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# Comparative biology of susceptible and naturally-resistant *Pseudosuccinea columella* snails to *Fasciola hepatica* (Trematoda) infection in Cuba: ecological, molecular and phenotypical aspects

Annia Alba Menendez

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# THÈSE

Pour obtenir le grade de  
Docteur

Délivré par

**UNIVERSITE DE PERPIGNAN VIA DOMITIA**

*En co-tutelle avec*

***Instituto “Pedro Kourí” de Medicina Tropical***

Préparée au sein de l'**ED305 Energie Environnement**

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**IHPE UMR 5244 / Laboratorio de Malacología**

Spécialité : **Biologie**

Présentée par

**Annia ALBA MENENDEZ**

**Comparative biology of susceptible and naturally-resistant *Pseudosuccinea columella* snails to *Fasciola hepatica* (Trematoda) infection in Cuba: ecological, molecular and phenotypical aspects**

Soutenue le 12 décembre 2018 devant le jury composé de

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‘Science is not only a discipline of reason  
but, also, one of romance and passion.’

*Stephen Hawking*



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---

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Alba A.

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## ABSTRACT

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*Pseudosuccinea columella* is one of the main intermediate hosts of *Fasciola hepatica*, a cosmopolitan snail-borne trematode that affects humans, livestock and wildlife. The occurrence in Cuba of susceptible and naturally-resistant populations of this species to *F. hepatica* infection (host's hemocytes encapsulate the parasite upon penetration) offers an interesting Mollusca - Trematoda model with applications on evolutionary biology, health sciences and vector control strategies. Thus, here we explored different ecological, molecular and phenotypical aspects of this system to better understand *P. columella* resistance. We determined that while susceptible and resistant snails share similar ecological requirements, resistant populations occurred only at low pH (6.5-6.5) and total hardness (TH; 4°-10°d) waters, with low snail species richness, suggesting a high ecological cost of resistance. Comparison of life history traits between susceptible and resistant snails, experimentally-reared at low or common pH/TH conditions, showed that resistant populations display higher tolerance to pH/TH variations and a lower reproductive potential. To gain more insights on the molecular bases of the features associated to resistance in *P. columella*, we performed comparative "omics" approaches on naïve snails from both phenotypes at whole snail level (RNAseq) and at the albumen gland level (2D-electrophoresis). The molecular differences found were fundamentally associated with an enrichment on biological process/functions and a particular abundance of proteins/transcripts related with immune defense/stress response in resistant snails. These results indicate a differential allocation of resources, at the molecular level, to self-maintenance and survival on resistant individuals, that possibly trades-off against reproduction, and that could endorse the enhanced responsiveness against stressors (*F. hepatica* or pH/TH variations) and the ecological cost of resistance observed in resistant population. On the other hand, we explored the effect of high infective doses, successive parasite exposures and different geographic origin of *F. hepatica* (Europe and the Caribbean) on the infection outcomes on both *P. columella* phenotypes and observed no reversion of the resistance independent of the experimental approach tested. Additionally, we found both phenotypes equally susceptible to another digenean trematode, *Trichobilharzia* sp., indicating a certain specificity of *P. columella* resistance, so far, to *F. hepatica*. Finally, we performed comparative studies on the cellular immune response following *F. hepatica* exposure. The significant differences found in resistant (*i.e.* high adherence and aggregation, and higher proliferation and encapsulation capacity of the hemocytes, and higher cytokine granulin expression) compared to susceptible snails endorse the differential response towards *F. hepatica* that develops on both phenotypes. This thesis presents the latest efforts to broadly characterize this model, which constitute building steps for the comprehension of *P. columella* resistance and for its application to tackle parasite transmission.

**Keywords:** liver fluke, Lymnaeidae, compatibility, ecology, immunobiology, cost of resistance

# RÉSUMÉ

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*Pseudosuccinea columella* est un des principaux hôtes intermédiaires de *Fasciola hepatica*, un trématode cosmopolite infectant l'Homme et les animaux domestiques et sauvages, et transmis par des mollusques d'eau douce. L'existence exclusive à Cuba de populations de *P. columella* qui sont sensibles et naturellement résistantes (encapsulation hémocytaire du parasite) à l'infection par *F. hepatica* offre un modèle Mollusque-Trématode très intéressant en termes de biologie évolutive, de santé humaine et de stratégies de contrôle vectorielle. L'étude des aspects écologiques, moléculaires et phénotypiques est essentielle pour comprendre les particularités de ce système. Ainsi, nos résultats ont montré que les mollusques sensibles et résistants partagent des exigences écologiques similaires mais chez les résistants la distribution est limitée aux localités avec des eaux acides et peu dures (pH/dureté totale (DT), 6-6.5/4°-10°d) et une richesse des communautés de mollusques très faible, ceci semblant indiquer coût élevé à la résistance. La comparaison de traits d'histoire de vie en laboratoire a permis de confirmer une haute tolérance aux variations de pH/DT de l'eau chez les mollusques résistants mais a montré également une diminution du potentiel reproductif des souches résistantes par rapport aux sensibles. Sur la base de ces observations, des approches «omiques» comparatives ont été entreprises entre souches sensibles et résistantes. Ces approches se sont focalisées sur l'étude de (i) la glande de l'albumine par protéomique comparative et (ii) par une approche de transcriptomique massive sur mollusques entiers. Nous avons observé une grande richesse de processus et fonctions biologiques et une forte expression de molécules en lien avec l'immunité et la réponse au stress chez les individus résistants. Ces différences moléculaires identifiées entre les deux phénotypes indiquent un trade-off entre la survie et la reproduction chez les individus résistants et peuvent expliquer en partie leur haute réactivité vis à vis des agents de stress biotique et abiotique (*F. hepatica* ou variations de pH/DT) et le coût de la résistance observée. Ainsi, nous avons exploré l'effet de doses parasitaires croissantes, des infections sérielles et des souches allopatriques de *F. hepatica* (Europe et Caraïbe) sur la compatibilité, sans modification du statut de résistance des mollusques. De plus, les deux phénotypes sont sensibles à un autre trématode (*Trichobilharzia* sp.), ce qui indique que la résistance chez *P. columella* est dirigée et ou spécifique de *F. hepatica*. Enfin, nous avons observé des différences significatives chez les souches résistantes concernant la réponse immunitaire cellulaire (*i.e.* haute adhérence et agrégation des hémocytes, haute activité proliférative et d'encapsulation et expression de la cytokine granuline) pour rapport aux individus sensibles. Ces résultats peuvent expliquer, en partie, la réponse immunitaire différentielle à *F. hepatica* qui se développe chez les deux phénotypes. Ce travail de thèse nous a aidé à comprendre les particularités de ce modèle hôte-parasite, mais présente également une avancée en termes d'utilisation de la résistance de *P. columella* à des fins de contrôle de la transmission parasitaire.

**Mots clé :** douve du foie, Lymnaeidae, compatibilité, écologie, immunobiologie, coût de la résistance

## RESUMEN

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*Pseudosuccinea columella* es uno de los principales hospederos intermediarios de *Fasciola hepatica*, un trematodo cosmopolita que afecta a humanos, animales domésticos y salvajes. La exclusiva ocurrencia en Cuba de poblaciones de *P. columella* susceptibles y naturalmente resistentes a *F. hepatica* (los hemocitos del hospedero encapsulan al parásito), ofrece un modelo fascinante, en el contexto Mollusca-Trematoda, con aplicaciones en biología evolutiva, las ciencias médicas y el control de vectores. En la presente tesis, se exploraron diferentes aspectos ecológicos, moleculares y fenotípicos de este sistema para comprender mejor la resistencia en *P. columella*. Se determinó que, aunque poblaciones susceptibles y resistentes comparten requerimientos ecológicos similares, la distribución restringida de *P. columella* resistentes a sitios de bajo pH (6-6,5), dureza total (TH; 4°-10°d) y riqueza de especies sugiere un costo ecológico significativo de la resistencia. Comparaciones de los parámetros de vida en el laboratorio, entre *P. columella* susceptibles y resistentes criadas en valores bajos o comunes de pH/TH, mostraron que las poblaciones resistentes son más tolerantes a las variaciones de pH/TH y tienen menor potencial reproductivo. Para explorar las bases moleculares de las características fenotípicas y ecológicas asociadas a la resistencia, se compararon, por técnicas “ómicas”, el proteoma de la glándula del albumen (2D-electroforesis) y el transcriptoma completo (RNAseq) entre individuos susceptibles y resistentes sanos. Las diferencias encontradas se refirieron fundamentalmente a un enriquecimiento de funciones y procesos biológicos y a una abundancia significativa de moléculas relacionados con la respuesta inmune/estrés en *P. columella* resistentes. Esto indican que existe, a nivel molecular, una asignación diferencial de los recursos hacia el mantenimiento y la supervivencia en individuos resistentes, posiblemente en detrimento de la reproducción, y podría explicar la reactividad incrementada frente a agentes estresantes (*F. hepatica* o variaciones en el pH/TH), y el costo ecológico asociado a la resistencia observados. Por otra parte, se constató que no hubo reversión de la resistencia frente a altas dosis parasitarias, infecciones múltiples con *F. hepatica* y con cepas alopatricas provenientes de Europa y el Caribe, lo que avala su estabilidad y universalidad. Adicionalmente, se encontró que los dos fenotipos de *P. columella* son igualmente sensibles a otro trematodo, *Trichobilharzia*, lo que apoya la especificidad de la resistencia de *P. columella*, hasta el momento, a *F. hepatica*. Finalmente, se realizaron estudios comparativos sobre la respuesta celular entre susceptibles y resistentes expuestos a *F. hepatica*. Las diferencias significativas encontradas en resistentes respecto a susceptibles (*i.e.* adherencia, agregación e incremento de la proliferación y encapsulación de los hemocitos, incremento de la expresión de la citoquina granulina) respaldan las diferentes respuestas a *F. hepatica*, que ocurren en cada fenotipo. Esta tesis contribuye a la caracterización de este modelo y constituye un pilar esencial para entender la resistencia de *P. columella* y para su futura aplicación en el control de la transmisión parasitaria.

**Palabras clave:** duela, Lymnaeidae, compatibilidad, ecología, inmunobiología, costo de la resistencia

# GENERAL INTRODUCTION

---

The interaction between hosts and parasites is one of the hot topics in ecology and evolutionary biology since (1) parasites exert density-dependent regulation of host populations and contributes to diversity patterns, (2) host–parasite co-evolution may lead to the maintenance of genetic diversity and possibly to the maintenance of sexual reproduction, and (3) it can lead to co-speciation of hosts and parasites (Joachim et al. 2001). Concomitantly, its study is also essential for the comprehension of the dynamic of infectious diseases since coevolution of host–parasite interactions is considered a reciprocal natural selection between host resistance and parasite infectivity (Hawley & Altizer, 2011).

Particularly, mollusks and their digenean parasites have existed for over 200 million of years thus, they have also evolved under each other selective pressures. In the next chapters, some general aspects of the relationship between mollusks and their digenean parasites will be reviewed with emphasis on compatibility, immunobiological interactions and ecological features affecting transmission. Focus is particularly given to the overview on the global burden of fasciolosis, a worldwide distributed trematodosis of major public and veterinary health importance, and its transmission by lymnaeid snails. Finally, insights into the peculiarities of the Cuban epidemiological scenario of fasciolosis transmission will be discussed to introduce the fascinating model of naturally-resistant and susceptible *Pseudosuccinea columella* snails in their interaction with the trematode *Fasciola hepatica*.

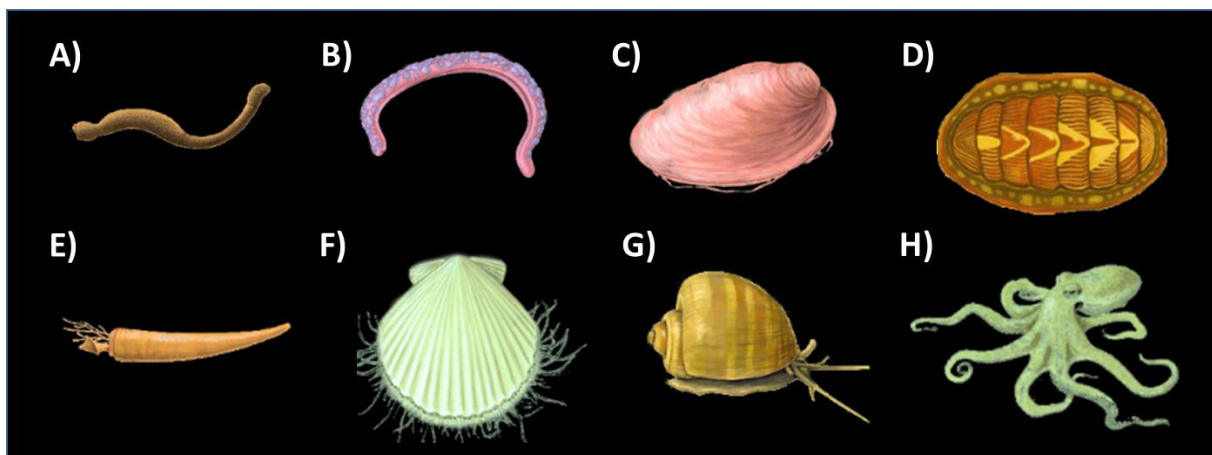
## I. A glance at the Phylum Mollusca

*“(...) In the space of the tide, with the sea and the shore  
And the sunshine, the Molluscs successfully fit;  
Some on land, some in lakes which were seas, they exist  
And though tideless for ages, the Molluscs persist”*

***Adapted from William Pallister (Poems of Science)***

The phylum Mollusca (Latin term for soft body) is one of the largest phyla of animals with over 93 000 living species and nearly 70 000 known fossils species. Mollusks are a very

diverse group including chitons, tusk shells, snails, slugs, sea butterflies, mussels, squids and octopuses; ranging from tiny microscopic species to the giant squid. Species of mollusks are found in a great range of habitats from the tropics to polar seas at altitudes over 7000 m or abyssal depths. Common habitats include ponds, lakes, streams, mud flats, rocky shores, rain forests, reefs and open oceans (Brusca & Brusca, 2003). Today, eight extant taxonomical classes are included within the Mollusca (Figure 1): from the primitive vermiform Caudofoveata and Solenogastres, to the Monoplacophora, Polyplacophora (chitons), Scaphopoda (tusk shells) and the more notorious taxa like Bivalvia (mussels, clams, oysters), Gastropoda (snails and slugs), and Cephalopoda (octopuses, squids and nautilus) (Sigwart & Lindberg, 2015). Primitive classes such as Rostroconchia are only known from the fossil record (Campbell, 2006). The phylum Mollusca has existed since the early Cambrian, more than 542 million years ago, and is the most diverse group in marine ecosystems where the eight classes are represented, with bivalves able to colonize freshwater habitats and gastropods both freshwater and land (Brusca & Brusca, 2003).



**Figure 1.** Representation of the eight extant classes of the phylum Mollusca. A) Caudofoveata, B) Solenogastres, C) Monoplacophora, D) Polyplacophora, E) Scaphopoda, F) Bivalvia, G) Gastropoda and H) Cephalopoda.

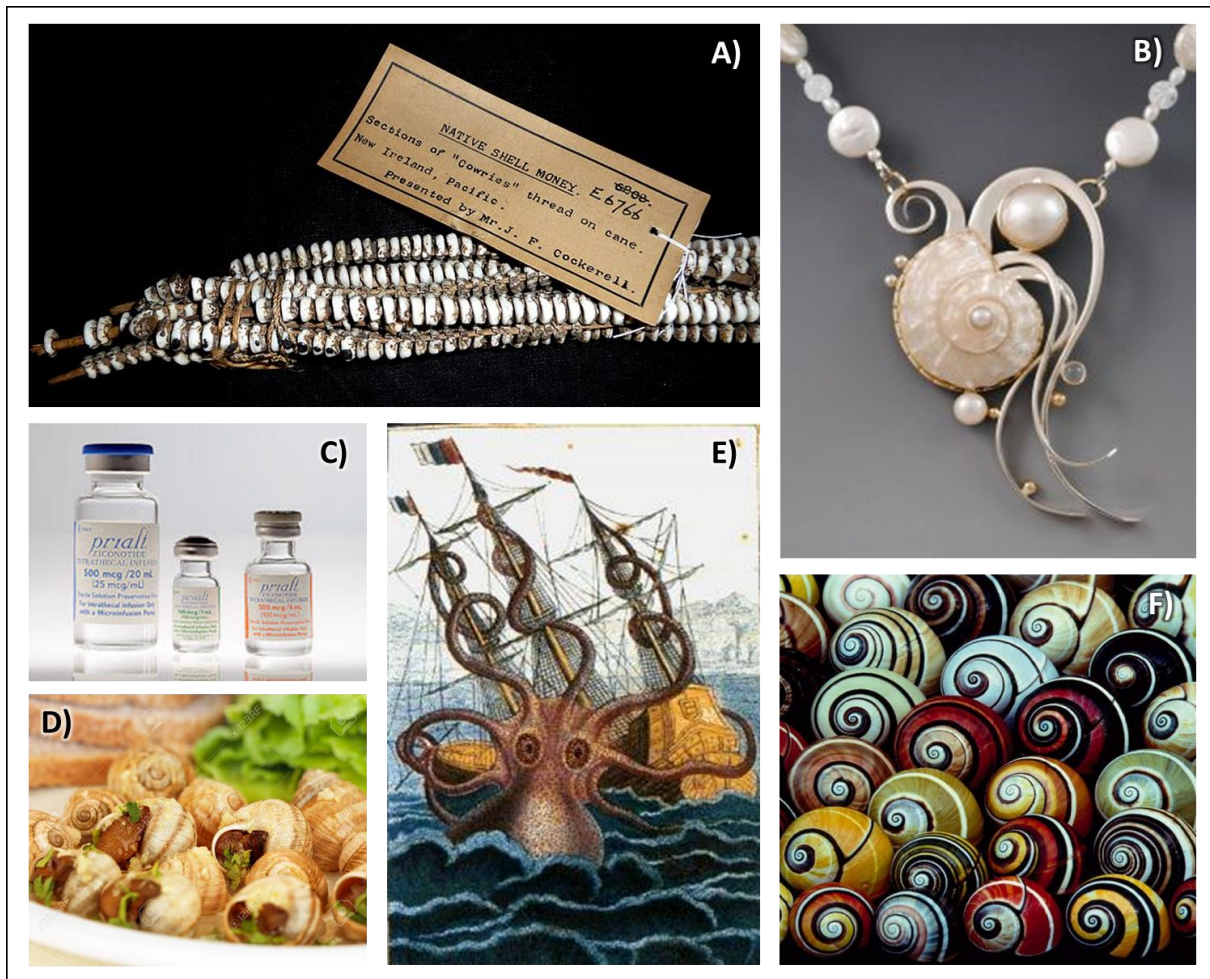
Mollusks are complex and highly diversified animals. They vary not only in their size, morphology and ecology, but also in their feeding habits including herbivorous grazers, predaceous carnivores, filter and detritus feeders, and parasites. Reproduction strategies are also diversified in mollusks with most species being dioecious and some hermaphroditic with both allo- or auto-fecundation adaptations. In many mollusks, a free swimming trochophore larva emerge from the eggs transforming directly to small juveniles (such as in chitons) or into another larva known as veliger (as in many gastropods and bivalves). Others, such as



cephalopods and many gastropods and bivalves, have no larval stages and hatch directly as juveniles. However, all mollusks are bilaterally symmetrical, true coelomate from the protostome branch. They are known to have the body covered by a thick epidermal-cuticular sheet of skin, the mantle, which forms a cavity (the mantle cavity) sheltering the internal organs. The mantle has special glands that secrete calcareous epidermal spicules, shell plates or shell. The body has a head with sensory structures, a centrally-concentrated visceral mass and a ventral muscular foot, used in locomotion, which can be modified, reduced or absent in certain species. The buccal region, at the anterior end, contains a radula (absent in bivalves), which is a tongue-like strap of teeth used for feeding that may be modified in certain species depending on their habits (Brusca & Brusca, 2003).

The elegant and diverse design of shapes and colors of mollusks' shells, the beauty of nacre and pearls and the legendary magnificence of octopuses and giant squids have fascinated mankind since early days. Thus, mollusks have been used by humans throughout history in a variety of applications: as food, trade goods and tools, in jewelry, mythology and religion, and medicine, while serving as inspiration for scientists, artists, architects and collectors (see Figure 2 for some examples).

However, in nature, mollusks have crucial roles in the community dynamics, and in the engineering and health of ecosystems. Beyond being predators or important source of food for a long list of species from different taxa, mollusks are considered as key players in recycling nutrients and biological waste (Jones & Shachak, 1990). Moreover, their shells are important substrata for attachment of epibionts, provide refuges from predation, physical or physiological stress, and control the transport of solutes and particles in the benthic environment (Gutiérrez *et al.* 2003).



**Figure 2.** Examples of the use of mollusk in different areas of human activities throughout history. A) shells of species like *Cypraea moneta* were used as trade goods and currency, B) shells and the nacre and pearls produced by gastropods and bivalves are extensively used in jewelry, C) Ziconotide (Prialt™), an atypical painkiller drug approved by the Food and Drug Administration in 2004 to be used on humans, is derived from the venom of the snail *Conus magnus*, D) edible snails boiled and served with garlic and fine spices, considered a French “*delicatessen*”, E) the painting “Colossal octopus” by Pierre Denys de Montfortin was inspired in the Scandinavian legend of the mythological Kraken monster, F) collection of beautiful *Polymita* shells, a critical endangered genus exclusive of eastern Cuba.

In addition, another ecological niche of mollusks concern hosting a plethora of parasites from microbes to helminths. This phylum has existed for so long that they eventually evolved with their own specialized lineage of pathogens which restrictedly depends on them: the digenetic trematodes (Loker *et al.*, 2004). So far, around 18 000 nominal species of digenetic parasites are reported for which only very few marine species are an exception to the following rule: the exclusive use of mollusk, *i.e.* scaphopods, bivalves, and mainly gastropods, as first intermediate host during their life cycle (Cribb *et al.*, 2003).

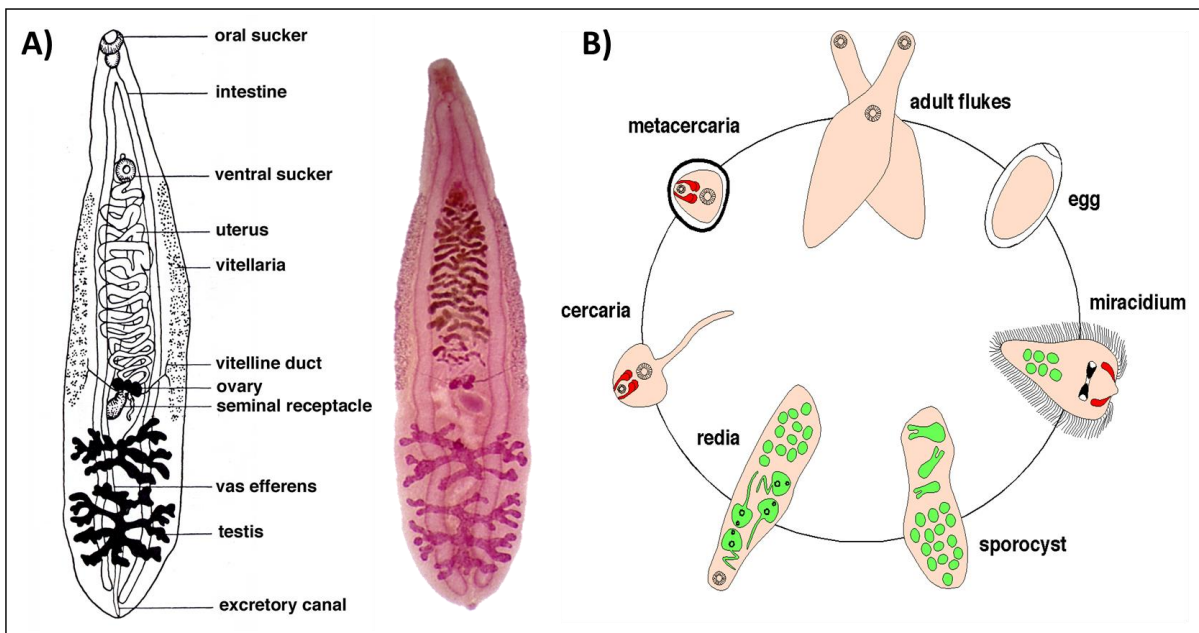
## II. Class Trematoda: mollusk-specialized parasites

*“Parasitic “puppet masters”, with their twisted, self-serving life history strategies and impressive evolutionary takeovers of host minds, capture the imagination of listeners”*

***Lafferty & Kuris, 2012. Host manipulation by parasites***

Even when parasitism is a way of life rather than a feature related to a particular taxon, the class Trematoda, within the phylum Platyhelminthes, comprises around 20 000 species of endoparasites, most of them grouped in the subclass Digenea, which stands as the most diversified parasites within the Neodermata clade (Cribb et al., 2003).

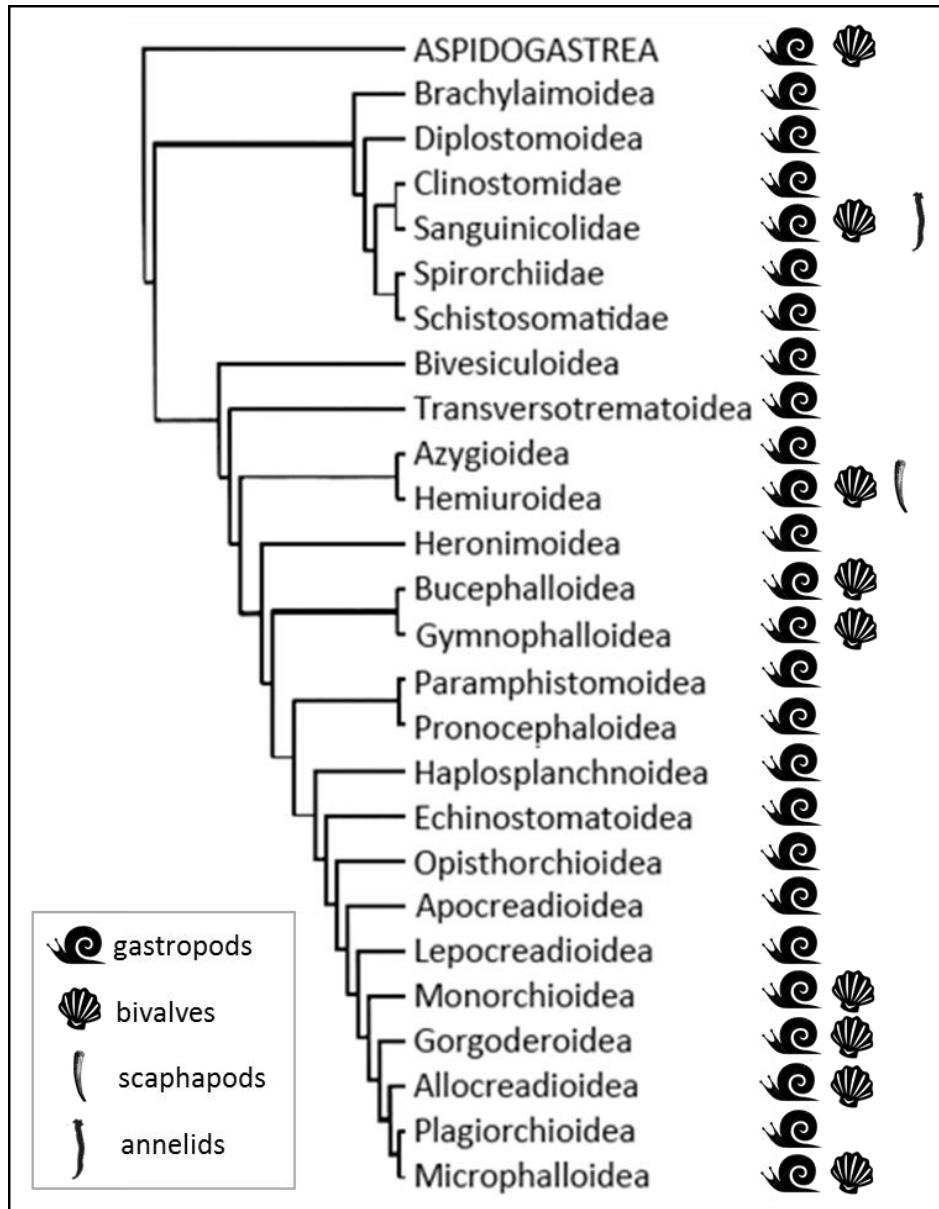
Particularly, digenean trematodes are small dorso-ventrally flattened acoelomate worms with simple anatomy lacking segmentation (Figure 3A). They present simple ladder nervous system (a pair of anterior ganglia with long longitudinal nerve cords transversally connected), simple sense organs (some present eyespots) and no respiratory or circulatory systems. Digenean parasites also possess two suckers (oral and ventral acetabulum) which they use for attachment within the host (Brusca & Brusca, 2003). The oral sucker contains the mouth that is linked to a muscular pharynx that allows the worm to pump food into a blind ending gut that can be quite extensive and highly branched in some genus like *Fasciola* (Brusca & Brusca, 2003; Andrews, 1999). The food uptake is performed through the gut and through the tegument (“skin”), which is a functional syncytium that covers the parasite’s body and has a plethora of functions; from the absorption of nutrient and sensorial activity, to the synthesis and secretion of functional molecules and the protection against the defense molecules of the hosts (Smyth & Halton, 1985; Dalton et al. 2004). Trematodes can produce an enormous amount of offspring by combining sexual reproduction inside the final host and asexual multiplication in the primary intermediate host. Trematodes are generally hermaphrodites with well-developed gonads, ducts and accessory organs and may perform internal cross and self-fertilization inside the definitive host. However, larval forms are capable of asexually multiply in the primary intermediary hosts (Esch et al. 2002).



