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

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

2. Material and methods

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

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

2.2 Experimental exposures of snails to *F. hepatica*

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

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

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

2.3 Hemolymph sampling

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

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

2.4.1 Flow cytometry

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

2.4.2 Confocal Fluorescence microscopy

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

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

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

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

2.6 Assessment of in vitro hemocyte proliferation

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

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

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

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

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

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

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

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

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

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

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

2.9 Data analysis

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

3. Results

3.1 Morphological observations of *P. columella* hemocytes

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

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

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

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

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

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

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

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

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

3.3 *In vitro* encapsulation assays

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

4.2.1 Resistant snails: higher cellular immune competence following exposure

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

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

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

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

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

4.2.2 Susceptible snails: evidence of parasite-driven immune modulation

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

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

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

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

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

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

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

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

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

Conclusions

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

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

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Author's contributions:

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

Declarations of interest:

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).

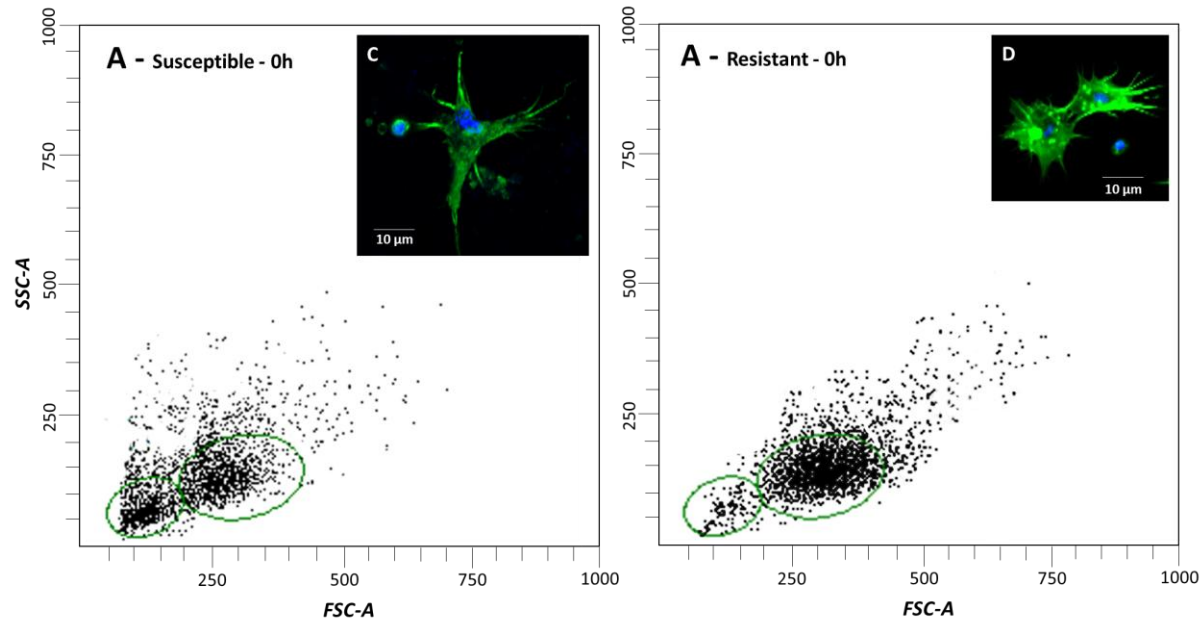
815 **Figure 3.** (A) Number of circulating hemocytes in the hemolymph of resistant and susceptible *Pseudosuccinea*
816 *columella* at different time points post-exposure to *Fasciola hepatica* miracidia. B) Hemocytes of 24h-
817 challenged resistant snails with DAPI-labelled nucleus (in blue) in some 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

