each line is a DE transcript (padj value <0.05), each  
1153 row the strain of parasite used and the colours depend on the level of expression,  
1154 green for down-regulated and blue for up-regulated transcripts. Last column of the  
1155 heat map indicates the immune receptor or recognition transcripts identified in each  
1156 cluster. Transcript annotations were defined using Blast2Go annotation, Blastp  
1157 against opened protein databases, domain searches and data from the literature.

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1159 **Figure 4. Vaccination with *Schistosoma* parasite extracts.**

1160 (A) Representation of *Schistosoma* parasite stages development into snail tissues  
1161 (B) Prevalence observed following *SmBRE* infection in *BgBRE* snails previously  
1162 vaccinated with different parasite native extracts (Miracidia, Sp1: Primary sporocyst,  
1163 Sp2: Secondary sporocyst, Cercaria and an equimolar pool of Miracidia, Sp1, Sp2  
1164 and Cercaria.)

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1166 **Figure 5. Homologous versus heterologous vaccination with sporocyst**  
1167 **extracts**

1168 Prevalence observed following *SmBRE* infection in *BgBRE* previously vaccinated  
1169 with sporocyst (Sp1) native extracts from homologous (*SmBRE*) or heterologous  
1170 (*SmVEN*, *SROD*) parasites.

1171  
1172 **Figure 6. Protection acquired following hemolymph transfer**

1173 Protection against *SmBRE* infection observed in naïve *BgBRE* snails following  
1174 different hemolymph transfer treatments. Naïve *BgBRE* snails were subjected to  
1175 injection with, (i) saline solution (CBSS); (ii) cell-free hemolymph (plasma) of naïve  
1176 snails, (iii) plasma from *BgBRE* snails primed with *SmBRE*; or (iv) plasma from  
1177 *BgBRE* snails challenged with *SmBRE* and recovered at 1 day, 4 days or 15 days  
1178 after challenge. Protection has been estimated using the following formula: (control  
1179 prevalence-experimental sample prevalence)/(control prevalence). Control  
1180 prevalence correspond to prevalence calculated for untreated snail following infection  
1181 with 10 *SmBRE* miracidia.

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1183 **Supplementary figure 1. Q-RT-PCR expression of innate immune memory**  
1184 **candidate genes following vaccination.**

1185 *BgBRE* snails were either vaccinated or primo-infected with *SmBRE* Sp1 and gene  
1186 expression was quantified 15 days after treatment. Q-RT-PCR was conducted after  
1187 vaccination and gene expression was compared to RNAseq data following primo-  
1188 infection. See supplementary table 1 for gene details.

1189  
1190 **Supplementary Figure 2. RNAseq-based FREP expressions following**  
1191 **homologous or heterologous challenges.**

1192 Histogram displays cumulative RNAseq Log2FC of differentially expressed FREP-  
1193 related transcripts identified in *BgBRE* primo-infected by *SmBRE* and challenged with  
1194 homologous (*SmBRE*) or heterologous (*SmVEN* and *SROD*) parasites.

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1200 Table 1. Long-lasting immunological memory in *Biomphalaria glabrata* snails.

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<b>Prevalence primo-infection</b>		
<b>BgBRE/SmBRE</b>		
<b>BgBRE</b>	N=15	100%
<b>Prevalence challenge</b>		
<b>BgBRE/SmBRE/SmBRE</b>		
<b>15 DPI BgBRE</b>	N= 15	0%
<b>25 DPI BgBRE</b>	N=15	0%
<b>56 DPI BgBRE</b>	N= 15	0%
<b>72 DPI BgBRE</b>	N= 15	6%
<b>140 DPI BgBRE</b>	N= 15	0%
<b>162 DPI BgBRE</b>	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.