**Figure 3.** A) Anatomical overview of a digenean adult. B) Typical developmental stages of the trematodes during their life cycle. Adult flukes live and develop within the definitive host where they become sexually mature and produce massive number of offspring (eggs). The eggs leave the definitive host at different degrees of maturity depending on the species but each contains an embryo called miracidium. The miracidium is a ciliated (=motile) larvae that hatches from the egg usually through a preformed “door”, the operculum, which is present in most species. This larva presents several chemical and mechanical receptors, and penetration glands that mediate infection upon contact with the appropriate mollusk host (first intermediate host). The miracidium usually metamorphosed, after primary host penetration, into a sporocyst, which acts as a germinal sac. The sporocyst develops into daughter sporocysts where the cercariae are developed. Depending on the species, sporocyst can produce a next amplification generation called redia. Redia have features of the adult fluke like a muscular pharynx, a gut and “birth pore” to release the cercaria. Cercaria are the larvae that leave the intermediate host and, depending on the digenean species, can either directly penetrate and infect the final host or suffer an encystation process on plants, water or within the tissues of another intermediate host to become a metacercariae. The cycle is completed once the cercariae (or encysted (meta)cercariae) gets into the final host where the adult stage develops.

Trematodes, and particularly digeneans, are an extraordinarily ubiquitous group with complex life cycles usually involving three host species and several free-living and parasitic larval stages (see Figure 3B for details into the different developmental stages within a trematode life cycle). Despite such complexity, these animals are highly adapted to their parasitic way of life and in fact, the subclass Digenea has managed to parasitize all major vertebrate groups (Brusca & Brusca, 2003). However, it is significant that the class Trematoda is defined, in part, by the fact that all known species parasitize mollusks, mainly gastropods, that acts as first intermediate host (Cribb *et al.*, 2003, see Figure 4). It is inside this primary mollusk host where a larva called miracidium, the infective stage for the

intermediate host, transforms into other larval stages (sporocyst and rediae) that asexually multiply within its host and produce cercariae (Cribb et al., 2003). For digeneans, the relation with the mollusk host is in fact so tight that even those species that have managed to simplify their life cycle up to only one host, the development inside the snail host remains obligatory (see Poulin & Cribb, 2002).



**Figure 4.** Phylogenetic tree of the class Trematoda (subclass Aspidogastrea and all the superfamilies within the major subclass Digenea) regarding life cycle variation in relation to the first intermediate hosts (*i.e.* gastropods, bivalves, scaphopods (from Mollusca) and annelids). Adapted from Cribb et al. 2003.

Therefore, this is, beyond doubt, a very interesting model for evolutionary biology studies but, also, for health sciences since many of these parasites species affects humans and

domestic animals. The final outcome after a parasite exposure depends on the ecological features, the genetic specificity ruling the interaction and, ultimately, in the resulting crosstalk of host defenses and parasite counter defenses thus, a briefly overview on mollusk – digenean dynamic is presented in the following section.

### III. Where Malacology meets Parasitology: snail – trematode interaction

*“Snail-digenean systems are excellent models to study the “give and take” involved in intimate, long-term host-parasite associations.”*

**E. Sam Loker, 2010; Adv Exp Med Biol. 708: 17-43**

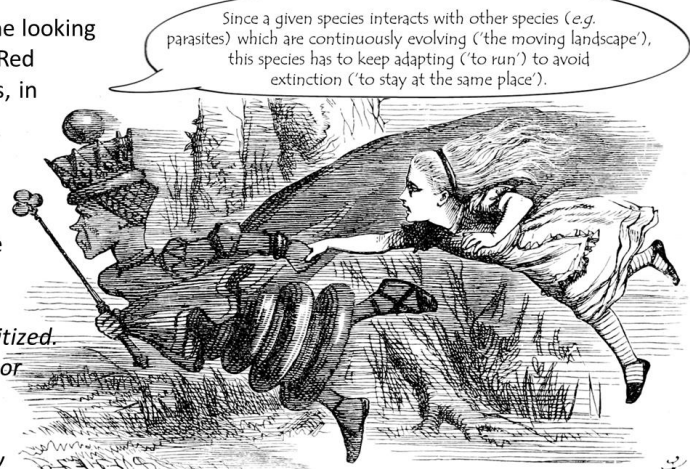
Parasites continuously evolve to improve their fitness for infecting, surviving, and being transmitted to the next host and, in turn, hosts must adapt to parasite changes to resist or tolerate the aggression in an evolutionary “race” (Hurtrez-Boussès et al. 2001). This type of interaction explains how parasites are selected to overcome host resistance while, consequently, hosts carrying rare resistant genes have a selective advantage. This co-evolutionary process is known as the Red Queen hypothesis (Van Valen 1973; see box 1). The existence of genetic variations determining host resistance or parasite virulence, as well as the specificity for particular host genotypes, are premises for the functioning of this system (Joachim et al. 2001). Thus, evolving under such antagonistic pressures of “Red Queen species” should favors the selection for sexual reproduction (Hamilton et al. 1990) and the local adaptation between parasites and hosts (Lively & Dybdahl, 2000).

The obligatory character of any digenean – mollusk interaction has resulted in shaping amazing strategies for the parasite to infect, survive and develop in its host with varying levels of specialization of such interaction (Niewiadomska & Pojmańska, 2011). Host restriction can differ depending on the digenean species, and examples can range from *Schistosoma mansoni* infecting only some *Biomphalaria* species to *Clonorchis sinensis* parasitizing snails within five different gastropod families. However, there is a degree on the specificity in mollusk-digenean interaction since the capacity of any given digenean to infect snails is not limitless across all snail species. Unambiguous evidences exist that genetically defined digenean parasites can colonize and successfully reproduce asexually only within caenogastropods or pulmonate snails (Adema & Loker, 2015). Several features, mechanisms and conditions which involve not only the digenean parasite and the molluscan host but also the environmental factors, relevantly contribute in shaping the landscape of host specificity

and infection success; from the pre-penetration step to the final outcome of each interaction.

**BOX 1 - The Red Queen hypothesis:**

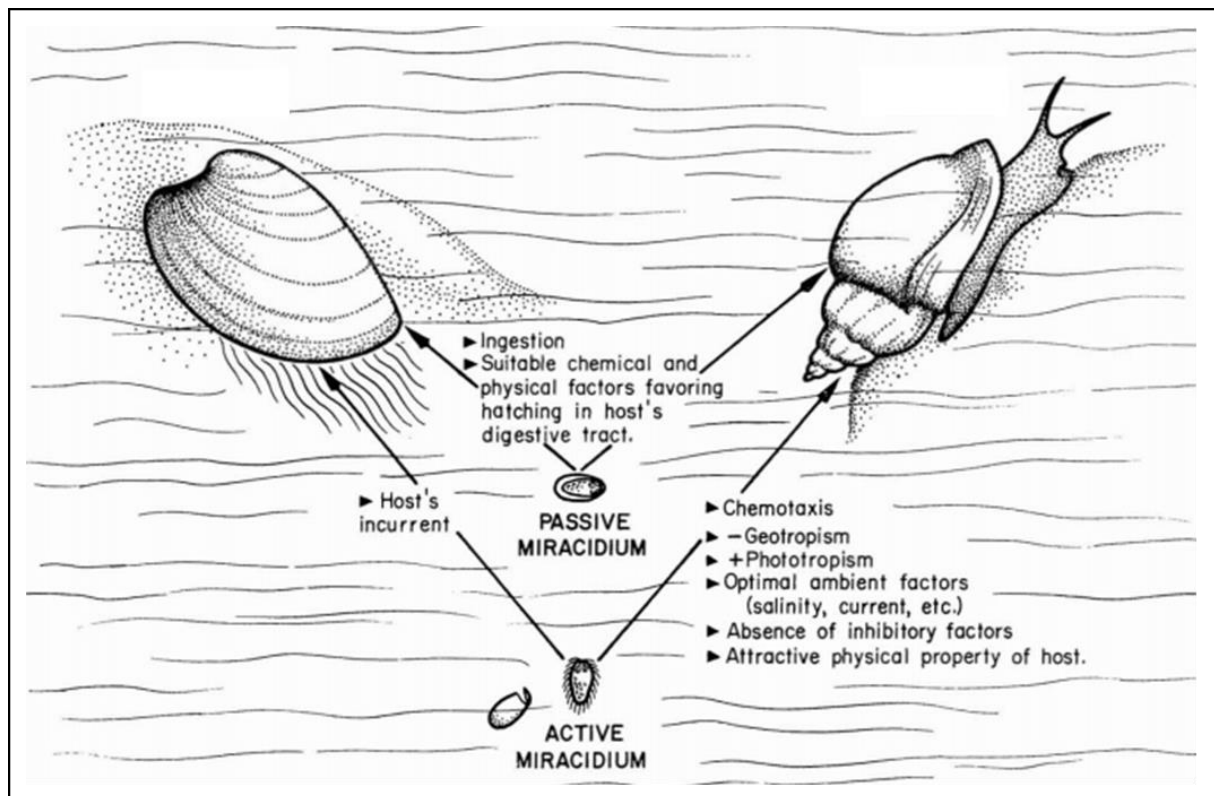
The passage in Lewis Carroll's book "Through the looking glass and what Alice found there" in which the Red Queen runs and explains to Alice that she needs, in a perpetually-moving background, to run continuously in order to stay in the same place, inspired in 1973 Leigh Van Valen to propose a new evolutionary theory. Van Valen applied this principle to evolutionary biology in what he named "The Red Queen's hypothesis": *"It is selectively advantageous for a prey or host to decrease its probability of being eaten or parasitized. It is often selectively advantageous for a predator or parasite species, and much more often for a predator or parasite individual, to increase its expected rate of capture or food. It is selectively advantageous for a competitor for resources in short supply, both to increase its own effect on its competitors and to decrease the effect of its competitors on itself. Every species does the best it can in the face of these pressures (...). We can think of the Red Queen's Hypothesis in terms of an unorthodox game theory. To a good approximation, each species is part of a zero-sum game against other species. Which adversary is most important for a species may vary from time to time and for some or even most species no one adversary may ever be paramount. Furthermore, no species can ever win, and new adversaries grinningly replace the losers"* (Van Valen, 1973).



**Making contact – a trematode's encounter with its snail host:** One of the main steps for a successful miracidia infection to occur involves host habitat selection, tightly linked to ecological compatibility of parasites and their hosts (both intermediate and definitive), from which the trematode makes its way to the environment of its vector snail (Christensen, 1980). This is in fact the Red Queen in action as presented above, where the host-parasite interplay is dictated by the continuous adaptation to each other's conditions. In fact, ecological preferences rather than phylogenetic affinities might explain the specificity for snail host of several digeneans parasites like some schistosomatids and paragonimids (Blair et al. 2001), and has been suggested for the *Fasciola hepatica* use of a wide variety of snail hosts (Lofty et al. 2008). In some cases, phylogenetic tracking rather than an arms-race relationship might be a common alternative that helps parasites to switch to new host species, most likely within related taxa but, for sure, ecologically equivalent (Blair et al. 2001).

Particular adaptations for parasitism to succeed, from morphological and biochemical to behavioral features, can be cited among miracidium from digeneans (Cheng, 1968).

Depending on the species, the miracidium would either hatch inside the host once the eggs are eaten by the mollusk (*e.g. Dicrocoelium, Clonorchis*; passive miracidium), or hatch in the environment to, afterwards, “track” and infect the intermediate host (*e.g. Schistosoma, Fasciola*; actively-infecting miracidium (Cheng, 1968; Figure 5). The former strategy could ensure longer survival time of miracidia within eggshell and guarantees the transmission of digenean trematodes occurring on terrestrial environments, although it can be also observed on species with aquatic or semi aquatic life cycles (Niewiadomska & Pojmańska, 2011). In this case, miracidia hatching upon egg ingestion is an active process stimulated by physical and biochemical factors present in the intestine of suitable host. For example, the hatching of *Dicrocoelium dendriticum* miracidia can be stimulated *in vitro* by the intestinal juice of *Helix pomatia* snails and CO<sub>2</sub> (Mitterer, 1975 cited in Mitterer, 2008; Mitterer, 2008).



**Figure 5.** Diagrammatic drawing illustrating the main factors that govern or influence miracidium-mollusk contact during the pre-invasion phase (Cheng, 1968).

However, for those water-associated digeneans whose eggs hatch in the environment, the need to find a suitable host to penetrate into is urgent since miracidia are short-lived non-feeding larvae with age-dependent infectivity (Anderson et al. 1982; Andrew, 1999). Even when it has not been described for most species, the tracking of the snail host by actively-



infecting miracidia is generally assumed and it is facilitated by particular behavioral, biochemical and morphological features. In this sense, actively-swimming miracidia are characterized by the presence of four functional complexes of organs: locomotory, sensory, penetration and generative, which mediate finding, infecting, developing and growing in the intermediate host (Galaktionov & Dobrovolskij, 2003). The highly ciliated surface of the miracidia is needed for the kinesis of the larva and such locomotory apparatus is functionally tied in with the nervous and sensory systems which facilitate the reaction of the larvae to the physical (*e.g.* light, direction of gravity) and chemical factors (*i.e.* ions, biomolecules) of the environment (Andrews, 1999; Galaktionov & Dobrovolskij, 2003). Other structures of the miracidium like eyespots, peripheral sensory endings, single apical and paired lateral glands and terebratoria are formed prior hatching, during the embryonic development of the larvae, and mediate location, adhesion, and penetration of the snail host (Galaktionov & Dobrovolskij, 2003; Born-Torrijos et al. 2017). Particular molecular sensors are believed to exist in the miracidium since evidences of discriminatory chemotaxis are reported when the larvae occur in the proximity of a suitable host, through the gradient of biochemical factors segregated by the snail (Wilson & Denison, 1970a, b). Chemical attraction of *F. hepatica* and *Trichobilhariza ocellata* miracidia by species-specific mucin-type glycoproteins released by *Galba truncatula* and *Lymnaea stagnalis* mediates host-finding in these snail-parasite systems (Kalbe et al. 1997; 2000).

In addition, epithelium features, particularly the mucus, has been pointed as a mediator of snail-digenean compatibility when contact is established. The visco-elastic properties of mucus may act as an adhesive, providing an initial physical trap and preventing colonization by pathogens (Vieira et al. 2004). Also, its biochemical nature may have anti-pathogenic activities (Ehara et al. 2002; Guo et al. 2009). Particularly, the *in vitro* effect of snail mucus on *Fascioloides magna* miracidia was assayed on two different snail species (Coyne et al. 2015). While no damage was observed when parasite larva was exposed to mucus from *Lymnaea elodes* (compatible host), significant tegumental damage and/or larval death was recorded on miracidia when tested against the mucus from an incompatible snail species (*Helisoma trivolvis*) and related with the presence of a cytotoxic protein-like factor (Coyne et al. 2015).

**The battle – parasite’s breaking through host defenses:** Penetration of a digenean parasite into its snail host is accompanied by the shedding of a highly antigenic components

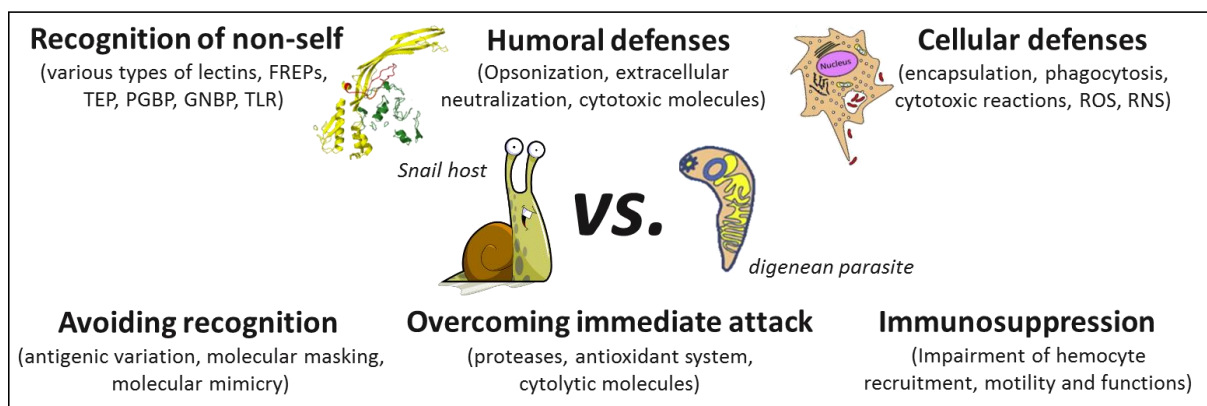
of its surface, such as the miracidial ciliated plates, that trigger immune response. Mollusks, as all invertebrates, rely only on innate mechanisms to defend themselves but it is noteworthy that their immune strategies are far from being simple or non-effective (Loker et al. 2004). Three basic components from the snail host's immunity are included in the multistep infection processes that determines the outcome of any host – parasite interaction (see Figure 6 for some examples): (1) physical, chemical and biological barriers (*e.g.* shell, mucus, microbiota), which impose the first challenge to overcome for any given pathogen, (2) elements to discriminate self from dangerous non-self (*e.g.* pattern recognition receptors, PRR) and (3) an arsenal of cellular and humoral effectors (*e.g.* hemocytes, reactive oxygen and nitrogen species (ROS, RNS), opsonins, proteases) and defense mechanisms which are triggered to actively control the injury. The communication and integration between the different immune pathways allow orchestrating an effective and multifactorial response towards the trematode (Pila et al. 2017), while evidences of specificity and innate immune memory within snail's immune system also exist (Sire et al. 1998; Portela et al. 2013; Pinaud et al. 2016).



**Figure 6.** Examples of some of the components of mollusk defense system. A) Visco-elastic slime secreted by a land snail. B) Pearls, which are formed by bivalves and some gastropods as mechanism

to localize and isolate an injury. C) Multi-layer hemocyte (immune cells)-formed capsule surrounding a dead sporocyst 48 h after parasite penetration into its freshwater snail host (Coustau et al. 2015).

Thus, for an infection to be established, a digenean trematode must circumvent the defense response of the snail to afterwards, multiply inside the host, taking advantage from host resources. Even when the specific molecules and mechanisms may vary in each host-parasite system, survival of digeneans in snails depends, at least in part, on the ability of the formers to evade host recognition, to overcome the attack of host immune cells (hemocytes) and of the molecular effectors and/or to induce long-term down-regulation of immune functions (Figure 7).



**Figure 7.** Overview of the arsenal of defensive strategies of the snail host (on top) and of the evasion mechanisms of the digenean parasite (on the bottom) mainly described from *Biomphalaria glabrata* – *Schistosoma mansoni* system. FREPS: fibrinogen-related proteins, TEP: thioester-containing protein, PGBP: peptidoglycan recognition protein, GGBP:  $\beta$ -glucan binding proteins.

*Recognition vs. avoiding recognition* - The mollusk's arsenal of discovered recognition elements includes a variety of molecules and domains that are present all along the animal kingdom; e.g.  $\beta$ -glucan binding proteins and peptidoglycan recognition proteins (Zhang et al. 2007), thioester-containing protein (TEP; Moné et al. 2010); scavenger receptors (Liu et al. 2011), different types of lectins (Dheilly et al. 2015) and molecules that feature one or two immunoglobulin superfamily domains associated with different C-terminal domains like fibrinogen domain (FREPs; Adema et al. 1997). Since these molecules participate in the interaction with the different pathogens, it is not surprising that avoiding host recognition is one of the main tactics of parasites.

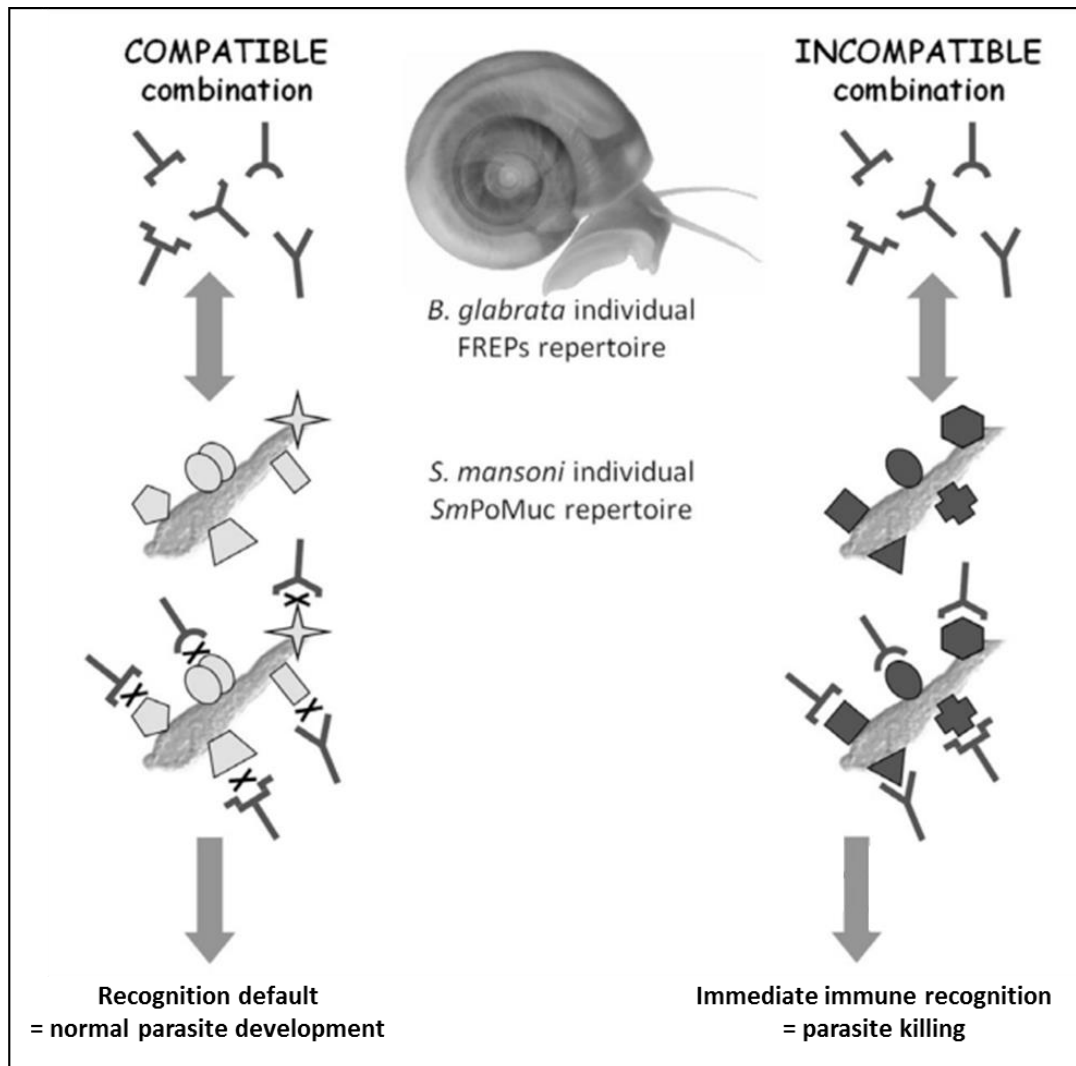
Model analyses using a genetically explicit model reveal that polymorphism is much greater at recognition loci than at the effector loci, and that recognition loci are better candidates for genomic hotspots of coevolution (Nuismer & Dybdahl, 2016). Particularly, infection and

development of digenean within the snails implies the variation of behavioral, morphological, metabolic and, ultimately, antigenic traits during the miracidia-sporocyst-(redia)-cercaria metamorphosis. In this sense, epitopes present in one phase can be lost or become underrepresented in another, as it has been suggested for *F. hepatica* (Alba et al. 2015).

Antigenic variation in *S. mansoni* particularly regarding the high level of polymorphism of its mucins (*SmPoMuc*; Roger et al. 2008), might be an example of the latter. *SmPoMuc* are coded by a multigene family that frequently recombine, transcribed in an individual-specific manner, to afterward suffer different post-translational modifications, that impact also in the glycosylation pattern of each resulting protein (Roger et al. 2008). According to these authors, this “controlled chaos” occurring in *S. mansoni* efficiently generates a high intra and inter parasite strain antigenic diversity. However, co-evolutionary dynamics of host-parasite interaction could have possibly helped in selecting a high polymorphism of *B. glabrata* FREPs (particularly FREP2, FREP3 and FREP12) which are relevant in the recognition and defense against *S. mansoni* and other digeneans (Hanington et al. 2010; Moné et al. 2010). These molecules are highly polymorphic at the genome level and can be further diversified by different mechanisms (*e.g.* alternative splicing and processed retrosequences [Zhang & Loker, 2003], exon loss, gene conversion and point mutation [Zhang et al. 2004]). This increases the range of germline-encoded immune recognition of each *B. glabrata* (and of the population) distinct FREP repertoires in an anticipatory (but not adaptive) manner that can counter antigenic-variable parasites (Adema, 2015; Mitta et al. 2012; Portet et al. 2017; see Figure 8).

Moreover, molecular masking (*i.e.* parasites acquire host epitopes during the infection) and molecular mimicry (*i.e.* antigens produced by the parasite present shared epitopes with those from the host) have been suggested to occur in the *S. mansoni* – *B. glabrata* system as survival strategies of the parasite (Yoshino & Bayne, 1983; Bayne et al. 1986). Molecular mimicry in *S. mansoni* has been further studied and tightly related to glycan determinants present in miracidia and sporocyst that are shared by the host (Lehr et al. 2008; Yoshino et al. 2013). In *F. hepatica* – Lymnaeidae, cross-reactivity with snail antigens of anti-rediae polyclonal antibodies raised in mouse and rabbit are suggestive evidences of the existence of

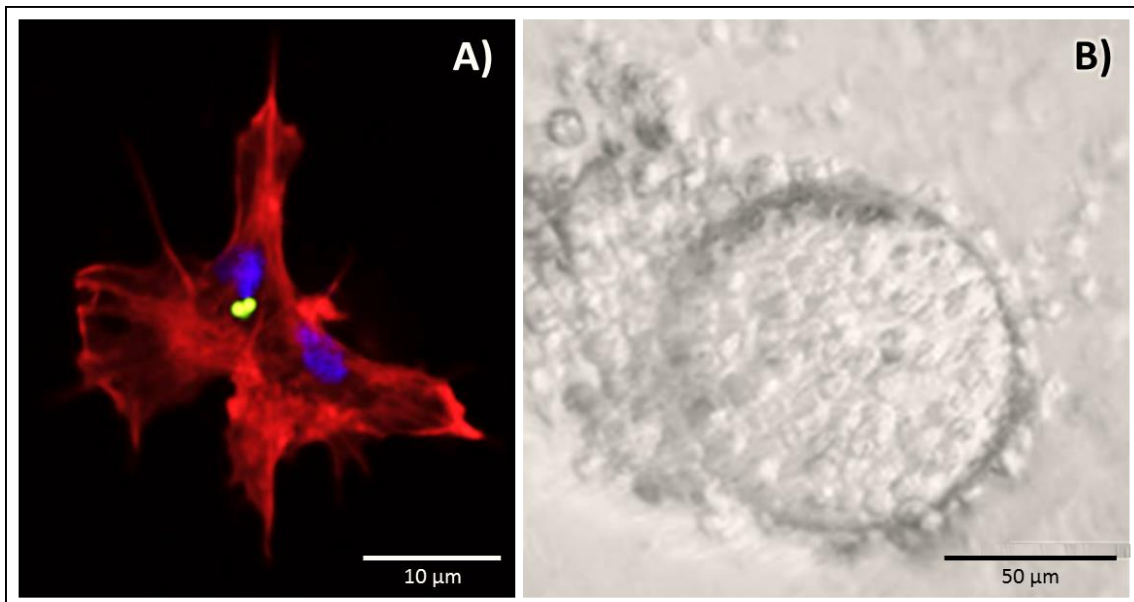
some immunogenic determinants of the parasite, mainly associated with low molecular weight proteins, similar to those of the host (Alba et al. 2014).



