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Natural resistance to *Fasciola hepatica* (Trematoda) in *Pseudosuccinea columella* snails: A review from literature and insights from comparative "omic" analyses

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Abstract :

The snail *Pseudosuccinea columella* is one of the main vectors of the medically-important trematode *Fasciola hepatica*. In Cuba, the existence of natural *P. columella* populations that are either susceptible or resistant to *F. hepatica* infection offers a unique snail-parasite for study of parasite-host compatibility and immune function in gastropods. Here, we review all previous literature on this system and present new "omic" data that provide a molecular baseline of both *P. columella* phenotypes from naïve snails. Comparison of whole snail transcriptomes (RNAseq) and the proteomes of the albumen gland (2D-electrophoresis, MS) revealed that resistant and susceptible strains differed mainly in an enrichment of particular biological processes/functions and a greater abundance of proteins/transcripts associated with immune defense/stress response in resistant snails. These results indicate a differential allocation of molecular resources to self-maintenance and survival in resistant *P. columella* that may cause enhanced responsiveness to stressors (i.e. F. hepatica infection or tolerance to variations in environmental pH/total water hardness), possibly as trade-off against reproduction and the ecological cost of resistance previously suggested in resistant populations of *P. columella*.

Highlights

► Comparative "omic" analyses of *P. columella* offer clues for resistance to *F. hepatica*. ► Naïve resistant snails display a higher molecular competence for defense/stress responses. ► Constitutively broad expression of immune factors associates with resistance to *F. hepatica*. ► Overrepresented pH/osmotic regulators endorse the pH tolerance of resistant snails. ► Resource allocation to defense/stress response endorse reproductive trade-offs.

Keywords : transcriptome, albumen gland, allocation of resources, response to stress, immune defense, cost of resistance

Abbreviations

Bge cells	*Biomphalaria glabrata* embryonic cell line
G-CSFR	granulocyte colony stimulatory factor
IRF	interferon regulatory factors
LBP/BPI	lipopolysaccharide-binding protein/bactericidal permeability-increasing protein
LRR	leucine rich repeats
MIF	macrophage migratory inhibitory factor
Myd88	myeloid differentiation primary response
PKC	protein kinase C
SOD	superoxide dismutase
TGF	transforming growth factor
TH	Total hardness
TLR	toll-like receptor
TBH	tyramine β-hydrolase

1. Introduction

Among digenean parasites that are transmitted by snail vectors, those causing fascioliasis (*Fasciola* spp.) and mainly *Fasciola hepatica* have gained particular interest due to their wide distribution, their impact on veterinary health and on the economy, in association with livestock infections (Khan et al., 2013, Mehmood et al., 2017), and their re-emergence as human pathogens (Mas-Coma et al., 2009). *Fasciola* spp. have a two-host life cycle (*i.e.* mammals and snails as definitive and intermediate hosts, respectively), and transmission in a specific geographical area is mostly dependent on presence of vector snails of the family Lymnaeidae. In this sense, host efficiency may be influenced by the local adaptation between parasite and snail species (Dar et al., 2013, Vázquez et al., 2014).

Worldwide, 30 lymnaeid species are known as intermediate hosts of *Fasciola* spp. (Vázquez et al., 2018). Particularly, *Pseudosuccinea columella* (formerly known as *Lymnaea columella*), considered one of the main intermediate host species for transmission of *F. hepatica*, is an efficient invasive snail species that has attained wide geographical distribution (Lounnas et al., 2017; Vázquez et al., 2018). Presumed to have originated in North America, the high adaptability and invasive capacity of few genotypes have allowed establishment of *P. columella* in several parts of the world (for details, see Lounnas et al. 2017). Nowadays, *P. columella* is reported in South America and the Caribbean (Cucher et al., 2006, Gutiérrez et al., 2011), as well as in Africa (Brown, 1994), Australia (Molloy and Anderson 2006), several Pacific islands (Cowie 2001, Pointier and Marquet, 1990) and Europe (Pointier et al., 2007).

Concomitantly, the global distribution of this snail species has also complicated the epidemiological scenario of fascioliasis (Lounnas et al. 2017). Transmission of *F. hepatica* by *P. columella* is well documented in and outside of its native range (e.g. Gutiérrez et al. 2011; Cruz-Reyes and Malek, 1987; Cucher et al. 2006) and *P. columella* snails have been found also naturally infected with *Fasciola gigantica* (Grabner et al. 2014). *Fasciola hepatica* infection in this species does reduce snail fecundity (Gutiérrez et al., 2002, Salazar et al., 2006), but is also characterized by higher redial and metacercariae productivity compared to *Galba truncatula* (Dar et al., 2014, Vignoles et al., 2015) and greater survival when compared to species of *Galba* (Salazar et al., 2006, Vignoles et al., 2015).

1.1 Lymnaeid snails and *F. hepatica* transmission in Cuba: resistant and susceptible populations of *P. columella*

In Cuba, *F. hepatica* is transmitted by two species of snails: *Galba cubensis*, considered the main intermediate host, (natural prevalence ranged from 1 to 34%; Alba et al., 2016, Vázquez et al., 2015) and

P. columella (Alba et al., under review, Gutiérrez et al., 2011). The latter has a more discrete distribution than *G. cubensis* (Vázquez et al., 2009); to date reported from only 68 localities in western and central Cuba with no records in the easternmost region of the island (Alba et al., under review). *Pseudosuccinea columella* shows no preferences for anthropic or natural habitats (Vázquez et al., 2009). Considered to have a secondary role as intermediate host of *F. hepatica* in Cuba (Vázquez et al., 2014), only two populations, located in Pinar del Rio province (*i.e.* IPA and Pilon, western Cuba), have been found naturally-infected with *F. hepatica*, with prevalence of 3 and 10%, respectively (Alba et al., under review, Gutiérrez et al., 2011). Despite so relatively few natural infections with *F. hepatica* as compared to *G. cubensis*, snails from most of the *P. columella* field populations can be successfully infected in the laboratory with Cuban *F. hepatica* isolates (Calienes et al., 2004; Gutiérrez et al., 2002, Vázquez et al., 2014). The prevalence of infection following lab-exposure of *P. columella* to *F. hepatica* varies depending on the snail (population) – parasite (isolate) combination used, indicating the existence of a polymorphism of compatibility in this parasite – host system (Vázquez et al., 2014, Alba et al., 2018).

Remarkably, *P. columella* snails from particular localities on Cuba, *i.e.* La Palma, El Azufre, Babiney, La Playita, La Coca and Candelaria (see Alba et al. (under review)) have never been found with naturally occurring *F. hepatica* infection, nor do they incur infection after experimental exposures to field-derived parasites, regardless of the local *F. hepatica* isolates used, challenge with increasing doses of the infective larvae (from 5 to 30 miracidia/snail), through single or serial exposures, and with allopatric parasites from Dominican Republic and from France (Alba et al., 2018, Calienes et al., 2004, Gutiérrez et al., 2002, Gutiérrez et al., 2003b, Vázquez et al., 2014). A previous study informed that the immune cells (hemocytes) of these *P. columella* snails encapsulate *F. hepatica* larva shortly after parasite penetration (at 24h post-exposure; Gutiérrez et al., 2003b). The above results indicate the occurrence of an effective immunological response rather than an unsuitability of these snails to *F. hepatica* (Alba et al., 2018, Gutiérrez et al., 2003b, Vázquez et al., 2014). Interestingly, as with *F. hepatica*-susceptible *P. columella* successful natural and experimental infections of resistant snails with other trematodes occur suggesting a certain specificity of the resistance (authors' unpublished data).

1.1.1. One species, two phenotypes

Significantly, resistant and *F. hepatica*-susceptible *P. columella* snails show no differences concerning the anatomy of their reproductive system, and share the same reliable conchological characters that are defined for *P. columella*: big shell, small spire, large aperture of about two thirds of the shell height and characteristic micro-sculptures in the periostracum (Correa et al., 2011, Pointier, 2008, Vázquez and

Sánchez, 2015). In addition, amplification and sequencing of nuclear ribosomal genes (1170 bp comprising the 3' region of the 18S, ITS-1, 5.8S, ITS-2 and the 5' region of the 28S) from resistant and susceptible snails showed a slight difference between phenotypes in only two bases, one in each ITS fragment (Gutiérrez et al., 2003a). Such differences represented only 0.17% of sequence variation between the two strains of *P. columella* and thus, are insufficient to segregate both phenotypes into different species (Gutiérrez et al., 2003a) especially considering the high level of self-fertilization observed in this species (Lounnas et al., 2017). All the above criteria can be used to discriminate certain species of molluscs, even within the family Lymnaeidae (*e.g. P. columella*; Bargues and Mas-Coma, 1997, Correa et al., 2011) but only when differences are evident. The high similarity encountered between these strains at morphologic, anatomic and genetic levels points at the occurrence of two different phenotypes concerning *F. hepatica* infection within *P. columella* species (Gutiérrez et al., 2003a). To our knowledge, this is a unique report of field-occurring resistance/susceptibility in a snail – trematode system with perhaps, the exception of the anti-digenean persistent resistance in the snail isolate BS90 of *Biomphalaria glabrata* versus *Schistosoma mansoni* (Paraense and Correa, 1963).

It is important to mention that the inheritance pattern of the specific markers concerning susceptibility and resistance of *P. columella* remains to be established. However, this issue is difficult to resolve by mating experiments as it has been demonstrated, by microsatellite markers-based analysis, that self-fertilization constitutes the almost exclusive reproductive strategy of this species, irrespective of its phenotype (Alba et al., under review, Lounnas et al., 2017, Nicot et al., 2008). A high-selfing rate is, in fact, a common feature of the family Lymnaeidae and has been reported also for other species including *G. truncatula*, the main vector of fasciolosis worldwide (Meunier et al., 2004).

1.1.2. Morphological and genetic differences between P. columella phenotypes

The resistant and susceptible *P. columella* populations can be readily and reliably discriminated by a morphological marker consisting of characteristic pigmentation pattern of the mantle (Gutiérrez et al., 2003b). All resistant snails display a band of small sharp spots in the mid-region of the mantle with bigger spots uniformly distributed on the upper and lower sides (Alba et al., under review, Gutiérrez et al., 2003b). Contrastingly, susceptible snails have diffused and sparser mantle spots, scattered without a clearly defined pattern (Gutiérrez et al., 2003b; see Figure 1 for details).