1239 Table 3. Cell-free hemolymph toxicity

1240

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

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1243 a, Fisher exact test < 0.05 compared to controls

1244 b, Fisher exact test < 0.05 comparing SmBRE or SmVEN sporocysts with SmBRE/SmBRE

1245 hemolymph

1246 c, Fisher exact test < 0.05 comparing SmBRE or SmVEN sporocysts with SmVEN/SmVEN

1247 hemolymph

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1249 NA: not available

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1256 Supplementary Table 1. Primers of Q-RT-PCR

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Transcript names	Annotations	Primer names and sequences <sup>§</sup>	
BBVBR_Locus_52081_Transcript_1/1_Confidence_0.000_Length_987	fibrinogen-related protein 3.3 [Biomphalaria glabrata]	Frep3.3	Forward: CCTCTGTCGAAGCGGAAGCTTATT Reverse: AACGTCGGCCTTTCAACAGTTATT
BB_Locus_14654_Transcript_1/1_Confidence_0.333_Length_433	PREDICTED: c-type lectin	C-type Lectin	Forward: TAATCGGAGCTGTACACACAA Reverse: AGCGTCAGCCGTTACAATACA
BBVBR_Locus_2588_Transcript_1/2_Confidence_0.667_Length_595	uncharacterized protein [Aplysia californica]_&_selectin partial	Selectin	Forward: GGGCGTTTGAATGGCGATGAAT Reverse: TGCATTCGTCGAGGAGGTGT
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: GGGGTGGAATCAATCTGGTCTA Reverse: TGGAAGTGAGTCTGATGGTGT
BB_Locus_4989_Transcript_4/4_Confidence_0.000_Length_1252	biomphalysin [Biomphalaria glabrata]	Biomphalysin	Forward: CGTGGAGGAATGTTGGGTAGT Reverse: TCAACGACAAGCAGCTGGAAGA

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1261 § Synthesized by ©Eurogentec, Belgium Sepop Desalt

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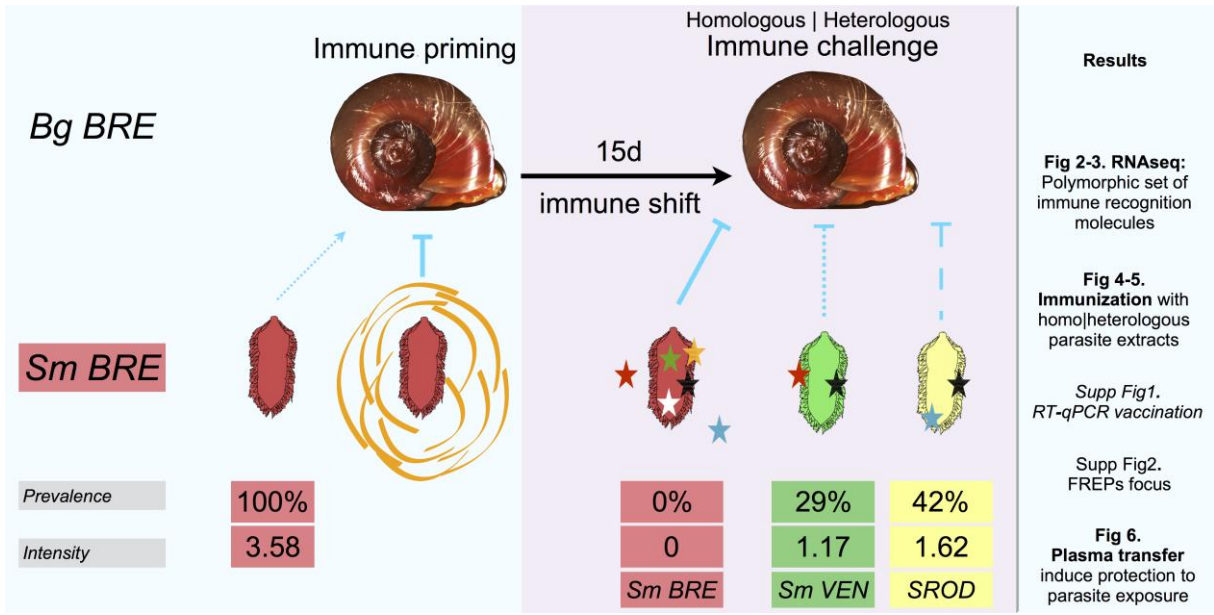
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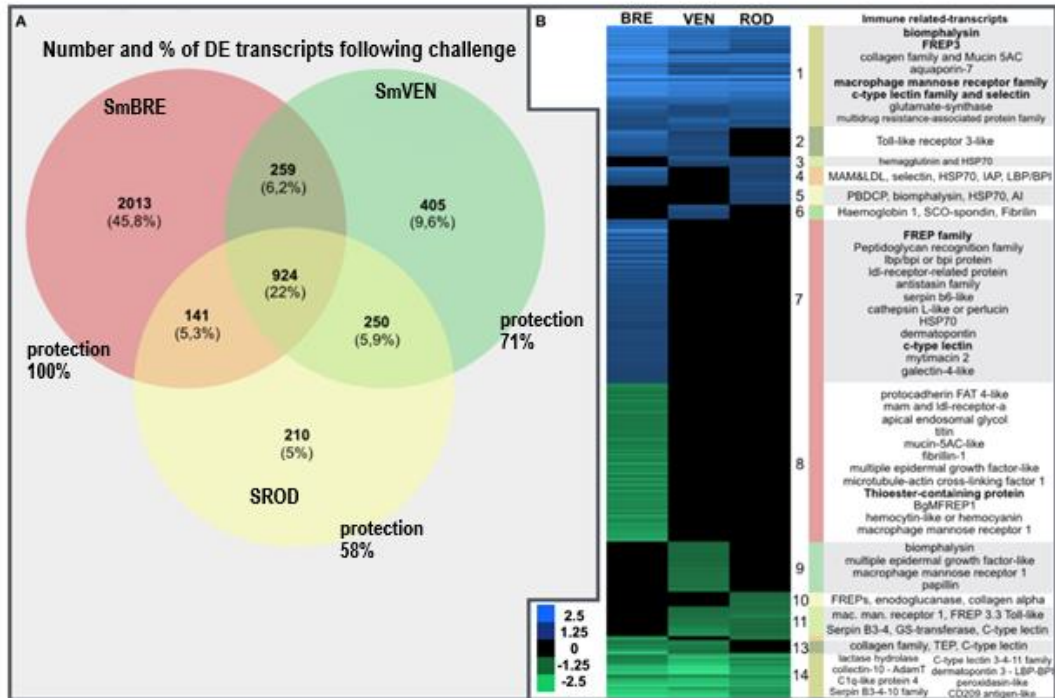