**Figure 8.** Representation of polymorphism of antigen leading to the compatibility/incompatibility hypothesis in *Schistosoma mansoni*/*Biomphalaria glabrata* interactions in which only recognized parasite (matching antigen – receptor; incompatible combination) are killed (adapted from Mitta et al. 2012).

*Defenses vs. counter defenses* - It is accepted that parasite encapsulation by snail's hemocytes is a protective defense response that involves (i) the isolation of the parasite from host signals and nutrients, and (ii) a secure and controlled environment for the hemocytes to release cytotoxic and humoral effectors that will help destroying the parasite (Loker et al. 1982; Gutiérrez et al. 2003a; Coustau et al. 2015). Being the leading actors of the cellular response, hemocytes functions go beyond surveillance, phagocytosis of small elements (Ataev et al. 2016; Cueto et al. 2015; Figure 9a) and encapsulation process (Gutiérrez et al. 2003a; Figure 9b) to cytotoxic reactions (Humphries & Yoshino, 2008) and to

the production of a plethora of molecules involved in signal transduction, defense regulation or effector capacities (Baeza-García et al. 2010; Galinier et al. 2013; Pila et al. 2016a). It is generally believed that they differentiate into different subsets that specialize in exerting more efficiently some particular functions (Pila et al. 2016b, see box 2 for details on hemocyte morphotypes). The generation and regulation of ROS and RNS is similar to the respiratory burst observed in vertebrate macrophages and dependent of NADPH-oxidase (Adema et al. 1994). During parasite encapsulation, cytotoxic reactions of hemocytes are triggered and involve the production of toxic ROS (Hahn et al. 2001a; Humphries & Yoshino, 2008) and RNS (Hahn et al. 2001b). The production of H<sub>2</sub>O<sub>2</sub> and NO is particularly important for *S. mansoni* killing by *B. glabrata* hemocytes (Hahn et al. 2001a;b; Moné et al. 2011).



**Figure 9.** A) Phagocytosis of fluorescently-labelled latex beads (in green) by hemocytes of *Pomacea canaliculata* (nuclei appear in blue and actin filaments of the cytoplasm in red; Cueto et al. 2013). B) Encapsulation of Sephadex beads by hemocytes of *Lymnaea stagnalis* (Kryukova et al. 2014).

Additionally, the role of plasma (cell-free hemolymph) in digenean – snail compatibility has proved crucial since significant damage to sporocyst tegument and diminishing of its survival can be seen after parasite exposure to plasma from incompatible snail host (Sapp & Loker, 2000). Besides its physical and chemical properties, the pattern of soluble molecules within snail's internal fluids can drive substantial effects on digenean survival. Several humoral effectors have been identified in gastropods e.g. PRR (Wu et al. 2017), opsonins and agglutinins (Sanchez et al. 2006; Hanington et al. 2010), antimicrobial proteins (Guillou et al. 2007; Lopez-Abarrategui et al. 2012) and cytotoxins (Galinier et al. 2013). The discovery of

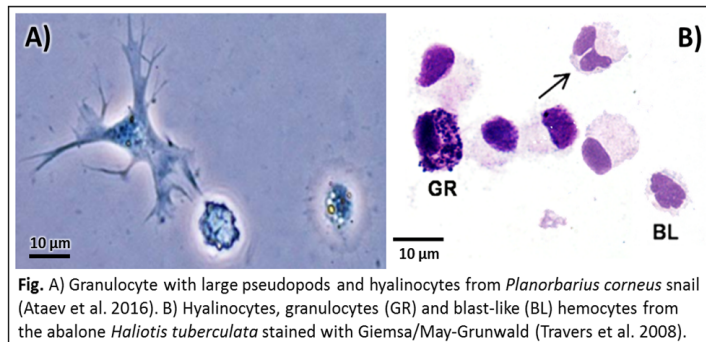
biomphalysin, a  $\beta$ -pore forming toxin produced by *B. glabrata* hemocytes, provides the first functional description of a humoral effector that binds to *S. mansoni* sporocyst and mediates parasite killing (Galinier et al. 2013).

**BOX 2 – Hemocytes: the “vedettes” of the cellular immune response in mollusks**

Investigations aiming at discriminating hemocytes comes mainly from bivalves and less from gastropod and cephalopod species. Without no specific molecular markers to deeply discriminate among cell lineages or maturation stages, attempts have been made to segregate hemocytes mainly based on morphological, cytochemical, and functional characteristics (Pila et al. 2016b). Key features for actual classification of hemocytes include their spreading and adherent capabilities, the presence/quantity and type of cytoplasmic granules, and phagocytic activity.

Concerning spreading capabilities, two main types of cells can be described: (1) cells that typically spread, forming filopodia or lamellopodia in the process, are believed to be the mature effectors in phagocytosis and encapsulation processes and constitute the majority (if not all) of hemocytes population, and (2) cells that do not spread or spread minimally when placed on artificial surfaces, often called as round or blast-like cells (Loker, 2010). Whether they represent different subtypes of cells or different developmental stages of a single type of hemocyte, morphological differences observed in these cells have been associated with certain functional differences (Matricon-Gondran & Letocart, 1999; Travers et al. 2008).

On the one hand, spreading cells can be further classified into granulocytes or hyalinocytes accounting for the number and conspicuity of cytoplasmic granules, and their proportion and particular features vary depending on the species (Pila et al. 2016b). On the other hand, round/blast-like cells are small, agranular hemocytes with high nucleus/cytoplasm ratio (Loker, 2010). This subset is mainly described from bivalves and is frequently referred as pro-hemocytes with certain stem-cell capacity (Wang et al. 2012; Rebelo et al. 2013).



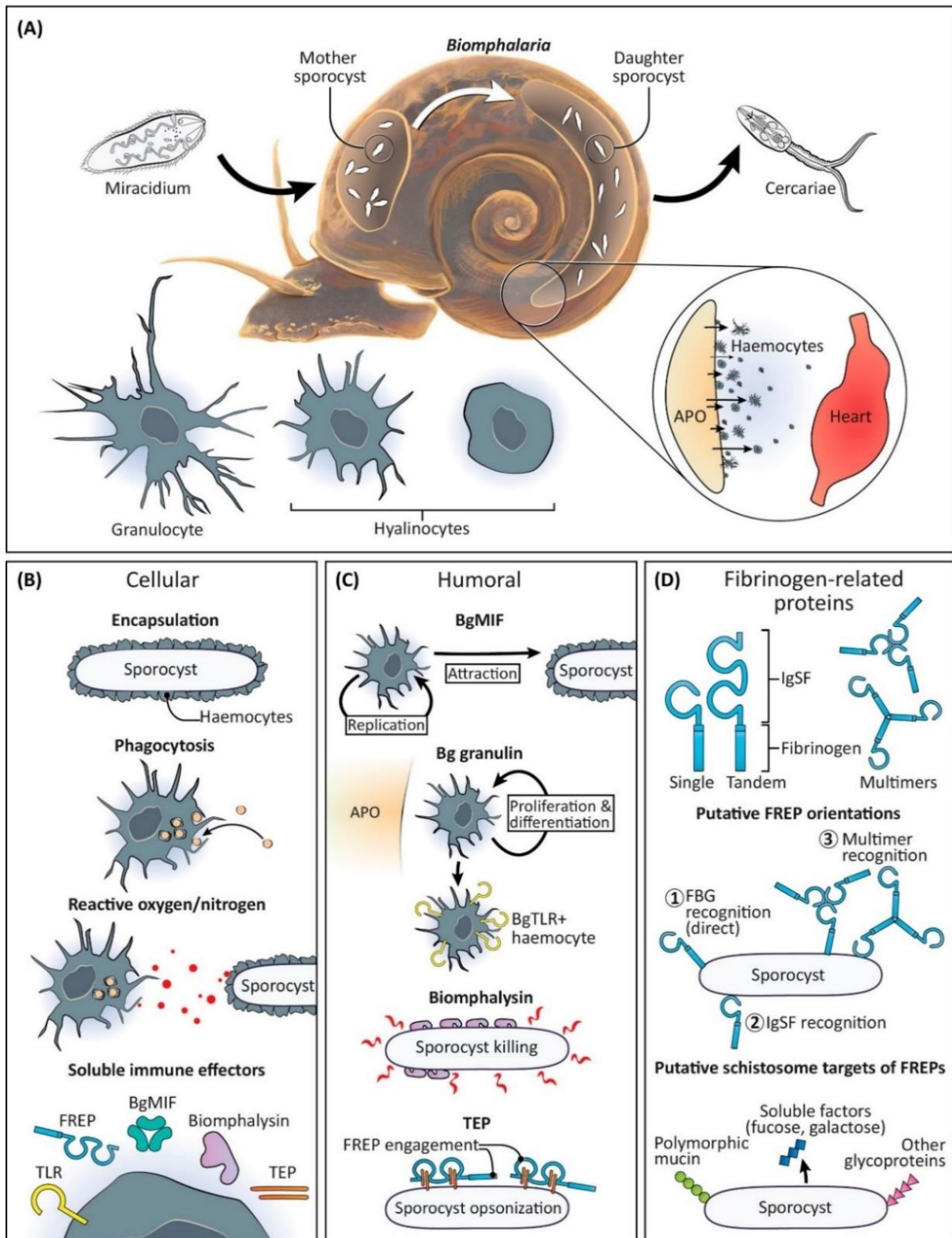
**Fig.** A) Granulocyte with large pseudopods and hyalinocytes from *Planorbarius corneus* snail (Ataev et al. 2016). B) Hyalinocytes, granulocytes (GR) and blast-like (BL) hemocytes from the abalone *Haliotis tuberculata* stained with Giemsa/May-Grunwald (Travers et al. 2008).

In this sense, digenean trematodes possess an arsenal of proteases and antioxidant molecules that could help to overcome the humoral effectors and the hemocyte driven-cytotoxic effects of snail hosts' defenses (Zelck & Von Janowsky, 2004; Gourbal et al. 2008; Wu et al. 2009). Schistosomes produce important quantities of proteins that can scavenge ROS, which is possibly a strategy to avoid the oxidative damage that is known to be crucial for hemocyte-mediated killing of parasite larvae (Guillou et al. 2007; Mourão et al. 2009). Additionally, direct immunomodulation also figures in the list of parasite countermeasures to snail immune system. Excretory–secretory products (ESPs) from *S. mansoni* decrease the generation of NO (Zahoor et al. 2009) and superoxide (Connors et al. 1991) from *B. glabrata* hemocytes cultures, as well as its motility (Lodes & Yoshino, 1990). ESPs from *E. paraensei* sporocyst interfered with the functions (Loker et al. 1992) and spreading of *B. glabrata* hemocytes *in vitro* which reflect observed patterns in age- and strain-related susceptibility (DeGaffé & Loker, 1998).

Other examples of immunomodulation within snail-trematode interactions can be cited. Hemocytes of *Trichobilharzia regenti*-infected *Radix lagotis* increase in number and accumulate nearby the parasite after penetration but, interestingly, they do not destroy it (Skála et al. 2014). In the same study, phagocytic activity and H<sub>2</sub>O<sub>2</sub> production of these cells were found diminished compared to those from non-infected snails, showing also lower levels of phosphorylation (*i.e.* activation) of protein kinase C and of extracellular signal-regulated kinase (ERK; Skála et al. 2014). Exposure to *Plagiorchis mutationis* causes a severe immunosuppression of the cellular immune response in *L. stagnalis*. Counts of circulating hemocytes were two-fold less in *P. mutationis* – infected snails compared to *in vitro* assays where these cells showed lower spreading, phagocytic and encapsulation capacities (Kryukova et al. 2014). Suppression of hemocytes' phagocytic ability *in vitro* by the digeneans *Himasthla elongata* and *Cryptocotyle lingua* (and other related species) ESPs was reported on blood cells derived from the snail host *L. littorea* but is absent in bivalve species (Iakovleva et al. 2006). Hemocytes from *H. elongata* naturally-infected snails also showed lower phagocytic activity compared with cells from healthy individuals. Such inhibitory effect was found to be related with high molecular weight proteins from parasite's ESPs (Iakovleva et al. 2006).

***The outcome – the frontier between successful or failed parasite establishment:*** As presented here, there is a variety of factors contributing to the outcome of each host-parasite encounter, ranging from encounter filters to compatibility filters (Combes, 2001). Particularly, unless the genetic profiles of the mollusk and the trematode are concordant in some critical attributes, the parasite is killed by the host (Bayne et al. 2001; King et al. 2011; Mitta et al. 2012). Figure 10 (from a recent paper of Pila et al. 2017), summarizes the current knowledge concerning how *B. glabrata* snails successfully fight back *S. mansoni* infection, in an integrative and coordinated manner, when incompatible combinations occurred.



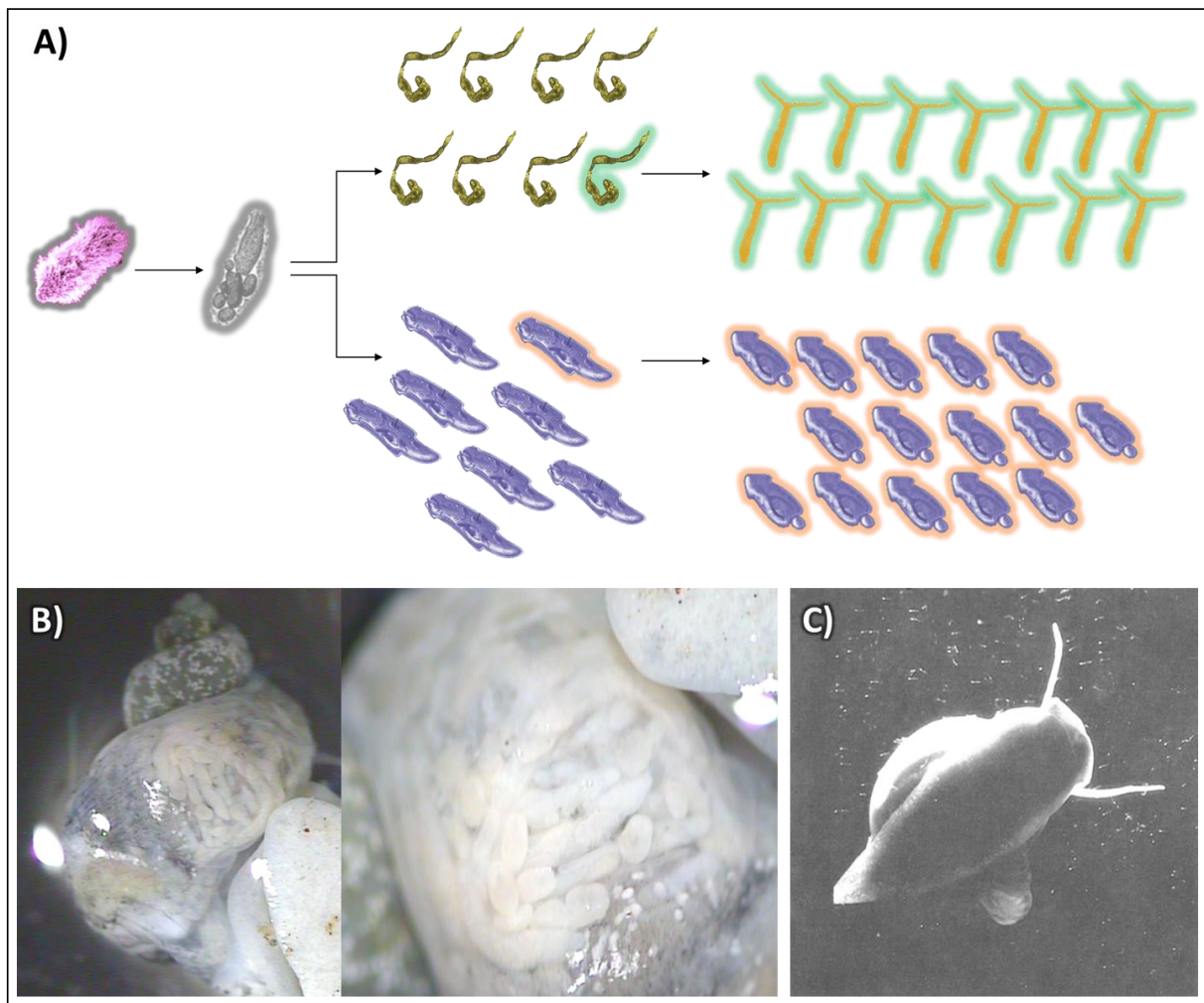


**Figure 10.** Integrative model of the immune response in *Biomphalaria glabrata* snails toward the digenean *Schistosoma mansoni* from Pila et al. (2017). A) Parasite-specific antigens stimulate hematopoiesis in the amoebocyte-producing organ (APO). B) Encapsulation and phagocytosis lead to parasite clearance in which the activation or production of cytotoxic compounds by host hemocytes and involvement of soluble immune effectors is essential. C) Humoral response includes the stimulation of hemocyte activation and proliferation by macrophage migration inhibitory factor (MIF)

and granulin cytokines, the production of soluble cytotoxic proteins (*i.e.* biomphalysin) and parasite opsonization by immune complexes of fibrinogen-related proteins (FREPs) - thioester containing protein (TEP). D) Antigenic determinants secreted by the parasite or exposed on its surface are recognized by host receptors in compatible host-parasite combinations. These targets may be directly recognized by single or tandem-immunoglobulin superfamily (IgSF) FREPs, which can interact with each other to form multimers (antigen recognition may be dependent or enhanced by multimer formation) either through their immunoglobulin superfamily domain (IgSF) or through their fibrinogen (FBG) domains.

Even when much remains to be discovered from *B. glabrata* – *S. mansoni* interaction and other snail-trematode systems need to be deeply studied, the existing data endorse the occurrence of a complex network of immune factors, from cellular to humoral defenses, that acts coordinately against the infection. In this sense, while phagocytosis also contributes to eliminate the debris derived from parasite infection (Loker et al. 1982), lectins and humoral effectors such as ROS, NO, opsonins and cytotoxins offer crucial assistance for parasite elimination (Hahn et al., 2001a,b; Moné et al. 2011; Hanington et al. 2010; Galinier et al. 2013). This is regulated by complex signaling cascades that control the activation and induction of certain elements and the direct action of chemokines and cytokines *e.g.* macrophage migration inhibitory factor (MIF) and granulin (Baeza-García et al. 2010; Pila et al. 2016a).

The dependence, for a digenean trematode, of its molluscan host together with the fact that they either infect or die in the attempt pose a strong selection against those parasites unable to infect the local host genotypes (King et al. 2011). Parasites, in general, present high population numbers, short generational times and higher dispersal capabilities than their hosts, which could be additional advantages in the evolutionary “arms race” to keep adapting to the hosts. In compatible host-parasite combinations, the successful infection of the primary snail host by a digenean, once established, concomitantly leads to the occurrence of the asexual reproduction of the parasite and the continuity of its life cycle. This allows a significant amplification of the next generation of infective stage, the cercariae (see Figure 11), and facilitates further transmission of the parasite to subsequent hosts.



**Figure 11.** A) Schematic representation of the asexual multiplication of digenean parasites that takes place within the primary snail host. A single miracidium transforms into sporocyst and (according to the trematode species) gives place to numbers of daughter sporocysts (on the top) or rediae (on the bottom), each one eventually producing tens of cercariae. B) Zoom of a mantle cavity of a lymnaeid snail prior cercarial shedding where a number of redial larva of *Fasciola hepatica* can be observed (Photo: Laboratory of Malacology, IPK). C) Shedding of tens of cercaria (small white dots surrounding the snails) from and infected snail.

To support their development, parasites profit from host resources while the degree of damage caused by the parasite is kept in check by the requirement that the host survive long enough until the time comes for the larva to leave the snail (Sorensen & Minchella, 1998). Such energy constraints during infection affects host life history traits, as they also attempt to use the same limited resources to maximize their own growth, reproduction and survival. At the light of host-parasite interaction, host's traits must be interpreted in terms of the life history strategies of both the parasite and the host (Sorensen & Minchella, 2001). Examples involving infection-associated effects that somehow contributes to reproduction and transmission of the parasite e.g. modulation of stress and neuroendocrine metabolism (Hoek

et al. 2005; Knight et al. 2016), behavioral changes (Wesołowska & Wesołowski, 2013), dramatic host castration (Joosse & Van Elk, 1986; Faro et al. 2013) and gigantism (Joosse & Van Elk, 1986; Sorensen & Minchella, 1998) or growth stunting (Krist & Lively, 1998; Muñoz-Antoli et al. 2007), are reported from digenean infections. From the abovementioned, fecundity reduction or total parasitic castration of the host is one of the most frequently reported effects from a number of different trematode-snail systems (see Sorensen & Minchella, 2001 for review).

Reduction in the reproductive activity of the host could be directly linked to the redirection of limited host resources to better support infection (Schallig et al. 1991; Wang et al. 2017) or a direct result of biochemical, histopathological and/or mechanical damage caused by parasite development in snail reproductive organs (Wilson & Denison, 1980; Faro et al. 2013). Regarding the former, there is debate concerning whether parasite-mediated fecundity reduction reflects “strategy” directed by hosts or parasites. However, parasitic castrators that actively shift host resources away from reproduction can catalyze another phenomenon: enhanced growth of infected snails (Sorensen & Minchella, 2001; Hall et al. 2007). Thus, it is not surprising that examples of both, a significant decrease of reproductive traits and an increased shell size, in parasitized snails has been observed in *Trichobilharzia ocellata* – *L. stagnalis* (Schallig et al. 1991), *Echinostoma revolutum* – *Lymnaea elodes* (Sorensen & Minchella, 1998), *Echinostoma friedi* – *B. glabrata* (Muñoz-Antoli et al. 2007), *Schistosoma mansoni* – *B. glabrata* (Faro et al. 2013; see Hall et al. 2007 for other examples). Significantly, evidence of gigantism in digenean-infected mollusk has been observed both in field and experimental studies, mostly related to freshwater snails (see Sorensen & Minchella, 2001 for review). Nevertheless, as in other cases, the final effect of a digenean infection (thus its transmissibility) on host traits is a conjunction of biological (including host-parasite compatibility) and environmental factors. Parasitic infection resulting on increase growth of the molluscan host after the cessation of reproduction is assumed to be more frequent in snails occurring in less stable habitats (or in singly housed experimentally-reared snails) since a reallocation from reproduction to growth rather than to maintenance is more probable in these populations (Sorensen & Minchella, 2001).

#### **IV. Comments on ecological and environmental features affecting trematode transmission**

As it has been already mentioned, host-parasite relationships cannot be separated from natural or anthropic changes, so different environments in which hosts and parasites develop, strongly affect specificity and genotype selection (Wolinska & King, 2009). Factors such as probability of encounter and of infection and the probability of transmission are tightly linked to the ecological requirements and niches of parasites and its hosts (Sandland & Minchella, 2003). Detwiler & Minchella (2009) studied the relation between host availability and host compatibility for *E. revolutum* and *Echinoparyphium* sp. They found that while parasite colonization was always stronger in *H. trivolvis* under experimental conditions, the patterns of infection in nature fluctuated in different snail species. Infection was predominantly driven by the probability of encounter in relation to the abundances of the potential host species, rather than been primarily guided by compatibility (Detwiler & Minchella, 2009). Even when high levels of parasite exposure do not necessary lead to high frequencies of patent (cercaria shedding) infections in the field (Allan et al. 2013), the probabilistic and multifactorial character of field transmission determines an increased chance for a parasite population to find compatible hosts if host abundances and exposure to parasite are high. In this sense, high host density and aggregation enhance the probability of encounter between snails and parasites increasing the chances of transmission. However, in the case of egg-transmitted digenean (those that infect the snail upon egg ingestion) this is accompanied by a decrease of per-snail infection risk within the host population, particularly if low prevalence in the definitive host exists. For active miracidium-transmission parasite recruitment and infection risk increase with miracidial input (Buck et al. 2017).

Even in compatible snail-digenean systems, changes in environmental and ecological variables (*e.g.* temperature, droughts, pollution, other infections) might significantly affect the outcome of parasite exposure through the variation of immunological and/or life history traits in the host (Sandland & Minchella, 2003; Seppälä & Jokela, 2010; Abrous et al. 2001; Ittiprasert & Knight, 2012; Leicht et al. 2013; Halstead et al. 2018) or by affecting crucial features of parasite's infectivity and/or survival (Morley & Lewis, 2015; Ford et al. 1998; Echaubard et al. 2017). Variation in key pathogen life-history features (*e.g.* mode of

reproduction, dispersal capacity, life cycle complexity) also drives epidemiological and genetic dynamics within and between pathogen populations (Barret et al. 2008).

## **V. What can the study of snail – trematode interaction brings to the control of disease transmission?**

*“(...) “Co-evolution of snails and trematodes is becoming increasingly apparent at both cellular and population levels; the implications of which are only beginning to be understood for disease control. Untangling the complex interactions of trematodes and snails promise fresh opportunities for intervention to relieve the burden of parasitic disease.”*

***Lockyer et al. 2004. Canadian J Zool. 82: 251-259.***

Up to this chapter, the relevance of snail – digenean relationship was explored from evolutionary, biological and immunological perspectives. The relevance of such ancient interaction could serve as a model to understand commitment of internal defense systems to symbionts or to explore the impact of orchestrating immune responses on life history traits, and to study the interface between immunity and behavior (Loker, 2010). However, since some digenean parasites cause significant diseases in humans and domestic animals, the study of the intermediate host snails and of their particular interaction may have a great impact on veterinary and human health sciences.