Resistant populations of *P. columella* can be also distinguished from susceptible snails by randomly amplified polymorphic DNA; RAPD (Calienes et al., 2004, Gutiérrez et al., 2003a), mitochondrial

haplotypes (Lounnas et al., 2017) and nuclear microsatellites (Alba et al., under review; Lounnas et al. 2017). These studies have demonstrated that resistant populations clustered separately from most *P. columella* populations from Cuba (Alba et al., under review, Calienes et al., 2004) and from the rest of the world (Lounnas et al., 2017), supporting the notion of a genetic determinism of the resistance to *F. hepatica*. Such segregation suggests that resistant *P. columella* have been selected from different pools than the susceptible snails that have spread widely, and that *P. columella* snails grouped outside of the "resistant" cluster could be primarily thought as susceptible to *F. hepatica*. Additionally, resistant populations (*i.e*: La Palma, El Azufre, Babiney and La Coca), showed higher overall allelic richness and a marked differentiation of microsatellites-based genetic population structure (Alba et al., under review; Lounnas et al. 2017) compared to susceptible *P. columella* from Cuba that were determined to be predominantly monomorphic (Alba et al., under review). These findings point to a more ancient introduction of the resistant populations and a detached evolutionary history from the susceptible phenotype in Cuba (Alba et al., under review). However, despite the indications of an earlier arrival on Cuba, resistant *P. columella* present a very discrete geographical distribution (only six localities listed above) compared to susceptible populations, and this suggest the existence of an ecological cost of the resistance that constraints range expansion in nature (Alba et al., under review).

1.1.3 Ecological patterns associated to the resistant phenotype: fitness cost of resistance

A follow-up study on snail abundance conducted in two nearby water bodies harboring either resistant (El Azufre) or susceptible (IPA) *P. columella* populations showed stable densities for both populations throughout the year. However, abundance of resistant *P. columella* was always lower compared to the susceptible population (Gutiérrez et al., 2005a).

Recently, ecological patterns associated with the distribution of each phenotype in nature have been analyzed separately for resistant and susceptible *P. columella* populations (Alba et al., under review). It was determined that while susceptible and resistant snails share similar ecological requirements, resistant populations only occurred in sites with low richness of other snail species (3.2 ± 1.02), characterized by slightly acid (pH = 6-6.5) and soft (total hardness (TH) = 4°-10°d) water (Alba et al., under review). Experiments in the laboratory showed that lowered pH/TH water conditions negatively affect *P. columella* species regardless of the phenotype, but resistant strains were significantly more tolerant to such conditions, as evidenced by higher survival rates, and both greater life expectancy and percentage of viable eggs as compared to susceptible strains (Alba et al., under review). However, lower fecundity rates, delay in egg hatching, late reproductive peaks (Alba et al., under review) and diminished

158 net reproductive rate (Gutiérrez et al., 2002) are indistinctively reported in resistant *P. columella* isolates
159 suggesting the occurrence of a certain trade-off against reproduction. Particularly, resistant snails (*i.e.* La
160 Palma) incur a diminished net reproductive rate if they are reared in the presence of susceptible *P.*
161 *columella*. By contrast, susceptible snails raised in competition with resistant individuals increase of shell
162 growth and of net reproductive rate (Gutiérrez et al. 2005b). These results have led to the following
163 hypothesis to explain the restricted distribution of resistant *P. columella* in nature. It is postulated that
164 the existence of an ecological cost of the resistance, possibly manifested as reproductive impairments,
165 results in a less competitive potential of resistant compared to susceptible snails (Alba et al., under
166 review). However, the higher tolerance to environmental pH/TH stress likely provide the resistant
167 phenotype with an ecological advantage in sites with lower pH/TH conditions that are less suitable for
168 other snails, and provide a concomitantly reduced competition from other (species of) snails (Alba et al.,
169 under review).

170 Overall, regarding life traits following experimental challenge with *F. hepatica*, exposed resistant snails
171 exhibited higher survival rates than infected-susceptible isolates (Alba et al., 2018, Gutiérrez et al.,
172 2002). In addition, no significant variations were observed in the fecundity rates between non-exposed
173 and exposed resistant snails (Gutiérrez et al., 2002).

174 **1.2 Comparative molecular screening identifies differences between resistant and susceptible *P.***
175 ***columella***

176 An understanding of the fine mechanisms that mediate *P. columella* resistance to *F. hepatica* is of
177 interest for the development of new strategies aiming at unveiling and controlling *F. hepatica*
178 transmission but also for study of determinants of pathogen virulence and for comparative immunology.
179 However, despite the gathering of extensive phenotypical, genetic and ecological data, no study has
180 been conducted to decipher the molecular scenario that give rise to each distinct *P. columella*
181 phenotype relative to *F. hepatica*. To undertake this task, we sought to investigate differences between
182 naïve susceptible and resistant *P. columella* at both the transcriptome and proteome levels.

183 Firstly, we constructed a *de novo* assembled transcriptome for this species from whole snails by RNAseq
184 and performed comparative analysis to identify differential patterns of abundance of transcripts that
185 may possibly account for the phenotypic and ecological features of *P. columella* resistance to *F. hepatica*
186 and tolerance to pH/TH stress. Secondly, we performed 2D-electrophoresis, and MS/MS spectrometry,
187 to identify differentially expressed proteins in a particular anatomical compartment of the female snail

reproductive system, the albumen gland. Apart from its role of producing the perivitelline fluid that is deposed in eggs to nurture the embryos (Duncan, 1975, Geraerts and Joosse, 1984), the albumen gland is also involved in the synthesis of a number of immune effectors and defense proteins (Guillou et al., 2007, Hathaway et al., 2010, Mu et al., 2017, Sen et al., 1992). Therefore, comparative analysis of the proteome of this multifunctional organ can give insights into the suggested trade-off between resistance to *F. hepatica* and reproduction in resistant snail isolates (Gutiérrez et al., 2002, Alba et al., under review).

In this study, we found similar patterns from the transcriptomic and the proteomic approaches: a significant bias towards an overall increased abundance of defense and stress-related process transcripts/proteins was associated with resistant snails versus the *F. hepatica*-susceptible phenotype of *P. columella*. These results support self-maintenance as favored strategy of resistant *P. columella*. Differences in metabolic organization to the detriment of protein synthesis/folding was also observed from both "omic" analyses. This may be particularly significant in the case of the albumen gland, potentially contributing to the reproductive constraints observed in resistant *P. columella* populations. The detected differences in expression are discussed in regard to the differential phenotypic and ecological features of the resistant and susceptible strains. In addition, this paper presents a list of candidate factors for further functional validation as predictors of susceptibility to infection and/or for potential inroads toward control of infection and transmission of fascioliasis by boost snail immune function.

2. Material and methods

2.1. *Laboratory-reared P. columella* snails

Five-week-old *P. columella* snails (average size 5.09 ± 0.64 cm) from *F. hepatica*-resistant (La Coca) and -susceptible (Aurora) populations, reared in the Laboratory of Malacology of the IPK (see Sánchez et al. 1995 for details on rearing) were used for the transcriptomic and the proteomic experiments. To gain an overview on constitutive differences between phenotypes, only naive snails (non-exposed to parasite infection) were sampled.

2.2. Comparative transcriptomic approach on whole snail

2.2.1. *RNA extraction and sequencing by Illumina*

Sixty *P. columella* snails per strain were used to obtain three biological replicates of 20 snails each. Briefly, snails were separated from the shell and immediately placed in individual vials containing RNA*later*® (Ambion) at a ratio 10 µL solution to 1 mg of snail tissue. Samples were kept overnight at 4°C and then stored at -20°C until use. For RNA extraction, RNA*later*® was discarded and whole-tissues of 20 snails per strain (three biological replicates per strain) were pooled, immediately frozen in liquid nitrogen and homogenized. RNA extraction was performed with TRIzol® Reagent (Ambion) following manufacturer's guidelines. Extracted RNA was purified using RNeasy® Minikit (Qiagen) and treated with DNase following the in-column procedure of Turbo DNA-*free*TM kit (Ambion). The quality and quantity of the extracted RNA was assessed on a 2100 Bioanalyzer using an RNA 6000 Nano kit (Agilent Technologies) and by Qubit™ 2.0 fluorometry (Invitrogen).

One µg of total RNA from each biological replicate was used for library preparation using Illumina TruSeq stranded mRNA kit and RNA sequencing was performed by Fasteris SA (Geneva, Switzerland), collecting paired-end, 75-bp read length reads, with three samples multiplexed per lane, using the Illumina HiSeq™4000 platform.

2.2.2. De novo assembly and annotation of P. columella transcriptome

A *de novo* transcriptome of *P. columella* was assembled using high-quality reads (quality > 38 phred score) from all six sequenced samples using the default options (which includes the normalization step) of Trinity 2.0.6.1 method (Grabher et al., 2011) on Galaxy Project Server (Giardine et al., 2005) at the instances of the IHPE laboratory (http://bioinfo.univ-perp.fr). From a total of 592 million raw reads, the first consensus transcriptome for *P. columella* resulted into 158 837 contigs, named full-transcriptome. To reduce its complexity, Trinity Super Assembly (Grabher et al., 2011) was applied to the *de novo* assembled transcriptome, and all transcripts shorter than 300 bp were removed, with 78 774 transcripts remaining for further analysis. Afterwards, as two phenotypes (and populations) with genetic differences within *P. columella* were included for RNAseq (see Alba et al., under review; Calienes et al., 2004; Lounnas et al., 2017), hypervariable families were reduced by CD-Hit-est (Li and Godzik, 2006) by clustering transcripts with matches above 95% identity. This results in 72748 transcripts, designated by the name simplified transcriptome. We used BUSCO tool (available http://busco.ezlab.org/v1; Simão et al. 2015) to assess the quality and completeness of the assembled transcriptomes. We obtained 909 out of 978 complete BUSCOs; no information losses resulted from the reduction steps. All 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). Transcriptomes are also available at the laboratory database: http://ihpe.univ-perp.fr/acces-aux-donnees/.

