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The immunobiological interplay between *Pseudosuccinea columella* resistant/susceptible snails with *Fasciola hepatica*: Hemocytes in the spotlight

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14 **Abstract**

15 The *Fasciola hepatica*/*Pseudosuccinea columella* interaction in Cuba involves a unique pattern of phenotypes;
16 while most snails are susceptible, some field populations are naturally resistant to infection and parasites are
17 encapsulated by snail hemocytes. Thus, we investigated the hemocytes of resistant (R) and susceptible (S) *P.*
18 *columella*, in particular morphology, abundance, proliferation and *in vitro* encapsulation activity following
19 exposure to *F. hepatica*. Compared to susceptible *P. columella*, hemocytes from exposed resistant snails
20 showed increased levels of spreading and aggregation (large adherent cells), proliferation of circulating blast-
21 like cells and encapsulation activity of the hemocytes, along with a higher expression of the cytokine granulin.
22 By contrast, there was evidence of a putative *F. hepatica*-driven inhibition of host immunity, only in
23 susceptible snails. Additionally, (pre-)incubation of naïve hemocytes from *P. columella* (R and S) with different
24 monosaccharides was associated with lower encapsulation activity of *F. hepatica* larvae. This suggests the
25 involvement in this host-parasite interaction of lectins and lectins receptors (particularly related to mannose
26 and fucose sensing) in association with hemocyte activation and/or binding to *F. hepatica*.

27 **Keywords:** host-parasite interaction; proliferation; parasite encapsulation; glycan; granulin; immune
28 modulation

29

30 **1. Introduction**

31 *Fasciola hepatica* is a digenetic trematode parasite with worldwide distribution and long-established in many
32 countries that causes an important liver disease known as fascioliasis, a severe burden particularly for bovine
33 and ovine livestock, and an emerging disease affecting 17 million people (Mas-Coma et al., 2009). Small
34 freshwater snails are a key factor in the parasite life cycle, several species of the gastropod family Lymnaeidae,
35 including *Pseudosuccinea columella*, transmit this trematode (Andrews, 1999). Therefore, the study of the
36 particular interactions established between the parasite and each host species may give insights into the ways
37 of transmission, epidemiology and vector-control opportunities, and reveal aspects of snail internal defenses
38 toward broader understanding of animal immunity as afforded by comparative immunology.

39 The *F. hepatica*/*P. columella* system in Cuba presents an attractive and accessible model to study
40 immunobiology within a trematode/snail interaction because two distinct phenotypes exist in naturally
41 occurring Cuban snails. While most *P. columella* populations are susceptible to the parasite infection and thus
42 support transmission, some field populations are resistant to *F. hepatica* (Gutiérrez et al., 2003b). To our
43 knowledge, this is the only lymnaeid snails/*F. hepatica* system in which persistent, naturally occurring
44 resistance to the parasite exists within the same snail species (Gutiérrez et al., 2003b, Gutiérrez et al., 2003a),
45 and that is effective irrespective of the geographical origin or the infective load of the parasite isolate (Calienes
46 et al., 2004, Vázquez et al., 2014, Alba et al., 2018). Hemocytes (circulating cells specialized in immune
47 response) of resistant snails encapsulate *F. hepatica* larvae shortly after penetration and well-formed capsules
48 can be observed, by histological sections, as early as 24 hours (h) following exposure (Gutiérrez et al., 2003b).
49 The observed cellular immune response is supposed to mediate parasite killing and resolution of infection, and
50 has been associated typically with the resistant phenotype of *P. columella* (Gutiérrez et al., 2003b).

51 **1.1 Hemocytes: the “vedettes” of the cellular immune response in mollusks**

52 Host responses against invading pathogens are mainly linked with cellular response activation. In
53 invertebrates, hemocytes figure among the main arsenal of the innate immune system, playing crucial roles in
54 both effecting cellular cytotoxicity and releasing humoral defense factors (Hillyer, 2016, Pila et al., 2016b).
55 Notably, innate immune cells are highly conserved among vertebrates and various invertebrate phyla
56 (Buchmann, 2014, Hartenstein, 2006). Even though the designations vary among the different taxa (*e.g.*
57 amebocytes, hemocytes, coelomocytes, granulocytes, monocytes, macrophages), these are macrophage-like
58 cells with similar phagocyte functions (Buchmann, 2014), that are thought to have evolutionary diverged from
59 a common type of ancestral progenitor cell-type, the hemocytoblast (Hartenstein, 2006).

60 In mollusks, as in other invertebrates, circulating hemocytes are the leading effectors of the cellular immune
61 response, and beyond immune-surveillance, phagocytosis (Cueto et al., 2015, Ataev et al., 2016) and
62 encapsulation (Gutiérrez et al., 2003b), their functions include cytotoxic reactions (Humphries and Yoshino,
63 2008) and the production of a plethora of molecules involved in signal transduction, regulation of immune
64 defenses or immune effector activities (Baeza-Garcia et al., 2010, Galinier et al., 2013, Pila et al., 2016a). To
65 date, no consensus exists regarding classification of hemocytes lineages, morphotypes and subtypes in
66 mollusks, due to the use of diverse criteria for differentiation and the lack of standardized biological markers,
67 see Pila et al. (2016b). However, a general description of the hemocytes based on their spreading capabilities
68 defines two main types: (a) cells that typically spread, forming filopodia or lamellopodia in the process,
69 believed to be the mature effectors in phagocytosis and encapsulation processes and that constitute the
70 majority (if not all) of hemocyte populations, and (b) cells that do not or minimally spread when placed on
71 artificial surfaces, often called round cells or blast-like cells (Loker, 2010). The production, differentiation and
72 activation of these immune cells are essential and regulated by various molecular signaling pathways, as yet to
73 be characterized in detail, to respond effectively to different stimuli (Pila et al., 2016b, Zhang et al., 2016).

74 Encapsulation of an invading parasite by snail hemocytes, as observed particularly, in resistant *P. columella*-*F.*
75 *hepatica* interaction, is an important defense response that involves the isolation of the parasite from host
76 signals and nutrients, and generates a secure and controlled environment for the hemocytes to release
77 cytotoxic and humoral effectors that will help in destroying the parasite (Loker et al., 1982, Coustau et al.,
78 2015). Several studies, involving snail species from a diverse phylogenetic background, report that increased
79 numbers of circulating hemocytes associates with lower parasite susceptibility (e.g. Gorbushin and Iakovleva,
80 2008, Larson et al., 2014, Oliveira et al., 2010) or where parasite killing is effected by hemocyte-mediated
81 cytotoxicity (e.g. Sullivan and Yeung, 2011, Gutiérrez et al., 2003b, Coustau et al., 2015). Therefore, the study
82 of hemocytes is essential to fully understand the immunological interplay between snails and trematode
83 parasites.

84 Here, we explore immune features of the hemocytes of resistant (R) and susceptible (S) *P. columella* following
85 parasitic challenge. We compare morphology, total numbers, proliferative activity, and *in vitro* encapsulation
86 capacity of hemocytes in each phenotype on naïve snails and after *F. hepatica* exposure. In addition, as
87 granulins among other cytokines offer crucial assistance for hemocyte activation, proliferation and
88 differentiation within the context of snail-trematode interaction (Pila et al. 2016a), we analyzed its
89 relative expression in *F. hepatica*-exposed S and R-*P. columella* snails. The present study provides novel
90 insights into the immunobiology of *P. columella* – *F. hepatica* interaction.

91 **2. Material and methods**

92 **2.1 Laboratory-reared susceptible and resistant *P. columella* snails**

93 All snails used were obtained from the Laboratory of Malacology of the Institute of Tropical Medicine “Pedro
94 Kouri”, Cuba. Susceptible (S) snail cultures were established with parental *P. columella* sampled from two field
95 populations, S-Negrines and S-Aurora, located in Havana and Mayabeque province, respectively. The resistant
96 (R) snail cultures (R-La Coca and R-La Palma) were established with snails originating from La Coca locality
97 (Havana province) and, from La Palma, (Pinar del Rio province). These R- *P. columella* snails display the
98 phenotypic marker of typical mantle pigmentation patterns, associated with resistant populations (Gutiérrez
99 et al., 2003b, Alba et al., 2019). All snails were reared according to Sánchez et al. (1995), in Petri dishes,
100 containing growing algae in 26°C de-chlorinated water supplemented with crushed shells as calcium carbonate
101 source. The snails were fed on the algae *ad libitum* relocating snails to new Petri dishes with cultured algae
102 once the food was depleted.

103 **2.2 Experimental exposures of snails to *F. hepatica***

104 *Fasciola hepatica*, adult flukes were obtained from a local cattle abattoir at Jaruco (Mayabeque province) and
105 kept alive for 4h in a solution of 0.85% NaCl, 5% glucose (Sigma, USA), for egg laying. Eggs were preserved at
106 4°C until use.

107 For complete maturation, eggs of *F. hepatica* were incubated for 15 days in distilled water, in total darkness, at
108 28°C. At day 15, mature eggs induced to hatch by exposure to light to obtain miracidia. Experimental exposure
109 to the parasite were carried out using five freshly hatched miracidia per snail, according to Vázquez et al.
110 (2014).