Given that it is mandatory for digenean parasites to develop inside the snails, controlling the populations of the latter appears to be a suitable strategy to control snail-borne parasites (Alba et al. 2016). Their complex life cycle and the tight relation with their transmitting-mollusk determine that for properly understanding, predicting and acting, in an integrative manner, on the epidemiological patterns of digeneans' transmission a thorough look into snail – parasite interaction is mandatory (Adema et al. 2012). The deep study of the immunobiological features of each particular digenean – mollusk system could allow to identify molecules with potential biomedical relevance, and could also serve as model to study the impact of pollutants or other stressors on immune function, to monitor climate change and record its impacts, or to ultimately predicts the effects on parasite transmission (Loker, 2010). In spite of the variety of models to study, the exciting investigations and discoveries that lay ahead, the applicability of such knowledge in different disciplines and their usefulness for science and society, scientist working on snails and the diseases they transmit are scarce (Adema et al. 2012). However, questions like “*what determines the*

*population structure and geographical distribution of snails that define endemic areas for parasite transmission, and how will global warming affect these? To what extent is the number of infected snails dictated by immuno-compatibility between parasite and snail versus the ecological factors that limit infections? (...). For how long infected snails continue to shed cercariae, and what is the number of cercariae produced per snail?” How “(...) immunity and virulence have evolved to influence the snail–parasite compatibility”? or which are the genetic markers for such compatibility or the regulators of parasite transmission in natural snail populations? These issues are undoubtedly crucial to move securely towards the control of human trematodosis and can only be answered through the study of snail-digenean interactions (Adema et al. 2012).*

## **VI. Snail-borne trematodosis: the example of fasciolosis**

*“(...) trematode infections are among the most neglected tropical diseases. Scarce attention is given to them by ministries of health or indeed the medical community. Their difficult names, complex life-cycles and specific geographical distribution simply add to their anonymity.”*

**WHO, 2007. Newsletter; 10.**

Snail-transmitted diseases affect around 300 million disadvantaged people worldwide (Adema et al. 2012). Considering the diversity of Digenea, only a few species are significant human parasites and yet they account for a considerable impact on public health, mainly in low-income countries (see Table 1). However, even when a substantial number of the human world population lives at risk of snail-borne trematodosis, these infections figure in the list of the neglected tropical diseases, most are not included within the priorities of the World Health Organization and received scarce attention and funding (Toledo et al. 2012; WHO, 2014). Table 1 summarizes some important aspects of the major species parasitizing humans: the blood flukes (*e.g. S. mansoni, S. haematobium, Schistosoma japonicum*), in which the human-infective larvae penetrate through the skin, and the large group of the food-borne trematodes: the liver flukes (*C. sinensis, Fasciola gigantica, F. hepatica, Opisthorchis felinus, and O. viverrini*), the lung flukes from the genus *Paragonimus*, and the intestinal flukes (*e.g. Fasciolopsis buski*, and the echinostomes and heterophyids). This list is far from be complete. For example, a total of 70 species (14 families and 36 genera) of intestinal flukes have been isolated from humans (Chai, 2007).

**Table 1.** Geographic distribution, vector snails, infection sources and at-risk populations of the major snail-borne trematodes that affect humans.

Trematode	Geographic distribution	Main primary snail host (Family: Genus)	Common infection source	People at risk (x10 <sup>6</sup> )*	Reference
<i>Clonorchis sinensis</i>	Eastern Asia	<b>Assimineidae:</b> <i>Assimineae</i> <b>Bithyniidae:</b> <i>Parafossarulus</i> , <i>Bithynia</i> <b>Hydrobiidae:</b> <i>Allocinma</i> <b>Thiaridae:</b> <i>Melanoides</i> <b>Semisulcospiridae:</b> <i>Semisulcospira</i>	Freshwater fishes	601	Hung et al. 2013
<b>Echinostomes</b>	Mostly in Asia	<b>Lymnaeidae</b> <b>Planorbidae</b> <b>Physidae</b> <b>Pomatiopsidae</b> <b>Viviparidae</b> <b>Pilidae</b>	Freshwater fishes, amphibians, mollusks, snakes	Not known	
<i>Fasciola hepatica</i>	All continents except Antarctica	<b>Lymnaeidae:</b> <i>Galba</i> , <i>Lymnaea</i> , <i>Pseudosuccinea</i> , <i>Austropeplea</i> , <i>Omphiscola</i>	Freshwater aquatic plants, water	180	Mas-Coma et al. 2009a
<i>Fasciola gigantica</i>	Tropical and subtropical Africa and Asia	<b>Lymnaeidae:</b> <i>Radix</i> , <i>Galba</i>	contaminated with metacercariae	Not known	
<i>Fasciolopsis buski</i>	Eastern Asia	<b>Planorbidae:</b> <i>Segmentina</i> , <i>Hippeutis</i>			
<i>Heterophyes heterophyes</i>	East and South Asia, North Africa	<b>Potamididae:</b> <i>Cerithideopsisilla</i> , <i>Cerithidea</i> , <i>Tympanotonos</i>	Fresh/brackish water fishes	Not known	Sripa et al. 2010
<i>Metagonimus yokogawai</i>	East and South Asia, Spain	<b>Semisulcospiridae:</b> <i>Semisulcospira</i>		Not known	
<i>Opisthorchis felineus</i>	Central and Western Europe	<b>Bithyniidae:</b> <i>Bithynia</i>	Freshwater fishes	80	
<i>Opisthorchis viverrini</i>	Southeastern Asia				
<i>Paragonimus</i> spp.	South and North America, East Asia, West Africa	<b>Assimineidae</b> <b>Bithyniidae</b> <b>Cochliopidae</b> <b>Hydrobiidae</b> <b>Semisulcospiridae</b> <b>Pomatiopsidae</b> <b>Thiaridae</b>	Freshwater crustacean, wild boar meat	292	Hung et al. 2013
<i>Schistosoma haematobium</i>	Africa, Eastern Mediterranean	<b>Planorbidae:</b> <i>Bulinus</i>			
<i>Schistosoma japonicum</i>	Eastern Asia	<b>Pomatiopsidae:</b> <i>Oncomelania</i>	Free-swimming cercariae	800	Colley et al. 2014
<i>Schistosoma mansoni</i>	Africa, South America, Eastern Mediterranean	<b>Planorbidae:</b> <i>Biomphalaria</i> ,			

\*based on data from WHO, 2014; Keiser &amp; Utzinger, 2009; Sokolow et al. 2018

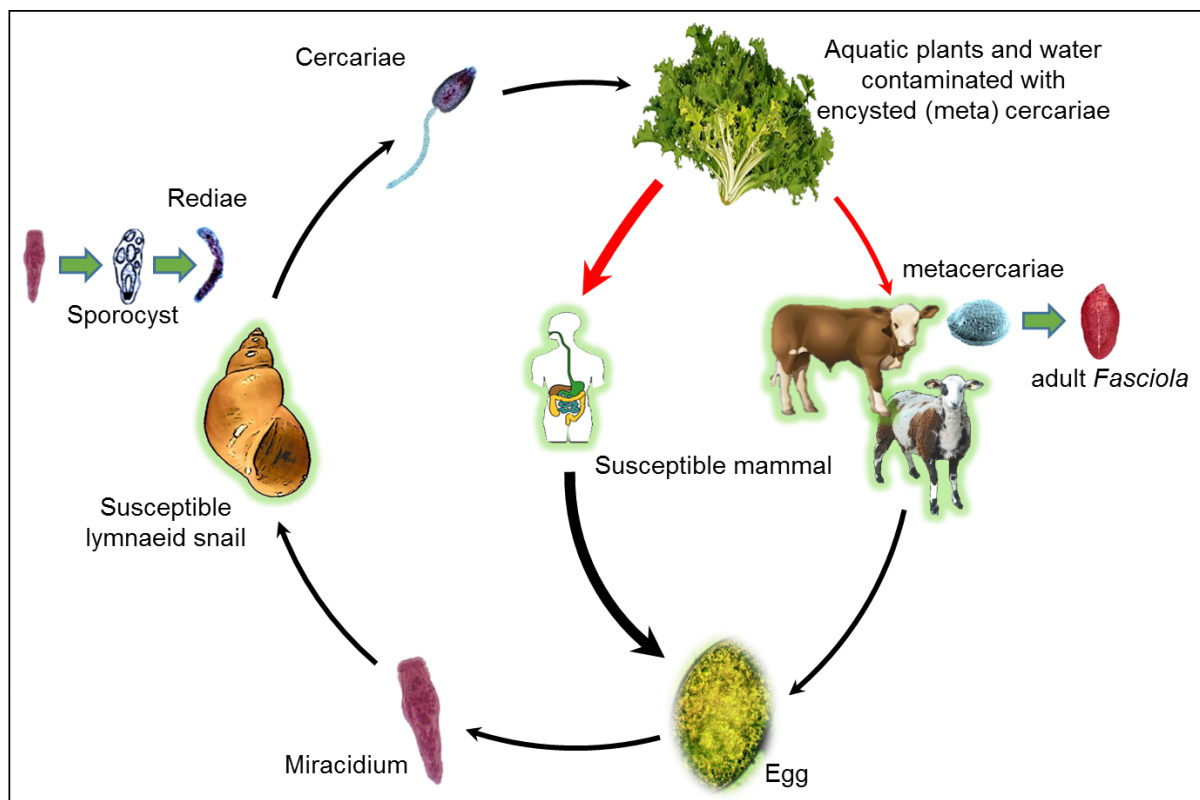


From the listed digeneans parasites, those causing fasciolosis (*Fasciola* spp.) and mainly *F. hepatica* are particularly interesting due to its wide distribution and huge impact on veterinary health and also on the economy in relation to the money losses associated to livestock infections (Khan et al. 2013). Additionally, the increased burden of human fasciolosis in barely two decades is alarming (Mas-Coma et al. 2009a). In 1992, the World Health Organization requested an update on the infection rate of human fasciolosis over a twenty-year period. From the reviewed literature, a prevalence of 2594 cases was calculated, with most cases coming from hospitalized patients from France, Spain, Cuba and Portugal (Chen, 1990). However, today estimation of human fasciolosis goes from 2.4 to 17 million infected people (WHO, 2013). Several factors such as the increased initiatives to study fasciolosis burden (WHO, 2007), the existence of better surveillance and diagnostic methods (Mas-Coma et al. 2014) and the effects of the climate change (Mas-Coma et al. 2009b), have been related to the current re-emergence. Furthermore, the high plasticity and level of success of *F. hepatica* within its snail and definitive hosts and the high worldwide risk of transmission increase the need to aim at its control.

## VII. Global impact, epidemiology, clinical aspects and etiological agents of fasciolosis

*Fasciola hepatica* and *F. gigantica* (Trematoda: Digenea: Fasciolidae), also known as liver flukes, are the main etiological agents of fasciolosis worldwide. The cosmopolitan biological features of *F. hepatica* determine a major role for this species in the global burden of the disease, while *F. gigantica* distribution is restricted to the tropical and subtropical regions of Asia and Africa (Mas-Coma et al. 2009a). Both parasites present a two-host life cycle in which more than 20 freshwater snails of the family Lymnaeidae and a large variety of mammals, including humans, can act as intermediate and definitive hosts, respectively (Mas-Coma, 2005; Figure 12). Briefly, *Fasciola* spp. are transmitted by susceptible lymnaeid snails after they get infected by a miracidium, a non-feeding parasite larva that hatches in the environment from eggs laid by adult flukes and is incubated under suitable conditions of temperature and humidity (temperature above 13°C; Andrews, 1999). The miracidium actively searches its compatible host, firstly by phototaxis and later, by following a gradient of certain species-specific molecules secreted by the snails into the snail conditioned water (Kalbe et al. 1997). Once inside the intermediate host, the miracidium – sporocyst – rediae

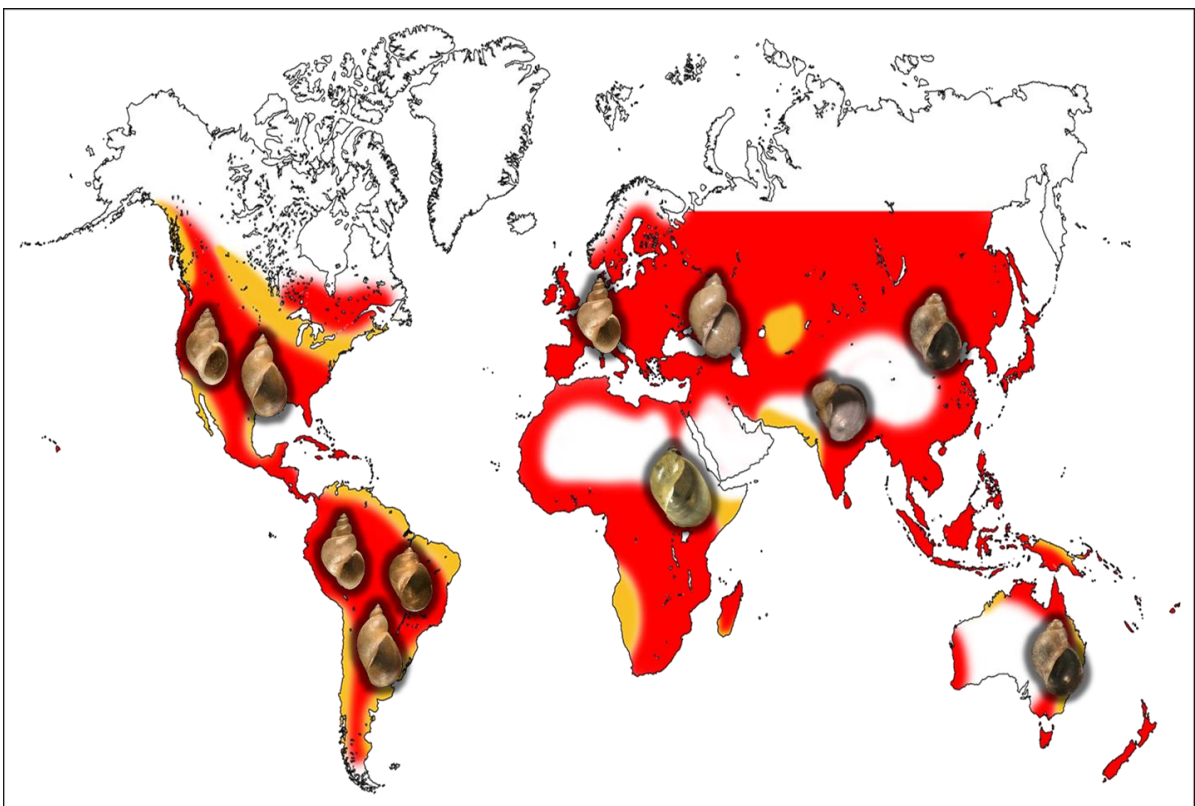
metamorphosis and the asexual reproduction and cercariae development of the parasite take place (Andrews, 1999). From 30 to 86 days post-infection, depending on the temperature and host-parasite compatibility, a tailed-cercariae actively emerges from the mollusk and encyst on aquatic or semi-aquatic vegetation or onto themselves to form the metacercariae (Mas-Coma et al. 2005; Morley, 2015). Metacercariae may last up to 1 year in suitable environmental conditions and remain viable to infect susceptible mammals after ingestion of contaminated vegetation (e.g. watercress) or water (Mas-Coma et al. 2009a). Adult flukes develop in the liver tissue and biliary ducts of its definitive hosts producing and secreting into the gall bladder thousands of eggs per day, which are ultimately expelled to the environment through the intestinal tract of the host, with the feces (Andrews, 1999).



**Figure 12.** Life cycle of *Fasciola* spp. which comprises freshwater lymnaeid snails acting as intermediate host and a variety of mammals, including humans, serving as definitive hosts.

As was previously mentioned, fasciolosis is a significant food-borne trematodosis that is globally transmitted, from temperate to tropical areas (in all continents except Antarctica), thanks to the worldwide distribution of the lymnaeid host snails and to parasite's adaptability. In fact, fasciolosis is considered to be the vector-borne disease with the widest altitudinal, latitudinal and longitudinal distribution (Mas-Coma, 2005; WHO, 2013; see Figure

13 for details). The highest prevalence of human fasciolosis and today's hot-spots have been reported in the Andean countries of Bolivia and Peru, at more than 4000 m above sea level (Esteban et al. 1997; 1999; Espinoza et al. 2010), and in the Nile delta, in Egypt (Dar et al. 2005). However, despite its increasing impact on public health, fasciolosis remains a neglected disease (WHO, 2007; 2013). On the other hand, its high prevalence in livestock (e.g. sheep, cattle, goat) results in negative effects on the health of domestic animals that leads to high economic losses due to deaths, liver condemnation, reduced milk and meat production, as well as a reduced fertility and high costs of drug therapy (Kaplan, 2001; Mezo et al. 2011).



**Figure 13.** Global distribution of fasciolosis (adapted from WHO, 2013) and some of the main lymnaeid species per geographic regions: North America – *Galba cubensis* and *Pseudosuccinea columella*; South America – *Galba truncatula*, *Galba viator*, *P. columella*; Europe – *G. truncatula*; Asia – *Stagnicola elodes*, *Austropeplea tomentosa*; Africa – *Radix natalensis*; Australia – *Austropeplea tomentosa*. Areas with confirmed transmission of *Fasciola* spp. appear in red. The zones where transmission might occur due to the presence of intermediate and definitive hosts are colored in yellow.

Since domestic animals act as major definitive host species of *F. hepatica* and *F. gigantica*, fasciolosis transmission is highly influenced by human activity (Mas-Coma et al. 2009a; Sabourin et al. 2018). However, a direct relation between high prevalence of infection in

human communities and that of their livestock is not always recorded, mainly due to differences in diet and food habits as well as in socioeconomic and sanitation conditions (Mas-Coma et al. 1999). In any case, the real number of animals and humans infected with fasciolosis in some countries is presumed to be higher than their reports considering that: a) infection can cause mild symptoms or go unnoticed for a long time, b) current diagnostic tools have sensitivity, reproducibility or cost-associated issues and c) coordinated and updated information concerning the disease is usually scarce, especially in low-income countries (Toet et al. 2014).

The disease progresses through four distinct phases in humans: 1) an initial incubation phase from the ingestion of metacercariae to the appearance of the first symptoms, which could last from a few days up to three months depending on the number of ingested parasites, and on the species of definitive host and its immunity; 2) an invasive or acute phase which may manifest with fever, malaise, abdominal pain, gastrointestinal symptoms, urticaria, anemia and jaundice; progressing to 3) a latent phase with fewer symptoms and ultimately, into 4) a chronic or obstructive phase, months to years later (Behm & Sangster, 1999; Mas-Coma et al. 2009a). In the chronic fasciolosis, inflammation of the bile ducts and gall bladder occurs and may cause gallstones and severe fibrosis of the hepatic tissue (Behm & Sangster, 1999; see Figure 14). The number and intensity of the symptoms depend on the quantity of worms, the stage of the infection and the species and immunity of the host (Mas-Coma et al. 2009a). The death rate could be significant in both sheep and cattle, but it is generally low among humans (Torgenson & Claxton, 1999).



**Figure 14.** Liver of a cow infected with *Fasciola hepatica* were gallstones, calcification and hypertrophy of hepatic ducts and severe fibrosis of the tissue characteristic of chronic fasciolosis can be observed. Also, a large quantity of worms can be seen (Photo: Alba A).

### VIII. Overview of the epidemiological scenario of fasciolosis in Cuba

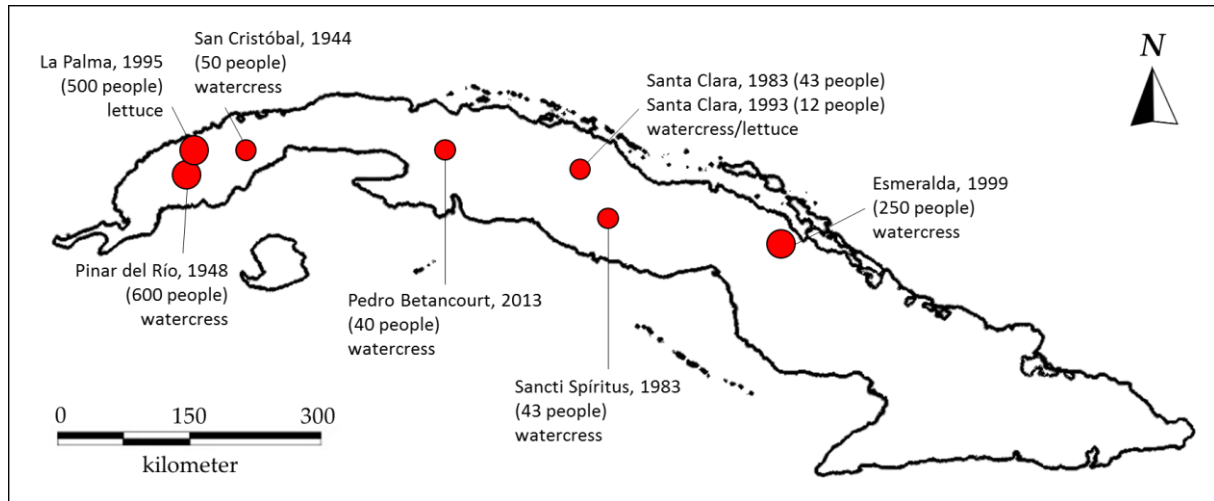
Cuba is the largest archipelago of the Caribbean basin and is located in the Americas, facing the peninsulas of Florida (at north) and Yucatan (at southwest), at the very entry of the Gulf of Mexico (for which it is known as the Key to the Gulf of Mexico; Figure 15). Essentially two main islands form the Cuban archipelago: Cuba, which is the biggest, and the Island of Youth, and also more than 4 000 islets and minor keys. Three major mountain systems occur in western, central and eastern Cuba while around 90% of the country is mainly plain and floodable lowlands suitable for livestock pasturing and for the occurrence of lymnaeid populations.



**Figure 15.** Cuba in the Caribbean basin.

The Cuban epidemiological scenario of fasciolosis, where only *F. hepatica* occurs, presents two different patterns depending on the definitive host. Human fasciolosis is characterized by reduced but repeated outbreaks (at least eight important outbreaks have been reported since the first human case in 1937) and several non-related cases are reported all year round (Rojas et al. 2010; Díaz et al. 2011; see Figure 16 for details). Conversely, it is accepted that the disease is an important veterinary problem with a hyper-endemic infection in cattle and sheep characterized by high prevalence and parasite burden (Brito et al. 2010; Rojas et al. 2010; Vázquez et al. 2016). The high number of infected animals in Cuban livestock imposes a high risk of occurrence of human fasciolosis due to elevated rates of transmission of the parasite in nature, mainly related to human activities such as cattle management (Vázquez et al. 2016; Alba et al. 2016). In addition, warm temperatures and frequent rainfall

throughout the year favor the existence of the two lymnaeid snails, *Galba cubensis* and *Pseudosuccinea columella*, both acting as intermediate hosts of *F. hepatica* in Cuba (Gutiérrez et al. 2011; Alba et al. 2016).



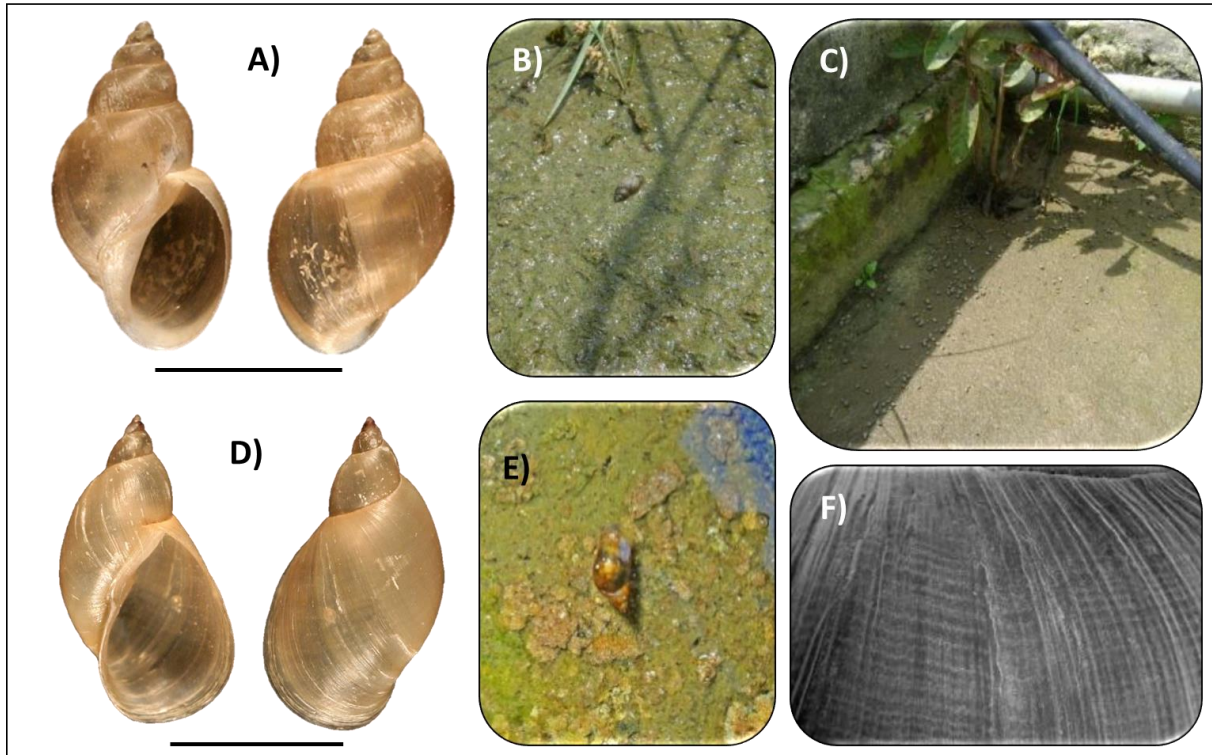
**Figure 16.** Epidemic outbreaks of human fasciolosis officially recorded. The size of the circle refers to the diagnosed cases.

### IX. Lymnaeid snails and *F. hepatica* transmission in Cuba: occurrence of naturally resistant and susceptible populations of *P. columella*

The transmission of *Fasciola* spp. is dependent on the lymnaeid hosts that occur in a particular geographical area, in which their roles as vectors are influenced by the local adaptation of the species (Dar et al. 2013; Vázquez et al. 2014). There are around 20 lymnaeid species known as intermediate hosts of fasciolosis worldwide (Correa et al. 2011). In Cuba, *F. hepatica* is transmitted by two species: *G. cubensis* (formerly known as *Fossaria cubensis*) and *P. columella* (formerly known as *Lymnaea columella*; Gutiérrez et al. 2011; Vázquez et al. 2015; Alba et al. 2016; Figure 17).

The wide distribution of *G. cubensis* all over the Cuban archipelago, its ecological features (*i.e.* amphibious snails with wide tolerance limits and habitat preference for anthropic environments) along with their strong compatibility with Cuban isolates of *F. hepatica* favor its role as main intermediate host to this parasite in Cuba (Vázquez et al. 2009; 2014; 2015). In fact, *G. cubensis* has been found naturally transmitting *F. hepatica* in Cuba in several surveys with a parasite burden of up to 76 rediae in a single snail (Vázquez et al. 2015) and prevalence of infection per transmission site of up to 34.32% (Alba et al. 2016). In the Caribbean area, as in Cuba, this species is considered the main vector of fasciolosis

(Gutiérrez et al. 2011). On the other hand, its presence in some regions of southern United States (Kaplan et al. 1997) and South America (Bargues et al. 2011; Medeiros et al. 2014, Alda et al. 2018), where it co-exists with other snail hosts leads to a shared role in *F. hepatica* transmission and therefore complicates the epidemiological scenario of fasciolosis in the continental Americas.

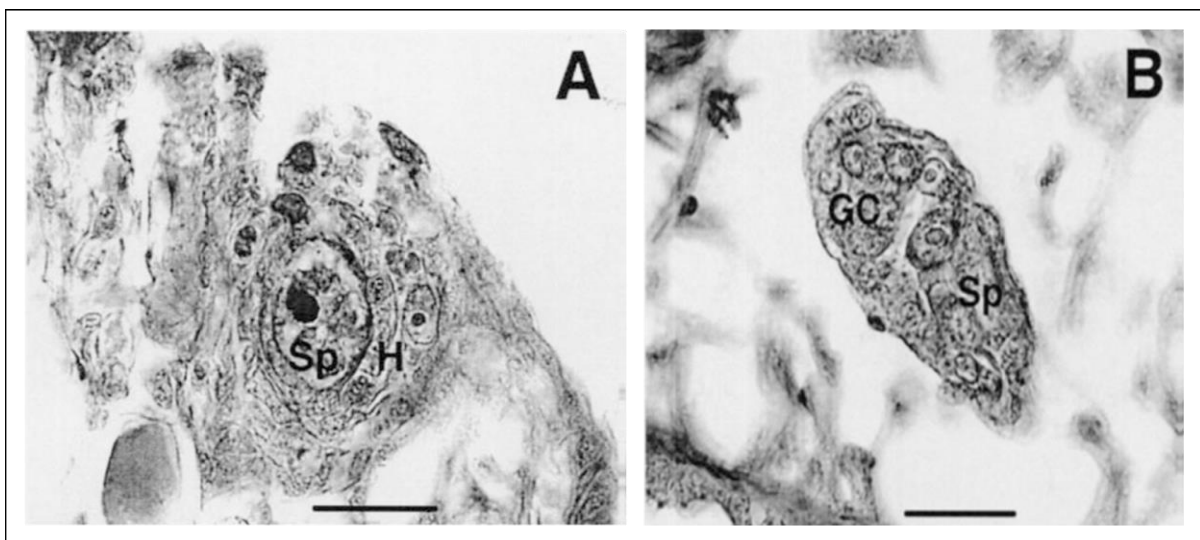


**Figure 17.** A) *Galba cubensis* shell. B, C) *G. cubensis* in the field. D) *Pseudosuccinea columella* shell. E) *P. columella* in the field. F) Typical spiral ridges in the periostracum characteristic of *P. columella* species. Scale bars = 5 mm (Photos: Laboratory of Malacology, IPK).