The automatic annotation of the simplified transcriptome was performed by Blast2GO version 2.4.2 (Conesa et al., 2005) using BLASTx against the National Center for Biotechnology Information (NCBI) non-redundant (nr) sequences database (with an E-value threshold set at 1E−03) was performed. Gene function, protein domain and enzyme annotation were also assigned by similarity searches using the Gene Ontology (GO) database and InterPro scan. Annotation results were obtained for only 38 968 transcripts (49.5%).

2.2.3 Comparative analysis between resistant and susceptible P. columella

For comparative analysis between susceptible and resistant *P. columella* phenotypes, quality reads (phred score > 38) were aligned to the reduced assembled transcriptome using Bowtie2 v 2.0.2 (Langmead and Salzberg, 2012) set in fast end-to-end mode. Transcripts were counted with Salmon (Patro et al., 2017) and differential expression was calculated by DESeq2 (Love et al., 2014), accounting for over or under-representation in the resistant phenotype. As threshold criteria, only differentially-expressed transcripts with adjusted $P < 0.05$ and displaying more than 2-fold changes were considered. All RNAseq analyses were run locally using the Galaxy Project server (Giardine et al., 2005).

Enrichment analyses of the transcriptome involving biological processes or molecular functions, considering separately over- and under-expressed transcript data, were performed using Fisher's Exact Test run on Blast2GO software. A particular GO term with *P* and false discovery rate (FDR) < 0.05 was considered enriched. In addition, differentially-expressed transcripts were evaluated combining Gene Ontology (GO) terms and keyword list of well-known defense molecules to select the genes putatively involved in immunity (*i.e.* defense response, wound healing and inflammation) and in relation to acid-base balance (*i.e.* carbon-bicarbonate buffering and ion transportation).

2.3. Comparative proteomic approach on the albumen gland

2.3.1. *Protein extraction of albumen glands*

The albumen gland of 15 naive snails from each strain (biological replicates) was removed by dissection, lyophilized and individually processed. Briefly, the crude extract of the albumen gland from each

individual snail was obtained by sonication on ice at 20% amplitude (Vibra Cell 75185 sonicator; Bioblock Scientific) for 20 s in 80 µL of ice-cold denaturing buffer (urea 7 M, thiourea 2 M, CHAPS 4% in Tris-HCl 30 mM, pH 8.5) and clarified by centrifugation at 2 000 x *g*, 15 min, 4°C. Protein concentration of the supernatant was estimated using the 2D Quant Kit (GE Healthcare) and samples were stored at -80°C until used.

2.3.2. 2D *gel electrophoresis of protein extracts from P. columella albumen glands*

Proteins extracted from each albumen gland were individually analyzed by 2D gel electrophoresis (15 gels per strain, each corresponding to different biological replicates). One-hundred µg of proteins from each extract were added to rehydration buffer (urea 7 M, thiourea 2 M, CHAPS 4%, DTT 65 mM) for a total volume of 350 µL. Individual samples were loaded onto 17 cm isoelectric focusing strips (BioRad) with a stabilized non-linear pH ranging from 3 to 10. Passive strip rehydration was achieved by 5h at 22°C, followed by an active rehydration of 14 h under a 50 V current (to help big proteins to enter into the strips) at the same temperature. Afterwards, isoelectric focusing of proteins was performed using the following program: 50 V for 1 h, 250 V for 1 h, 8,000 V for 1 h and a final step at 8,000 V for a total of 90,000 Vh with a slow ramping voltage (quadratic increasing voltage) at each step. Focused proteins were reduced by incubating the strip twice with equilibration buffer (Tris, 1.5 M; urea, 6 M; SDS, 2%; glycerol, 30%; bromophenol blue; pH 8.8) containing DTT (130 mM) at 55°C. Then, proteins were alkylated by an incubation with equilibration buffer containing iodoacetamide (135 mM) on a rocking agitator (400 rpm) at room temperature protected from light. Proteins were separated according to their molecular weight (second dimension) on 12% acrylamide/0.32% piperazine diacrylamide gels run at 25 mA/gel for 30 min followed by 75 mA/gel for 8 h using a Protean II XL system (Bio-Rad). Gels were stained with MS-compatible silver staining protocol and scanned using a ChemiDoc MP Imaging System (Bio-Rad) associated with Image Lab software version 4.0.1 (Bio-Rad).

2.3.3. *Comparative analysis of 2D-gels between susceptible and resistant P. columella*

Considering the complexity of the 2D gel profile of albumen gland, and quality of sample resolution, five most representative gels per strain were selected for comparative analysis on PD-Quest v. 7.4.0 (Bio-Rad) to identify differences in protein abundance between proteomic profiles of albumen glands from susceptible versus resistant individual snails. When a spot was reproducibly present in all five replicates from one strain and absent from all five replicates from the other strain, it was considered a qualitative change. For quantitative changes, only spots whose mean intensity across five replicates per strain was

four-fold higher or lower than those from the other strain, with a $P < 0.01$ (Mann-Whitney U-test), were considered as significantly differentially abundant between the two strains. Selection of spots for further analysis by MS/MS spectrometry was based on qualitative (*i.e.* present in only one of two phenotypes) or highly quantitative differences (*i.e.* > 4-fold differentially abundant; $P < 0.01$). Differentially represented spots were excised from the gels, destained, trypsin digested and the obtained peptides were identified by tandem mass spectrometry using the PISSARO platform facility (University of Rouen, France).

For the identification of the protein(s) present in each spot, the obtained peptides were matched to the consensus *de novo* assembled full transcriptome for *P. columella* (158837 transcripts). The transcript sequences confidently matching the peptides were used as a query for a xBLAST against non-redundant NCBI database to determine the protein identity of the best match. Up to the first three best hits were considered, when at least 4 peptides matched the sequence with a coverage > 10%. Identified transcripts sequences were translated into protein using Expasy server (Gasteiger et al., 2013) to identify conserved domains with CDD (Marchler-Bauer et al., 2015). The theoretical pI and molecular weight were also calculated using the Expasy server (Gasteiger et al., 2013) to cross-reference the protein sequence data with the location of the spot on the gel. Altogether, these complementary analyses allowed confident characterization of the protein identity of each spot.

3. Results

3.1. Transcriptomic differences between susceptible and resistant P. columella *phenotypes*

Overall, 97.49% of the Illumina RNAseq reads from both phenotypes of *P columella* were successfully realigned against the simplified transcriptome. A total of 6876 transcripts (9.45%; 4126 up-regulated and 2750 down-regulated) showed a significantly greater than 2-fold differential-expression between phenotypes (see supplementary data 1) from which 3290 transcripts (47.5%) had no annotation results.

Results from the enrichment analyses for both over- and under-represented transcripts (test data) relative to the annotated simplified transcriptome (reference data) are shown in Figure 2 and supplementary data 2. Analysis of the increased expression transcript data set showed that the resistant phenotype associated with five main categories of biological processes: biological regulation and homeostasis, defense/stress responses, primary signaling pathways/transduction, and nitrogen metabolism, particularly biosynthesis, maintenance and repair of DNA (Figure 2A). In addition, molecular functions related to signal transduction (*e.g.* transmembrane receptor/G-protein coupled receptor and

protein tyrosine phosphatase activities) and with Ca^{2+} binding were enriched in the resistant isolate (Figure 2B). Contrastingly, metabolic process that were related to protein synthesis were under-represented in resistant snails (*e.g.* ribosome biogenesis, structural constituent of ribosome, peptide biosynthetic process, translation; Figure 2C).

It was particularly significant to find an enrichment in biological processes related to defense and stress response in the resistant phenotype (see Figure 2A) supported by increased abundance in resistant snails of transcripts for pathogen receptor/interacting molecules, transcripts with regulatory roles in immunity and molecules involved in the activation and orchestration of defense responses (see Figure 3 A, B; supplementary data 1). These included several lectins, CD109, cytokines and cytokine-related molecules (*e.g.* macrophage migration inhibitory factor (MIF), granulocyte colony stimulatory factor receptor (G-CSFR), transforming growth factor 1-beta (TGF1β)), as well as of signaling/regulatory transcripts (*e.g.* Toll-like receptors (TLR), protein C kinase (PKC) members of the superfamily of tumoral necrosis factor (TNF) receptors and interferon regulatory factors (IRF), see (Figure 3B).

Additionally, potential anti-parasitic defenses like ferritin, nitric oxide synthase and antioxidant molecules such as catalase, superoxide dismutase (SOD) and probable deferrochelatase peroxidase were also more abundant in resistant snails (Figure 3B). Increased levels of immune surveillance in the resistant phenotype were suggested by transcripts that can function in leukocyte adhesion, rolling and tethering (interference hedgehog-like, Ras-like GTP-binding Rho1, rho-associated kinase 1), and wound healing (Ras-like GTP-binding Rho1).

Concordant with the association of greater tolerance for variations in pH and TH levels with snails from resistant *P. columella* populations (Alba et al., under review), several major ion regulatory factors, and other transporters that contribute to pH and osmotic regulation were observed more from resistant snails (Figure 3C; supplementary data 1). In addition, carbonic anhydrase transcripts were also found highly abundant in this isolate (Figure 3C).

3.2. Qualitative and quantitative differences between proteomes of albumen glands from susceptible and resistant P. columella *phenotypes*

Albumen glands of *P. columella* exhibited complex proteomic profiles notably consisting of highly abundant proteins of high molecular weight and acidic pI (upper left part of the gels in Figure 4). Such highly abundant proteins with several isoforms can mask nearby proteins and impede the quantitative analysis and proper protein identification. Therefore, this region of the gels, identified with a white

rectangle in Figure 4, was excluded from analysis. A total of 554 spots were identified from both proteomes, from which more than 80% were present and similarly abundant in both phenotypes.

However, 18 spots were uniquely to resistant snails while 13 were only observed from susceptible individuals (supplementary data 3; Figure 4). Twenty-eight spots differed significantly ($P < 0.01$, Mann-Whitney U-test, > 4-fold) in abundance between resistant and susceptible *P. columella* (Supplementary data 3; Figure 4), 12 and 16 spots of the resistant strain were over- and under-represented, respectively compared to the susceptible strain (Supplementary data 3; Figure 4). The ratios of resistant/susceptible quantity ranged from 0.24 (spot # 2107) up to 26.11 (spot # 7117).