111 Because the cellular response in the resistant host occurs shortly after parasite penetration, exposed snails
112 were sampled at different time points (depending on the experiments, as described below) within the first 96
113 h after parasitic exposure. Additionally, for each of the four *P. columella* strains, a group of 30 exposed snails
114 was used to monitor the outcome of infection and checked for parasite larvae by dissection at 25 days post
115 exposure. As expected, no infection developed in resistant snails from R-La Coca and R-La Palma, while
116 showed prevalence of *F. hepatica* infection in S-Aurora and S-Negrines populations was 76.3% and 54.67%,
117 respectively.

118 **2.3 Hemolymph sampling**

119 Five-to-six weeks old lab-reared *P. columella* snails (shell sizes ranged from 5.1 to 6.9 cm), sexually mature
120 adult as indicated by egg laying were used to obtain hemolymph. Snail shells were cleaned with de-chlorinated
121 water and cotton swabs to remove any surface debris and mucus, and wiped dry. Snails were bled by cardiac
122 puncture (Bayne et al., 1980) using sterile pipette tips to collect hemolymph samples, including hemolymph
123 that pooled on the shell, from individual snails.

124 **2.4 Morphology-based analysis of resistant/susceptible *P. columella* hemocytes**

125 *2.4.1 Flow cytometry*

126 Hemocytes from naïve snails were analyzed in a MACS Flow Cytometer equipment (Miltenyl Biotech), using
127 the MACS Quantity™ v. 2.5. (2003) software. Data acquisition was performed at a flow rate of 1 µL/s, with
128 analysis of 10,000 events. Hemolymph samples were pooled using 5 µL of freshly collected hemolymph from
129 each of four snails, see section 2.3) per strain combined with 180 µL of saline snail buffer (SSB: 5 mM HEPES,
130 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 4 mM CaCl₂; pH 7.9). Samples were placed in conical
131 skirted polypropylene tubes and kept on ice until measurement to minimize cellular adhesion. Flow cytometry
132 was performed using a blank sample containing only SSB and five replicate samples per strain. Cells were gated
133 by size (forward scattering; FSC) and complexity (side scattering; SSC) and visualized on SSC (linear)/FSC
134 (linear) density dot plots.

135 *2.4.2 Confocal Fluorescence microscopy*

136 Hemolymph samples collected from naive snails (0h) and at 24h (time point at which well-formed capsules of
137 hemocytes surrounding *F. hepatica* larvae are observed) post-exposure were applied onto polystyrene slides
138 (Evergreen) and incubated at room temperature for 45 min in a humid chamber to allow for cell
139 sedimentation, adherence and spreading to form hemocyte layers. Hemocytes were fixed with
140 paraformaldehyde 4% for 10 min and samples were rinsed with phosphate saline buffer (PBS: 8 g/L NaCl, 0,2
141 g/L KCl, 1,15 g/L Na₂HPO₄, 0,2 g/L KH₂PO₄, pH 7,2) for 30 s. Each sample was incubated (30 min, room
142 temperature) with 500 µL of Alexa Fluor® 488 Phalloidin (Invitrogen; 40X stock solution in PBS following
143 manufacture guidelines) diluted 1/40 in PBS, for actin labelling. The excess of phalloidin was removed by
144 rinsing with PBS (30 s) and 500 µL of 4',6-diamidino-2-phenylindole (DAPI: Invitrogen; 1 mg/mL stock solution
145 in H₂O) diluted 1/1000 in PBS was added to the slides for DNA (nucleus) staining and incubated for 2 min.
146 Samples were washed in PBS (30 s), dried to air and mounted in fluorescence mounting medium (Dako). The
147 stained hemocytes were inspected using a Zeiss LSM 700 confocal microscope. The associated software Zen

148 (2008) enabled the adjustment of acquisition parameters. The images were processed with ImageJ 1.41o.
149 Based on staining profiles, the overall percentage of different morphotypes (blast cells versus large
150 hemocytes) was estimated for naïve (0h) and at 24h post-exposure for snails of each strain.

151 **2.5 Hemocyte counts post-exposure of *P. columella* to *F. hepatica***

152 The hemolymph from individual snails (see section 2.3) was mixed 1:5 with SSB, loaded on a Neubauer
153 Improved hemocytometer, leaving the hemocytes to settle for 5 min. Total number of cells was counted in five
154 1 mm squares at 200X magnification and cell numbers were calculated as cells/ μ L of hemolymph. Cell counts
155 were performed at 6 time points: 0 h (naïve snails), 6 h, 12 h, 24 h, 48 h and 96 h after exposure of the snails to
156 *F. hepatica* miracidia. Samples of hemolymph of nine snails per strain were individually counted at each time
157 point. Counts were obtained from two independent experiments.

158 **2.6 Assessment of in vitro hemocyte proliferation**

159 The proliferation activity of circulating hemocytes from R- and S- *P. columella* strains was determined *in vitro*
160 by assaying for DNA synthesis with the Click-iT[®] EdU Imaging kit (Invitrogen) according to manufacturer's
161 instructions. Briefly, 5 μ L of freshly collected hemolymph from naïve (0h) and 24h-exposed snails (n = 6
162 snails/time point/strain) were applied as individual samples onto polystyrene slides (Evergreen) and diluted
163 1:2 with SSB. The EdU solution was added to each sample (0.1 mM final concentration) and incubated for 3h at
164 room temperature in a humid chamber. Afterwards, cells were fixed with 4% formaldehyde (100 μ L) for 15
165 min, washed twice with 3% bovine serum albumin in PBS (PBS-BSA) and permeabilized with 100 μ L of 0.5%
166 Triton X-100 for 20 min. After washing twice with PBS-BSA for 30 s, samples were incubated for 30 min in the
167 dark in freshly prepared Click-iT solution. DAPI was used for counterstaining of nuclei (see section 2.4 above),
168 and samples were mounted in fluorescence mounting medium (Dako). Hemocytes were inspected
169 microscopically using epifluorescence and phase-contrast to characterize morphology of proliferating (EdU-
170 marked) cells and to record the overall number of cells (DAPI-stained nucleus) and the number of proliferating
171 cells (EdU-marked nucleus) for each R and S snail strains (n = 4) under naïve conditions (0h) and at 24h post-
172 exposure.

173 **2.7 Granulin expression in *P. columella* resistant/susceptible after *F. hepatica* exposure**

174 The full transcriptome of *P. columella*, assembled de novo from 75 nt paired-end Illumina RNAseq, sequenced
175 from triplicate samples of two (R and S) strains (Alba et al. 2019; RNAseq data is available in the Sequence Read
176 Archive of NCBI under the following accession numbers: Submission number [SUB5947916](#); BioProject:

177 PRJNA555222: Pseudosuccinea columella RNA sequencing; BioSample: SAMN12305757: Pseudosuccinea
178 columella RNAseq (TaxID: 31228)), was used for RT-qPCR primers design. Briefly, the sequence of the
179 proliferation-related cytokine granulin from *Biomphalaria glabrata* snails (GenBank accession HQ661843.1) was
180 used in blast searches to identify the granulin ortholog gene in *P. columella* transcriptome (TR27784|c1_g1/4;
181 transcriptome available at <http://ihpe.univ-perp.fr/acces-aux-donnees/>). The ortholog transcript sequence
182 was verified through analysis of the primary protein sequence and functional domains with Blastx, ExPasy and
183 CDD servers and was deposited in the GenBank (accession number MN244715). Primer3Plus software was used
184 to design several sets of primers for RT-qPCR primers from the *P. columella* granulin sequence, further
185 evaluating the primer design with OligoProperties Calculator (Kibbe, 2007).
186 Initial testing led to selection of a primer set that generated a single qPCR product with an efficiency higher
187 than 1.9 for the quantification of granulin expression (F: 5' TGTCTGCCCTTGCTTACATTCA, R: 5'
188 GCAAGGGTATTTACCTTTGATTTC). The nuclear 18S ribosomal gene of *P. columella* (GenBank accession
189 EU241866.1) was used as standard for relative quantification (F: 5' AGGGACGAGTGGCGTTAGC, R:
190 5'CTTCGTGCTAGGGATTGGGG).

191 *RNA extraction*: Adult S and R *P. columella* snails were used at five time points: naive snails (0h) and 6 h, 12 h,
192 24 h and 96 h post-exposure to *F. hepatica*. RNA samples were collected from 30 snails from each of the four
193 (R and S) strains for every time point. After separation from the shell, snail bodies were immediately placed in
194 individual vials containing RNA*later*[®] (Ambion) solution at a ratio of 1 mg of snail : 10 µL of RNA*later*. Samples
195 were kept overnight at 4°C and then stored at -20°C until use. RNA*later* was discarded and whole snail tissues
196 were pooled into three biological replicates, each of 10 individuals per time point, immediately frozen in liquid
197 nitrogen and homogenized using a laboratory mixer mill MM400 (Retsch). RNA was extracted in TRIzol[®]
198 Reagent (Ambion) according to manufacturer guidelines. Following treatment with Turbo DNA-freeTM kit
199 (Ambion) to remove residual genomic DNA from the samples, the quantity of RNA was estimated
200 spectrophotometrically.