In contrast, in Cuba *P. columella* presents a more restricted distribution (western and central regions), it is highly abundant in rural ecosystems (Vázquez et al. 2009), and it is believed to play a secondary role as intermediate host of *F. hepatica* (Vázquez et al. 2014). So far, only one population, located in Pinar del Rio province (*i.e.* IPA), has been reported naturally-infected with the parasite in Cuba with 2% prevalence (also considered the first record of natural *F. hepatica* transmission by *P. columella* in the Caribbean; Gutiérrez et al. 2011). However, even showing less overall compatibility with Cuban *F. hepatica* than *G. cubensis* (in the laboratory and in nature), several populations of this lymnaeid species have been successfully infected for many years in the laboratory using different *F. hepatica* isolates (Gutiérrez et al. 2002; 2003a; Vázquez et al. 2014). Under experimental conditions, the

prevalence of *F. hepatica* infecting *P. columella* ranges from 0% to 100%, depending on the snail (population) – parasite (isolate) combination used, which points at the existence of a polymorphism of compatibility in this parasite – host system (Vázquez et al. 2014).

However, *P. columella* snails from certain localities from western and central Cuba (La Palma, El Azufre, Babiney, La Playita, La Coca and Candelaria) have never resulted infected by *F. hepatica* after experimental exposures with field-derived miracidia, regardless of the isolates of the parasite used (Gutiérrez et al. 2002; 2003a; Calienes et al. 2004; Vázquez et al. 2014). In this sense, it was noticed in histological sections that this phenomenon (of non-infection) involved the penetration of *F. hepatica* miracidium into the snail followed by a hemocyte-mediated encapsulation of the parasite at 24 h post-exposure (Gutiérrez et al. 2003a; see Figure 18). Both facts point at the occurrence of an immunological activation rather than an unsuitability of these snails to *F. hepatica* that, noteworthy, has been selected in nature (Gutiérrez et al. 2003a; Vazquez et al. 2014).



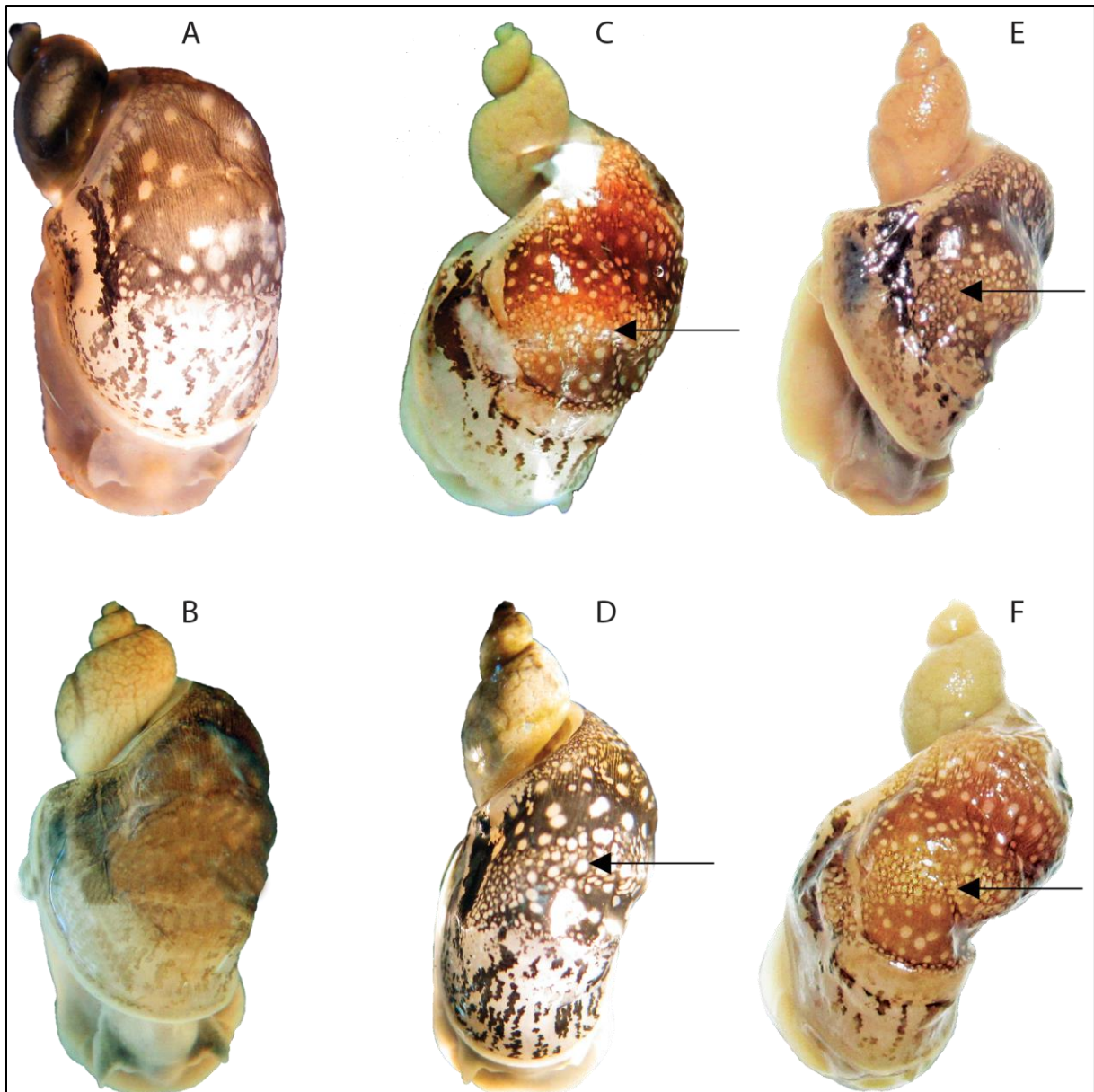
**Figure 18.** A) Encapsulated *F. hepatica* sporocyst (Sp) undergoing phagocytosis by hemocytes (H) can be seen within resistant *P. columella* snails at 24h post-exposure while B) normal development of sporocyst in a susceptible *P. columella* is observed in susceptible snails (Gutiérrez et al. 2003a).

Significantly, resistant snails from these populations share the same reliable conchological characters defined for (susceptible) *P. columella*: big shell, small spire, large aperture of about two thirds of the shell height and the characteristic micro-sculptures in the periostracum (Pointier, 2008; Vázquez & Sánchez, 2015; Correa et al. 2011). In addition, amplification and sequencing of nuclear rDNA ITS (1170 bp comprising the 3' region 18S, ITS-1, 5.8S, ITS-2 and the 5' region of the 28S) from resistant and susceptible snails showed only



a slight difference of two bases, one in each ITS fragment (Gutiérrez et al. 2003b). Such differences accounted for only 0.17% of sequence variation between the two strains and are insufficient to segregate both phenotypes into different species (Gutiérrez et al. 2003b). These morphological, anatomical and genetic criteria can be used to discriminate certain mollusk species, even within the family Lymnaeidae (e.g. *P. columella*; Bargues & Mas-Coma, 1997; Correa et al. 2011) but only when significant differences are found. The high similarity encountered between these strains points at the occurrence of two different phenotypes concerning *F. hepatica* infection within *P. columella* (Gutiérrez et al. 2003a) and constitutes, to our knowledge, the only report of field-occurring resistance/susceptibility on a snail – trematode system.

Nonetheless, it has been proved that both resistant and susceptible populations can be discriminated by phenotypic differences of mantle pigmentation: 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 (Figure 19); susceptible snails have diffused and sparser mantle spots, scattered without a clear pattern (Gutiérrez et al. 2003a). Resistant populations of *P. columella* can also be distinguished from susceptible snails by polymorphic genetic markers (Calienes et al. 2004; Lounnas et al., 2017). A random amplification of polymorphic DNA (RAPD)-based study using commercial primers of arbitrary sequence showed different genetic profiles between resistant (La Palma) snails and two isolates of susceptible *P. columella* from Havana with 17 of the 26 primers tested while no within-group variation was detected (Gutiérrez et al. 2003b). Additionally, when the same method was applied to 12 field-collected populations from western and central Cuba, including three resistant populations (La Palma, El Azufre and Babiney), the same reproducible patterns of RAPD profiles, each associated with resistance or susceptibility to *F. hepatica* infection, and little within-group polymorphism were observed (Calienes et al. 2004). These results clearly clustered the populations of *P. columella* into two genetically distant groups within the species that are consistent with the displayed phenotypes (Calienes et al. 2004). In addition, a recent study that analyzed *P. columella* at a global scale, by microsatellites markers and mitochondrial haplotypes, also showed that resistant populations segregate independently from (susceptible) *P. columella* (Lounnas et al. 2017).



**Figure 19.** Mantle pigmentation pattern of susceptible (A: Aurora, B: Negrines) and resistant (C: Babiney, D: La Coca, E: El Azufre, F: La Palma) *Pseudosuccinea columella* populations to *Fasciola hepatica* infection. (Photos: Laboratory of Malacology, IPK). The arrows indicate the characteristic band of small sharp spots in the mid-region of the mantle of resistant snails.

Regarding life-history traits, uncommon relationships have been demonstrated under experimental conditions between Cuban specimens of *P. columella* susceptible and resistant to *F. hepatica* (Gutiérrez et al. 2002). While *F. hepatica* is known to castrate its host and to induce an increase of shell growth in *G. truncatula* (Wilson & Denison, 1980), *F. hepatica*-infected susceptible *P. columella* showed an increased fecundity during the patent period of infection and no increase of size (Gutiérrez et al. 2002). On the other hand, exposed resistant snails exhibit a reduced survival and fecundity rates compared to non-exposed

resistant controls and to infected susceptible snails. Furthermore, non-exposed resistant snails showed lower fecundity rates compared to non-exposed susceptible *P. columella*. All these results suggest a possible fitness and physiological cost associated with the resistant phenotype of *P. columella* (Gutiérrez et al. 2002).

The inheritance pattern of the specific markers identified for susceptibility and resistance of *P. columella* remains to be established. However, mating experiments may not resolve this issue since it has been demonstrated, by microsatellite markers-based analysis, that self-fertilization constitutes the almost exclusive reproductive strategy of this species (Nicot et al. 2008; Lounnas et al. 2017). A high-selfing rate is, in fact, a common feature of the family Lymnaeidae and has been reported for several species including *Galba truncatula*, the main vector of fasciolosis worldwide (Meunier et al. 2004).

## **X. Prevention and control of fasciolosis transmission: are snail-based strategies a feasible answer?**

In a global scenario in which human fasciolosis reemerges and high prevalence of infection is recorded on livestock, an accurate control of parasite transmission is urgently needed. However, even when several approaches and molecular candidates have been evaluated for vaccine development, a commercially available formulation is unfortunately still lacking (see Molina-Hernández et al. 2015 for review). So far, control schemes are mostly based on anthelmintic treatment of infected humans and domestic animals. Nevertheless, this is problematic given the appearance of *F. hepatica* strains resistant to the drug of choice (triclabendazole) in several regions of the world and the absence of novel effective drugs to counteract parasite-spreading resistance to triclabendazole (Fairweather, 2011). In addition, the ability of *F. hepatica* to infect a wide range of wild and autochthonous animals (*e.g.* rodents, lagomorphs, Andean camelids, marsupials; Robinson & Dalton, 2009; Mas-Coma et al. 2009a), combined with the occasional inaccurate livestock management (including the application of anti-parasitic treatment; Arias et al. 2010), complicates transmission control and almost ensures persistence of the parasite wherever infected snails occur. These factors along with the increase of livestock production to fulfill market demands and the effect of global warming on vector populations, tackle fasciolosis control only through strategies focused on the definitive hosts (Knubben-Schweizer & Torgerson, 2015; Khan et al. 2013).

Alternatively, since *F. hepatica* requires particular snails to host some of its larval stages, such strict dependence can be used, like in the case of other vector-borne diseases, to overcome the mentioned difficulties through the development and application of feasible snail-based strategies for controlling fasciolosis transmission (Knubben-Schweizer & Torgerson, 2015; Knubben-Schweizer et al. 2010). However, the worldwide distribution of lymnaeid vectors of fasciolosis (Correa et al. 2010) combined with their high resilience and ecological plasticity, facilitate transmission and impose challenges for the success of snail-targeted control policies. In any case, all strategies aiming to be effective must suit the epidemiological scenario of each particular transmission focus and be integrative. In this sense, conducting applied research to develop ecologically friendly snail-control alternatives (Adema et al. 2012) and more reliable methods to survey intermediate host populations (= to detect active transmission foci; Alba et al. 2016) is essential. Also, basic investigations concerning ecology, immunobiology, genetics and evolution of snail – trematode interactions (= knowledge that may facilitate monitoring, predicting, and ultimately modifying transmission) are key factors to accomplish this goal (Adema et al. 2012). Fortunately, even when *Fasciola* spp. – snail host interactions have not been deeply explored, the existence of naturally-selected phenotypes of resistance and susceptibility to *F. hepatica* within the same snail species, a system so far unique concerning this snail – trematode model, constitutes a valuable asset to study the underlying mechanisms behind this phenomenon.

The invasive and adaptive capacities of the species *P. columella* (Lounnas et al. 2017) have favored its establishment in several parts of the world: from North America where it is presumed to have originated from (Cruz-Reyes & Malek, 1987; Correa et al. 2010) to South America and the Caribbean (Cucher et al. 2006; Gutiérrez et al. 2011), and more recently, to Europe (Pointier et al. 2007), Africa (Grabner et al. 2014), Australia (Molloy & Anderson, 2006) and several Pacific islands (Pointier & Marquet, 1990; Cowie, 2001). In this sense, the potential application of resistant *P. columella* strains as a biological control of fasciolosis transmission (e.g. introducing resistant snails in particular high-risk focus or genetically modifying the intermediate host populations), might be a feasible answer. As a first step in this path of great potential in alleviating the burden of fasciolosis, a deeper understanding of this particular snail – trematode interaction is mandatory.

In the present thesis we gathered our recent research efforts to further characterize resistant/susceptible *Pseudosuccinea columella* from Cuba in interaction with *Fasciola hepatica* in relation to ecological, molecular and phenotypic aspects.

## Specific aims of the thesis:

### Distribution and ecology of *P. columella* in Cuba

1. To compare the distribution and ecological patterns of resistant and susceptible *P. columella*.
2. To assess the effect of differential ecological factors associated with the occurrence of resistant and susceptible populations in the field on the life history traits of *P. columella* under experimental conditions.

### Overview of the molecular constitutive features of resistant over susceptible *P. columella*

3. To identify the constitutive molecular differences between susceptible and resistant *P. columella* by comparative transcriptomic and proteomic approaches.

### Phenotype of *P. columella* towards *F. hepatica* challenge

4. To determine the effect of increasing dose, serial exposure and geographic origin of *F. hepatica* on the infection outcome of resistant and susceptible *P. columella*.
5. To evaluate the sensitivity of susceptible and resistant *P. columella* to other digenean trematode.
6. To characterize the dynamic and functional activity of hemocytes in resistant and susceptible *P. columella* during *F. hepatica* exposure.

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## CHAPTER 1

### Distribution and ecology of *P. columella* in Cuba: insights into the cost of parasite resistance

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The distribution of parasites greatly depends on the distribution of their hosts and thus, this knowledge is essential to understand epidemiological patterns and predict parasite transmission. However, host-parasite relationships cannot be detached from natural or anthropic disturbances, so different environments in which host and parasites develop may strongly affect the specificity and genotype selection of both entities (Wolinska & King, 2009). Host populations displaying high genetic diversity and occurring on stable environments are supposed to decrease the chances for parasite transmission (Sandland et al. 2007; Tavalire et al. 2016) while the arrival of new invaders with different genotypes and usually a depleted genetic diversity could dramatically modify parasite success (Meunier et al. 2011; King & Lively, 2012).

On the other hand, differences in the outcome of parasitism result from the occurrence and selection of more resistant variants within host populations. However, such selection for resistance is thought to carry a cost in fitness in the absence of parasites, giving place to an arms race constrained by the ecological cost that usually comes with the resistance (Lively & Dybdahl, 2000; Carton et al. 2005). For example, the non-susceptibility of *B. glabrata* to the digenean *E. caproni* is genetically encoded and can be selected for generations but imposes a trade-off concerning reproductive maturity (Langand et al. 1998). With all these factors influencing transmission, only a deep knowledge of the distribution and ecological patterns of the host species, as well as the genetic structuration of their populations in the light of parasite transmission, could bring forward accurate hypotheses and predictions.

Here, we will be addressing these fundamental aspects of *P. columella* from Cuba, by analyzing data from the field to the laboratory (article 1). The facts that (1) this is an actual intermediate host species of *F. hepatica* in Cuba (Gutiérrez et al. 2011), (2) that its presence in Cuba (as elsewhere out of its North American native range) is probably associated with invasion events

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of either natural (*e.g.* via aquatic birds or strong meteorological events like hurricanes) or anthropic causes (aquatic plant trade) (Lounnas et al. 2017), and (3) the occurrence, so far only in Cuba, of resistant populations to *F. hepatica* in the field, make the approaching to this model through this study particularly attractive from both, the epidemiological and the evolutionary points of view.

We will present the updated distribution (up to 2018) and the population genetics of this species in Cuba, exploring the ecological patterns associated to the existence of both phenotypes (resistant and susceptible) combining field (long-term ecological follow up) and laboratory (experimentally life-history traits) studies. From these approaches, we expect to answer several important questions in this model: is the distribution of *P. columella* in Cuba homogenous for resistant and susceptible populations?; should we expect an enhanced transmission of *F. hepatica* following the introduction of certain genotypes?; which environmental factors could differentially influence the occurrence of each phenotype in nature?; if we are to use resistant snails as biological control, should we expect a cost for resistance?; and which conditions should be met for such case? These questions range from the most basic experimental research to the practical issues related to the further applicability of our results.

### **Article 1 (in preparation)**

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**Ecology and population genetics of a vector snail in Cuba: insights into the history and colonization of *Pseudosuccinea columella*, its relation with *Fasciola hepatica* and trade-offs associated to parasite resistance**

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**Ecology and population genetics of a host snail in Cuba: insights into the history and colonization of *Pseudosuccinea columella*, its relation with *Fasciola hepatica* and trade-offs associated to parasite resistance**

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## Abstract

*Pseudosuccinea columella* snails transmit the trematode *Fasciola hepatica* but in Cuba, six naturally occurring populations successfully resist to parasite infection. Here, we presented the updated distribution of *P. columella* in Cuba, which was verified on 68 localities with earliest records more abundant in the west-central Cuba while populations from central-east Cuba generally correspond to the newest samples. No records were found farther east. The study of the population genetics using microsatellite markers showed a low allelic and multilocus genotypic richness (MLGT), mainly in susceptible populations, strong deviations from panmixia and high self-fertilization rates. Susceptible individuals were grouped in one major cluster containing the majority of MLGT and two independent clusters gathered MLGT of resistant individuals from western and central populations respectively. From these, we propose that several introductions of *P. columella* occurred in Cuba, primarily in the west, with the formers deriving on the resistant populations. A more recent introduction of susceptible *P. columella* carrying MLGT T and Y may have occurred, with the latter spreading fast in the island and increasing the risk of parasite transmission since all *F. hepatica*-infected individuals recorded in Cuban fields carried the MLGT Y. Interestingly, even when resistant populations are supposed to be older in Cuba, they only exist in six localities, suggesting an ecological cost of resistance. Thus, we analysed the ecological patterns of occurrence of each phenotype in the wild and found that resistant populations colonized sites characterized by a soft (total hardness ranged from 4 to 9°d) and slightly acid (pH from 6 to 6.5) freshwater with low snail species richness. Such tendency was confirmed through a two-year follow-up ecological study in a farm where susceptible and resistant snails occurred in sympatry. Comparison of life traits of susceptible and resistant isolates reared at two different pH/total hardness conditions (*i.e.* 5.9/4°d and 7.8/14°d) showed that low pH/TH negatively affect *P. columella*, irrespective of the phenotype. However, a higher tolerance to such conditions was observed for resistant *P. columella* isolates. This could explain the restricted distribution of resistant populations in nature to sites less suitable to other snails where the impairment of their competitive capacities is then compensated.

**Keywords:** ecology, population genetic, invasion, liver fluke, Lymnaeidae, microsatellite markers, fitness cost, compatibility, resistance

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## 1. Introduction

The freshwater snails of the family Lymnaeidae (also known as pond snails) inhabit tropical, temperate and cold regions, from sea level to high altitudes [1]. Many of the recently accepted nearly 100 species [2] have become the target of taxonomical and parasitological studies in the past few years, due to their important roles as intermediate hosts of several medically relevant parasites, including the worldwide distributed liver fluke *Fasciola hepatica* (e.g. [3, 4]). Within the Lymnaeidae, several species have been incidentally carried out of their native range and introduced elsewhere (see the cases of *Galba cubensis* and *Austropeplea viridis* in Europe; [5, 6]). However, probably one of the most interesting examples is that of *Pseudosuccinea columella* (Say, 1817), a worldwide introduced species with a considerable invasive ability [7]. The transmission of *F. hepatica* by *P. columella* is well documented either in its native range (North America; see [8]) and in places where it has been introduced such as Brazil [9], Argentina [10] or Australia [11]. Moreover, this species is also presumed to enhance transmission in remote regions where it has invaded, like Egypt [12] and the Pacific islands, including Hawaii and the French Polynesia [13, 14].

In Cuba, the first report of *P. columella* dates back to 1858 in the west and the latest malacological surveys depict a western-central distribution [15]. Although this species is thought to play a secondary role in *F. hepatica* transmission in Cuba (with *Galba cubensis* being the main host; see [16, 17]), it was recently found naturally infected in the field [18]. Important investigations have been carried out on *P. columella* in the late decades due to the existence of resistant phenotypes against *F. hepatica* infection in certain natural populations from Cuba [19, 20]. Experimental exposures of these resistant snails to different sympatric and allopatric snail-fluke combinations always result in the failure of parasite larval stage development [19-22] associated to an effective encapsulation of the parasite by the snail immune cells [19].

Interestingly, resistant *P. columella* can be easily differentiated from susceptible individuals in the field by a phenotypic marker consisting on a belt-like pattern of small, sharp and whitish spots in the mid-region of the mantle with bigger spots scattered in the upper and lower sides [19]. Attempts to genetically differentiate susceptible from resistant individuals have independently clustered both phenotypes by RAPD markers [21]. More recently, Cuban resistant populations were segregated from other *P. columella* world populations using

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mitochondrial markers [7]. Yet, it is important to stress that observed molecular and phenotypic differences do not stand enough to separate resistant populations as a different species. Resistant snails displayed the same reliable features of the shell and the same reproductive system anatomy defined for *P. columella* with only a slight difference of two-nucleotide changes (0.17%) within the ITS1 and ITS2 when ribosomal genes were sequenced and compared [23].

Undoubtedly, the natural occurrence of susceptible and resistant populations to a parasite within the same host species provides a unique model to be addressed from several disciplines, with an incidence on evolutionary biology and host-pathogen interaction, environmental and health sciences and snail control. Here, we bring forward for the first time, a global overview on the ecology of *P. columella* in Cuba, considering both phenotypes separately, from different approaches. We present its updated distribution, study its infection status and its genetic structure by microsatellites markers, and elucidate the ecological patterns associated with the occurrence of resistant and susceptible populations through the analysis of field data and experimental life history traits. From these, we gain insight into the history, colonization and population structure of *P. columella* in Cuba, its relation with *F. hepatica* and the increasing risk for parasite transmission that represents certain widespread genotypes. Moreover, we provide further knowledge related with habitat preferences of *P. columella*, the ecological cost associated to resistance to *F. hepatica* and the environmental conditioners that contribute to determine its occurrence in the wild. All these aspects are essential to understand and predict the distribution pattern and further spreading of *P. columella* snails in Cuba. This would be helpful to envision potential risks for conservation biology, parasitology and epidemiology and to devise intermediate host control strategies based on the potential application of *P. columella* resistance.

## **2. Material and Methods**

### **2.1. Distribution of *P. columella* in Cuba**

The updated distribution of *P. columella* was obtained from the National Reference Malacological Database for freshwater snails in Cuba maintained by the Laboratory of Malacology of the Institute of Tropical Medicine in Havana through the revision of 496 sampled localities from 1982 to 2018. Each record includes the presence of a given freshwater

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snail, geographic coordinates, habitat type, date and collector. Nation-wide malacological surveys are carried out by our laboratory on a 10-year interval basis complemented with additional shorter expeditions every year. All positive sites to *P. columella* were plotted in a map differentiating each record according to a 11-year lapse, to account for historical and fresh data, using MapInfo v.15 (Pitney Bowes Software Inc., New York, USA, 2015). The frequency of each habitat type where *P. columella* was observed was used as a measure of habitat preference. Populations resistant to *F. hepatica* infection were differentiated based on the presence of the typical mantle pigmentation pattern associated with the resistant phenotype [19]. Also, individuals from resistant and susceptible populations were always checked in the laboratory by experimental infection using several sympatric and allopatric strains of *F. hepatica* from Cuba following the methodology described previously [20].

## 2.2. Population genetics of *P. columella* from Cuba

### 2.2.1 Snail sampling, DNA extraction, amplification and genotyping of microsatellite loci

We sampled 20 populations of *P. columella* (17 susceptible, 3 resistant according to the phenotypic markers described by Gutiérrez et al. [19]). Up to 30 individuals were collected when possible from each population up to a total of 329 snails. The snails were carefully dissected in the laboratory for screening of possible parasite infection. A piece of tissue from the foot was used for DNA extraction to avoid foreign genetic contamination (*e.g.* sperm from cross-fertilization). The extraction of DNA was performed using Chelex methodology following [24] and slightly modified for 96-well plates. Briefly, a small portion of tissue was added to a mixture of 100  $\mu$ L of 5% Chelex<sup>®</sup>100 (Bio-Rad) and 5  $\mu$ L of proteinase K (50 mg/mL; Promega) in each well. The plate was mixed in vortex, incubated overnight at 56°C and placed for 10 min at 95°C. The mixture was centrifuged at 6000  $\times g$  for 6 min and the supernatant containing the DNA was collected and stored at -20°C until use.

PCR amplifications were performed in a MJ-Research PTC 100 96-well in all samples. Six microsatellite loci, previously described for *P. columella* species by Nicot et al. [25], were amplified (GenBank Access number): PCO01 (EU04295), PCO02 (EU049296), PCO07 (EU049299), PCO12 (EU049303), PCO13 (EU049304) and PCO20 (EU049309). Each locus was amplified through PCR using 1  $\mu$ L of extracted DNA in a 10  $\mu$ L of reaction volume containing 2  $\mu$ L buffer 5X (Promega), 1  $\mu$ L of 25 mmol/L MgCl<sub>2</sub>, 0.5  $\mu$ L of 2mmol/L dNTP (Invitro-gen/Life

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Technology), 0.2  $\mu$ L of each primer (10 pmol) and 0.2  $\mu$ L of *Taq* DNA polymerase (Promega). Thermocycling consisted of 10 min of initial denaturalization at 94°C, 30 cycles at 94°C for 30 s, annealing at 55°C (for each locus) for 30 s, 60 s at 72°C and a final elongation step at 60°C for 30 min.

Primers were fluorescently labelled to be used in an ABI automated sequencer (ABI Prism 310 Genetic Analyser, Applied Biosystem, Perkin-Elmer, USA). Amplified products for each individual were used for sequencing adding 1  $\mu$ L of diluted amplicon (1/100) to a mix containing 15  $\mu$ L of Hi-Di Formamide and 0.27  $\mu$ L of internal size standards (GENESCAN 500 LIZ, Applied Biosystems). Reading of each allele's length was performed using GeneMapper® v.4.0 software (Applied Biosystems™).