The selection of 59 spots representing differentially expressed proteins for MS characterization yielded successful identification of 49 proteins and several isoforms (Table 1; Supplementary data 3). No identification was made from spots 7107, 7108 and 6515. Spot 6002 contained two protein isoforms with 67% sequence similarity (E-value = 2E-67 in BLASTp output; Supplementary data 3) to an uncharacterized protein (LOC106073623) predicted from genome sequence of *B. glabrata* snails (NCBI *Biomphalaria glabrata* Annotation Release 100), which has been associated with certain lysozyme and peptidoglycan binding activities according to BLASTp consensus (Buddenborg et al. 2017). However, no putative conserved domains were detected in the isoforms of spot 6002 (Supplementary data 3). The presence of more than one protein or of different protein isoforms into the same spot in the same gel was expected due to the complexity of the electrophoretic profiles (see Figure 4).

Table 1 summarizes the proteins (from one up to four hits per spot) that were identified by MS analysis from the 59 spots excised from the 2D gels. Overall, defense/stress and metabolism-related molecules were more abundant in resistant snails (Table 1). Particularly, G-type lysozyme and lipopolysaccharide-binding protein/bactericidal permeability-increasing protein (LBP/BPI) were only observed in resistant snails (Table 1). Conversely, proteins related to protein anabolism were underrepresented in resistant compared to susceptible snails (Table 1). It is worth noting some qualitative differences between isolates that concern the proteins that were identified as involved in signaling processes. In this sense, abundance of tyramine β-hydrolase like (TBH) in resistant snails, an enzyme that catalyzes the conversion of the neurotransmitter tyramine into octopamine, contrasts with the lower representation of dopamine β-hydrolase-like monooxygenase (which mainly converts dopamine in norepinephrine) as compared with susceptible snails (Table 1).

393 **Table 1.** Characterization by 2D electrophoresis/Mass Spectrometry of components that differ
394 qualitatively (underlined spot #) or quantitatively between proteomic profiles of albumen gland from
395 resistant and susceptible *Pseudosuccinea columella* strains.

Primary Function	Protein Annotation	Present/abundant (spot #)	
		Susceptible	Resistant
Defense / stress	G-type lysozyme	-	6118; 7117
	Glutathione S-transferase	2107	-
	L-amino acid oxidase –like	2005; 5004; 8301; 8305; 7203	3615; 4826; 4824; 5709
	Lipopolysaccharide-binding protein/bactericidal permeability-increasing protein (LBP/BPI)	-	3615
	Pathogenesis related protein 1 like	-	8011
	Pathogenesis related protein 1-3 like	7001	-
	Programed cell death protein 6-like isoform X2	-	108
	Protein disulfide isomerase A3-like	-	5709
	Protein disulfide isomerase A6	-	3615
Matrix / adhesion	Cartilage matrix protein-like	7114	-
	Dermatopontin 2	1025	-
	Filaggrin-like/ cartilage acidic protein 1-like	4503; 1025; 1001; 1114	5208; 7117; 1409; 8011
	Matrilin/Cartilage matrix protein	6119	-
	TGF-β-induced protein ig-h3	3005; 2107; 8301; 8305	4306; 1409
Metabolism and acid-base balance	3-oxoacyl - [acyl carrier protein] reductase FabG-like	-	9303
	4-hydroxyphenylpyruvate dioxygenase-like	-	5509
	Adenylate kinase 2 mitochondrial-like	7203	-
	Arginine kinase	-	5509
	Bifunctional purine biosynthesis protein PURH-like	6818; 5808; 5713	-
	Carbonic anhydrase 7-like	-	4306
	Cytochrome b-c1 complex subunit 2 mitochondrial-like	-	8608
	Citrate synthase mitochondrial like	6602	5618
	Purine nucleoside phosphorylase-like	-	4306
Protein synthesis / assembly / folding	40S ribosomal protein S2	8301; 8305	-
	40S ribosomal protein S7-like	6110	-
	40S ribosomal protein S12-like	2005	-
	60S ribosomal protein L7a-like	8301; 8305	-
	60S acidic ribosomal protein PO-like	-	9303
	Elongation factor 1-gamma like	6602	5618
	Peptidyl-prolyl-cis-trans isomerase 5-like	1114	-
	Peptidyl-prolyl-cis-trans isomerase B-like	8108; 8106	9203; 9204
Protease and protease	Serine peptidase 2/ fibrinolytic enzyme, isozyme C-like	3210	4210

inhibitors	Serpin B-like protein 2	-	5005; 3214; 5106
	Serpin B3-like	6602	5618
	Serpin Z2B-like	4503; 7114; 2011; 2005; 1114; 6818; 2107; 6119; 5808; 5713	108; 1409; 4601
Signaling process and transduction	Dopamine β-hydrolase (DBH)-like monooxygenase protein 1	6818; 5808; 5713	-
	Guanylate kinase like	-	9203
	Neurogenic locus Notch protein	4102; 7009, 7001; 6110; 4111; 2107	2112; 108
	Tyramine-beta hydroxylase like	-	4824; 4826
Other functions	Endo-beta-1,4 glucanase	7009; 8305	-
	Glutamyl tRNA (Gln) amidotransferase subunit A-mitochondrial like	-	1409
	Hemocyanin-like	6818; 5808; 5713	-
	Histone H4	-	4117
	Lamin B1-like	-	4826
	Probable ATP-dependent RNA helicase DDX 43	3210	-
	Ribonuclease UK-144-like	3002	4006
	Protein DGCR14-like	7203	-
	WD repeat-containing protein 36-like	2011	-

4. Discussion

4.1. Omics provide a molecular baseline for phenotypic and ecological features of *P. columella* snails that are naturally resistant to *F. hepatica*

4.1.1. Molecular clues for parasite resistance and high tolerance to pH/TH variations

Resistance to *F. hepatica* in *P. columella* has been associated with an active encapsulation of the invading parasite shortly after penetration that develops fully within 24h post-exposure and that eventually leads to the death of the larvae and to the resolution of the infection (Gutiérrez et al., 2003b). The consistent nature of the rapid commitment to this immune response in resistant *P. columella*, and the lack thereof in snails from susceptible populations reasonably support the thought that some features involved in the susceptibility/resistance to the parasite *F. hepatica* are constitutively evident in each snail phenotype. In the present paper, both mRNA- and protein-centered "omic" approaches showed an overall enrichment in resistant snails, of biological functions associated with defense and stress responses. It is worth to mention that proteomic differences could be more

extensive, due the exclusion of the top left region of gels (lots of large proteins) from the study. However, these finding are possibly related to the fact that both defense and stress responses are linked by several molecular functions, effectors and signalling pathways (Demas et al., 2011, Matozzo et al., 2012). In fact, both the effective hemocytic encapsulation of *F. hepatica* (Gutiérrez et al., 2003b) and the higher tolerance to pH/TH variations associated with resistant *P. columella* (Alba et al., under review) are responses toward (biotic or abiotic) stressors.

On the one hand, the greater abundance of immune-related transcripts/proteins (*e.g.* several lectins, pro-inflammatory signaling pathways, cytokines, effector molecules) compared to susceptible snails, may provide significant advantage to resistant snails, enabling the accelerated mounting and regulating of an early and efficient immune response to *F. hepatica*. Similarly, elevated constitutive molecular processes were proposed to be the base for snail refractoriness to parasite immunosuppressive factors in *B. glabrata* strains, experimentally-selected for resistance to *Echinostoma caproni* (Humbert and Coustau, 2001). Notably, representation of plasma proteins differed significantly in these susceptible and resistant *B. glabrata* strains and was linked to a differential gene regulation expressed in the albumen gland (Vergote et al., 2005).

On the other hand, overall higher abundance in resistant snails of several carbonic anhydrase isoforms, both at the transcriptomic or the proteomic level (see Figure 3C, Table 1) suggests a higher potential to compensate for pH variations (Freitas et al., 2006). Likewise, given that water acidification negatively affects osmotic regulation in aquatic invertebrates (Freitas et al., 2006, Mattews, 2017), constitutive over-representation of different ion regulators and transporters (see Figure 3C) may, in part, provide the molecular bases the for increased tolerance to pH/TH stress that was observed from resistant *P. columella* (Alba et al., under review).

Furthermore, there was enrichment in molecular functions related to Ca^{2+} binding. This ion is a common secondary messenger inside the cells with important roles in signal transduction during several processes including immune response in molluscs (Tunholi-Alves et al., 2014). In addition, it is essential for shell formation and alteration of calcium levels in hemolymph during infection or due to pH regulation can result in shell hypocalcification (Tunholi et al., 2011, Tunholi et al., 2017, Tunholi-Alves et al., 2014). Thus, it is hypothesized here that enhanced capabilities to manage calcium balance is also linked with the resistance to *F. hepatica* and the tolerance to pH/TH stress in *P. columella*, and might be somehow related to the characteristic pigmentation patterns displayed in the mantle of resistant snails as it is the site of shell formation (see Gutiérrez et al. (2003b)).

4.1.2. Resistance trade-off against reproduction: molecular clues from the albumen gland

The differential transcription effort towards stress/defense response observed in naïve resistant *P. columella* suggests that self-maintenance and survival is the preferred life strategy in this phenotype. However, energy budget constraints cause trade-off against other biological processes, as exemplified by reproductive constraints observed in some pathogen-resistant hosts, (*e.g.* Langand et al., 1998). The limited reproductive output specifically of the resistant phenotype of *P. columella* (Alba et al., under review) may be associated with the underrepresentation of biological process/functions/proteins related to protein synthesis, evident from both "omic" approaches (see Figure 2C and Table 1). Enhanced immune responsiveness may reconfigure intermediate metabolism towards a relative increase of the respiration rate, glycolysis, proteolysis and lipolysis (Lochmiller and Deerenberg, 2000), and increased defenses against stress (*e.g.* antioxidative enzymes (Freitas et al., 2006) may cause lower energy reserves (protein and glycogen content) and higher metabolic rates.