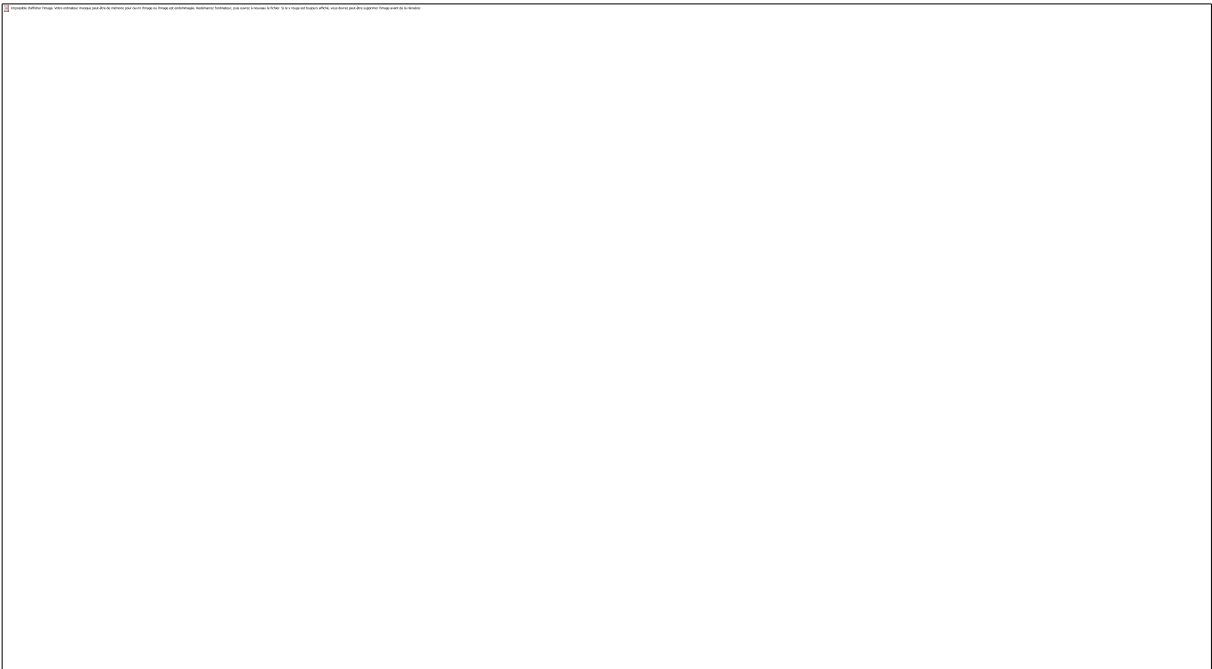
responsiveness and to activation of specific pathways resulting in parasite elimination through hemocytic encapsulation. During interaction, the early molecular crosstalk between parasite-specific antigens and host receptors increases the hematopoiesis of circulating blast-like cells, the immune activation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

Figure 1



857 Figure 2

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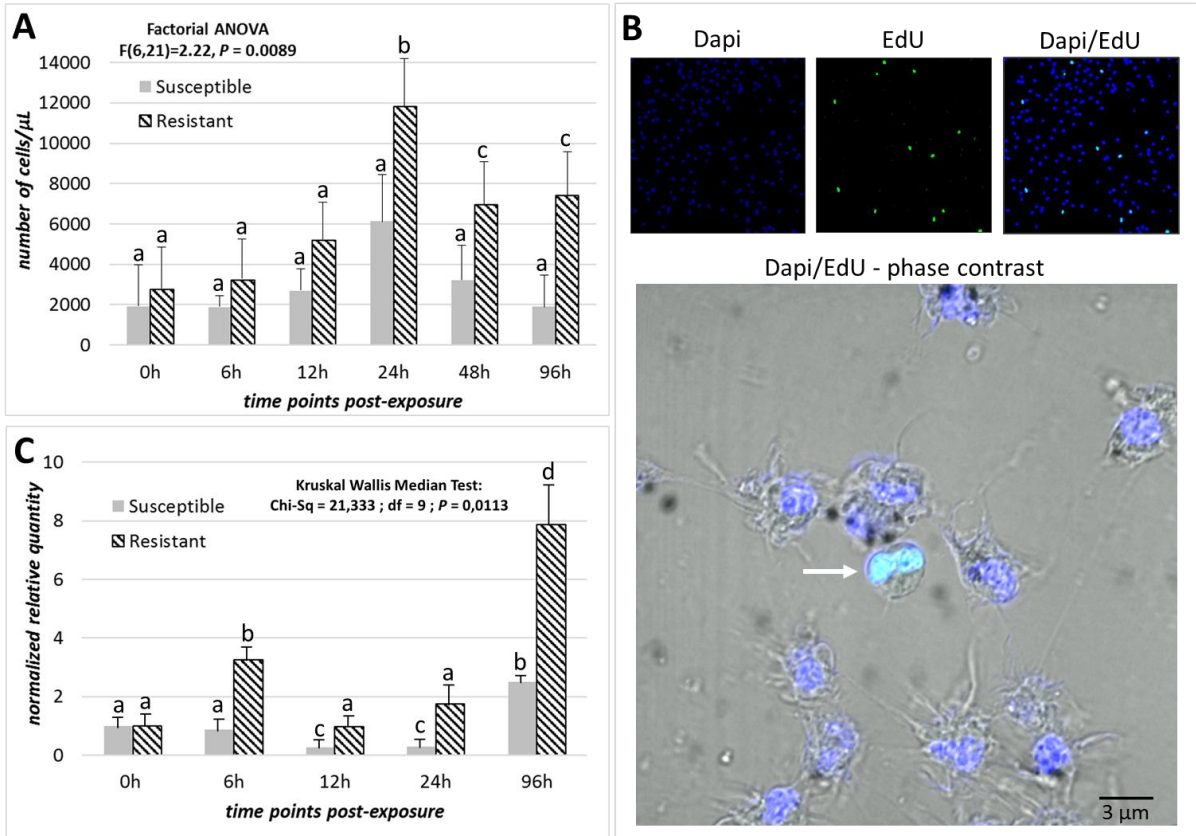