201 *RT-qPCR*: RNA (0.5 µg) of each biological replicate was reverse transcribed to cDNA using random primers and
202 the Maxima[™] H Minus First Strand cDNA Synthesis kit (Thermo Fisher), simultaneously including RT negative
203 controls (RT-) for sample that were stored at -20°C until use as (negative) controls. Then, qPCRs were
204 performed in a LightCycler[®] 480 Instrument II system (Roche) using 10 µL reactions consisting of 1X of SYBR
205 Green Master Mix Takyon[®] qPCR kit (Eurogentec), 0.5 µM of each primer, 2 µL of 10-fold diluted cDNA as
206 template and molecular grade water. Three technical replicates for each biological replicate were done.
207 Negative controls contained water or RT- products instead of cDNA template. Standard curves of cDNA from

208 naïve snails (two-fold diluted from 1/10 to 1/640) were generated to calculate the primer efficiencies. The
209 amplification program was set as: denaturation at 95°C for 10 s followed by 35 cycles of hybridization at 60°C
210 for 20 s and elongation at 72°C for 30 s. The relative quantity (RQ) of granulin was estimated versus the
211 housekeeping gene (18S) using the LightCycler® 480 Instrument II software v. 1.5.0 (Roche), considering and
212 efficiency of 2. The resulting RQ of each time point post-exposure were normalized against the RQ calculated
213 at naïve conditions (0h) for each strain.

214 **2.8 Functional characterization: *in vitro* encapsulation assays of *F. hepatica* sporocyst**

215 Hemolymph from naïve (0h) and 24h-exposed R and S snails was obtained by cardiac puncture (see section
216 2.3) to assess the propensity of snail hemocytes to encapsulate *F. hepatica* sporocysts *in vitro*. The activity of
217 the cells toward parasitic larvae was tested under two conditions: (1) naïve (0h) hemocytes were (pre-
218)incubated with monosaccharides (*i.e.* the experiment combined pre-incubation of the hemocytes and
219 encapsulation of the parasite in presence of the monosaccharides), to assess the effect of specific glycans on
220 the encapsulation activity, and (2) hemocytes were collected from snails at 24h post-*F. hepatica* exposure to
221 evaluate for variations in the performance of the cells after parasitic challenge. Untreated, naïve control
222 hemocytes were used as baseline for comparing the results from the two experimental conditions. Six snails
223 for the R and S strains were used and results were independently recorded for the different assays and *P.*
224 *columella* strains.

225 Briefly, hemolymph samples (20 µL) from naïve and parasite-exposed (24h) snails were individually added to
226 wells of 96-well culture plates previously filled with sterile SSB (110 µL/well). The plates were kept in ice to
227 prevent aggregation and attachment of the hemocytes until all samples were added to the wells. Counting
228 hemocytes and assessment of cell viability (Trypan blue exclusion) were performed in a Neubauer chamber,
229 with aliquots taken from 2 randomly selected wells for each experimental group. Aliquot of naïve hemocytes
230 were pre-incubated separately by addition of one of each of the following monosaccharides: D-manose, D-
231 glucose, L-fucose and D-galactose (from Sigma), at a final concentration of 150 mM (20 µL of 1M stock) under
232 agitation (100 rpm) for 30 min, at 27°C. To standardize the volumes for all aliquots, 20 µL SSB was added to
233 wells containing hemocytes that were not treated with monosaccharides. Afterwards, one newly *in vitro*-
234 transformed *F. hepatica* sporocyst obtained according to Gourbal et al. (2008) was placed in each well (in 20
235 µL) with the help of a stereoscope, and plates were then incubated at 27°C, for 2h, in agitation (100 rpm).
236 Then, encapsulation was evaluated for each condition. Based on pilot studies, categories had been established
237 to score the state and thicknesses of the hemocytes capsules around the parasite larvae into five grades (from

238 1 to 5: from almost none to complete encapsulation), to express and compare observed differences in
239 encapsulation on a semi-quantitative scale, as done elsewhere (Kryukova et al., 2014, Loker et al., 1989).

240 **2.9 Data analysis**

241 Means and standard deviations were plotted for each value of cell count per strain, discarding outlier values
242 (points beyond standard deviation). Data was checked for normality (Shapiro-Wilk test) and variance
243 homogeneity (Levene test) and Factorial ANOVAs were carried out to assess the significance between the
244 amount of hemocytes (dependent variable) with the snail phenotype and time post-infection as factors
245 (section 2.5). A post hoc Tukey test followed the ANOVAs was used to obtain a multiple comparison matrix for
246 each mean. The percentage of specific morphotypes and of proliferative hemocytes per strain per time point
247 were compared by Fisher's exact Tests (sections 2.4.2 and 2.6). A Kruskal-Wallis Test and Duncan's Tests were
248 used to estimate statistical differences between RQs of granulins at each time point in each strain (section 2.7).
249 The encapsulation grade showed by the hemocytes from each strain was associated to the assayed conditions
250 (incubation with monosaccharides or parasitic challenge) by means of a multivariate correspondence analysis
251 using the previously established qualitative scale (see section 2.8). All analyses were performed in Statistica
252 v.12 (StatSoft. Inc., Tulsa, OK, USA 2014). Differences were considered statistically significant for values of $P <$
253 0.05.

254 **3. Results**

255 **3.1 Morphological observations of *P. columella* hemocytes**

256 Two well-defined morphotypes of circulating cells, differing in size/granulocytic content and size/spreading
257 capacity, were observed in the hemolymph of both S and R strains of *P. columella* (Figure 1). Flow cytometry
258 analysis performed on cells from naïve snails showed two main, dense clusters of *P. columella* hemocytes
259 segregated by size and granulometry: large cells highly granular and smaller cells with lower cytoplasmic
260 complexity (Figure 1 A, B). Consistent with the flow cytometry results, inspection of from phalloidin-DAPI
261 stained adherent hemocyte preparations also showed two morphotypes (i) large adherent cells (7 to 15 μm)
262 displaying pseudopodia and (ii) roundly-shaped small cells (3 to 5 μm), with high nucleus/cytoplasm ratio and
263 low cytoplasmic complexity, here called blast-like cells, in both R and S phenotypes of *P. columella* (Figure 1 C,
264 D).

265 Extensive evaluation indicated that large adherent hemocytes are the main morphotype, and these hemocytes
266 assume a typically spreading morphology with formation of pseudopods, similarly for samples obtained from

267 naïve snails of both R and S phenotypes (Figure 2 A-D). However, qualitative differences were observed from
268 large hemocytes from S and R snails at 24h-post exposure to *F. hepatica* (Figure 2 E-H). Specifically, large
269 adherent hemocytes of R snails tended to aggregate, and displayed increased length of broad-based
270 cytoplasmic projections, extending these into a sheet-like spreading pattern (Figure 2 E, F). By contrast, large
271 adherent cells from S snail strains appeared more scattered, without a clear tendency to aggregate and
272 showed a discrete spreading morphology with shorter pseudopodia, as in the case of S-Aurora snails (Figure 2
273 G, H). Intra and inter-strain variability regarding the morphology of large hemocytes morphology was more
274 pronounced in S snails (Figure 2). The percentage of large adherent hemocytes was always higher in R snails
275 (ranging from 89 to 97%) compared to snails from S strains snails (62 to 78%), irrespective of experimental
276 conditions (at 0h RR vs. SS Fisher's Exact Tests $P < 0.05$; at 24h R-La Palma vs. SS Fisher's Exact Tests $P < 0.05$).
277 This difference in relative percentage of this large hemocyte morphotype between resistant and susceptible
278 snails was also indicated by dot plots of flow cytometry profiles (see Figure 1 A, B).

279 Overall, the percentages of the blast-like cell morphotype ranged from 3 to 36% of the total cell counts in
280 naïve *P. columella*; (Figure 2A-D), with significantly higher percentages of blast-like cells in susceptible snails (S-
281 Aurora - 26.3%, S-Negrines – 36.5%) compared to resistant strains (R-La Coca – 3.7%, R-La Palma – 10.1%;
282 Fisher's Exact Tests $P < 0.05$). At 24h post-exposure, an increase of blast-like cells was observed in resistant
283 strains (11 and 11.2% in R-La Coca and R-La Palma, respectively) that was significant in R-La Coca (Fisher's
284 Exact Tests $P = 0.014$).

285 **3.2 Hemocyte counts, proliferation rate and granulin expression following exposure to *F. hepatica***

286 The cellular response of susceptible and resistant *P. columella* to *F. hepatica* displayed basically the same trend
287 at early times post-exposure characterized by a gradual increase of hemocyte counts starting within the first
288 hours to reach a maximum at 24h post parasite-exposure, and beginning to decrease thereafter (Figure 3A).
289 Nevertheless, the actual number of hemocytes was significantly higher in R snails compared to S isolates, with
290 cell counts almost two-fold higher at 24h post-exposure ($P < 0.001$; Figure 3A). Relative to naïve control R
291 snails, the number of circulating hemocytes in exposed-resistant snails was still significantly higher at 48 and
292 96h post-exposure (Figure 3A).