### 2.2.2 Population genetics analysis

Current parameters of population genetics like the mean number of alleles ( $a$ ), the observed ( $H_o$ ) and expected ( $H_s$ ) heterozygosity and  $F_{IS}$  were estimated for each locus. Pairwise differentiation between populations ( $F_{ST}$ ) was also tested. Calculations were computed using FSTAT v2.9.3.2 [26] and Bonferroni corrections were applied for multiple tests [27]. The selfing rate ( $s$ ) for each population was calculated using the equation  $s = 2F_{IS}/(1+F_{IS})$  [28]. Identical multilocus genotypes (MLGT) were identified based only on individuals with all microsatellite loci amplified and coded using a combination of letters (one letter or letters' combination = one MLGT = unique combination of alleles across all analysed loci). Nei's [29, 30] genetic distances between populations were estimated using Genetix [31]. The obtained distance matrix was used to build a neighbour-joining network of all MLGT in SplitsTree4 [32]. The genotypic diversity was calculated in each population with an adaptation of the Simpson index using GENCLONE 2.0 [33].

## 2.3. Ecological patterns of resistant and susceptible *P. columella*

The ecological patterns of the two phenotypes of *P. columella* were explored using three approaches: laboratory field records, field study and experimental life tables.

### 2.3.1 Revisiting laboratory field records of resistant and susceptible *P. columella* localities

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A thorough literature review accounting for published papers and unpublished data from field notes of our laboratory during the last 37 years was conducted. We gathered data of species composition of the mollusc assemblage and abiotic (phosphates, nitrates, dissolved oxygen, pH and total hardness) factors measured in the environment of the resistant and susceptible populations investigated herein.

### 2.3.2 Ecological follow up of resistant and susceptible populations of *P. columella*

We performed a two-years ecological follow-up in a cattle farm (La Coca) from south-western Havana harbouring resistant and susceptible populations of *P. columella* (see Figure 1A). The region is a lowland meadow commonly flooded during the rainy season, which extends for nearly 10 km<sup>2</sup>. Several channels used for irrigation and cattle drinking cross over the area usually not deeper than 60 centimetres. Gramineous vegetation is abundant but aquatic plants in the channels are scarce. Freshwater snail populations are scattered all over the area in all water bodies. We selected two sites for the ecological follow-up considering the existence of a population of susceptible individuals (Negrines), and another of resistant individuals (La Coca) located in a nearby site called Segundo Potrero. Resistant snails were easily identified in the field by the phenotypic marker of mantle pigmentation [19]. Both sites are 500 m afar but connected by one of the channels. Each site was sampled at monthly intervals from January 2011 to December 2012 always during the first hours in the morning by the same person using soft forceps and sieves (1 mm mesh). We used the capture per unit of effort method during an interval of 15 minutes [34] and relative abundance was measured as individuals/15' for each species. Aquatic and shore vegetation were always swept in every sample. The abiotic factors pH, temperature, total hardness (TH), carbonate hardness (CH), dissolved oxygen, phosphates and nitrites were measured using a field kit (Merck). Species diversity was calculated for each site using the Simpson index [35] and a canonical correspondence analysis [36] was done to detect unimodal response of mollusc species abundances to ecological factors.

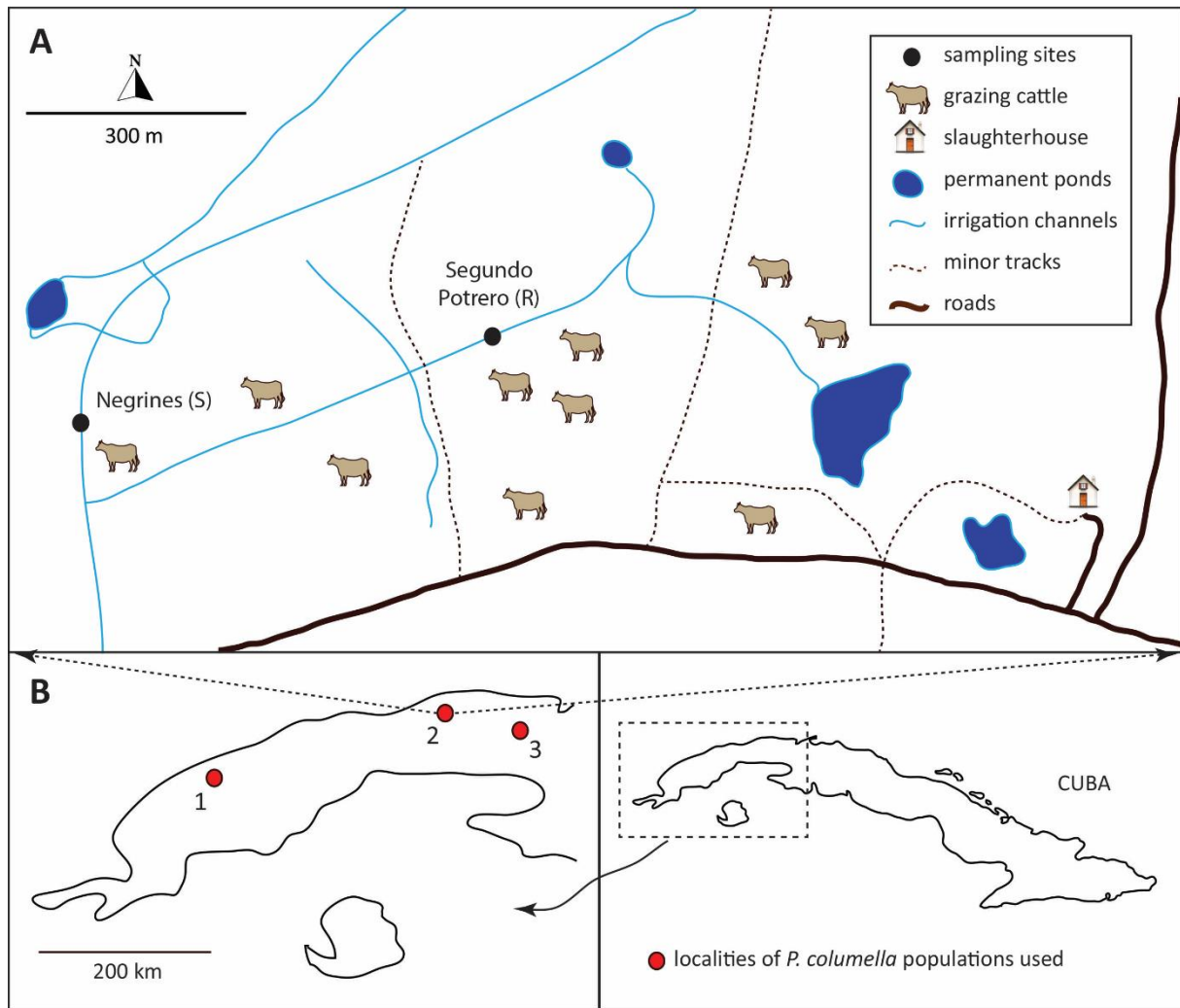
Additionally, all collected lymnaeid snails were brought to the laboratory and dissected to account for *F. hepatica* larval stage infections and calculate field prevalence.

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### 2.3.3. Laboratory life tables of resistant and susceptible *P. columella*

Four natural populations of *P. columella* of which two are composed of susceptible (Negrines, 22.9577°N; -82.4649°W and Aurora, 23.0789°N; -81.9176°W) and two of resistant (La Coca, 22.9554°N; -82.4598°W and La Palma, 22.7695°N; -83.5443°W) individuals (see Figure 1B), were sampled and reared under laboratory conditions for one generation. Given the results derived from field data concerning the ecological patterns related to the occurrence of both phenotypes in nature, two different settings of pH/total hardness (TH) were set up: low (5.9/14°d) and common (7.6/14°d) conditions (according to Perera [34], the latter are the most common conditions of Cuban freshwater habitats). Snails were then reared up in the two conditions using separate 25 L aquariums. Five-day-old snails from each isolate (n=30 per population at each condition) were placed in the aquariums and that moment was considered to be time zero for the experiment. All aquariums were provided with 50 mg each of CaCO<sub>3</sub> and MgCO<sub>3</sub> to help with shell formation. Water acidification down to pH 5.9 (low condition) was achieved through an automatic CO<sub>2</sub> pump connected to a setter pH meter. Total hardness conditions were adjusted to the required range for each aquarium by differentially mixing aged tap water with distilled water. Both pH and TH were systematically checked along with other abiotic parameters (Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, carbonate hardness) with a commercial aquarium kit to guarantee optimal and reproducible conditions during all the experiments. Temperature was always maintained at 25-26°C and the snails were fed three times per week with a mixture of algae cultured for this purpose following Sánchez et al. [37]. Live and dead individuals, the numbers of eggs, egg masses and viable eggs/newly hatched snails (NHS) were counted at week intervals. The experiment was ended after all initially set individuals from all populations were dead. At the end of the experiment, the following life table parameters were determined [38]: proportion of living individuals (lx), probability of survival (px), life expectancy (ex), fecundity rate (mx), net reproduction rate (R0), and intrinsic (r) and finite (λ) rates of natural increase.

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**Figure 1.** A) Overview of La Coca locality showing the two resistant (R) or susceptible (S) *Pseudosuccinea columella* occurring sites, Segundo Potrero and Negrines, that were sampled during the two-year ecological follow-up study. B) Localities from western Cuba where the four populations of resistant (1: La Palma, 2: La Coca) and susceptible (2: Negrines within La Coca locality, 3: Aurora) *P. columella* used in the life table experiments were collected from.

Survival curves of exposed snails (from day 0 to day 25 post- (first) exposure) were constructed based on the number of alive snails at each time point, divided by the number of exposed individuals and expressed in percentage. We performed log-rank tests of Kaplan-Meier curves to assess the statistical differences on survivorship data. The factorial ANOVAs followed by the *post hoc* multiple comparison Tukey test were carried out to assess statistical significance between the mean values of eggs, egg masses and egg/egg masses per population. The overall percentage of viable eggs per strain at each condition was compared by a Fisher's Exact Test. Similarly, the percentage of NHS within the first, second and third week after egg laying was



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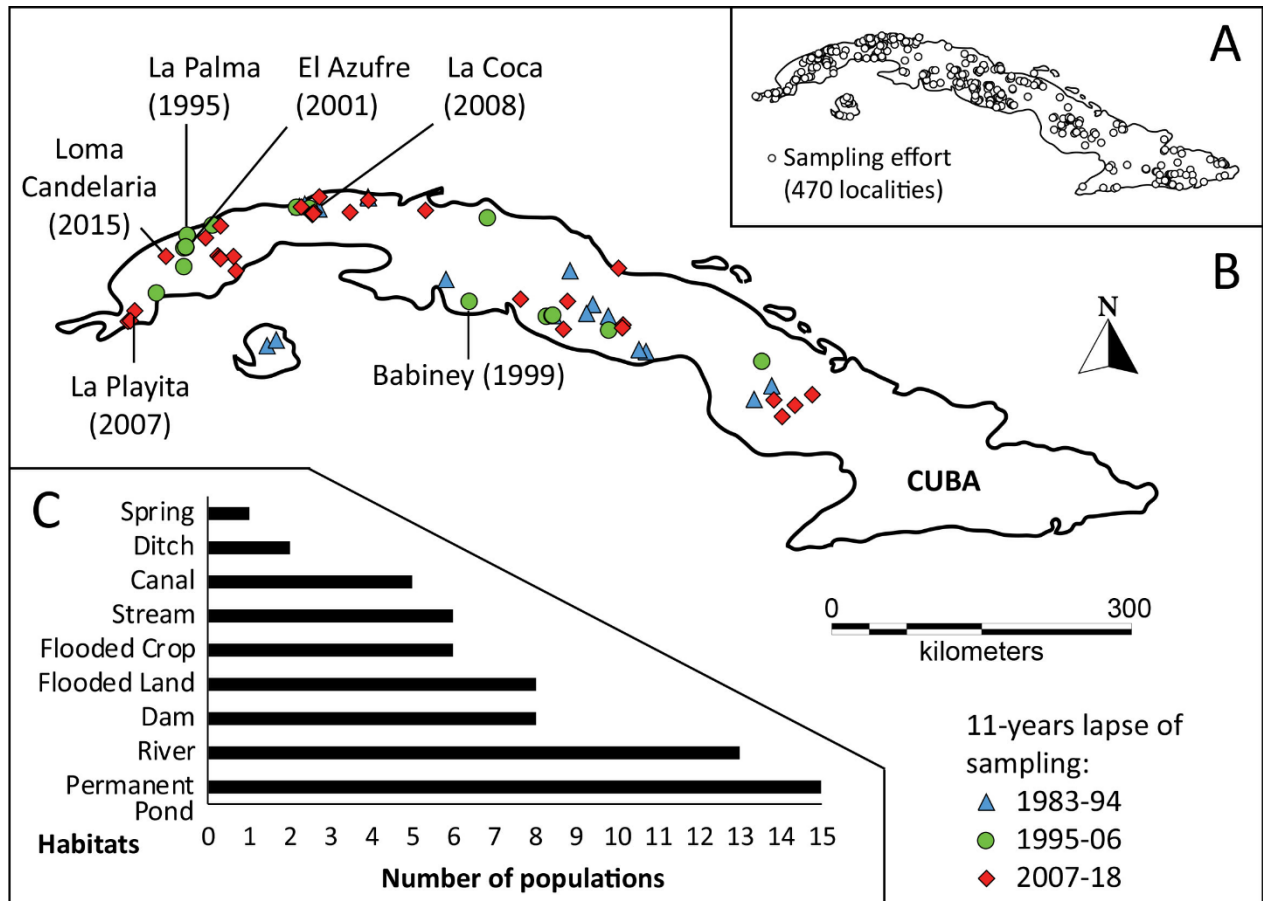
determined in each strain and compared between experimental settings to assess possible delays. Statistica v.12 (StatSoft. Inc., Tulsa, OK, USA 2014) software was used for calculations and differences were considered significant when  $P < 0.05$ .

### 3. Results

#### 3.1. Distribution of *P. columella* in Cuba

Knowing the distribution of a snail species is particularly important for assessing its epidemiological significance and for aiming at its effective control. The National Reference Database contains nearly 500 records in Cuba (Figure 2A), from which 68 verified localities harbour *P. columella* (Figure 2B). The distribution of *P. columella* ranges from the western most region (Pinar del Río province) to the central-east (Camagüey province) of Cuba (Figure 2B). No records have ever been found within the most eastern provinces. Notably, 59% sampled populations were recorded in the last 15 years and, globally, six populations of phenotypically resistant individuals to *F. hepatica* infection are acknowledged. Overall, earliest records are more abundant in the central-west while populations from central-eastern Cuba correspond mostly to newer samplings. Populations of resistant individuals are mostly recorded in the west (Pinar del Río – Havana provinces) with the only exception of Babiney in Cienfuegos (central Cuba; Figure 2B). Concerning the habitat type preferences, permanent ponds, rivers and dams harbour 62.5% of all *P. columella* records, with up to six different habitats concentrating the remaining populations (Figure 2C). We did not find a clear pattern of habitat types for resistant populations which were observed in permanent ponds (2), flooded lands (1), springs (1), ditches (1) and canal (1). Globally, the number of populations recorded in open or connected water systems (*e.g.* rivers, streams, canals) is fewer than those of closed habitats (*e.g.* dams, ponds, flooded lands; Figure 2C).

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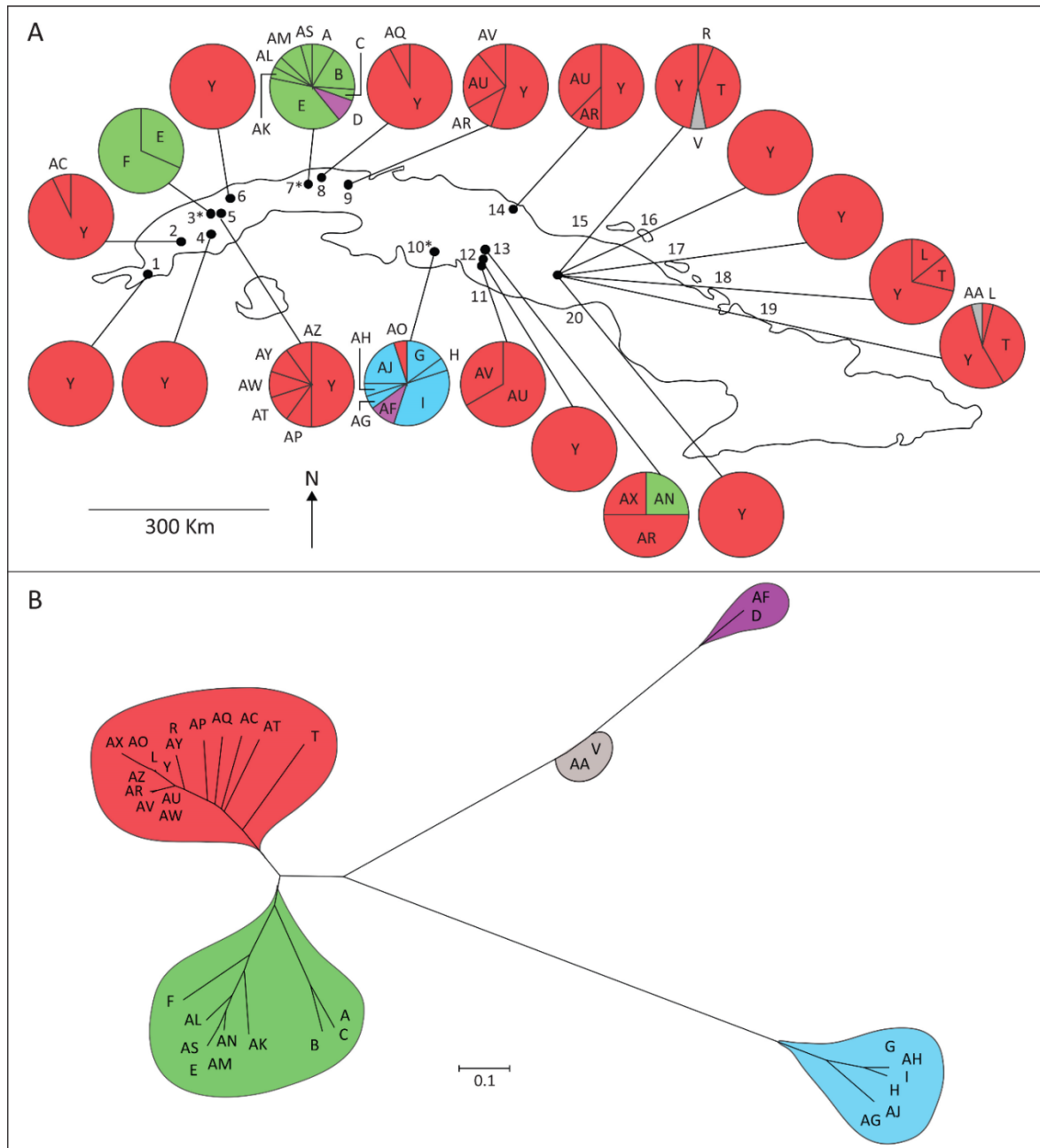
**Figure 2.** A) Distribution of all registered records in the National Malacology Reference Database accounting for freshwater mollusc species from 1982-2018. B) Updated distribution of *Pseudosuccinea columella* from Cuba. Positive sites were plotted with different points representing 11-year time lapses to account for historical and newly recorded data. Positive sites harbouring resistant snails are named in the map, consistently with previous published works. The year of first record of resistant populations is also indicated. C) Number of records of *P. columella* per type of habitat. (Laboratory of Malacology, IPK).

### 3.2. Population genetic structure of *P. columella* in Cuba

Population genetic studies on *P. columella* could give insights into important questions for biological and health sciences; its evolutionary history in Cuba, its pattern of colonization, the genetic structure of its populations and its relation with *F. hepatica* transmission. Thus, from the distribution of *P. columella* presented above, we selected 20 localities (see Figure 3 for details) to analyse the population genetic structure of this species in Cuba. Dissection of 329

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snails evidenced infection of *F. hepatica* in two individuals from the locality of IPA. No infection by other trematode species was observed in any other locality.



**Figure 3.** A) Map showing the distribution of the different multilocus genotypes found (MLGT; one letter or letters' combination = one MLGT = unique combination of alleles across all analysed loci) on sampled *Pseudosuccinea columella* populations genotyped through 6 microsatellite loci (3, 7, 10: \*localities with resistant populations). B) MLGT of *P. columella* clustered following a joining-neighbour network using Nei's genetic distance from each MLGT (different filling pattern correspond to different clusters). 1: Tio Pancho, 2: SJM, 3: El Azufre\*, 4: Río Hondo, 5: IPA, 6: Pilón, 7: La Coca\*, 8: Parque Lenin, 9: V7, 10: Babiney\*, 11: Vega

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Grande, 12: El Antojito, 13: El Cacao, 14: Río Manaquita, 15: Río Yayabo, 16: Puesto de Mando, 17: Guillén, 18: Río Central, 19: Matadero Aves, 20: Arroyo.

The six explored microsatellite loci were polymorphic in *P. columella*, especially PCO12 which displays seven different alleles in Cuba. Population genetic parameters are presented in table 1.

**Table 1.** Mean allelic number ( $a$ ), observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity,  $F_{IS}$  (level of significance after Bonferroni correction) and estimated auto-fecundity rates ( $s$ ) for resistant ( $R$ ) and susceptible ( $S$ ) populations of *Pseudosuccinea columella* ( $n$ : number of analysed individuals).

Population	N	$a$ ( $\pm DS$ )	$H_o$ ( $\pm DS$ )	$H_e$ ( $\pm DS$ )	$F_{IS}$ ( $P$ )	$S$
$R$ La Coca	37	2.17 $\pm$ 0.75	0.056 $\pm$ 0.074	0.304 $\pm$ 0.227	0.816 (<0.01)	0.9
$R$ Babiney	26	2.17 $\pm$ 0.75	0.068 $\pm$ 0.059	0.249 $\pm$ 0.197	0.726 (<0.01)	0.84
$S$ IPA	19	2 $\pm$ 1.1	0.068 $\pm$ 0.121	0.127 $\pm$ 0.164	0.469 (<0.01)	0.64
$S$ Parque Lenin	26	1.67 $\pm$ 0.82	0.019 $\pm$ 0.045	0.087 $\pm$ 0.104	0.786 (<0.01)	0.88
$S$ Río Arimao	13	1.83 $\pm$ 0.75	0.249 $\pm$ 0.259	0.239 $\pm$ 0.267	0.038 (NS)	0.08
$S$ Río Hondo	15	1.5 $\pm$ 0.84	0.025 $\pm$ 0.039	0.092 $\pm$ 0.191	0.733 (<0.01)	0.85
$S$ Río Manaquita	15	1.5 $\pm$ 0.84	0.028 $\pm$ 0.044	0.149 $\pm$ 0.25	0.814 (<0.01)	0.9
$S$ Río Yayabo	20	1.33 $\pm$ 0.52	0.01 $\pm$ 0.025	0.174 $\pm$ 0.27	0.94 (<0.01)	0.97
$S$ Tío Pancho	22	1.17 $\pm$ 0.41	0.008 $\pm$ 0.019	0.008 $\pm$ 0.02	0 (NS)	1
$S$ V7	13	1.33 $\pm$ 0.52	0.031 $\pm$ 0.049	0.101 $\pm$ 0.212	0.689 (<0.05)	0.82
$S$ Vegas Grandes	10	1.33 $\pm$ 0.52	0.05 $\pm$ 0.083	0.117 $\pm$ 0.24	0.571 (NS)	0.73
$S$ Arroyo	16	1 $\pm$ 0	0	0	-	1
$S$ Puesto de Mando	6	1 $\pm$ 0	0	0	-	1
$S$ El Antojito	6	1 $\pm$ 0	0	0	-	1
$R$ El Azufre	19	1.17 $\pm$ 0.41	0	0.076 $\pm$ 0.186	1 (<0.01)	1
$S$ Guillén	15	1 $\pm$ 0	0	0	-	1
$S$ Río Central	7	1.5 $\pm$ 0.84	0.048 $\pm$ 0.073	0.091 $\pm$ 0.163	0.478 (NS)	0.65
$S$ Matadero de Aves	24	1.5 $\pm$ 0.84	0.007 $\pm$ 0.017	0.17 $\pm$ 0.263	0.959 (<0.01)	0.98
$S$ Pilón	6	1 $\pm$ 0	0	0	-	1
$S$ SJM	14	1.17 $\pm$ 0.41	0	0.024 $\pm$ 0.058	1 (NS)	1

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Allelic richness is very low in the 20 studied populations (mean  $2.034 \pm 0.646$  SD) and five populations (Pilón, El Antojo, Puesto de Mando, Guillén and Arroyo) are completely monomorphic at the six loci. The highest allelic richness was observed in the two resistant populations of La Coca and Babiney ( $2.17 \pm 0.75$  SD). However, the third analysed resistant population (El Azufre) showed a lower allelic richness ( $1.17 \pm 0.41$  SD). Expected heterozygosity (mean  $0.1 \pm 0.09$  SD) and observed heterozygosity ( $0.03 \pm 0.056$  SD) were very low. Most populations had high and significant values of  $F_{IS}$  after Bonferroni correction (mean  $0.667 \pm 0.313$  SD) for which estimated selfing rates were also high ( $> 64\%$ ). Although an overall value of  $F_{ST}$  of 0.339 ( $P > 0.05$ ) was observed, significant differentiation was only recorded in resistant populations (La Coca, Babiney and El Azufre) that not only differed from susceptible populations but also from each other suggesting an *in-situ* evolution.

Multilocus genotyping at the six microsatellites loci showed the existence of 36 MLGT. Observed mean number of MLGT in susceptible populations was 2.764 ( $\pm 1.562$  SD) while a higher average of 6.333 ( $\pm 3.785$  SD) was attained in resistant populations. Differences between each group of populations were found statistically significant (t-Student value 2.93;  $P = 0.008$ ). Mean genotypic diversity was 0.397 ( $\pm 0.357$ ) with the highest values in the resistant populations of La Coca (0.893) and Babiney (0.836). Particularly, in the case of resistant individuals, 18 MLGT were observed of which only two (*E* and *F*) are shared in two populations (El Azufre and La Coca). Figure 3A shows the distribution of the 36 MLGT in Cuba with the MLGT *Y* widespread in most sampled localities and the only observed in susceptible monomorphic populations. This MLGT is absent in resistant populations (Figure 3A). Interestingly, the two infected individuals found in the IPA population and the population from Pilón previously reported as infected (see Gutiérrez et al. [18]), shared the same MLGT *Y*. A different MLGT (*T*) was observed only in the easternmost populations (Río Manaquita, Río Central and Matadero Aves) within several individuals. The rest of the MLGT are considered rare and differ from one another by a single or few alleles. The obtained MLGT network from Nei's genetic distance displays three majoritarian clusters (Figure 3B). Susceptible MLGT gathered within one major cluster. Two different major clusters and a small cluster contain most MLGT from resistant individuals (Figure 3B). Only MLGT *AN* from Río Arimao (susceptible) pooled together with other MLGT from resistant populations (La Coca and El Azufre).

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### **3.3. Ecological patterns associated with the occurrence of resistant and susceptible *P. columella* in Cuba: field and laboratory observations**

The higher allelic and MLGT richness of resistant populations presented herein contrasts with their restricted distribution, as they have been identified only in six localities from the 68 reported harbouring *P. columella* and suggests an ecological cost of such resistance. In this sense, studies depicting more general ecological interactions of both susceptible and resistant *P. columella* in the mollusc assemblage and their interaction with the abiotic factors, which could explain the differential distribution observed are lacking. Considering that such resistance could promise worthy as control strategy to disengage *F. hepatica* transmission in the field it is important to account for possible trade-offs between resistance and ecological or demographical patterns. Thus, we analysed the ecological patterns of occurrence of each phenotype in the wild and we carried out experimental life tables to address this topic.