In the albumen gland of resistant snails, energy production through the respiratory chain and energy storage (synthesis of fatty acid) were indicated particularly active by the abundance of cytochrome b-c1 complex subunit 2 mitochondrial-like isoforms and of 3-oxoacyl- reductase FabG-like (catalyzes the first step of the fatty acid synthesis), respectively (see Table 1). Elevated representation of 4-hydroxyphenylpyruvate dioxygenase-like may indicate an association with catabolism of aromatic amino acids, whose intermediates enter into the Kreb's cycle. These processes likely constrain the production of the protein components of the perivitelline fluid, that are normally deposited in eggs and directly involved in the sustainability of the embryos, a primary reproductive function of the albumen gland (Duncan, 1975, Geraerts and Joosse, 1984). Consequently, the reproductive output of resistant snails, whether in quantity (fecundity rates, net reproductive rate), quality (viable eggs) or in time (late reproductive peaks), might be affected especially because the albumen gland is also devoted to the synthesis of proteins involved in immunity and other functions (see Table 1). This then results in a trade-off against reproduction, particularly egg production/viability (Stearns, 1992). Thus, results from this study may provide, at least in part, insights into the molecular basis of the reproductive constraints of resistant compared to susceptible snails (see Alba et al. (under review); Gutiérrez et al. (2002) for details).

Speculatively, the metabolic rearrangement and allocation of resources in the albumen gland of resistant snails, as proposed here, may relate to differences in neurotransmitter metabolism observed between *P. columella* phenotypes (see signaling proteins in Table 1). The presence of TBH suggests that

tyramine and, mainly, octopamine are preferentially involved in neuroendocrine control of the albumen gland of resistant snails. To date, however, only a few studies (from bivalves) inform regarding a functional role of octopamine in molluscan reproduction (Blais et al., 2010). Yet other studies refer to potential involvement of octopamine in regulation of feeding and locomotion in gastropods (Ormshaw and Elliott, 2006, Vehovszky and Elliott, 2001, Wentzell et al., 2009) and, octopamine participates in the regulation of metabolism, ovulation and egg laying in insects and nematodes (Monastirioti, 2003, Monastirioti et al., 1996, Tao et al., 2016). Additionally, this neurotransmitter was also associated with stress responses in invertebrates. Increased TBH concentrations may be a stress marker in insects (Châtel et al., 2013). In free living nematodes a rise in octopamine levels allows metabolic rearrangements to maintain energy homeostasis during stress induced by starvation (Tao et al., 2016). Similarly, a significant increase of octopamine has been observed in the central nervous system of severely food-deprived *Lymnaea stagnalis* snails (Aonuma et al., 2017). The results of this study certainly warrant further studies of neurotransmitters concentration and their role in regulating allocation of resources and metabolic functions in the reproductive and immune functions of resistant and susceptible *P. columella.*

4.2. *Elevated representation of immune-related molecules in resistant snails*

Compared to susceptible snails, naïve resistant *P. columella* display an overall enhanced immune capacity based on novel and/or elevated expression of an arsenal of defense factors, including pathogen recognition/interacting molecules, particularly C-type lectins as wells as mannose and galactose binding lectins (see Figure 3B). The variety of up-regulated transcripts potentially able to interact with pathogens displayed by resistant snails may be advantageous given that the first step of any defense response requires recognition of the threat (Pinaud et al., 2019). C-type lectins, in particular, are known for recognition of carbohydrates that constitute pathogen-associated molecular patterns (PAMPs) like LPS and peptidoglycan. Once bound, lectins mediate immune activities ranging from microbe agglutination and opsonization to triggering signaling pathways for induction of phagocytosis and encapsulation (Vázquez-Mendoza et al. 2013; Pees et al., 2016). In snail-digenean interactions, C-type lectins from *B. glabrata* interact with antigens of *S. mansoni* sporocysts (Wu et al., 2017) and are expressed by resistant snails in response towards *E. caproni* infection (Guillou et al., 2007). Potentially, the great abundance of mannose binding lectins (see Figure 3B for details) increases the potential of resistant *P. columella* for rapid effective immune detection of *F. hepatica,* because the surface of sporocyst is mainly covered with N-acetyl-D-glucosamine and α-mannose (Georgieva et al., 2016). More

effective parasite recognition in resistant *P. columella* may be contributed to further by highly abundant CD109. The *B. glabrata* homolog of this thioester-containing protein family member interacts with *S. mansoni* larvae (Wu et al., 2017).

Furthermore, abundant transcripts encoding cytokine such as G-CSFR, TGF1B and MIF, involved in the proliferation, recruitment and activation of immune cells provide a likely explanation for the effective cellular response that is reliably triggered in resistant snails in response to *F. hepatica* infection (Gutiérrez et al., 2003b). TGF1B belongs to a family of multifunctional cytokines found in different phyla that share a highly conserved signal transduction pathway and participates in the regulation of different biological functions including stress response and immune modulation (Huminiecki et al., 2009). MIF was recorded from several species of mollucs, the homolog of *B. glabrata* is present in hemocytes, and promotes cell aggregation and hemocyte proliferation, while inhibiting NO-dependent p53-mediated apoptosis. RNAi-mediated knockdown confirmed immune function of *B. glabrata* MIF, yielding increased parasite burden following exposure to *S. mansoni* (Baeza-Garcia et al., 2010). Likewise, in *Oncomelania hupensis* a more distantly related prosobranch gastropod, expression of MIF increased after challenge with *Schistosoma japonicum* and its involvement in the activation, differentiation and recruitment of hemocytes was demonstrated: RNAi knockdown of MIF decreased the proportion of phagocytic circulating hemocytes and restrains the migration of blood cells from the host towards the site of infection (Huang et al., 2017).

The abundance, in resistant snails, of transcripts related to pro-inflammatory signaling pathways (*e.g.* TLR/Myd88 and PCK) and with regulatory activities (*e.g.* LRR and IRF) is particularly significant and indicates that increased amounts of signaling components are available for a higher immune responsiveness as is associated with the resistant phenotype. Notably, PKC regulates differentiation and activation of snail hemocytes towards a phenotype more prompt to cellular reactions, particularly promoting increased production of NO and H_2O_2 levels (Humphries and Yoshino, 2008, Lacchini et al., 2006, Wright et al., 2006) and cell spreading (Humphries et al., 2001). According to Walker et al. (2010), PKC and ERK pathways are involved with focal adhesion kinase in cell adhesion, spreading and formation of lamellipodia, necessary for phagocytosis in *L. stagnalis*. TLRs are widely studied primary mediators of innate immunity that following recognition of PAMPs, activate highly conserved immune signaling pathways across many different animal phyla. Interestingly, the downstream components (Myd88 and NF-*k*B) of this signaling pathway were also significantly abundant in resistant snails (see Figures 2B and 3B). In molluscs, TLRs can be activated, and expression up-regulated, after septic injury to promote

hemocyte activation (for review see Brennan and Gilmore, 2018, Nie et al., 2018). Involvement in snail-trematode interactions was confirmed by a report showing that a TLR was highly expressed in hemocytes from a *S. mansoni*-resistant strain of *B. glabrata* and siRNA-mediated knockdown significantly reversed the resistant phenotype (Pila et al., 2016).

Other molecules involved in acute phase response and redox killing like ferritin, nitric oxide synthase and antioxidant enzymes are also significantly abundant in resistant snails. These may contribute to the orchestration of effective cellular cytotoxic immune responses that lead to parasite elimination in resistant *P. columella*. Hemocytes of a *B. glabrata* strain resistant to *S. mansoni* highly express ferritin prior parasite infection (Lockyer et al., 2012). Ferritin binds and regulates iron distribution. As part of an acute phase response, ferritin is up-regulated after microbial challenge for iron sequestration in order to deprive infecting microorganisms from iron acquisition and multiplication of pathogens (Ong et al., 2006). Ferritin also acts as antibacterial agent in mollusks (Zheng et al., 2016, Chen et al., 2016), remarkably different ferritin isoforms are induced in *B. glabrata* in a pathogen-specific manner (Deleury et al., 2012). Ferritin may affect the cellular balance of reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) and hydroxyl free radical (OH) thereby regulating oxidative killing of trematode parasites in *B. glabrata* (Lockyer et al., 2012). In vertebrates and invertebrates, various nitric oxide synthase enzymes catalyze production of highly reactive nitric oxide (NO) for immune defense to effect killing of pathogens (Nathan and Shiloh, 2000; Wright et al. 2006). In *Drosophila melanogaster*, NO mediates signal transduction to activate a defense response to Gram-negative bacteria (Foley and O'Farrell, 2003). Particularly in snail-trematode interactions, killing of *S. mansoni* by *B. glabrata* hemocytes involves significant production of NO and H_2O_2 (Hahn et al., 2001b, Hahn et al., 2001a).

Resistant *P. columella* may benefit from higher constitutive antioxidant potential, as afforded by catalase and SOD, prior to parasite infection because up-regulation of catalase is essential to maintain redox balance and to resist bacterial infection in the clam *Meretrix meretrix* (Wang et al., 2013). Additionally, high constitutive levels of Cu/Zn SOD are positively related to anti-parasite resistance in *B. glabrata* snails as this enzyme helps to focus the production of oxygen reactive species from superoxide radical toward H_2O_2, which is more effective at killing trematode larvae (Goodall et al., 2004, Lockyer et al., 2012).

The comparative proteomic analysis on products from the albumen gland, showed an overall higher abundance in resistant *P. columella* of two immune-effectors: LBP/BPI and G-type lysozyme. The G-type lysozymes are lytic proteins with potent activities against pathogens, mainly bacteria. These lysozymes

564 are ubiquitously distributed across animal phylogeny, and have also been reported from several species
565 of molluscs where they can be induced to function as immune effectors (Bathige et al., 2013, Guo and
566 He, 2014, Zhang et al., 2012). A significant increase of the expression of three G-type lysozymes was
567 observed in *S. japonicum*-infected *O. hupensis* snails and suggests involvement in the defense against
568 trematode parasites also (Zhang et al., 2012).

569 For the resistant strain of *P. columella*, the expression of LBP/BPI is also of interest as this protein has
570 been associated with snail parental investment in immune protection of offspring (Baron et al., 2013).
571 Particularly, the albumen gland of *B. glabrata* produces an isoform of LBP/BPI that is contributed to the
572 perivitelline fluid of snail eggs (Hathaway et al., 2010) and provides protection against oomycete
573 infections (Baron et al., 2013). Thus, elevated levels of both immune effectors, lysozyme and LBP/BPI if
574 contributed by the albumen gland of *P. columella* to the perivitelline fluid, may provide enhanced
575 immune protection for snail embryos that develop inside eggs to potentially compensate for the limited
576 reproductive outputs observed from resistant phenotype snails (Gutiérrez et al., 2002, Alba et al., under
577 review).