293 Positive EdU staining of hemocytes, indicative of DNA synthesis, was recorded for resistant snails, 24 h post
294 exposure (R-La Coca - 6.25%; R-La Palma - 7.14%, $P = 0.8496$), suggesting the active proliferation within the
295 circulating hemocyte population (Figure 3B). Conversely, no EdU staining was observed in hemocytes from S-
296 Aurora snails, either naïve or at 24h post-exposure. A single individual snail from the S-Negrines strain 24h

297 post exposure) displayed EdU-signal indicative of proliferation in a modest 1.96% of hemocytes. This
298 proportion, however, was significantly lower ($P < 0.05$) than the numbers of proliferative hemocytes of R-*P.*
299 *columella* strains. EdU marked-cells were always of the small and round-shape hemocyte morphotype,
300 referred here as blast-like cells (Figure 3B, lower panel).

301 Lastly, a significant increase in expression of the cytokine granulin was recorded only from resistant *P.*
302 *columella*, early after parasite exposure (6h; $P = 0.041490$). By contrast, in susceptible snails, granulin
303 expression decreased below constitutive levels at 12h and 24h post parasite exposure ($P < 0.05$; Figure 3C).
304 Comparatively, the relative expression of granulin between R and S phenotypes accounted for an almost 3-fold
305 difference, at 24h post-exposure. Additionally, an increase of expression was recorded for both phenotypes
306 four days after parasite exposure, however, significant differences were also observed between resistant and
307 susceptible snails at this time point (Figure 3C).

308 **3.3 *In vitro* encapsulation assays**

309 The observations from pilot *in vitro* assays (Figure 4A), were used to establish a quantitative scale to compare
310 the encapsulation grade of *F. hepatica* sporocysts by *P. columella* hemocytes under different conditions, (pre-)
311 incubation of naïve hemocytes with monosaccharides or *F. hepatica* challenge in a multivariate
312 correspondence analysis (Figure 4B). Encapsulation activities of naïve hemocytes from resistant strain snails
313 were higher (strong association with Grade 4) than those from snail of the susceptible strains (association with
314 Grade 3). In addition, encapsulation grade 5, corresponding to the observation of a thickest capsule of cells
315 around the larvae (Figure 4A), was associated only with hemocytes from R-strain snails at 24h post exposure to
316 *F. hepatica* (Figure 4B). Notably, while naïve hemocytes from susceptible *P. columella* strongly associated with
317 an encapsulation grade 3, no clear pattern was observed from hemocytes at 24h post exposure with
318 encapsulation activities ranging between grades 2 to 4 (Figure 4B).

319 On the other hand, (pre-)incubation of naïve hemocytes with monosaccharides was associated with lower
320 encapsulation degrees (Figure 4B). Particularly, (pre-)incubation with D-mannose was associated with
321 encapsulation grade 1 regardless of the phenotype (R or S) and of *P. columella* population. In both susceptible
322 populations, (pre-)incubation with L-fucose was also associated with the lowest encapsulation grades (Figure
323 4B).

324 **4. Discussion**

325 **4.1 Hemocytes from naïve *P. columella* snails: morphological and functional observations**

326 In the present study, the analysis of differences in spreading properties, size and cytoplasmic complexity (see
327 Loker (2010) of *P. columella* hemocytes revealed two main morphotypes: i) non-adherent small and roundly-
328 shaped hemocytes with low cytoplasmic complexity, characteristic features described for blast-like cells and ii)
329 large adherent cells with higher cytoplasmic complexity. The latter morphotype may potentially be further
330 segregated into granulocytes and hyalinocytes after thorough analyses of cell granularity, similar to hemocytes
331 in *Biomphalaria* spp. and *Lymnaea palustris* (Russo and Lagadic, 2000, Cavalcanti et al., 2012), but such
332 classification requires additional staining and functional approaches.

333 In any case, distinct cell morphologies are proposed to reflect certain functional differences among snail
334 hemocytes, representing either different subtypes of cells or different developmental stages of a single lineage
335 of hemocytes (Boisseaux et al., 2016, Matricon-Gondran and Letocart, 1999, Mohandas et al. 1992). In this
336 sense, high numbers of large, adherent (spreading) hemocytes in the hemolymph of *B. glabrata* snails were
337 suggested to be a predictive parameter of lower susceptibility to *Schistosoma mansoni* (Larson et al., 2014). In
338 the present study, large adherent cells were highly predominant in snail from both R- versus S- *P. columella*
339 strains. Additionally, naïve hemocytes from R snails were observed to have higher encapsulation activity of *F.*
340 *hepatica* sporocysts compared to both S strains. Both results suggest that R- *P. columella* snails have a higher
341 constitutive baseline of circulating hemocytes more competent of effective immunity against this parasite than
342 those from S-*P. columella* snails (Figure 5). A likely molecular basis for this enhanced immune competence is
343 provided by a previous comparative transcriptomic study that disclosed, compared to S-strain snails, a
344 significantly greater resource allocation in naïve *P. columella* snails from resistant strains to express an overall
345 greater abundance of transcripts encoding for several pathogen-interacting molecules, pro-inflammatory
346 signaling pathways and cytokines (for details, see Alba et al., 2019).

347 Interestingly, in both *P. columella* phenotypes, the involvement of molecules with different carbohydrate
348 specificities (lectins) appears to be fundamental for hemocyte functionality; monosaccharides generally led to
349 lower encapsulation grades (see Figure 4B). Similarly, adhesion of cells from the *B. glabrata* embryonic (Bge)
350 cell line to *S. mansoni* sporocyst was inhibited by carbohydrate compounds, including mannose-6-phosphate,
351 heparin, dextran sulfate, and mainly, fucoidan (Castillo and Yoshino, 2002). The significantly diminished
352 encapsulation activity of *P. columella* hemocytes due to incubation with mannose or fucose (Figure 4B) may be
353 explained with two non-exclusive hypotheses: i) monosaccharides saturate glycan-binding molecules that
354 function as hemocyte receptors for sensing exogenous entities, thereby involved in non-self recognition
355 (binding and subsequent immune activation) of the complex carbohydrate surface motifs of a parasite like *F.*

356 *hepatica*, or ii) monosaccharide binding alters hemocyte activity through over-activation of particular glycan-
357 binding molecules that are involved in the normal regulation of hemocyte activity.

358 Lectins, and their activation by specific carbohydrate ligands, have fundamental roles in host-parasite
359 interaction, either associated with parasite binding/recognition (Adema et al., 1997, Yoshino et al., 2001, Wu
360 et al., 2017) or as target for parasite-driven immune modulation/suppression (Guasconi et al., 2011, Rodríguez
361 et al., 2015, Rodríguez et al., 2017). In *B. glabrata*, stimulation with mannose, fucose and galactose promotes
362 the generation of reactive oxygen species by hemocytes (Hahn et al., 2000), fucosylated glycans are crucial for
363 interaction of *S. mansoni* sporocyst with *Bge* cells (Castillo et al., 2007), and fucoidan promotes division and
364 proliferation of hemocyte precursor cells (Sullivan et al., 2014). By contrast, exposure of *B. glabrata* to
365 carbohydrate PAMPS (pathogen-associated molecular patterns), like immunogenic fucoidan, led
366 downregulation of expression in the amebocyte producing organ of many genes, including several that are
367 directly involved in immune response (Zhang et al., 2016). In addition, fucosylated-motifs are proposed to
368 participate along with other carbohydrates in molecular (glycan) mimicry employed by *S. mansoni* parasites to
369 evade the immunity of *B. glabrata* (Lehr et al., 2008, Yoshino et al., 2013). It is of note that evasion by *F.*
370 *hepatica* of immune responses from the mammalian definitive hosts is thought to involve carbohydrates like
371 mannose, fucose and galactose (Rodríguez et al., 2017, Rodríguez et al., 2015, Guasconi et al., 2011).

372 Immune-recognition of different groups of digenetic trematodes appears to require detection of unique
373 carbohydrates. In contrast to the highly fucosylated tegumental antigens of *S. mansoni* sporocysts, (Johnston
374 and Yoshino, 1996, Peterson et al., 2009), intramolluscan *F. hepatica* sporocysts display no fucose motifs, but
375 mainly N-acetyl-D-glucosamine and α -mannose, with minor proportions of galactose and N-acetyl-D-
376 galactosamine at the surface (Georgieva et al. 2016). Although the parasite larvae may present other
377 fucosylated glycans without a terminal fucose--galactose-motif, mannose glycans, because of significant
378 presence at the parasite surface may be particularly important for non-self recognition/binding of *F. hepatica*
379 sporocyst by snail hemocytes. The strong inhibitory impact of mannose on encapsulation of *F. hepatica*
380 sporocysts by snail hemocytes from both R- and S- strains also points at a central role of mannose and
381 mannose-receptors in the *P. columella-F. hepatica* interaction.