1282 Figure 1  
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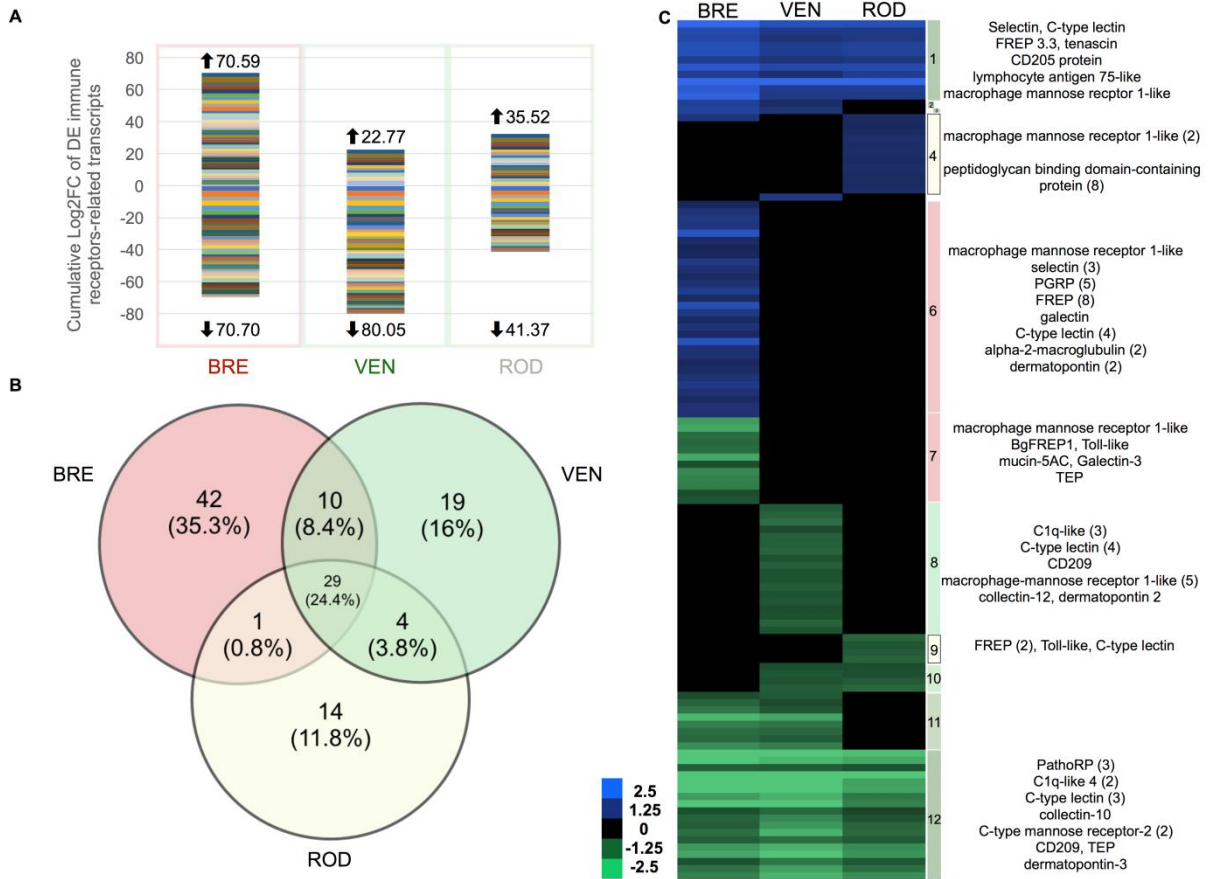


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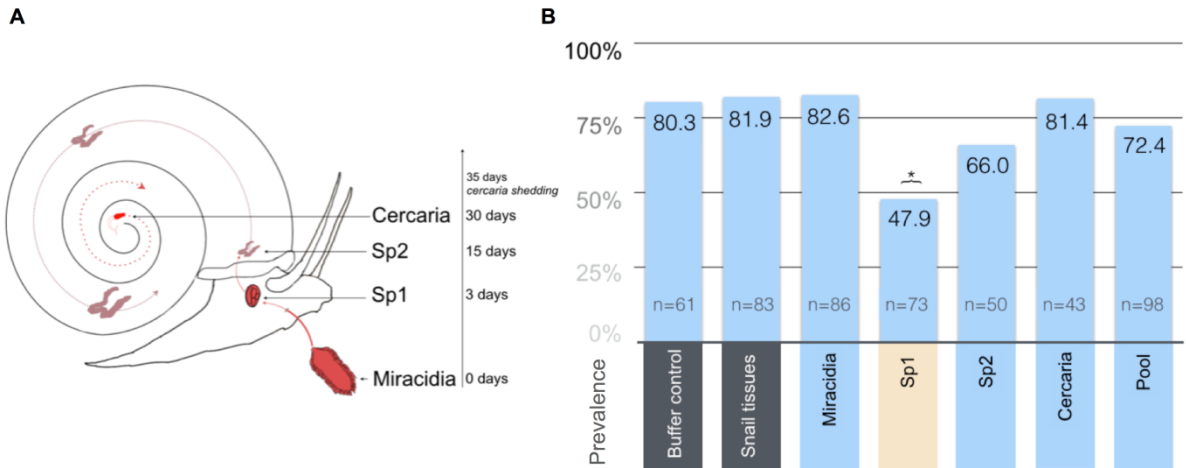