578 **Conclusions**

579 This review of the complex interaction of *P. columella* with its trematode parasite *F. hepatica*, focused
580 on the phenotypical, genetical and ecological features of the resistant snail phenotype that occurs in
581 Cuban field populations, underscores the value of in-depth "omics" to reveal the underlying
582 immunobiology. Accordingly, this paper also presents for the first time, a transcriptome of *P. columella*
583 and, by combining different comparative omic analyses of resistant and susceptible snails, we
584 characterized the molecular baseline of the constitutive biological profile behind the resistance to *F.*
585 *hepatica* in naïve *P. columella* prior to infection.

586 Similar patterns observed from resistant snails at both the transcriptomic and the proteomic levels, from
587 whole snails and albumen glands, respectively converged to reveal increased abundance of biological
588 processes/transcripts/proteins involved in immune and stress responses and under-representation of
589 functions/molecules involved in protein synthesis), that is interpreted to indicate an overall allocation of
590 resources towards survival and self-maintenance in these snails. Specifically, the enhanced immune and
591 stress responsiveness found at the molecular level offer a link between parasite resistance in *P.*
592 *columella* and the increased tolerance observed in resistant populations to stress due to low pH and TH
593 variation in the environment. The dedication of metabolic effort to immune and stress response, is

indicated to be in detriment of protein synthesis/folding that, especially significant in case of the albumen gland, may amount to reproductive trade-offs and may explain, at least in part, the suggested fitness cost of heightened immune function in snail from Cuban populations of *P. columella* that are consistently resistant to *F. hepatica* infection.

However, characterization of differences between naïve resistant and susceptible *P. columella* snails is only a first step toward the elucidation of the molecular processes and specific mechanisms involved in the resistance to *F. hepatica.* Analyses of the immunobiological response following parasitic exposure is warranted, progressing to detailed investigation of the role of *P. columella* hemocytes in response to *F. hepatica* infection.

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Author's contributions:

AA, GT, CC, JS, AAV, BG designed and performed the experiments and analysis, and participated in the reviewing process. AA, AAV, JS participated in snail breeding, and sample recollection. AA, GT: conducted the proteomic experiment and analyses. AA, CC, BG conducted the transcriptomic experiment and analyses. AA, GT, 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 [and its supplementary information files]. All RNAseq data is available online under the following accession numbers: NCBI-SRA submission number: SUB5947916; BioProject: PRJNA555222 : Pseudosuccinea columella RNA

sequencing; BioSample: SAMN12305757: Pseudosuccinea columella RNAseq (TaxID: 31228). Full and simplified transcriptomes of *P. columella* snail are also available at: http://ihpe.univ-perp.fr/acces-aux-donnees/

Declarations of interest:

Author's declare that there no conflict of interest exists

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908 **Legends to figures and supplementary data**

909 **Figure 1.** Mantle pigmentation pattern of susceptible (A: Aurora, B: Negrines) and resistant (C: Babiney,
910 D: La Coca, E: El Azufre, F: La Palma) *Pseudosuccinea columella* populations to *Fasciola hepatica*
911 infection. The arrows indicate the characteristic band of small sharp spots in the mid-region of the
912 mantle of resistant snails. (Photos: Laboratory of Malacology, Institute “Pedro Kourí” of Tropical
913 Medicine).

914 **Figure 2.** A) Enriched biological process and B) molecular functions of *Pseudosuccinea columella*
915 transcriptome that are involved (overrepresented; in red) in the resistant phenotype. C) Enriched
916 biological process and molecular functions of *P. columella* transcriptome particularly underrepresented
917 (in blue) in the resistant phenotype. Each bar represents the percentage of unisequences found in the
918 test data (over or under-represented transcripts in resistant *P. columella*) according to their GO terms.

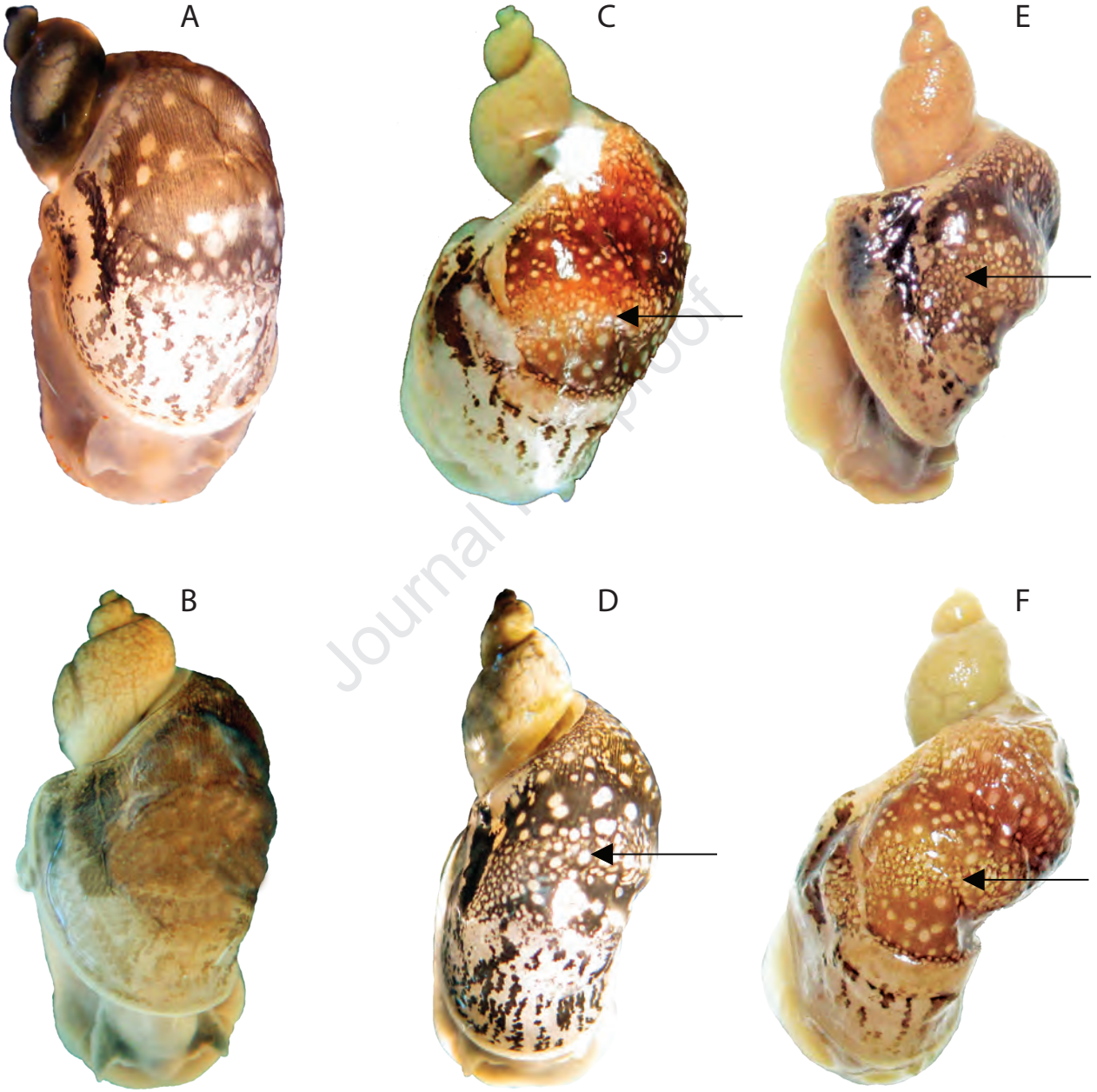
919 **Figure 2.** Immune-related A) under- (in blue) and B) over- (red, right) represented transcripts in resistant
920 *Pseudosuccinea columella*. C) Overrepresented transcripts related to acid-base regulation in resistant *P.*
921 *columella*. Different colors within the same bar account for different variants within the same family of
922 transcripts. Only transcripts with known homologs are shown. A2M: α-2-macroglobulin; BANK: B-cell
923 scaffold with ankyrin repeats-like isoform X1; BPI: bactericidal/permeability increasing protein; G-CSFR:
924 granulocyte colony stimulatory factor; Ig: immunoglobulin; IL: interleukin; IFN: interferon; IRF:
925 interferon regulatory factors; LRR: leucine rich repeats; MIF: macrophage migratory inhibitory factor;
926 Myd88: myeloid differentiation primary response; MZB1: marginal zone B- and B1-cell-specific -like;
927 PKC: protein kinase C; PTAFR: platelet-activating factor receptor-like; PECAM: platelet endothelial cell
928 adhesion molecule; SAMHD1: deoxynucleoside triphosphate triphosphohydrolase SAMHD1; TGF:
929 transforming growth factor; TLR: toll-like receptor; TNF: tumor necrosis factor.