382 It is of note that hemocyte (pre-)incubation with monosaccharides (galactose, glucose and L-fucose in
383 particular) impacted individual snails of both R- and S-strains differently, producing different intrapopulation
384 patterns with regards to encapsulation grade; Figure 4b. This observation points at the occurrence of
385 qualitative/quantitative differences concerning lectin receptors for specific carbohydrates on the surface of
386 naïve hemocytes among *P. columella* strains and phenotypes. Similar differences in hemocyte surface

387 determinants have been reported for susceptible and resistant *B. glabrata* snails, suggesting that these may
388 contribute to determine the ultimate phenotypic response after parasite infection (Agner and Granath, 1995,
389 Yoshino et al., 2013). This notion is supported by the demonstration of significant differences in abundance of
390 several immune-factors involved in antigen recognition and pro-inflammatory signaling pathways (C-type
391 lectins, CD109, and mannose-binding protein C, protein kinase C) between R and S phenotypes of *P. columella*
392 prior exposure to *F. hepatica*; Alba et al., 2019). Adding this result to the previous line of thought, an hemocyte
393 surface enriched, for example, in lectins potentially involved in parasite recognition (*e.g.* through mannose
394 interaction) and in the further activation of a protective response, as it appears to be the case of resistant
395 snails, could increase the chances for cell-parasite interaction to occur. The latter was demonstrated here as
396 naïve hemocytes from both resistant populations showed higher encapsulation grades than those from
397 susceptible snails (Figure 4B). In contrast, enriched hemocytes on receptors with regulatory activities are more
398 likely to be driven towards a less responsive phenotype upon stimulation by certain parasite antigens involved
399 in immune modulatory mechanism of host defenses.

400 **4.2 Differential immune-related patterns behind *P. columella* phenotypes during *F. hepatica*** 401 **challenge**

402 *4.2.1 Resistant snails: higher cellular immune competence following exposure*

403 Considering the major role of cellular immunity, at least in part, in resolving *F. hepatica* infections in resistant
404 *P. columella* (Gutiérrez et al., 2003b), the significantly higher hemocyte numbers, particularly of large
405 spreading hemocytes, (compared to susceptible snails) could facilitate the orchestration and maintenance of
406 an effective immunoprotective response against the parasite. Indeed, low numbers of hemocytes may
407 associate with increased susceptibility to infection in the *B. glabrata* – *S. mansoni* model (Oliveira et al., 2010,
408 Larson et al., 2014), and immune stimulation leads to increased hemocyte counts in parasite-resistant *B.*
409 *glabrata* (Noda, 1992, Sullivan et al., 2004). Significantly, hemocyte counts reached a significantly maximum
410 increase in resistant *P. columella* at 24h-post parasite challenge which is also the same time point at which
411 hemocyte encapsulation of *F. hepatica* sporocyst is complete in resistant snails (see Gutiérrez et al., 2003b).

412 Although hematopoiesis in snails is generally believed to occur in a primary hematopoietic site, like the APO of
413 planorbid snails (Pila et al., 2016b), concordant with previous reports of proliferation of circulating hemocytes
414 from lymnaeid snails (Dikkeboom et al., 1988), proliferative blast-like hemocytes were recorded from the
415 hemolymph of *P. columella* snails from R-strains in response to infection. These mitotic events in the immune
416 cells of resistant snails represent a further differential response versus the susceptible phenotype. This

417 increase in hemocyte proliferation may directly contribute to the overall significant increase of cell counts in
418 resistant *P. columella* during infection. While the functional role of blast-like cells remains unclear, perhaps
419 these results indicate a potential stem-cell capacity in *P. columella*, considering that blast-like cells in molluscs
420 from other taxa (caenogastropoda and bivalves) were proposed to be an immature stem cell-like
421 developmental stage (Gorbushin and Iakovleva, 2008, Wang et al., 2012). Alternatively, blast-like cells have
422 been also associated with crucial roles in pathogen interaction during infection through the production of
423 molecular signals (Cima et al., 2000, Travers et al., 2008, Portet et al., 2018).

424 However, an increase in the number of circulating hemocytes and/or proliferative rate does not always
425 provide increased resistance, (for example consider parasite-snail associations of *Himasthla elongate* -
426 *Littorina littorea* (Gorbushin and Iakovleva, 2008) and *F. hepatica* – susceptible *P. columella* (Ribeiro et al.,
427 2017). For effective encapsulation to occur, hemocytes must recognize and adhere to the surface of a non-self
428 target and to each other, and form layers of overlapping cells to surround and isolate the parasite (Loker et al.,
429 1982). At 24h-post exposure, the large adherent hemocytes from R-strain *P. columella* snails showed a strong
430 *in vitro* tendency to aggregate into sheet-like cell layers of spreading, cohesive hemocytes that are
431 interconnected by an abundance of numerous and long, broad pseudopods (see Figure 2), likely behaviors that
432 contributes to effective cellular immune response against *F. hepatica*. Remarkably, the highest encapsulation
433 grade was strongly associated with hemocytes from R snails at 24h post-exposure suggesting that immune
434 activation in response to *F. hepatica* further enhances the considerable baseline immune effector capabilities
435 of hemocytes from R strains of *P. columella* (see Figure 4B).

436 Lastly, resistant *P. columella* significantly increased the expression of granulin, a proliferative cytokine,
437 following exposure to *F. hepatica*. This evolutionary-conserved cytokine regulates hematopoiesis and
438 inflammation (Jian et al., 2013), *B. glabrata* granulin not only promotes hemocytes proliferation but also drives
439 differentiation of these cells towards a more adherent and highly phagocytic phenotype, more prompt to
440 cellular response and associated with a lowered susceptibility to *S. mansoni* infection (Pila et al., 2016a). Thus,
441 as result of overall higher expression during 24h after parasite challenge observed here in R-snails (Figure 3C),
442 granulin may function in proliferation and regulation of the phenotypical and functional changes of hemocytes
443 (Figures 2-4) also observed in R-*P. columella* - *F. hepatica* interaction. Of course, functional validation through
444 gene silencing/editing is needed to demonstrate the role of granulin in the activation of *P. columella*
445 hemocytes following *F. hepatica* exposure. In addition, multiple determinants and molecular signals from both
446 host and parasite probably contribute to the different profiles recorded from parasite-exposed *P. columella*
447 and these remain to be elucidated also.

448 4.2.2 Susceptible snails: evidence of parasite-driven immune modulation

449 Study of hemocytes from susceptible *P. columella* provided insights that contrast strongly with the indications
450 of potent cellular immunity from resistant snails. Following infection with *F. hepatica*, hemocyte layers from
451 exposed-susceptible snails showed that large hemocytes remained mostly scattered, displaying slender or
452 short pseudopodia compared to naïve cells. In addition, no significant proliferative activity was recorded in
453 blast-like cells (or any other hemocytes). Compared to naïve snails, there was a significant decrease in
454 expression levels of the cytokine granulin at 12 and 24h post exposure, and this might relate to the differential
455 morphological and functional patterns in exposed-hemocytes from susceptible *P. columella*. Possibly, the
456 depressed immune features evident from S-strains after infection is relate to modulation of snail host
457 immunity by the parasite, through as yet to be characterized signals (molecules) that only (or mostly) interact
458 with molecular targets of the susceptible phenotype snails. Trematode countermeasures against host defenses
459 responses include direct immune modulation (interference), and this has been reported for several parasite-
460 host models, including *S. mansoni*/*E. paraensei* – *B. glabrata* (e.g. DeGaffé and Loker, 1998, Loker et al., 1992,
461 Zahoor et al., 2008), *H. elongata* – *L. littorea* (Iakovleva et al., 2006), *Trichobilharzia regenti* – *Radix lagotis*
462 (Skála et al., 2014) and *Plagiorchis mutationis* – *Lymnaea stagnalis* (Kryukova et al., 2014). In case of *F.*
463 *hepatica*, parasite antigens are known to mediated potent immune suppression/modulation in the definitive
464 host (e.g. Prowse et al., 2002, Hamilton et al., 2009, Dowling et al., 2010), and the results of this study indicate
465 that *F. hepatica* also interferes with immune function in the susceptible snail intermediate host.

466 It is also significant that no clear pattern was evident regarding encapsulation activity of hemocytes from
467 parasite-exposed snails of the susceptible *P. columella* strains, particularly S-Aurora, with encapsulation grades
468 ranging between 2 to 4. This differential impact of parasite infection upon hemocyte immune function may be
469 explained by different degrees of compatibility, sensitivity to parasite-mediated interference among snails of
470 the same susceptible population. In this sense, while both the S-Aurora and S-Negrines populations of *P.*
471 *columella* are susceptible, infection does not develop following exposure to *F. hepatica*, in 20% and 45% of
472 snails, respectively. Genotype-genotype interactions may drive different infection outcomes for single,
473 individual level host-parasite interactions. Such a compatibility polymorphism has been applied to the *B.*
474 *glabrata* – *S. mansoni* model (see Theron and Coustau, 2005) and is proposed to also occur in susceptible *P.*
475 *columella* – *F. hepatica* system (Vázquez et al., 2014, Alba et al., 2018).