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1290 Figure 3



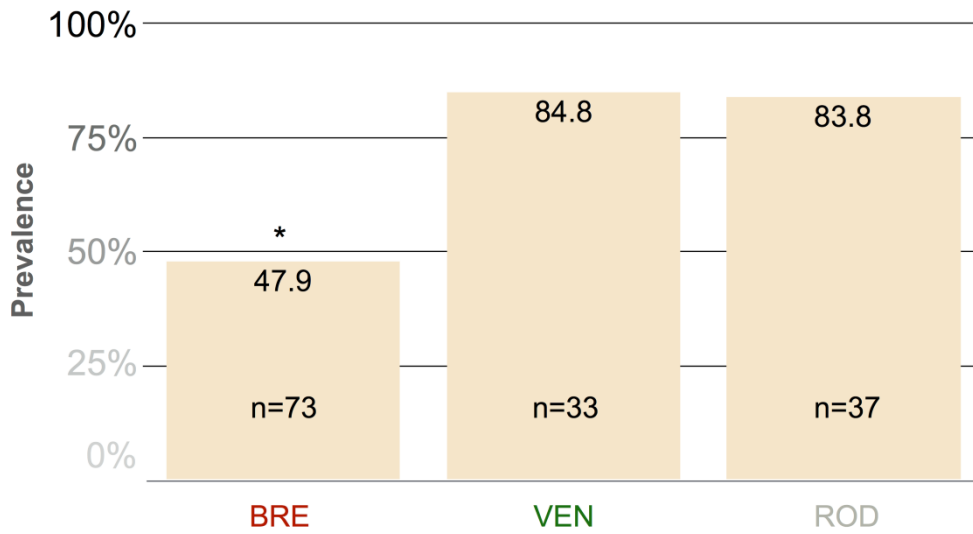
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Figure 4