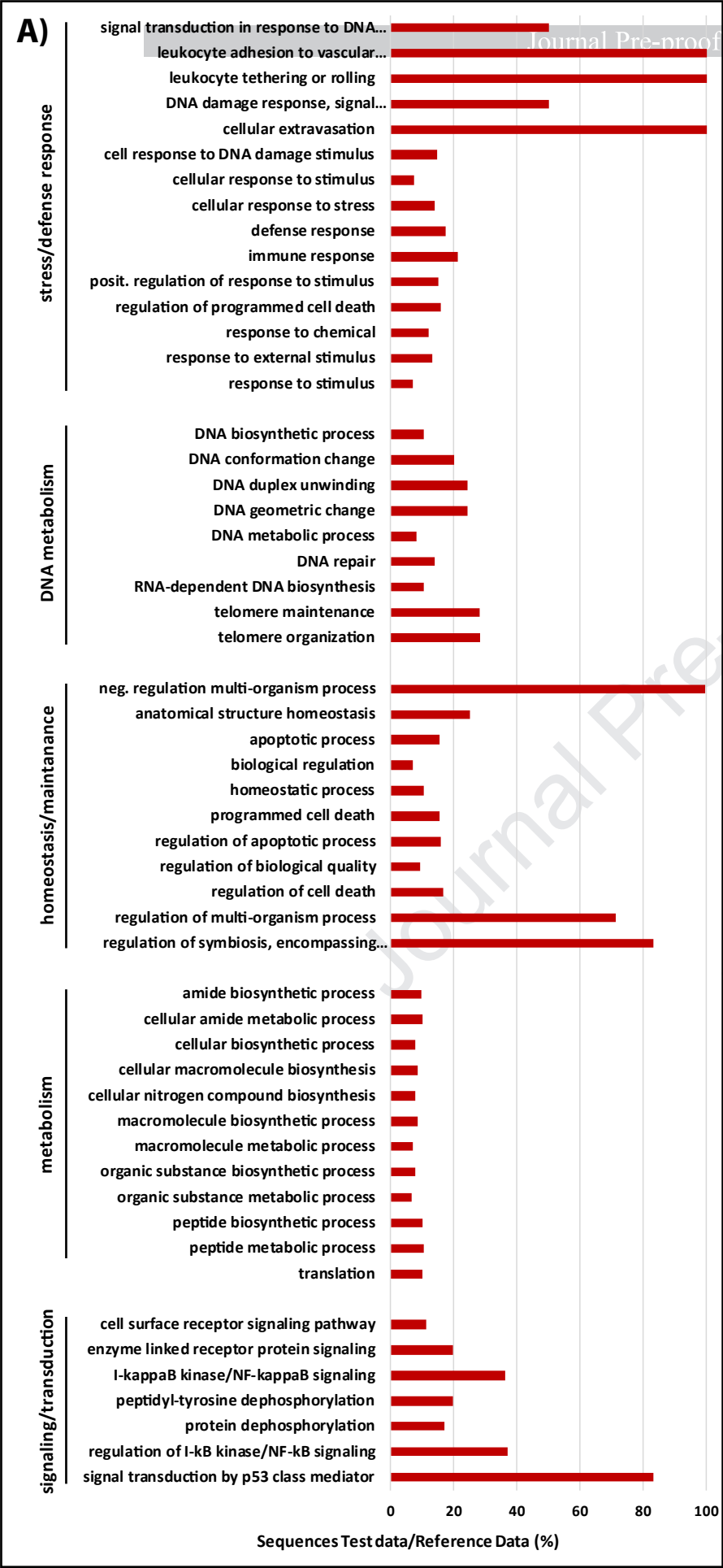
930 **Figure 3.** 2D-electrophoretic profiles of the albumen gland of *Pseudosuccinea columella* resistant (gel on
931 the left) and susceptible (gel on the right) to *Fasciola hepatica* infection. Spots identified only in the
932 resistant or susceptible strains are indicated in red and green, respectively. Spots with significant four-
933 fold difference between the two strains are indicated in blue, only on the gel corresponding to the strain
934 in which they are overrepresented. Next to each spot is written the four-digit identification code used in
935 the Table 1 and Supplementary File 3. The white rectangle within the gels represents an area of highly
936 abundant proteins with several isoforms left aside of the differential analysis as its complexity impede
937 the quantitative analysis and proper spot and protein identification.

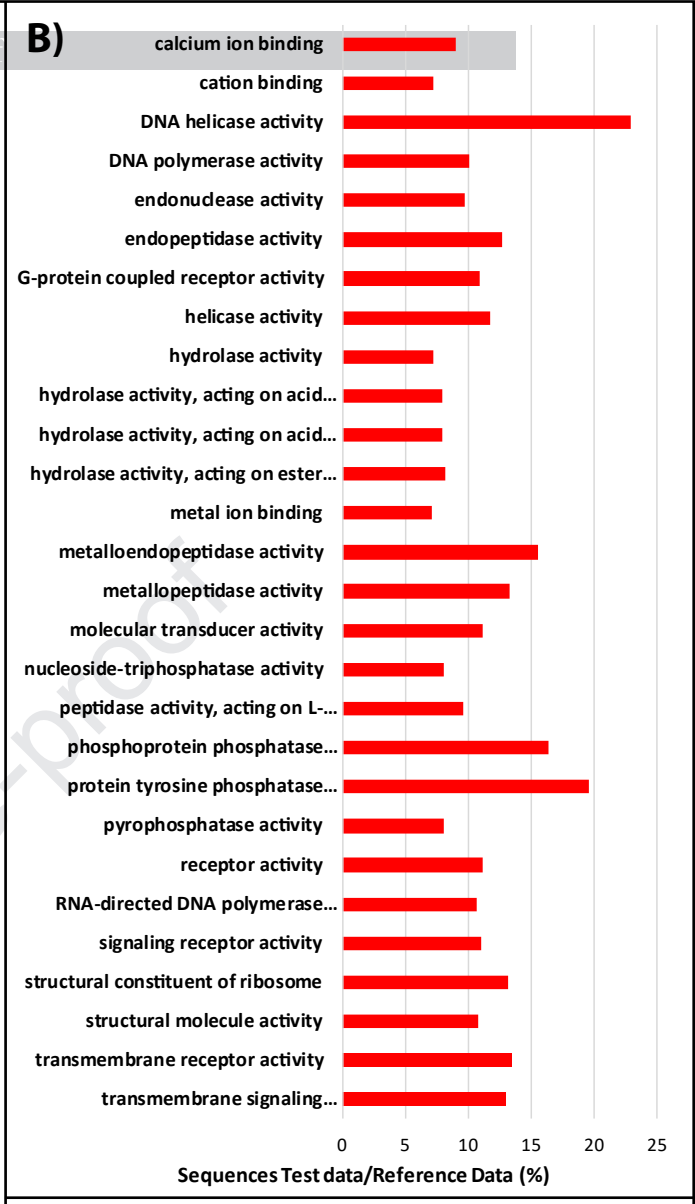
938 **Supplementary data 1:** Excel files with the detailed DESeq2- information and annotation description
939 (Blast2GO information) of the 6876 over- (S1A) and under- (Table S1B) represented transcripts of
940 *Pseudosuccinea columella* showing more than 2-fold differential-expression and *P* adjusted < 0.05
941 between resistant and susceptible phenotypes to *Fasciola hepatica* infection. In addition, lists relating
942 the differentially-expressed transcripts identified as putatively involved in immunity (*i.e.* defense
943 response, wound healing and inflammation) and in relation to acid-base balance (*i.e.* carbon-
944 bicarbonate buffering and ion transportation) are also included in different sheets.

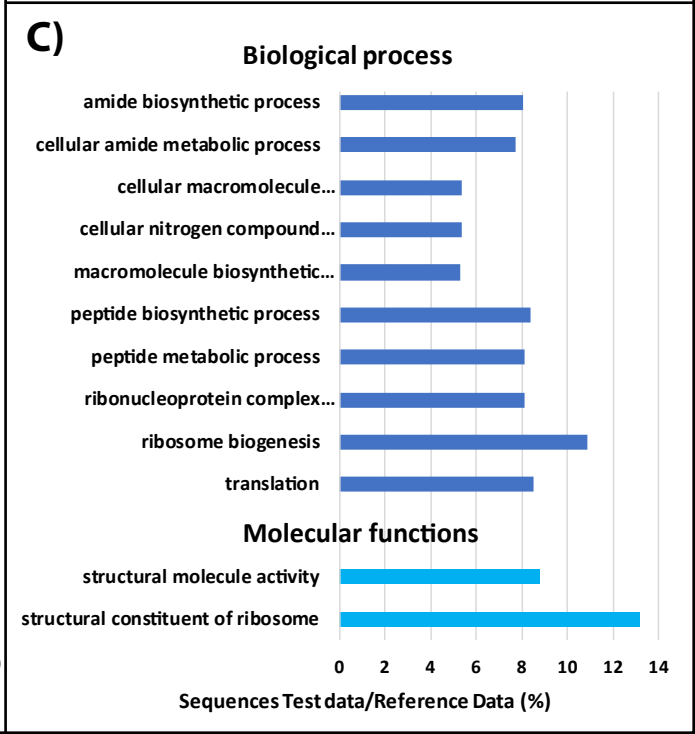
945 **Supplementary data 2:** Excel files showing the table results of the enrichment analyses on
946 *Pseudosuccinea columella* transcriptome involving biological processes or molecular functions, and
947 considering separately over- (S2A) and under (S2B) -expressed transcript data on resistant snails to
948 *Fasciola hepatica* infection. Fisher's Exact Test run on Blast2GO software was used for analyses and a
949 particular GO term was considered to be enriched when *P* and false discovery rate (FDR) < 0.05.

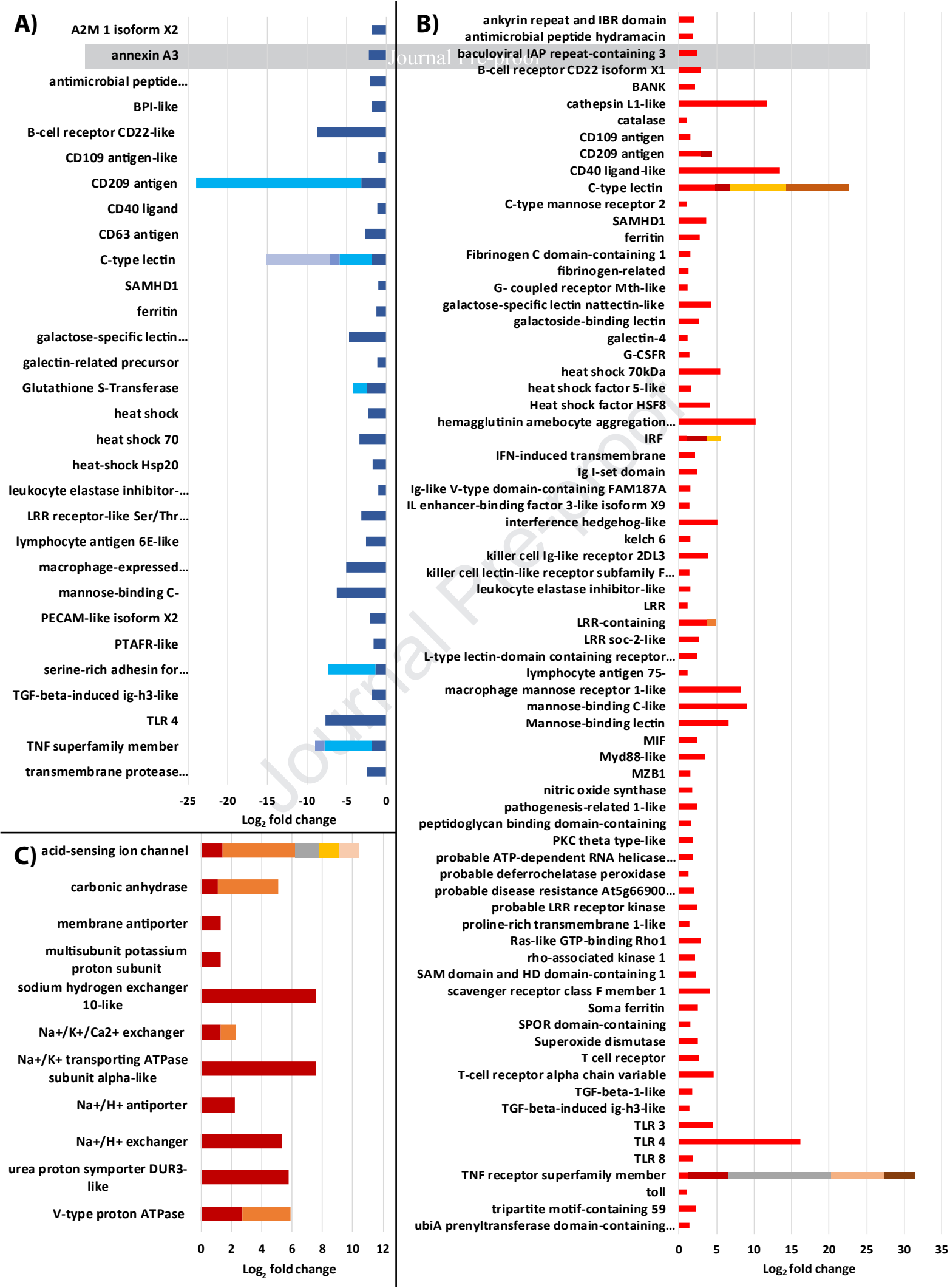
950 **Supplementary data 3:** Excel file showing details on the analysis and further identification of the
951 differentially represented proteins of the albumen gland between *Pseudosuccinea columella* susceptible
952 and resistant to *Fasciola hepatica*, identified on 2D-electrophoresis (more than 4-fold of quantitative
953 differences; $P < 0.01$). Html files containing the information on MS/MS spectrometry analysis from the
954 PISSARO platform facility (University of Rouen, France) are also included.