476 4.3 A model for immunobiology of resistant *P. columella* – *F. hepatica* interactions

477 About the genetic bases of the interplay: Remarkably, the resistant *P. columella* field populations always defeat
478 *F. hepatica* infection, regardless of the parasitic load (Alba et al. 2018) or the geographic origin of the parasitic
479 strain used in the challenge, either Cuban (e.g. Calienes et al., 2004, Vázquez et al., 2014) or more
480 geographically-distant (see Alba et al. 2018). This suggest that resistance in *P. columella* is not tightly restricted
481 to a host genotype – parasite genotype interaction, especially because *F. hepatica* is recognized as a
482 genetically diverse parasite (Cwiklinsky et al., 2015, Vázquez et al., 2016). The hypothesis postulated here is
483 that the outcome (infection versus non-infection) after *F. hepatica* penetration is determined by
484 absence/scarcity or presence/abundance of a major contributing factor, possibly associated in *P. columella*
485 with a single locus or few low-diversified loci. This major host factor would be involved proximately or
486 ultimately in the immunobiological interplay, interacting/interfering with factor/function(s) determined by a
487 single or few highly conserved loci of the parasite, that are fundamentally crucial for establishment within
488 snails, that accordingly are displayed by most, if not all, *F. hepatica* strains. While all snails of resistant
489 populations likely are selected for genotypes that possess the above-mentioned major contributing factor, it
490 appears that lack of this trait in other genetic lineages determines susceptibility in *P. columella*. Recent studies
491 have demonstrated the influence of allelic variations in a single 1 Mb sized-genomic region containing 15
492 protein coding loci of *B. glabrata* (i.e. Guadalupe resistance complex) susceptibility to schistosome infection
493 (Allan et al., 2017) and its effect on other biological roles like microbiome diversity (Allan et al., 2018b), and
494 humoral (Allan et al., 2019) and cellular (Allan and Blouin, 2018a) immune properties of the hemolymph.
495 However, the outcome and intensity of infection in *S-P. columella* snails is ultimately dictated by yet other
496 molecular determinants, possibly coded by different loci with several allelic variants as to explain the proposed
497 polymorphism of compatibility. This is a plausible theory because several steps ranging from snail host finding,
498 parasite establishment and development, and molecular determinants (including immune factors) determine
499 compatibility in any host-parasite interaction (Chen, 1968, Combes, 2001, Tennessen et al., 2015, Portet et al.,
500 2017).

501 About the evolutionary and mechanistic bases of resistance: The limited information concerning the molecular
502 and physiological dialogue between *F. hepatica* and lymnaeid host snails challenges detailed speculations
503 about particular molecules and mechanisms coevolving and codetermining the outcome of infection.
504 However, *F. hepatica* is presumed to mount efforts to effect parasite-driven immune modulation/suppression
505 in the intermediate hosts, that will particularly affect susceptible but not resistant *P. columella* strains (see
506 section 4.2.2 for discussion). One of the major differences between both phenotypes at the molecular and
507 cellular level is the existence of enhanced immune responsiveness prior infection in resistant snails which
508 could have been influenced from host-parasite coevolutionary dynamics. Recently, it has been proposed that

509 coevolution of parasites with specific immune components (receptors for detection/interaction or effectors for
510 elimination) may alter energy allocation in the host to optimize defense against infection. Especially, if co-
511 evolutionary feedback between parasite and host populations renders induced immune responses less
512 efficient due to parasite counter-evolution, then the host will be driven to heavily invest in constitutive
513 immunity (Kamiya et al., 2016). In this sense, *F. hepatica* driven-immune modulation as a mean for parasite
514 establishment would certainly pose a difficult challenge to overcome for a snail host if it relies mainly on
515 induced immunity. Thus, an emphasis toward enhanced constitutive immune responsiveness could enable the
516 host to counter immediately, preventing the parasite to settle and activate an arsenal of immune modulatory
517 mechanisms. Recalling what was previously discussed, *F. hepatica* immunomodulatory/immunosuppressive
518 strategies may be the above-mentioned major highly conserved function fundamentally crucial for
519 establishment within snails, whereas the major factor determining resistance in *P. columella* circumvents
520 parasite's major infective function possibly by proximately or ultimately participating in the constitutively
521 increased resource allocation to immune defense (e.g. higher expression levels of pathogen interacting
522 molecules, pro-inflammatory signaling pathways, cytokines, antimicrobial and anti-oxidant molecules; Alba et
523 al., 2019). Apart of the nature of the antigen, the lineage/state of the host cells and the environment in which
524 the antigen is 'seen' by the immune system are crucial to decide the ultimate response thus, a differential
525 molecular and cellular baseline between *P. columella* phenotypes may contribute from the start to the
526 "insensibility" of resistant snails towards parasite immune modulation mechanisms. Similarly, constitutive
527 differences between *B. glabrata* strains, susceptible and experimentally-selected for resistance to infection
528 with *Echinostoma caproni*, were proposed as the base for refractoriness by preventing immunosuppression
529 efforts of the parasite (Humbert and Coustau, 2001). For instance, prior infection, enriched hemocytes in
530 surface determinants directly committed to non-self recognition and/or pro-activation signaling upon
531 stimulation in resistant snails may render them more prone to trigger protective anti-parasitic responses than
532 to be driven into a refractory or suppressed immunophenotype, as appears to be the case in *S-P. columella*
533 strains. As it was previously suggested, differences in hemocyte surface determinants are likely to occur
534 between resistant and susceptible snails and the higher propensity by naïve hemocytes to encapsulate *F.*
535 *hepatica* sporocysts (see Figure 4B) constitutes an experimental evidence of an enhanced immune
536 responsiveness prior infection in the resistant *P. columella* phenotype.

537 Finally, the indications for a highly efficient amplification of immune activities following parasite exposure in R-
538 compared to *S-P. columella* needs to be interpreted as the conjunction of both factors: the enhanced baseline
539 immune capabilities that contribute to recognize non-self and to trigger a potent protective response, and the

540 hijacked of *F. hepatica* immunosuppressive strategies that influence parasite's infective capacity to
541 migrate/colonize or to develop inside the host.

542 Figure 5 presents a model that integrates the current knowledge regarding molecular and functional
543 determinants that may contribute the immunobiological bases for resistance in this interesting host-parasite
544 interplay between *F. hepatica* in *P. columella* snails. Future studies aiming at comparing the transcriptomic
545 dynamics during *F. hepatica* – *P. columella* resistant/susceptible interactions will help focus further
546 investigations and bring forward new elements to confirm and/or expand this model.

547 **Conclusions**

548 Comparative approaches were used to explore the immunobiology behind interactions of predictably resistant
549 and susceptible phenotypes of *P. columella* with *F. hepatica*. After (pre-)incubation with different
550 monosaccharides, decreased encapsulation activities of *F. hepatica* sporocysts by naïve hemocytes, were
551 observed, irrespective of the *P. columella* phenotype. This suggests the involvement of carbohydrate-binding
552 receptor molecules (lectins) in regulation of functional behavior of these cells towards the parasite. Future
553 investigations will be aimed at in-depth exploration of the lectin – ligand interactome that governs the
554 hemocyte response to the parasite. Whereas naïve hemocytes from resistant *P. columella* strains showed
555 higher encapsulation grades compared to susceptible snails, parasitic challenge also brought about significant
556 phenotypical and functional differences at the hemocyte level between R and S snail phenotypes. These
557 changes may further enhance the differential resistant and susceptible phenotypes of *P. columella* in relation
558 to *F. hepatica* infection. Specifically, parasite infection resulted in immune modulation, rendering anti-parasite
559 immunity in susceptible snails even less effective while *F. hepatica* exposure boosted hemocyte activities in
560 resistant *P. columella*, possibly augmenting the rapid immune-elimination of parasites that associates with the
561 resistant phenotype of these snail strains. The augmented response was characterized (but may not be limited
562 to) a significant increase of overall hemocyte counts, enhanced proliferation of blast-like cells, morphological
563 changes of large adherent cells, a greater *in vitro* encapsulation activity from hemocytes (the latter also
564 observed *in vivo*; Gutiérrez et al., 2003b), and a significantly increased transcription of granulin, a cytokine
565 involved in proliferative, activation and differentiation of hemocytes.

566 In summary, snails from resistant strains of *P. columella* have an inherent ability to initiate and amplify a
567 powerful and protective response following *F. hepatica* exposure. This ability is likely due to an enhanced
568 allocation of resources to defense in resistant *P. columella* at baseline level and after parasitic exposure.

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578 **Author's contributions:**

579 AA, DD, JS, ABP, AAV, BG designed and performed the experiments and analysis, and participated in the
580 reviewing process. AA, AAV, JS participated in snail breeding, parasite exposures, hemocyte quantification and
581 *in vitro* assays (experiment and analysis). AA, ABP, DD: conducted the cytometer experiments, cell staining and
582 morphological descriptions. DD, RG, SP participated in qRT-PCR experiments. AA, DD, BG drafted the
583 manuscript. All authors read and approved the final version of the manuscript. All data generated or analyzed
584 during this study are included in this article.

585 **Declarations of interest:**

586 Author's declare that there no conflict of interest exists

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804 **Legends to figures**

805 **Figure 1.** Dot plots (A-B; pooled hemolymph from 4 snails/strain) and DAPI/Phalloidin stained (C-D) naïve
806 hemocytes from *Pseudosuccinea columella* susceptible and resistant to *Fasciola hepatica*. Green circles in A-B
807 indicates the two main morphotypes of cells, segregated by size and cytoplasmic complexity. Arrows in C-D
808 point at the small-round shape cell with high nucleus/cytoplasm ratio morphotype; phalloidin (actin labelling,
809 green) and DAPI (nucleus labelling, blue).