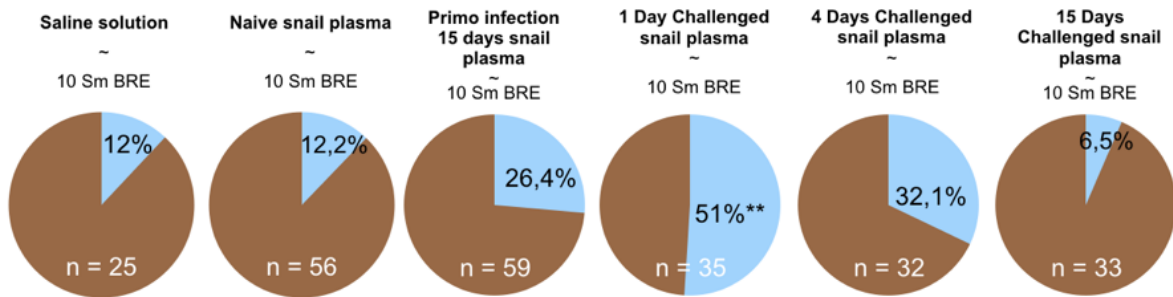


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1303 Figure 5

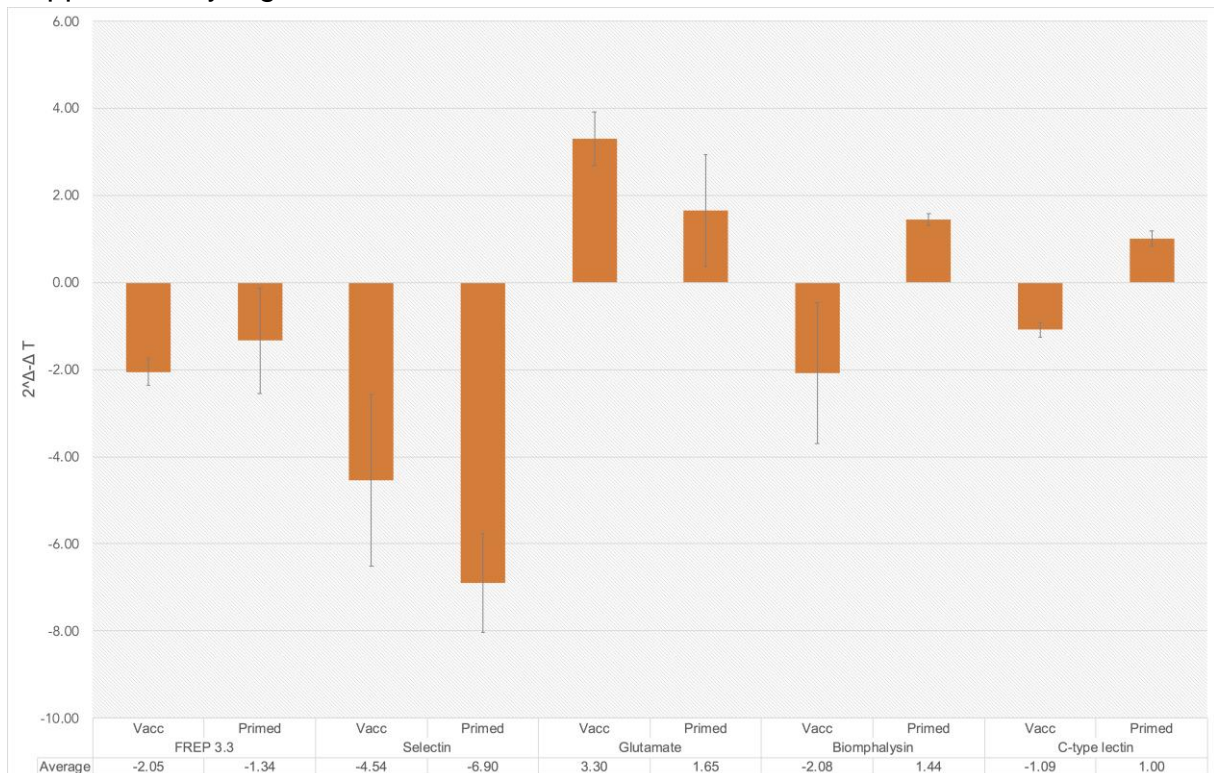


1304 Figure 6  