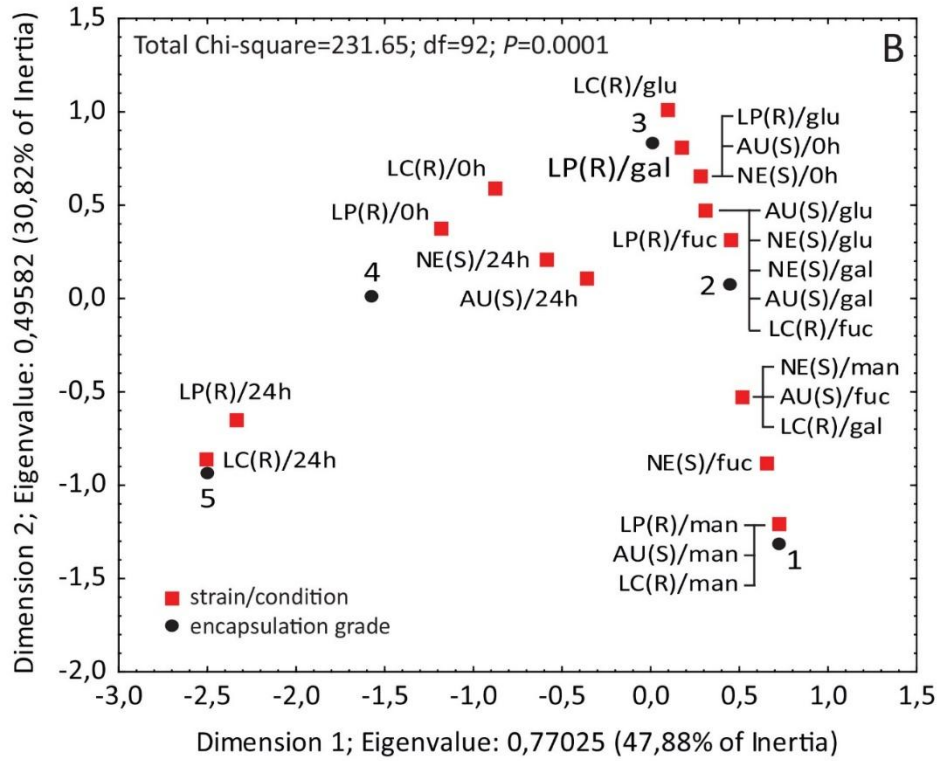
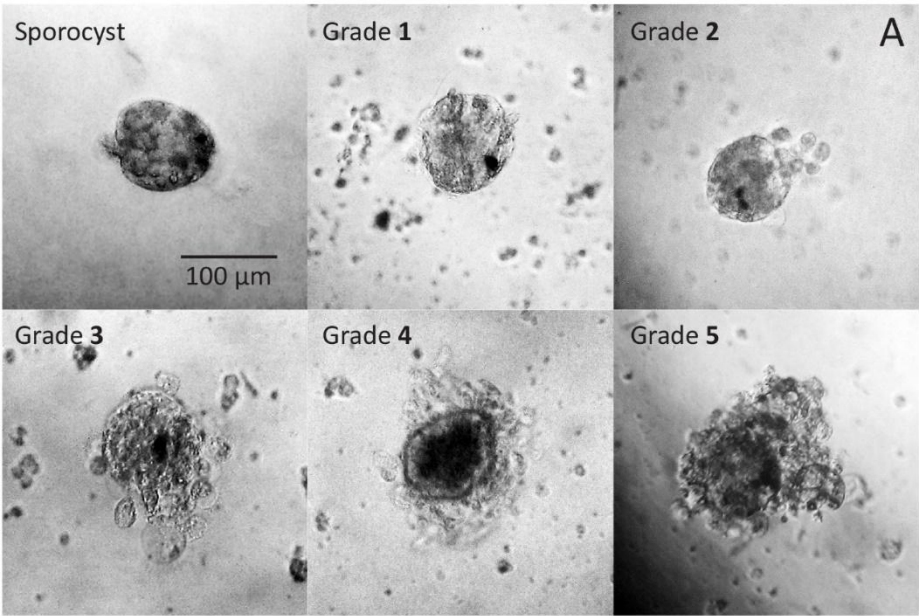
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Figure 3





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Figure 5