810 **Figure 2.** Confocal microscopy images of *Pseudosuccinea columella* hemocytes from naïve (upper panel, 0h)
811 and *F. hepatica*-challenged (lower panel, 24h post-exposure), resistant and susceptible snails from four
812 different populations. Arrows in A-D point at the small-roundly shape cell with high nucleus/cytoplasm ratio
813 morphotype. Cell staining was performed with Phalloidin (actin labelling, green) and DAPI (nucleus labelling,
814 blue).

815 **Figure 3.** (A) Number of circulating hemocytes in the hemolymph of resistant and susceptible *Pseudosuccinea*
816 *columella* at different time points post-exposure to *Fasciola hepatica* miracidia. B) Hemocytes of 24h-
817 challenged resistant snails with DAPI-labelled nucleus (in blue) in some of which active proliferation (EdU
818 labelling, green), corresponding only to small- roundly shape cells (arrow, lower panel), was observed. C)
819 Relative quantity of granulin expression in resistant and susceptible *P. columella* at different time points post
820 exposure to *F. hepatica*. In bars, normalized RQs at each time point against the RQ in naïve conditions (0h) for
821 each strain; different letters represent statistical significance among experimental points $P < 0.05$.

822 **Figure 4.** A) Quantitative scale used to represent the observed differences (encapsulation grade: 1 to 5) on the
823 *in vitro* encapsulation of *Fasciola hepatica* sporocyst by *Pseudosuccinea columella* hemocytes. B) Association
824 (through multivariate correspondence analysis), of the encapsulation grade of hemocytes from resistant (R)
825 and susceptible (S) *P. columella* with the assayed conditions (incubation of naïve hemocytes with
826 monosaccharides or *F. hepatica* challenge). *P. columella* populations: LP: La Palma, LC: La Coca, AU: Aurora,
827 NE: Negrines; Monosaccharides: man: mannose, fuc: fucose, gal: galactose, glu: Glucose; Time-points of
828 experimental infection: 0h: naïve hemocytes, 24h: 24h-challenged hemocytes.

829 **Figure 5.** Integrative model of the immune response in resistant (R) *Pseudosuccinea columella* snails toward
830 the digenean *Fasciola hepatica*. The significant immune competence in naïve snails prior to infection possibly
831 helps to negate and avoid *F. hepatica*-driven immune modulation, leading to enhanced immune

832 responsiveness and to activation of specific pathways resulting in parasite elimination through hemocytic
833 encapsulation. During interaction, the early molecular crosstalk between parasite-specific antigens and host
834 receptors increases the hematopoiesis of circulating blast-like cells, the immune activation of large-adherent
835 hemocytes to assume a more active morphotype and stimulates the production of the cytokine granulins. The
836 constitutive high expression levels of protein kinase C (PKC) and of several Toll-like receptors (TLR) in naïve
837 resistant snails may facilitate orchestration of the induced immune response that is triggered upon parasite
838 penetration. Likewise, the abundant expression of macrophage migration inhibitory factor (MIF) prior to
839 infection may also contribute to the immune stimulation of hemocytes. All the latter may contribute to build
840 up the cellular immune reaction; at 24h post exposure the increase in the encapsulation activity of the
841 hemocytes results in the visualization of well-formed capsules surrounding the parasite that will eventually
842 leads to the parasite death. In this sense, the abundance of superoxide dismutase and nitric oxide synthase
843 along with anti-parasite specific humoral or intracellular immune effectors (e.g. ferritin, lipopolysaccharide-
844 binding protein/bactericidal permeability-increasing protein), as reported in resistant *P. columella* prior
845 infection, might positively influence parasite clearance following exposure. Man: mannose, ROS: reactive
846 oxygen species, RNS: reactive nitrogen species.