#### *3.3.1 Reviewing data from natural populations*

Since no apparent pattern concerning the type of habitat was found in either resistant or susceptible *P. columella* (see section 3.1), we aimed to unfold particular ecological features in each locality. For this, we used 14 localities harbouring *P. columella* that also presented the most complete data concerning abiotic factors (Table 2). From this analysis, we could note that both susceptible and resistant populations seem to prefer water with low nitrites and ammonium concentration. Interestingly, the recorded ecological data showed a marked tendency of resistant populations settling in waterbodies with lower pH (6-6.5) and TH (<10°d) and a restricted species richness (Table 2).

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**Table 2.** Information on species richness (S) concerning freshwater molluscs values of dissolved ions ( $\text{PO}_4^{3-}$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^-$ ),  $\text{O}_2$  and pH and total hardness (TH) from sampled localities harbouring resistant (<sup>R</sup>) and susceptible (<sup>S</sup>) *Pseudosuccinea columella*. Data from unpublished field notes archived in the Laboratory of Malacology, IPK, Cuba are referred in this table as this study.

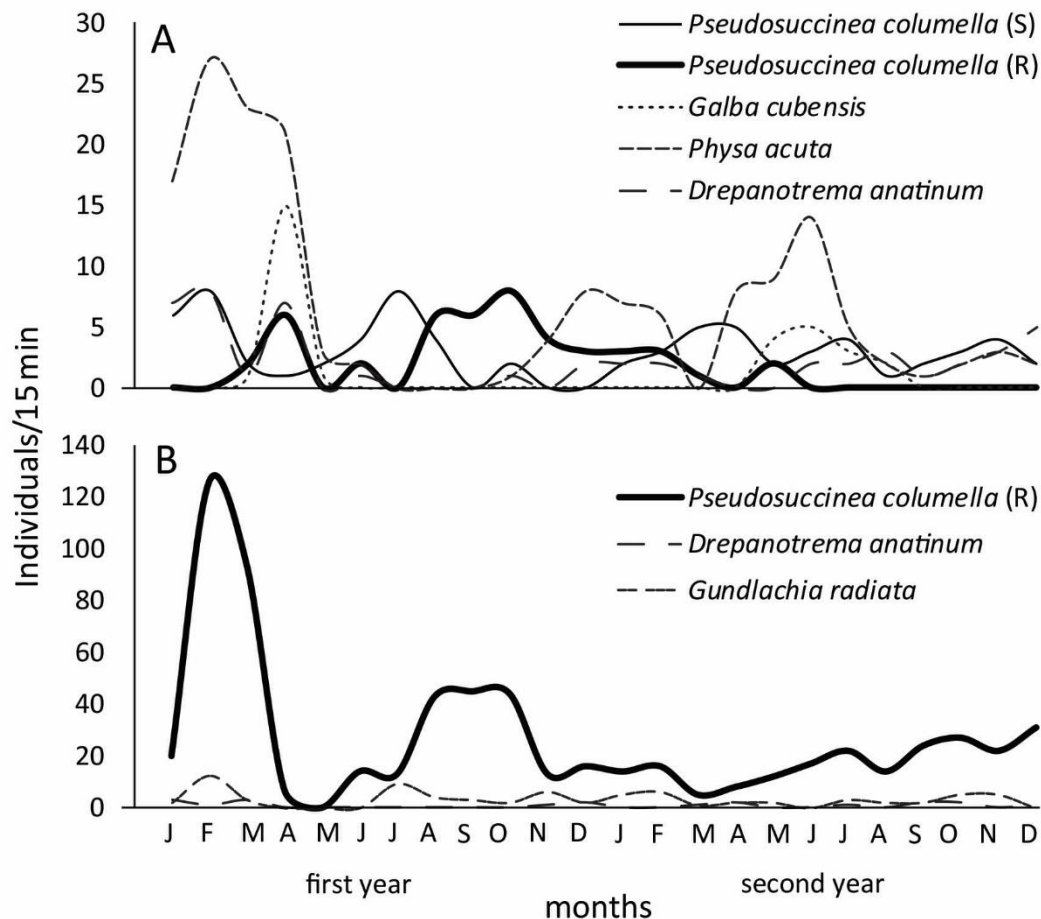
Locality	S	$\text{PO}_4^{3-}$	$\text{NO}_3^-$	$\text{NO}_2^-$	$\text{NH}_4^-$	$\text{O}_2$	pH	TH	Reference
<sup>R</sup> La Palma	3	-	50	0.1	0.2	-	6.5	6°d	This study
<sup>R</sup> El Azufre	7	0.5	10	0.05	0.2	-	6	9°d	[39]
<sup>R</sup> La Coca	3	0.25	-	0.05	0.2	15	6	4°d	This study
<sup>R</sup> La Playita	2	-	-	-	-	-	6.5	-	This study
<sup>R</sup> Candelaria	1	-	-	-	-	-	6	6°d	This study
<sup>S</sup> Negrines	5	0.5	-	0.05	0	20	7.5	20°d	This study
<sup>S</sup> Batabanó	11	-	-	-	-	-	8	20°d	[40]
<sup>S</sup> Botánico	7	-	0	0.1	0.4	-	8	11°d	[34]
<sup>S</sup> El Rubio	7	-	25	0.05	0.2	64	8	22°d	This study
<sup>S</sup> Parque Lenin	15	0.25	5	0.1	0	68	8	18°d	This study
<sup>S</sup> Bodegón	4	-	25	0	0	-	7.5	22°d	[34]
<sup>S</sup> San Juan Martinez	7	0.5	10	0.05	0.2	-	7.5	8°d	This study
<sup>S</sup> Camagüey	8	-	-	-	-	-	7	18°d	This study
<sup>S</sup> Hanabanilla	12	-	21	-	0.03	-	7.2	4°d	[41]

### 3.3.2 Ecological factors affecting the abundance of resistant and susceptible *P. columella* in the locality of La Coca

We found the two distinct phenotypes (resistant and susceptible) of *P. columella* occurring in La Coca, but with a marked degree of ecological segregation depending on the particular sampled site with Segundo Potrero only harbouring resistant snails. The species *Galba cubensis* (Lymnaeidae), *Physa acuta* (Physidae), *Drepanotrema anatinum* and *Drepanotrema lucidum* (Planorbidae) were recorded in Negrines. In this site *P. acuta* and *D. anatinum* showed similar variations while *G. cubensis* performed two ephemeral colonisations with rapid extinctions (Supplementary Figure 1). *Physa acuta* resulted dominant in this site but it did not

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seem to affect the population of *P. columella* (Supplementary Figure 1). Contrastingly, the scenario at Segundo Potrero was completely different with a very low snail species richness marked by the low abundances of *D. anatinum* and *Gundlachia radiata*, and a considerable dominance by *P. columella* (Supplementary Figure 1).



**Supplementary figure 1.** Monthly relative abundances of the freshwater mollusc species in La Coca locality where resistant (R) and susceptible (S) *Pseudosuccinea columella* inhabits in sympatry. Two sites were sampled: A) Negrines. B) Segundo Potrero.

Concerning the different phenotypes of *P. columella*, we should note that, while in Segundo Potrero only resistant individuals are found, in Negrines susceptible individuals occurred in sympatry with resistant snails at certain times of the study (Figure 4B). The canonical correspondence analysis performed with all the ecological data recorded in both sites showed a particular relationship between the abundances of resistant and susceptible individuals with the variations of pH and both TH and CH (Figure 4A). We found a strong negative association between resistant snails and pH evidencing certain tolerance for slightly acid waters. The

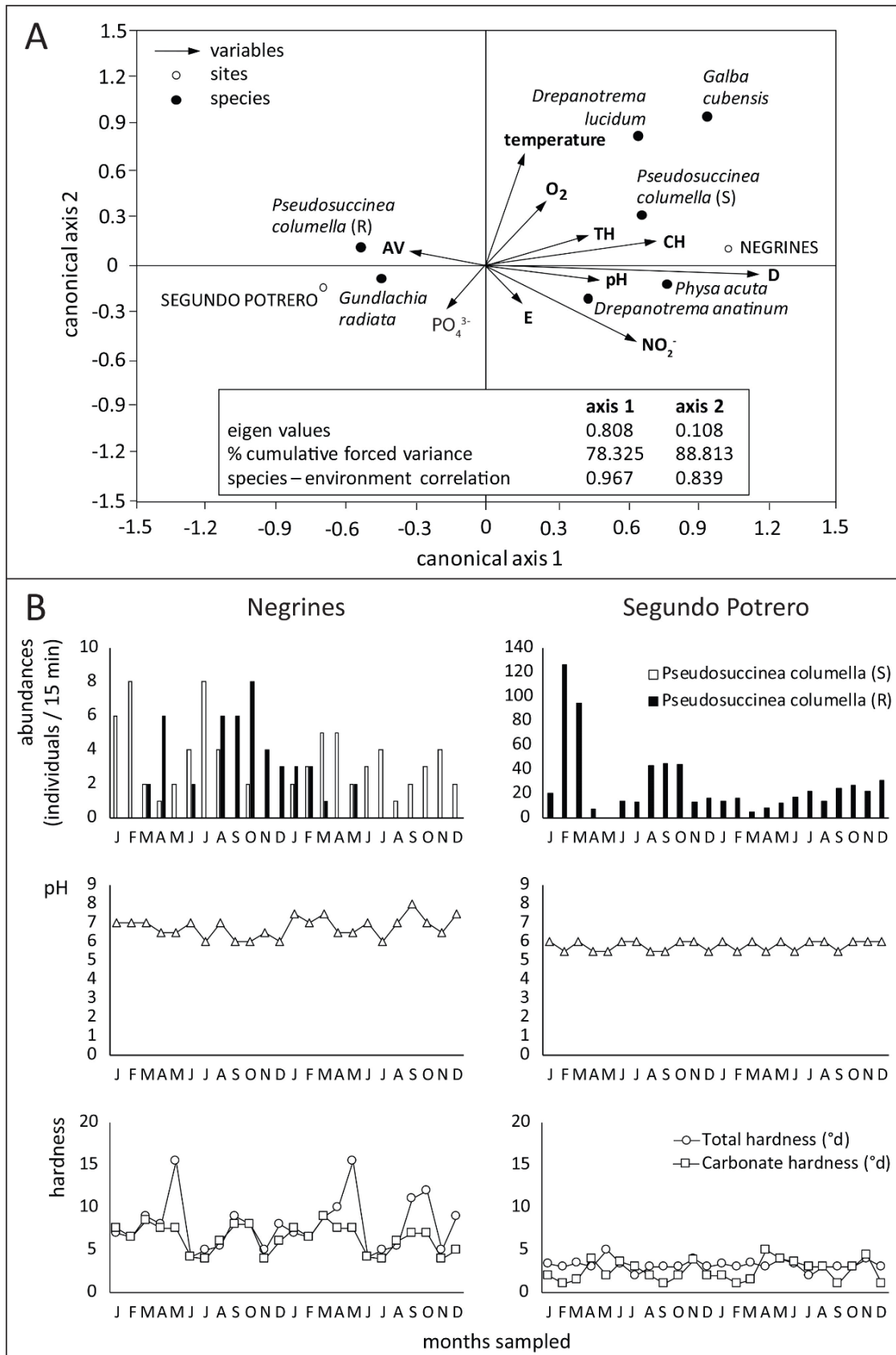


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remaining factors had a minor effect on species abundances. While in Negrines the mean values of pH and TH were around 7.5 and 14°d respectively, Segundo Potrero showed lower values (pH: 5.5-6; TH: 2°d-4°d) with a yearly stability and a high abundance of resistant *P. columella*. However, in Negrines, the population of *P. columella* remained stable at low effective size but with peaks of resistant individuals when susceptible snails decreased or disappeared. Interestingly, such colonization events by the resistant snails in Negrines matched a drop in pH and TH values bringing forward their particular association with water acidity and TH observed in the canonical analysis (Figure 4) and with the overall pattern recorded from the previous analysis (section 3.3.1). Globally, susceptible snails were more abundant when pH ranged between 7 and 8.

Notably, we failed to detect *F. hepatica* infection from *P. columella* during the two-year ecological follow-up study in La Coca. However, while susceptible snails from Negrines were experimentally infected, it was not possible to infect resistant snails with different *F. hepatica* isolates.

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within La Coca locality. AV: aquatic vegetation, CH: Carbonate hardness, D: Simpson's Diversity index, S: susceptible *P. columella*, R: resistant *P. columella*, T: temperature, TH: Total hardness.

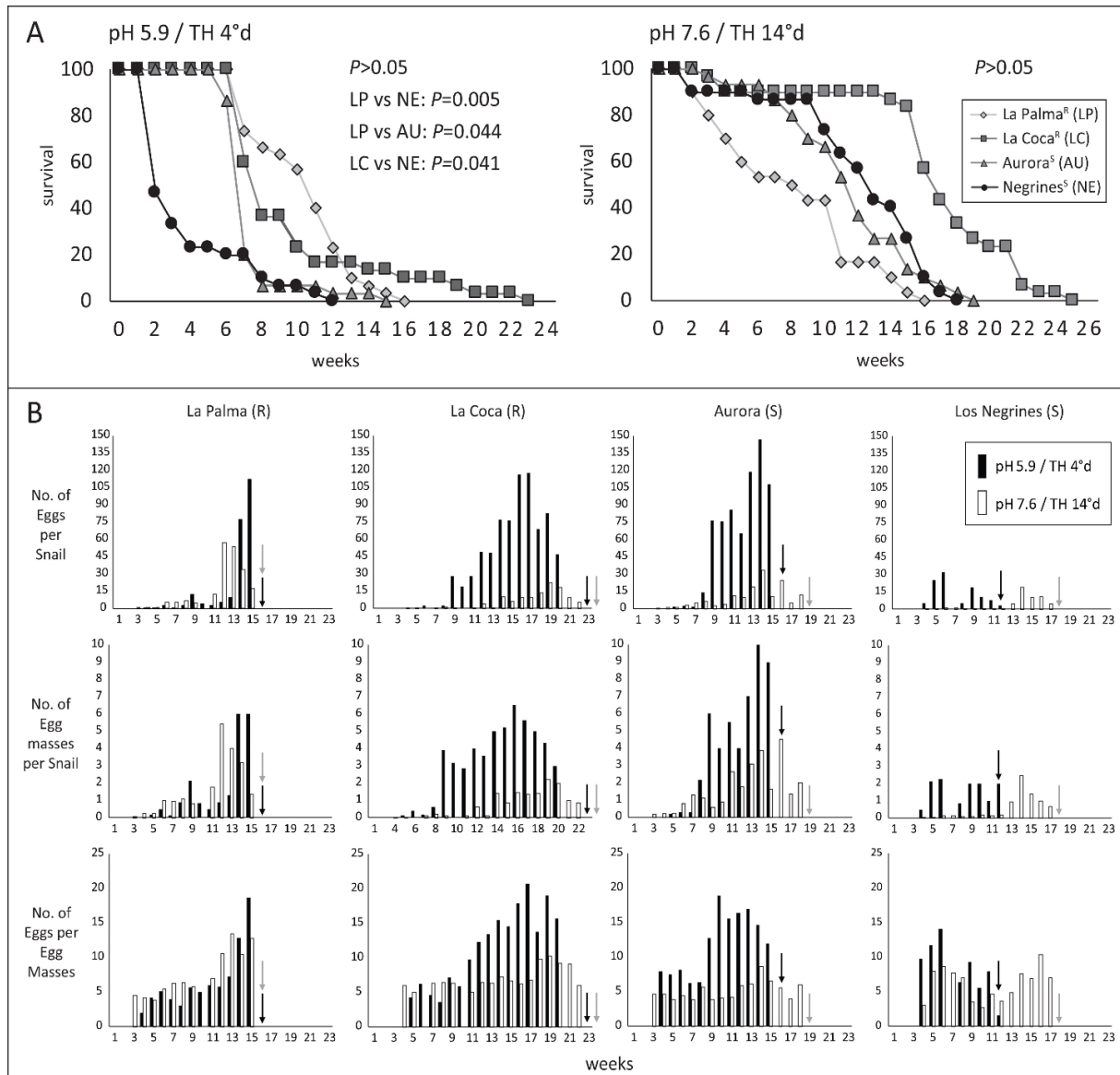
### 3.3.2 Influence of pH and total hardness on experimental life history traits of resistant and susceptible *P. columella*

Given that the evidences gathered from the historical field notes and from the population dynamics in La Coca suggested a differential relation between occurrence of susceptible and resistant individuals concerning the pH/TH (*i.e.* positive association of resistant populations with acidic and soft waters with low species richness), we performed an experimental life table study to assess the effect of these abiotic factors on several life-history traits of *P. columella*. Several age-dependent traits are shown in Table 3 for weeks 0, 5, 9 and 14 considering the start of the experiment, sexual maturity, middle and late snail developmental stages respectively. To facilitate comprehension and elucidate patterns, we will address the results from two different levels of analysis: a) the overall effects on the species and b) the differential effects on the resistant/susceptible phenotypes.

**Table 3.** Experimental life-history traits of resistant (R: La Palma and La Coca) and susceptible (S: Aurora and Negrines) *Pseudosuccinea columella* at different pH and total hardness (TH) conditions ( $e_x$ : life expectancy,  $m_x$ : fecundity rate, NHS within one week: snails that hatched within one week after egg laying, Ro: net reproductive rate, FR: week at first reproduction,  $r$ : intrinsic rate of natural increase,  $\lambda$ : finite rate of natural increase. Data correspond to weeks (W) 0, 5, 9 and 14. The (\*) represents significant differences ( $P < 0.05$ ) for each population when comparing the given trait between experimental settings by Fisher's Exact Tests.

Condition (pH/TH)	Strain	Age-dependent traits								Age-independent traits				NHS within one week (%)	Viable eggs (%)
		W0		W5		W9		W14		FR	Ro	r	$\lambda$		
		$e_x$	$e_x$	$m_x$	$e_x$	$m_x$	$e_x$	$m_x$							
5.9/4°d	La Palma <sup>R</sup>	10.9	5.9	0.7	3.7	12.7	2.3	115.5	4	37.4	0.72	2.06	72.9	99.37	
	La Coca <sup>R</sup>	10.3	5.3	0.6	5.5	27.8	6.1	96.7	5	116.8	0.79	2.20	68.4*	96.46	
	Aurora <sup>S</sup>	7.9	2.9	1.5	5.0	76.5	1.5	147.0	4	37.8	0.73	2.06	53.9*	95.4*	
	Negrines <sup>S</sup>	4.4	4.4	25.0	3.3	28.0	-	-	4	19.2	0.59	1.80	64.8*	67.5*	
7.6/14°d	La Palma <sup>R</sup>	8.6	6.7	1.1	4.0	5.3	2.2	56	3	45.1	0.95	2.59	71.5	98.3	
	La Coca <sup>R</sup>	17.4	13.8	0.2	9.8	0.7	5.0	10.6	4	56.9	0.81	2.24	97.4*	98.7	
	Aurora <sup>S</sup>	12.1	7.6	0.8	5.0	2.6	2.7	33.4	3	56.4	1.00	2.74	92.5*	96.6*	
	Negrines <sup>S</sup>	12.7	8.9	0.3	5.2	0.3	2.5	20.4	4	21.8	0.62	1.85	87.6*	99.2*	

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**Figure 5.** A) Survival curves of resistant (R) and susceptible (S) *Pseudosuccinea columella* populations in which pairwise comparisons of snail survival between population at each experimental setting, performed by log-rank Tests, are also shown. B) Egg production data of experimentally reared *P. columella* snails at pH and total hardness (pH/TH) of 5.9/5°d or 7.6/14°d. Arrows within the egg production data indicate the week at which 100% mortality was observed for the given population at each condition. Results of Factorial ANOVAs were  $P < 0.05$ .

*Overall life-history traits for Pseudosuccinea columella:* Globally, survival of *P. columella* was high during the first eight weeks with a lifespan ranging from 15 to 23 weeks (3 to 5 months). Sexual maturity was reached between the third and fifth weeks, depending on the population

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and experimental setting. Reproductive traits show a steady increase since first egg laying until reaching a peak between week 14 and week 16 (see Table 3 and Figure 5 for details) thus, the survival of most individuals up to late maturity ensures higher biotic potential. Higher overall fitness was observed when *P. columella* was reared at common conditions of 7.6/14°d regardless of the phenotype (Figure 5, Table 3). Low pH/TH values had a negative impact on snail survival with a mortality peak at week 1 for Negrines and around week 7 for the remaining populations, suggesting that it is less suitable for *P. columella* than common conditions (Figure 5A).

Furthermore, fertility traits were also negatively affected with this setting since an overall delay on snail hatching was recorded. At lower pH/TH, a significant delay on NHS within the first week after egg laying was observed for all populations except La Palma, with NHS numbers below 73%, while at common conditions it ranged from 71.5% to 97.4% (Table 3). Also, a delay of up to three weeks of snail hatching (on up to 3.7% of NHS) was only observed when eggs were laid and incubated at 5.9/4°d. In this condition, lower rates of population increase ( $r$  and  $\lambda$ ) were also recorded coupled with a week delay of first egg laying for all populations (except for Negrines) compared to 7.6/14°d settings. Net reproductive rate was also found diminished at 5.9/4°d except for La Coca which presented a two-fold higher  $R_0$  (Table 3). Interestingly, overall higher fecundity rate was observed at 5.9/4°d (Table 3) probably to compensate the negative effects on self-maintenance and reproduction observed when rearing *P. columella* in this condition. However, a significant increase of the mean number of eggs, egg masses and egg per egg masses was only attained by La Coca and Aurora snails at low pH/TH ( $P < 0.05$ ).

*Differential effects of pH and TH at the resistant/susceptible phenotype level:* Despite the overall decrease in *P. columella* fitness at 5.9/4°d compared to 7.6/14°d settings, major differences can be seen between phenotypes. Overall, higher survival ( $P < 0.05$ ; Figure 5) and life expectancy (Table 3) was observed in resistant snails compared to the susceptible isolates at low pH/TH conditions. Similar life spans to those observed at 7.6/14°d were also recorded for the resistant strains (Figure 5A, Table 3). On the other hand, at low pH/TH, resistant snails showed higher numbers of viable eggs compared to susceptible populations ( $P < 0.05$ ) while no significant variations were observed in this trait at common pH/TH (Table 3). Conversely,

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an overall significant decrease in egg viability was observed on susceptible *P. columella* populations at 5.9/4°d (Table 3).

*Overall differences on life traits between populations – possible trade-offs for resistant isolates:* Since trade-offs are expected to occur from selecting resistance and to contribute to its low occurrence in the wild (where common pH/TH conditions are frequent), we compared life traits between the different susceptible and resistant *P. columella* populations. Globally, the lowest biotic potential was recorded in Negrines (susceptible) independently of the condition used, giving little for comparison. On the other hand, the Aurora (susceptible) isolate was characterized by a high performance regarding reproductive traits (high values of  $R_0$ ,  $r$  and  $\lambda$  for both water conditions; Figure 5; Table 3). Particularly, Aurora snails showed a unimodal-like pattern of egg production after the first reproduction from which the highest output of laid eggs was recorded at 5.9/4°d setting (Figure 5). Contrastingly, age-independent reproductive traits of La Palma snails (resistant) were lower in relation to the susceptible population of Aurora, even after the increasing on  $R_0$ ,  $r$  and  $\lambda$  observed at 7.6/14°d (Table 3). In addition, La Palma population showed the lowest percentage of egg hatching when reared at common condition where the fitness for *P. columella* species was demonstrated to be higher (Table 3). In the case of the resistant population from La Coca, we recorded overall lower fecundity rates than those from Aurora and even Negrines (Table 3).

## 4. Discussion

### 4.1. Distribution and population genetics of *P. columella* in Cuba: insights into its history and colonization and its relationship with *Fasciola hepatica* transmission

*Pseudosuccinea columella* is considered native from North America but has been largely introduced out of its native range due to natural (e.g. aquatic birds, flooding) or human-mediated invasion [7]. The date of introduction of *P. columella* in Cuba is unknown, but it was first reported in 1858 by Poey under the name of *Lymnaea francisca*. It is then reasonable to think of a first introduction around the early 19<sup>th</sup> century that allowed *P. columella* to settle in the west of Cuba where earlier and more abundant records exist. The close position of Cuba with the North American continent supposes an easy opportunity for natural introductions, particularly in this particular region. However, from our results, several facts suggest that successive introductions of *P. columella* occurred successfully in Cuba, mainly in the west but

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also in the central region. A sluggish invasion of this species to the eastern region of the island, mostly by invasive genotypes more recently introduced (*e.g.* MLGT Y), is presumed to remain in course. Perhaps the most conspicuous evidences that can be brought forward to endorse the presented assumption are: (1) the increased number of records in the latest years from central-east and its complete absence in the easternmost region; (2) the higher allelic and multilocus genotypic richness in the west (particularly in resistant populations); (3) the existence of three major clusters of MLGTs and the presence of some MLGTs only found in the central region of Cuba, which contrasts with (4) the similar number of MLGTs shared by most susceptible *P. columella* populations (with five that were completely monomorphic) and the widespread distribution of MLGT (Y) in Cuba. The overall low genetic structuration and diversity found in *P. columella* can be explained by recent genetic bottlenecks that usually occur after introduction events, followed by the expansion of certain invasive genotypes such as MLGT Y (see Lounnas et al. [7]).

Particularly, regarding resistant *P. columella*, previous studies showed that these populations differ from those susceptible. Gutiérrez et al. [23] detected different RAPD profiles using 17 different primers. In another study, Calienes et al. [21] made the first approach to detect clear segregation when comparing polymorphic DNA of three resistant populations (La Palma, El Azufre, Babiney) and nine susceptible populations using RAPD profiles. In the present study, we found that the genetic structure of the three resistant populations analysed (El Azufre, La Coca and Babiney) is significantly different in terms of presence and amount of MLGTs from susceptible populations, with higher genotypic diversity. The clear segregation into different susceptible and resistant MLGT associated clusters observed here, support the idea of an early, different and detached history from susceptible *P. columella* populations. Contrastingly with most susceptible populations, MLGTs from west and central populations cluster separately from each other, thus it is likely that they originated after different introduction events. Additionally, most resistant isolates displayed significant  $F_{ST}$  values suggesting an *in-situ* evolution. Particularly, the lower genotypic diversity found in the population from El Azufre could be related to continuous genetic bottlenecks due to its occurrence in an isolated spring that could be severely affected by droughts. This effect may not be impacting resistant populations from La Coca and Babiney, both showing the highest genotypic diversity, probably because they are established in open flooded terrains more stables over time.

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Concerning parasite transmission, the observed distribution pattern (range and number of populations) in *P. columella* compared to the other lymnaeid *G. cubensis* (over 100 recorded populations all over the Cuban archipelago; see [15]) supports the presumed secondary role for this species as an intermediate host of *F. hepatica* in Cuba. A recent world-scale genetic study on *P. columella* considered one of our recorded MLGT (*T*) as invasive after it was detected in Colombia, Peru, Venezuela, South Africa and the Indian Ocean [7]. We observed this genotype in two of the easternmost populations in Cuba (Río Central and Matadero Aves). Thus, a possibility rises for this invasive MLGT to be able to fully spread and colonize eastern Cuba in the future. Additionally, the presumed recently introduced MLGT *Y* (only found in monomorphic populations) may in fact presented itself as ecologically adaptable (found in 15 out of 20 analysed populations) and highly compatible with *F. hepatica*. The, so far, only five individuals found naturally-infected with *F. hepatica* in Cuba correspond to this very same MLGT (two individuals from IPA and three from Pilón). From this, if populations showing certain biased inclinations to become infected match those also presenting a wide ecological tolerance, it can be presumed a highly favourable scenario for *F. hepatica* transmission in Cuba since it increases the probability for the parasite to find suitable host. In any case, the lack of genetic diversity has been already pointed out as an advantage for parasites to overspread in a given host population [42], and overall, susceptible populations of *P. columella* showed no significant differentiation (low  $F_{ST}$ ,  $P > 0.05$ ).