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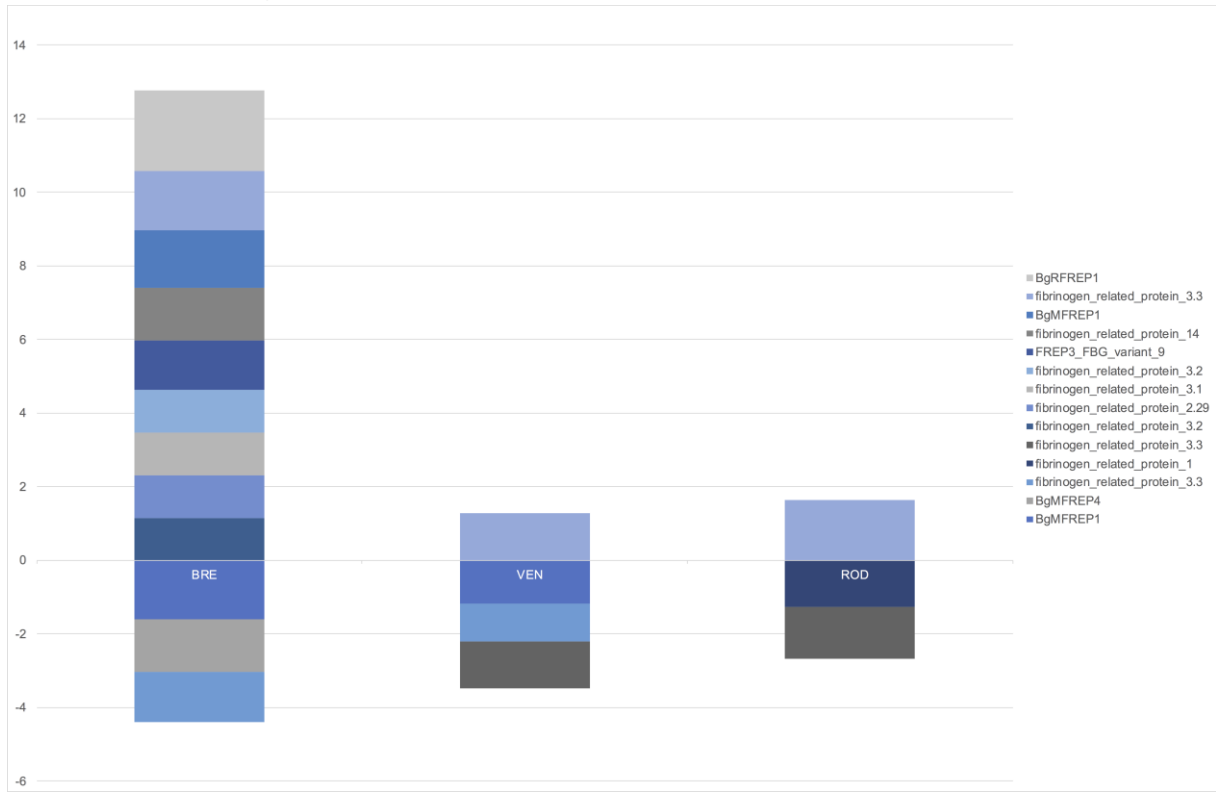
Supplementray Figure 1



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