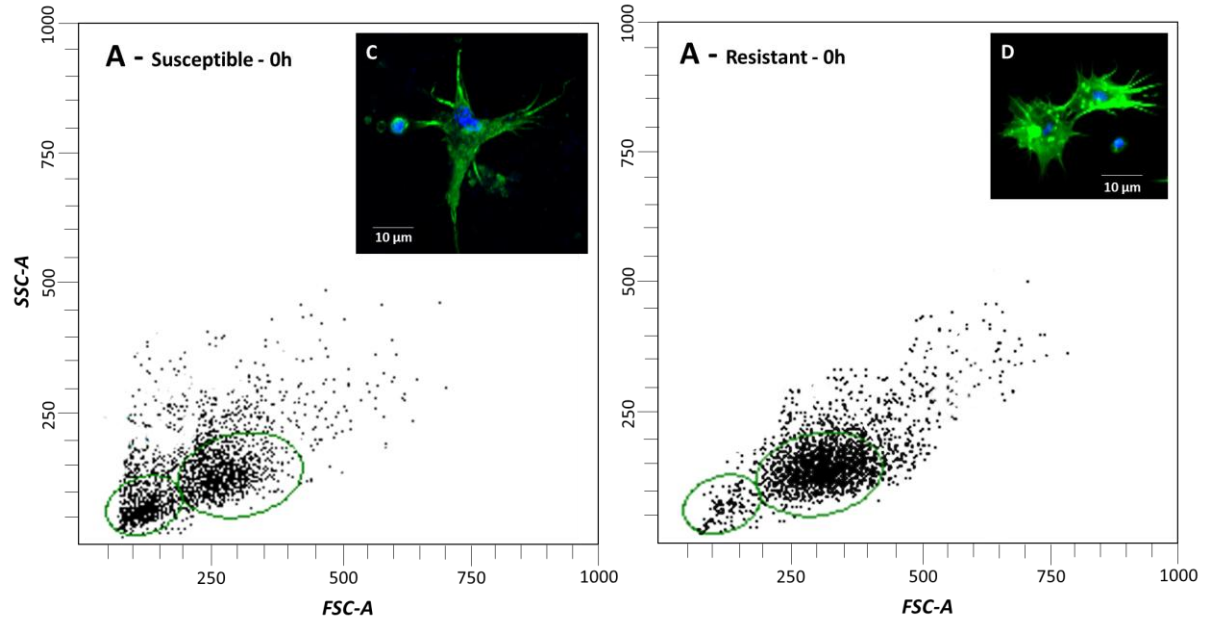
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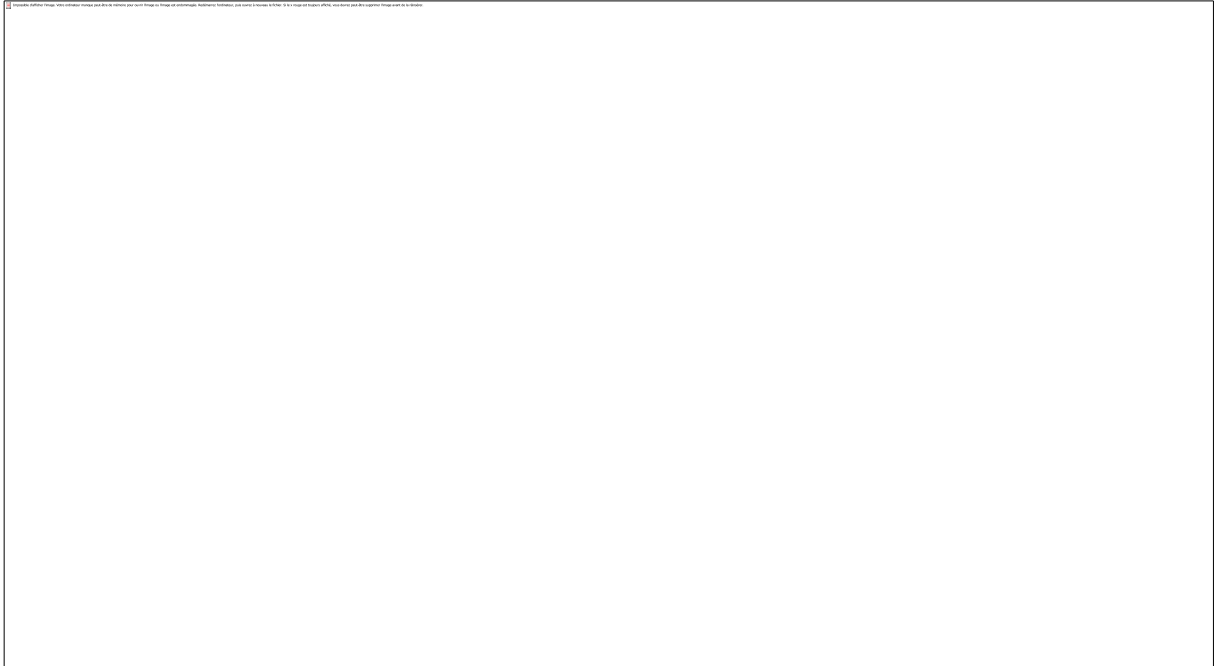
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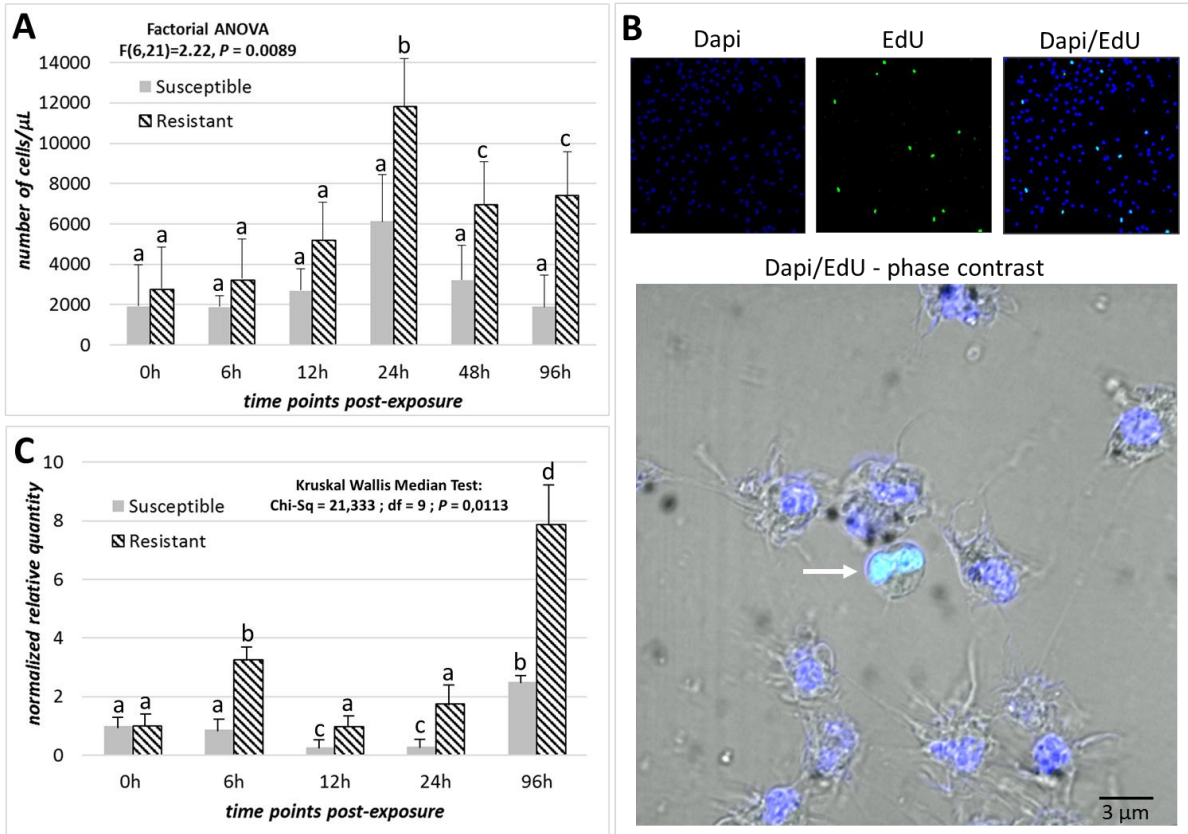
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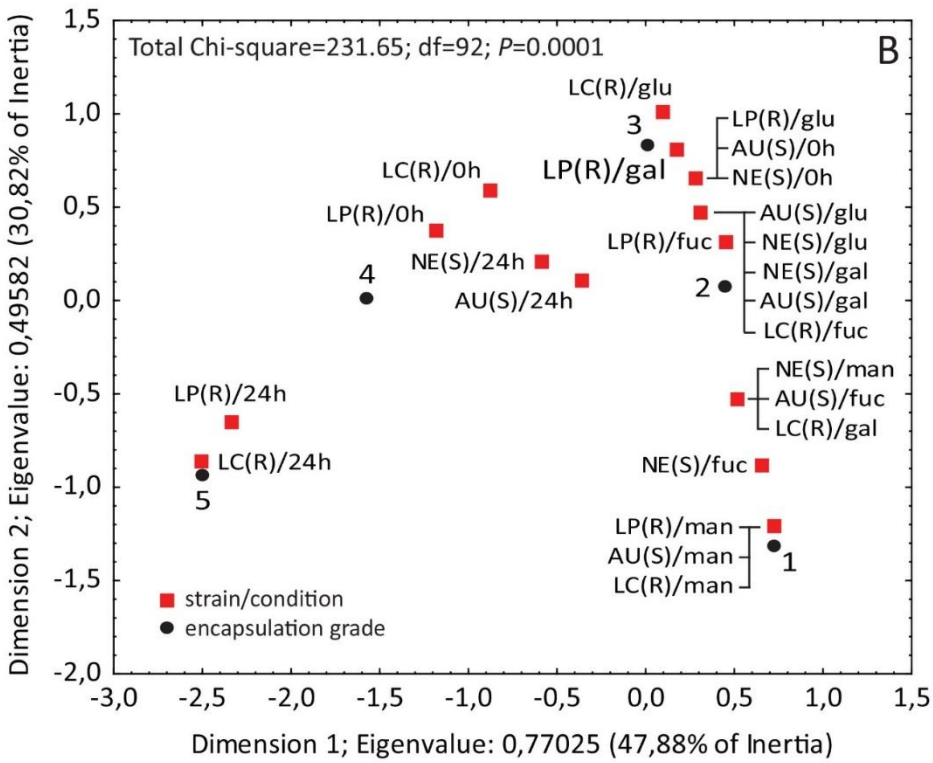
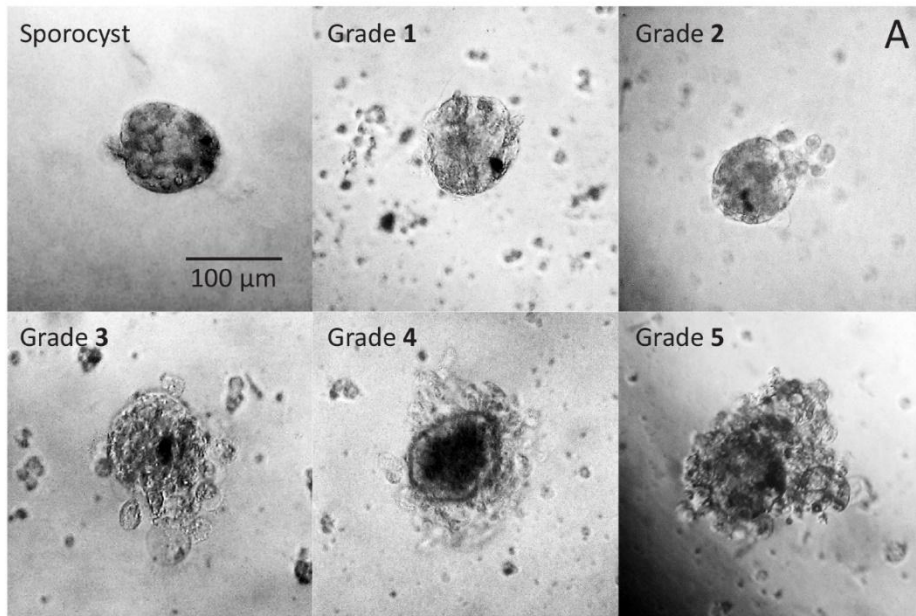


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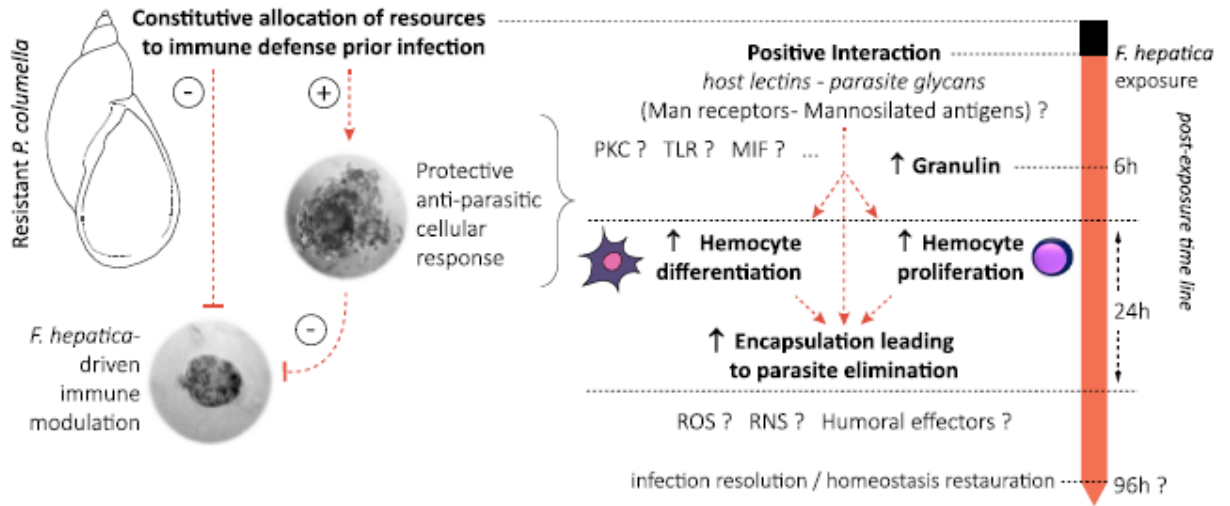
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