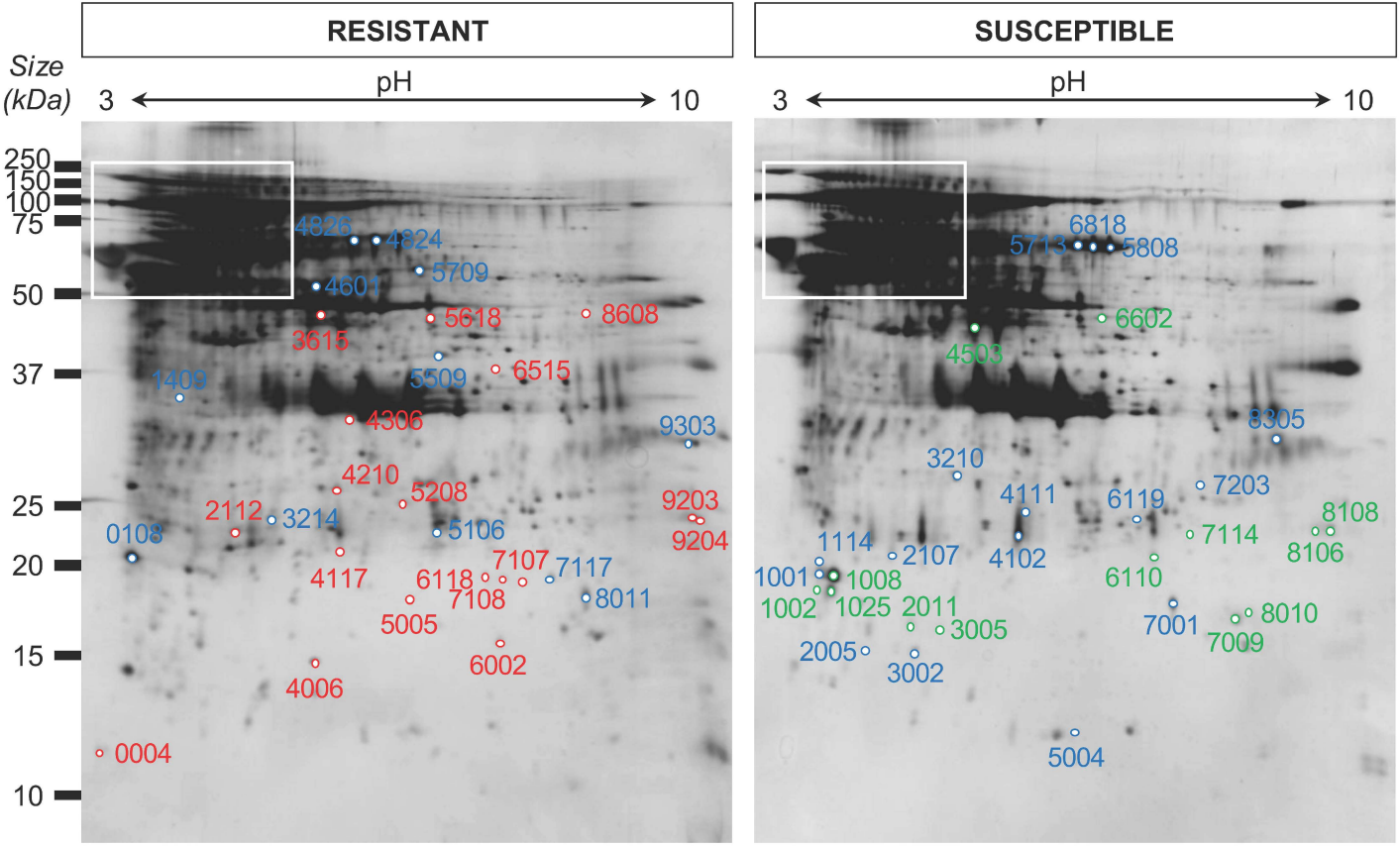









A)
A2M 1 isoform X2
annexin A3
antimicrobial peptide...
BPI-like
B-cell receptor CD22-like
CD109 antigen-like
CD209 antigen
CD40 ligand
CD63 antigen
C-type lectin
SAMHD1
ferritin
galactose-specific lectin...
galectin-related precursor
Glutathione S-Transferase
heat shock
heat shock 70
heat-shock Hsp20
leukocyte elastase inhibitor-...
LRR receptor-like Ser/Thr...
lymphocyte antigen 6E-like
macrophage-expressed...
mannose-binding C-
PECAM-like isoform X2
PTAFR-like
serine-rich adhesin for...
TGF-beta-induced ig-h3-like
TLR 4
TNF superfamily member
transmembrane protease...
-25 -20 -15 -10 -5 0
Log2 fold change
B)
ankyrin repeat and IBR domain
antimicrobial peptide hydramacin
baculoviral IAP repeat-containing 3
B-cell receptor CD22 isoform X1
BANK
cathepsin L1-like
catalase
CD109 antigen
CD209 antigen
CD40 ligand-like
C-type lectin
C-type mannose receptor 2
SAMHD1
ferritin
Fibrinogen C domain-containing 1
fibrinogen-related
G- coupled receptor Mth-like
galactose-specific lectin nattectin-like
galactoside-binding lectin
galectin-4
G-CSFR
heat shock 70kDa
heat shock factor 5-like
Heat shock factor HSF8
hemagglutinin amebocyte aggregation...
IRF
IFN-induced transmembrane
Ig I-set domain
Ig-like V-type domain-containing FAM187A
IL enhancer-binding factor 3-like isoform X9
interference hedgehog-like
kelch 6
killer cell Ig-like receptor 2DL3
killer cell lectin-like receptor subfamily F...
leukocyte elastase inhibitor-like
LRR
LRR-containing
LRR soc-2-like
L-type lectin-domain containing receptor...
lymphocyte antigen 75-
macrophage mannose receptor 1-like
mannose-binding C-like
Mannose-binding lectin
MIF
Myd88-like
MZB1
nitric oxide synthase
pathogenesis-related 1-like
peptidoglycan binding domain-containing
PKC theta type-like
probable ATP-dependent RNA helicase...
probable deferrochelatase peroxidase
probable disease resistance At5g66900...
probable LRR receptor kinase
proline-rich transmembrane 1-like
Ras-like GTP-binding Rho1
rho-associated kinase 1
SAM domain and HD domain-containing 1
scavenger receptor class F member 1
Soma ferritin
SPOR domain-containing
Superoxide dismutase
T cell receptor
T-cell receptor alpha chain variable
TGF-beta-1-like
TGF-beta-induced ig-h3-like
TLR 3
TLR 4
TLR 8
TNF receptor superfamily member
toll
tripartite motif-containing 59
ubiA prenyltransferase domain-containing...
0 5 10 15 20 25 30 35
Log2 fold change
C)
acid-sensing ion channel
carbonic anhydrase
membrane antiporter
multisubunit potassium proton subunit
sodium hydrogen exchanger 10-like
Na+/K+/Ca2+ exchanger
Na+/K+ transporting ATPase subunit alpha-like
Na+/H+ antiporter
Na+/H+ exchanger
urea proton symporter DUR3-like
V-type proton ATPase
0 2 4 6 8 10 12
Log2 fold change
Journal Pre-proof


RESISTANT
SUSCEPTIBLE
Size (kDa)
pH
3
10
250
150
100
75
50
37
25
20
15
10
4826
4824
5709
4601
5618
8608
3615
1409
5509
6515
4306
9303
4210
5208
9203
2112
3214
5106
9204
0108
7107
7117
4117
6118
8011
7108
5005
6002
4006
0004
6818
5713
5808
6602
4503
8305
3210
4111
6119
7203
8108
1114
2107
4102
7114
8106
1001
1008
6110
1002
1025
2011
3005
7001
8010
2005
7009
3002
5004

Highlights

- Comparative “omic” analyses of *P. columella* offer clues for resistance to *F. hepatica*
- Naïve resistant snails display a higher molecular competence for defense/stress responses
- Constitutively broad expression of immune factors associates with resistance to *F. hepatica*
- Overrepresented pH/osmotic regulators endorse the pH tolerance of resistant snails
- Resource allocation to defense/stress response endorse reproductive trade-offs