In the present study, the low allelic richness per population as well as the strong deviations from panmixia suggest a high self-fertilization rate in this species as also shown by Lounnas et al. [7]. Other lymnaeid snails such as *G. truncatula* [43] and *Omphiscola glabra* [44] also prefer self-crossing as a reproductive mode. Similarly, other studies have observed the same low allelic richness pattern in *P. columella* that we have found [25]. However, here, three populations of *P. columella* (IPA, Río Central and Río Arimao) showed lower self-fertilization rates than those previously reported. Several studies have indicated that cross-fertilization may represent a selective advantage when populations are under severe parasitic pressures [45, 46]. Conversely, self-fertilization can be selected in stochastic environments [47] securing reproduction even if only one individual survives the harsh conditions. Thus, we should expect lower self-fertilization rates in stable habitats or endemic areas for parasite transmission. The locality IPA matches both criteria since it was previously suggested as a fasciolosis



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transmission site by Gutiérrez et al. [48], and consists of a permanent pond used to stock water. In fact, the two infected individuals belonged to this locality. The other two localities (Rio Central and Rio Arimao) are recognised as fasciolosis areas as high bovine prevalence are reported [49].

It is important to note that, despite the fact that the establishment of resistant populations decreases the chances for transmission of the liver fluke to occur, the observed distribution pattern of such phenotype indicates that there is an ecological cost for resistance (particularly if we accept at least 200 years of its presence in Cuba) which was previously suggested by Gutiérrez et al. [50, 51]. Thus, a deeper look into the ecological patterns associated with the occurrence of this phenotype in nature is mandatory in the pursuit of using this phenomenon to develop novel control strategies for *F. hepatica* transmission.

#### **4.2. Ecological patterns associated with resistant and susceptible *P. columella* from Cuba: insights into the cost of resistance**

Lymnaeid snails are known to occur worldwide in an extensive range of habitat types [1]. In Cuba, it has been shown previously that *P. columella* does not exhibit particular preferences regarding natural or transformed habitats and contrasts with its relative *G. cubensis*, commonly established in anthropic sites [15]. *Pseudosuccinea columella* is highly aquatic and consequently could be more severely affected by the physical and chemical factors of the water, a fact that could be linked to its overall preference for closed freshwater systems and, particularly, to habitats such as the permanent ponds recorded herein. A previous study by Gutiérrez [39] in Pinar del Río, Cuba, showed a positive correlation between the abundances of *P. columella*, the pH and the concentrations of nitrites. Here, we bring forward a different effect considering separately the overall pattern of occurrence of resistant and susceptible individuals and following the ecological dynamics of those living in sympatry. As presented here, the conditions for occurrence of susceptible individuals in terms of pH, TH and the concentration of nitrites and phosphates are similar to those reported by Perera [34] elsewhere in Cuba. However, resistant snails present a marked association to lower values of water pH and TH and a tendency to colonize sites with low species richness. This result could be explained by two hypotheses:

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(1) A plausible hypothesis could be that those conditions would be essential for the survival of resistant snails in the field which would have a different ecology from susceptible snails. This ecological segregation might guide us to think in a possible stronger differentiation of each phenotype ending in speciation. However, some resistant *P. columella* have been kept in our laboratory for more than 20 years at pH/TH conditions ranging between 7-8/12-18°d. In addition, in France, invasive and *F. hepatica*-susceptible populations of *P. columella* have been reported in sites characterized by acidic soils and soft waters [52, 53]. Both facts suggest that, as belonging to the same species, both *P. columella* phenotypes can tolerate such pH and TH variations rather than be differentially-restricted to specific values of these parameters. Our experimental life-history traits also support the former since higher overall fitness was observed at common water conditions (pH/TH: 7.6/12°d) regardless of *P. columella* phenotype (Table 3; Figure 5). However, in this sense, an overall higher tolerance at low pH/TH conditions, in terms of self-maintenance (survival and life expectancy) and reproductive (egg hatching) traits, was observed to be associated with resistant isolates.

(2) Thus, a second hypothesis could be that low pH and TH are much less suitable for other species (with narrower tolerance levels) directly reducing competition. In this sense, our results have demonstrated that such conditions negatively affect some demographic traits of *P. columella* in favour of resistant phenotypes. Therefore, it is hard to explain a maintenance of resistant individuals in 'normal' (where common pH/TH exists) sites unless colonisation events by competitor species are rare and that resistant individuals had been the only settled. This was observed from our field results since colonization of Negrines by resistant snails coincided with a decrease of pH and TH, and lower abundances of susceptible individuals (Figure 4). This hypothesis can also be supported by the observed pattern of low species richness in slightly acid/soft water-sites where resistant populations are found. Particularly, in a colonisation event of such suitable sites by susceptible *P. columella* we should expect a replacement of the resistant snails. Under experimental conditions Gutiérrez et al. [51] reported that resistant *P. columella* are less competitive than susceptible snails in terms of reproductive traits (reduced  $R_0$  when raised in the presence of susceptible snails). The latter contrasted with an increase of shell growth and  $R_0$  on susceptible *P. columella* when reared with resistant individuals [51]. Summing up, the restricted distribution and the negative

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overall correlation of resistant *P. columella* and species diversity can be interpreted as an ecological cost of resistance, explained by a less competitive potential of resistant snails.

Assessing the competitive potential of resistant isolates in relation with susceptible *P. columella* and other freshwater snail species could be experimentally challenging. Yet, identifying potential trade-offs associated with this phenotype might bring forward some understanding. A previous study demonstrated lower  $R_0$  for La Palma (resistant) snails compared to susceptible *P. columella* populations [50]. Evidences of trade-off regarding reproduction for La Palma and La Coca resistant isolates are also reported in the present study (La Palma: lowest egg hatching at common conditions, lower  $R_0$  compared to Aurora snails, lower fecundity rates in La Coca strain compared to both susceptible isolates) and could account for the preclude of a natural dispersion of this phenotype in Cuba where the most common conditions of Cuban freshwater bodies include pH and TH ranging from 7-8 and 12-18°d [34].

### **Concluding remarks**

The introductions of vectors and intermediate hosts are significant issues because of the epidemiological risks they pose. In fact, recently introduced populations suffer strong genetic bottlenecks that drag off former genetic diversity preventing them from acquiring resistance to local parasites [42]. Genetic flow could act as a force that increases this diversity but, contrarily to what may be occurring in the liver fluke populations from Cuba (high cross-fertilisation and bad management of cattle that mixes the strains; [54]), it is unlikely to take place at effective rates in *P. columella* snails. However, as discussed above, populations carrying some highly invasive MLGTs are colonizing a number of available sites in Cuba, one of which (MLGT Y) was also directly related with *F. hepatica* transmission in the present study. Thus, it is plausible that the ability of *P. columella* individuals with this MLGT to invade represents a true danger regarding transmission of the liver fluke. In any case, it is of major importance to maintain an active surveillance of parasite prevalence in *P. columella* and of potentially new introductions of this species in different and distant regions of Cuba and of the world.

On the other hand, while the spread of resistant *P. columella* could definitively aid tipping the scales towards the effective control of *F. hepatica* transmission, our results have shown that

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a resistance-related cost exists, as previously suggested by Gutiérrez et al. [50, 51]. It is, thus, highly improbable that genotypes with invading abilities also correspond to those resistant to *F. hepatica*. However, possible variants to exploit the existence of resistant vectors can be tested for which the present work constitutes a building step. Knowledge regarding the ecological cost of resistance and the patterns associated with its occurrence in nature attained from our results could be used for planning a human-mediated introduction of resistant snails in particular high-risk transmission foci. Other strategies towards the incorporation of resistant features into susceptible populations by out-crossing could be challenging if we considered the high self-fertilization rates of *P. columella* showed herein and elsewhere [7] and the absence of evidences linked to such phenomena from the wild, where resistant and susceptible individuals inhabit in sympatry. On the other hand, other approaches like genetic manipulation, the controlled selection of resistant features or induction of resistance-mediated mechanisms in wild vector populations might turn out feasible. However, for any of these to be applied, a deeper and wider comprehension of the molecular and immunological bases mediating the natural resistance of *P. columella* to *F. hepatica* is mandatory.

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## CHAPTER 2

### Constitutive molecular features of *P. columella* resistant and susceptible to *F. hepatica*: comparative omics approaches

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The increasing accessibility of “omics” approaches combined with high throughput put methods belonging to next sequencing technology has made possible the generation of enough data as to enlarge our view of single species (Cwiklinsky et al. 2015) and host-parasite interactions (Westermann et al. 2017). Particular emphasis has been made on the usefulness of these technologies for the deeper comprehension of snail – trematode systems (Schultz & Adema, 2017; Portet et al. 2017) which have proved their worth in previous works (e.g. Pinaud et al. 2016; Buddenborg et al. 2017; Wu et al. 2017).

In this chapter we will be reviewing the differential constitutive molecular features associated with resistant and susceptible *P. columella* (article 2). Resistant snails are able to efficiently eliminate *F. hepatica* within the multilayered hemocytic capsule at early times after penetration (Gutiérrez et al. 2003a). Thus, we argue that analyzing constitutive differences between naïve snails using comparative transcriptomic and proteomic approaches could be a good strategy for identifying relevant resistant-dependent molecular processes. Through both omics approaches particularly relevant transcripts/proteins associated to each phenotype could be identified which will become the first candidates to further look for when unveiling the immunobiological bases of *F. hepatica* resistance/susceptibility in *P. columella*.

On an initial attempt, we will obtain the first whole-snail transcriptome of *P. columella* with samples from both phenotypes. From this, we aim at increasing the availability of molecular information on this species by generating a large dataset of sequences that could be used as a cornerstone for a wider variety of studies. Furthermore, the screening and further comparison of the constitutive transcripts expressed on naïve resistant and susceptible snails will allow us to gain insights into the molecular landscape and overall allocation of resources related to both *P. columella* phenotypes prior infection. In this sense, it could be

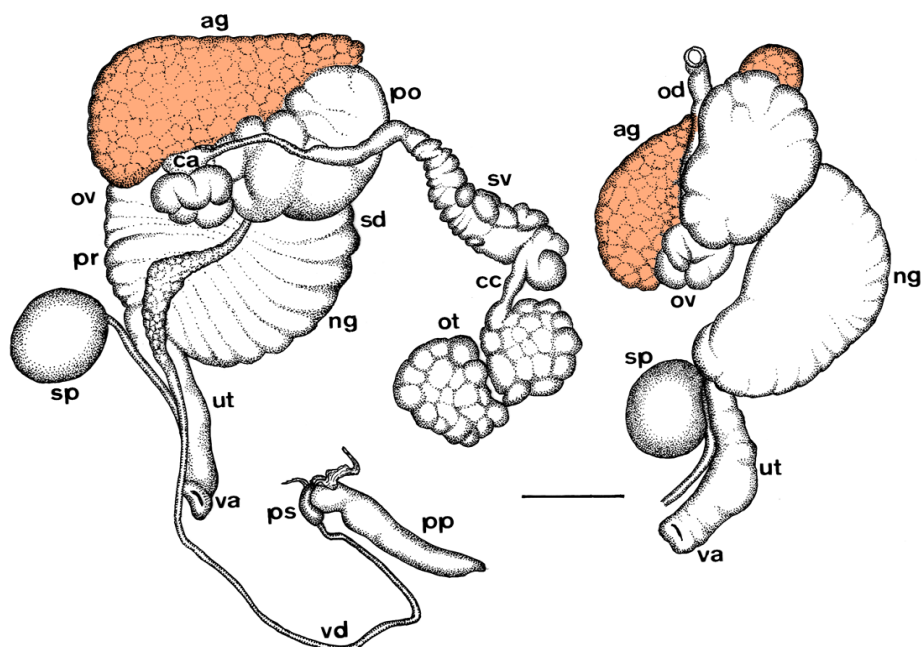


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expected that the significant differences observed between susceptible and resistant *P. columella* at the phenotypic and even ecological level should echo the differential molecular features even in naïve snails.

Additionally, we will focus the attention on the albumen gland, an accessory organ of the reproductive system of mollusks (see Fig. 20 for details on the reproductive system of *P. columella*) that has been associated with the production of molecules involved in immunity (Sanchez et al. 2006; Hathaway et al. 2010). Given such multifunctional character of this organ, the analysis of the differentially-represented proteins between susceptible and resistant snails on naïve conditions could give us information regarding the investment into reproduction against survival (as fighting back parasite infection) at the molecular level. In fact, differences are likely to exist as a fitness cost for resistance is expected to occur (see results of Chapter 1) and has been previously suggested in this model (Gutiérrez et al. 2002). In this sense, it is worth mentioning that the generation of the transcriptome of *P. columella* was essential for the identification of the differentially-represented proteins of the albumen gland of this species as former screening using available databases of the planorbid *B. glabrata* and of the lymnaeid *L. stagnalis* proved to be non-useful.



**Figure 20.** Schematic drawing of the reproductive system of *Pseudosuccinea columella* where the albumen gland is colored; ag: albumen gland, ca: carrefour, cc: collecting canal, ng: nidamental gland, od: ovispermiduct, ov: oviduct, ot: ovotestis, po: pouch of oviduct, pp: preputium, pr: prostate, ps: penis sheath, sd: spermiduct, sp: spermatheca, sv: seminal vesicle, ut: uterus, va: vagina, vd: vas deferens. Scale bar: 1 mm. Modified from Pointier et al. 2005.

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**Article 2 (in preparation)**

***Molecular bases of the resistance to *Fasciola hepatica* in *Pseudosuccinea columella* snails: insights from comparative omics approaches***

**Molecular basis of the resistance to *Fasciola hepatica* in naturally-occurring *Pseudosuccinea columella* snails: insights from comparative omics analyses**

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## Abstract

*Fasciola hepatica* is a worldwide distributed snail-borne trematode that affects humans, livestock and wildlife. In Cuba, the occurrence of naturally resistant and susceptible populations to *F. hepatica* infection within one of its snail vector species (*Pseudosuccinea columella*) has prompted us to carry out investigations directed to the understanding of this host-parasite system. To elucidate the molecular bases involved in the phenotypic and ecological features associated with resistance in *P. columella* (*i.e.* rapid parasite encapsulation by snail's immune cells and higher tolerance to pH and total hardness water variations), a comparative transcriptomic approach, by Illumina sequencing at whole snail level, was performed on naïve snails. In addition, as a fitness cost regarding reproduction exists in resistant *P. columella*, a comparative screening by two-dimensional electrophoresis of the albumen gland, an organ involved in both reproduction and immunity have been also conducted. Both omics approaches showed a constitutive enrichment on biological functions and proteins associated with defense/stress and a significant abundance of immune-related molecules (*e.g.* several lectins, pro-inflammatory signaling pathways, cytokines, antimicrobial molecules) on resistant compared to susceptible snails that could account for the early and efficient immune response that develops towards *F. hepatica*. This indicates a differential allocation of resources towards self-maintenance and survival of resistant individuals, that could be possibly linked to the underrepresentation of metabolic functions and proteins related with peptide/protein anabolism also observed from both approaches. All these could explain the trade-off against reproduction and the ecological cost of resistance associated with its restricted distribution in nature only to low pH/total hardness freshwaters. Concerning the latter, besides the overall abundance of functions related with response to stress and homeostasis maintenance, we also observed an over-representation of several transcripts related with pH and osmotic regulation (*i.e.* carbonic anhydrase and major ion regulators) on resistant *P. columella*. These points at a higher potential for acid-base regulation, at the molecular level, associated with these snails and also endorse these particular phenotypical and ecological features.

**Keywords:** lymnaeid snails, transcriptome, albumen gland, allocation of resources, response to stress, immune defense

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## Introduction

The species *Pseudosuccinea columella* is one of the widest distributed and more efficient invader snails within the Lymnaeidae family [1]. It participates as intermediate host in the transmission of *Fasciola hepatica*, a veterinary and medically-significant trematode which is the main etiological agent of fasciolosis, a worldwide distributed infection that is considered re-emergent [2]. So far, two *P. columella* phenotypes have been described concerning infection: while most snails can be infected with *F. hepatica* (susceptible populations), few natural populations found in Cuba appeared as resistant [3, 4]. Within resistant *P. columella*, the immune cells (hemocytes) encapsulate *F. hepatica* larva shortly after penetration, resulting in an active resistance [4] irrespective of the parasite isolates [5, 6]. In addition to the latter, results from previous field and experimental studies on resistant snails have brought forward interesting features of this *P. columella* phenotype that highlights the complexity of this phenomenon: while resistant populations showed a higher tolerance to pH and total hardness variations [7], trade-offs against reproduction has been suggested as resistant populations laid fewer eggs per snails than susceptible isolates [7, 8].

Understanding this host-parasite model and, particularly the resistance, is of high interest as it could eventually lead to the development of new strategies for controlling *F. hepatica* transmission. However, despite all the gathered phenotypical descriptions concerning *F. hepatica* resistance in *P. columella*, no studies have been undertaken to decipher the molecular basis behind each phenotype. Here, we explored for the first time the transcriptome of this species and informed on constitutive differences between resistant and susceptible snails that endorse the phenotypic features observed on the former concerning infection and osmotic and acid-base balance.

In addition, we also focused attention on the albumen gland, an accessory organ within the female reproductive system mainly committed with production of the perivitelline fluid to nurture the embryos [9, 10], but also with the synthesis of a number of immune effectors and defense proteins [11-14]. In this sense, we performed two-dimensional (2D) gel electrophoresis of this multifunctional organ and studied differentially abundant spots between susceptible and resistant snails to observe a possible distinct allocation of resources to defense/stress in resistant snails that could explain their reproductive impairments.

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Herein, we found overall similar patterns between the transcriptomic and the proteomic approaches with a significant bias towards an overall abundance of defense and stress-related process/ transcripts/proteins in resistant compared to susceptible snails. These results contribute to endorse self-maintenance as favored strategy of resistant populations. Differences in metabolic planning in the detriment of protein synthesis/folding was also observed from both “omics” analyses and could be particularly significant in the case of the albumen gland, as to the contribution to reproductive trade-offs. The molecular differences found between resistant and susceptible strains are discussed at the view of their differential phenotypic and ecological features. In addition, this paper presents a list of candidate proteins which roles can be further validated and used as predictors of infection or as potential targets for controlling infection.

## Material and methods

### *Laboratory-reared P. columella snails*

Five-week-old *P. columella* snails from *F. hepatica*-resistant (La Coca) and -susceptible (Aurora) populations, reared in the Laboratory of Malacology of the IPK (see Sánchez, Perera [15] for details on rearing) were used for the transcriptomic and the proteomic approaches. To gain an overview on constitutive differences between phenotypes, only naive snails (non-exposed to parasite infection) were sampled.

### ***Comparative transcriptomic approach on whole snail***

*RNA extraction and sequencing by Illumina:* Sixty *P. columella* snails per strain were carefully separated from the shell and immediately placed in individual vials containing RNA*later*<sup>®</sup> (Ambion) solution at a ratio of 1 mg of snail: 10 µL of RNA*later*<sup>®</sup>, w/v. Samples were kept overnight at 4°C and then stored at -20°C until use.

RNA*later*<sup>®</sup> was discarded and whole-tissue of 20 snails per strain (biological replicates) was pooled, immediately frozen in liquid nitrogen and homogenized. RNA extraction was performed with TRIzol<sup>®</sup> Reagent (Ambion) following manufacturer’s guidelines. Extracted RNA was cleaned up with RNeasy<sup>®</sup> Minikit (Qiagen) and an in-column DNase treatment with Turbo DNA-*free*<sup>™</sup> kit (Ambion) was also performed. The quality of the extracted RNA was assessed on a 2100 Bioanalyzer using an RNA 6000 Nano kit (Agilent Technologies) and its amount was estimated by Qubit<sup>™</sup> 2.0 fluorometric quantitation (Invitrogen).

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Equal volumes of total RNA from each technical replicate per biological replicate were pooled ( $n = 3/\text{strain}$ ) up to 1  $\mu\text{g}$  of RNA as final amount. Library preparation and RNA sequencing was performed by FASTERIS SA (Geneva, Switzerland) in paired-end, 75-bp read length, with three samples multiplexed per lane, using Illumina HiSeq™4000 platform.

*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 of Trinity 2.0.6.1 method [16] on the Galaxy Project server [17] and resulted in 158 537 transcripts. To reduce its complexity, Trinity Super Assembly [16] was applied to the *de novo* assembled transcriptome, and all transcripts shorter than 300 pb were removed, with 78 774 transcripts remaining for further analysis. Afterwards, we reduce hypervariable families by CD-Hit-est [18] to cluster transcripts with matches above 95% identity, resulting in 72748 transcripts. Transcriptomes are available at laboratory database: <http://cbetm.univ-perp.fr/blast/blast.html>.

The automatic annotation of the final transcriptome was performed by Blast2GO version 2.4.2 [19] using BLASTx against the National Center for Biotechnology Information (NCBI) non-redundant (nr) sequences database (with an E-value threshold set at  $1\text{E}-03$ ) was performed. Gene function, protein domain and enzyme annotation were also assigned by search similarities on Gene Ontology (GO) database and InterPro scan.

*Comparative analysis between resistant and susceptible P. columella - enrichment analysis and overview of expressed genes related with immunity and acid-base balance:* For comparative analysis between susceptible and resistant *P. columella* phenotypes, quality reads (phred score > 38) were aligned to the assembled transcriptome using Bowtie2 v 2.0.2 [20] set in fast end-to-end. Transcripts were counted with Salmon [21] and differential expression was calculated by DESeq2 [22], 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 change were considered. All RNAseq analysis was run locally using the Galaxy Project server [17].

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 was considered enriched when  $P$  and false discovery rate (FDR) < 0.05. In addition, differentially-

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expressed transcripts were revised based on keyword list developed in our laboratory and on Gene Ontology (GO) terms 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).

### ***Comparative proteomic approach on the albumen gland***

*Sampling the albumen gland:* The albumen gland of 15 naive snails from each strain was carefully removed by dissection, lyophilized and individually processed. Briefly, the crude extract of the albumen gland of each individual snail was obtained by sonication on ice at 20% amplitude for 20 s in 80  $\mu$ L of ice-cold denaturing buffer (thiourea 2 M, urea 7 M, CHAPS 4% in Tris-HCl 30 mM, pH 8.5) and clarified by centrifugation at 2 000  $\times$  *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.

*2D-electrophoresis of the albumen gland of P. columella:* 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  $\mu$ g 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  $\mu$ L. They were then individually loaded onto 17 cm isoelectric focusing strips (BioRad) with a stabilized non-linear pH ranging from 3 to 10. Strip rehydration was achieved by 5h of passive rehydration at 22°C, followed by an active rehydration of 14 h under a 50 V current (to help big proteins enter into the strips) at the same temperature.

Afterwards, migration of the proteins in function of their isoelectric points 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 V.h 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, they 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 also 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

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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).

*Comparative analysis of 2D-gels between susceptible and resistant *P. columella**: Considering the complexity of the profile of albumen gland, some samples were better resolved by 2D-gels than others and we kept the better and most representative ones for the analysis. Five gels per strain were then selected for comparative analysis on PD-Quest v. 7.4.0 (Bio-Rad) to identify changes in protein abundance between proteomic profiles of albumen glands from susceptible and resistant individuals. 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. Assortment of spots for further analysis by MS/MS spectrometry was based on qualitative (*i.e.* to be present in one of two phenotypes) or highly quantitative differences (*i.e.* differentially abundant in more than four-fold;  $P < 0.01$ ). Differentially represented spots were then 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 confronted to the consensus *de novo* assembled reference transcriptome for *P. columella* of 158 837 transcripts. The transcript sequences confidently matched by the peptides were retrieved and they were used as a query for a xBLAST against non-redundant databases to determine the protein identity of the best match. We considered for this, up to the first three best hits when at least 4 peptides matched the sequence with a coverage  $> 10\%$ . Identified transcripts sequences were translated into protein using Expsy server [23] to identify conserved domains with CDD [24]. Their theoretical pI and molecular weight were also calculated using the Expsy server [23] to compare them with the location of the spot on the gel. Altogether, these complementary analyses allow to confidently characterize the protein identity of each spot.



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## Results

### ***De novo transcriptome of P. columella: constitutive differences between susceptible and resistant P. columella phenotypes***

From a total of 592 million raw reads, the first consensus transcriptome for *P. columella* was assembled into 158 837 contigs, representing all the potential assembled mRNAs from both *P. columella* phenotypes: including spliced variants, non-overlapping fragments of the same mRNA, UTRs or mRNAs in different splicing stages. For analysis, the complexity of the transcriptome was reduced to 78 774 transcripts (see material and methods section for details) but annotation rendered results for only 38 968 transcripts (49.5%). Afterwards, 97.49% of the reads were successfully realigned against the reduced transcriptome and 6876 transcripts (9.45%; 4126 up-regulated and 2750 down-regulated) were accounted to significantly show more than 2-fold differential-expression between phenotypes (see supplementary file 1). From these, 3290 transcripts (47.5%) had no annotation results.

Results from the enrichment analyses performed with each over and under-represented set data of transcripts (test data) against the annotated transcriptome (reference data) are shown in Figures 1 and 2. Noteworthy, as it could be expected, several biological processes related with defense (*e.g.* immune response, defense response, apoptotic process, response to molecules of bacterial origin) and response to stress (*e.g.* cellular response to stress and to DNA damage, response to chemical, positive regulation of response to stimulus) were found to be particularly enriched and associated with the resistant phenotype. In addition, processes involved in nitrogen metabolism particularly biosynthesis, maintenance and repair of DNA, and with biological regulation and homeostasis were also particularly involved with resistant snails. All these are possibly linked to the enrichment observed on primary signaling pathways (*e.g.* I- $\kappa$ B kinase/NF- $\kappa$ B) and molecular functions related with signal transduction (*e.g.* transmembrane receptor activity, G-protein coupled receptor activity and protein tyrosine phosphatase activity). Interestingly, Ca<sup>2+</sup> binding was also found to be particularly enriched and associated to the resistant phenotype (Figure 1). Contrastingly, enrichment analysis derived from underrepresented transcripts showed that biological processes and molecular functions particularly related with protein biosynthesis (*e.g.* ribosome biogenesis, structural constituent of ribosome, peptide biosynthetic process, translation) were negatively associated with the resistant phenotype (Figure 2).

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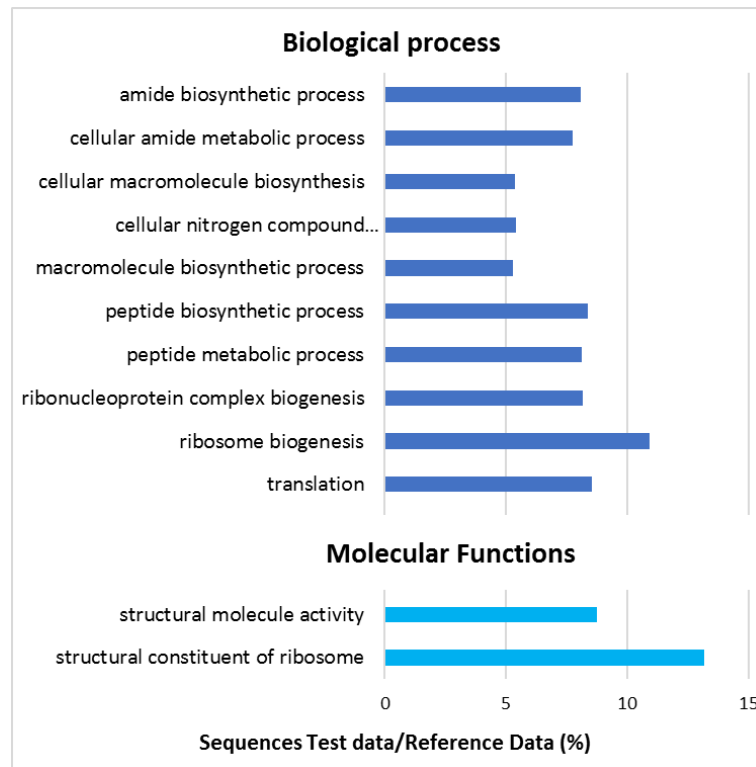


**Figure 1.** Enriched biological process (left) and molecular functions (right) of *Pseudosuccinea columella* transcriptome that are particularly involved in the resistant phenotype. Each bar

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represents the percentage of unisequences found in the test data (over-represented transcripts in resistant *P. columella*) according to their GO terms.



**Figure 2.** Enriched biological process and molecular functions of *Pseudosuccinea columella* transcriptome that are particularly underrepresented in the resistant phenotype. Each bar represents the percentage of unisequences found in the test data (under-represented transcripts in resistant *P. columella*) according to their GO terms.

### ***Differentially represented transcripts related with immunity and acid-base balance between resistant and susceptible P. columella***

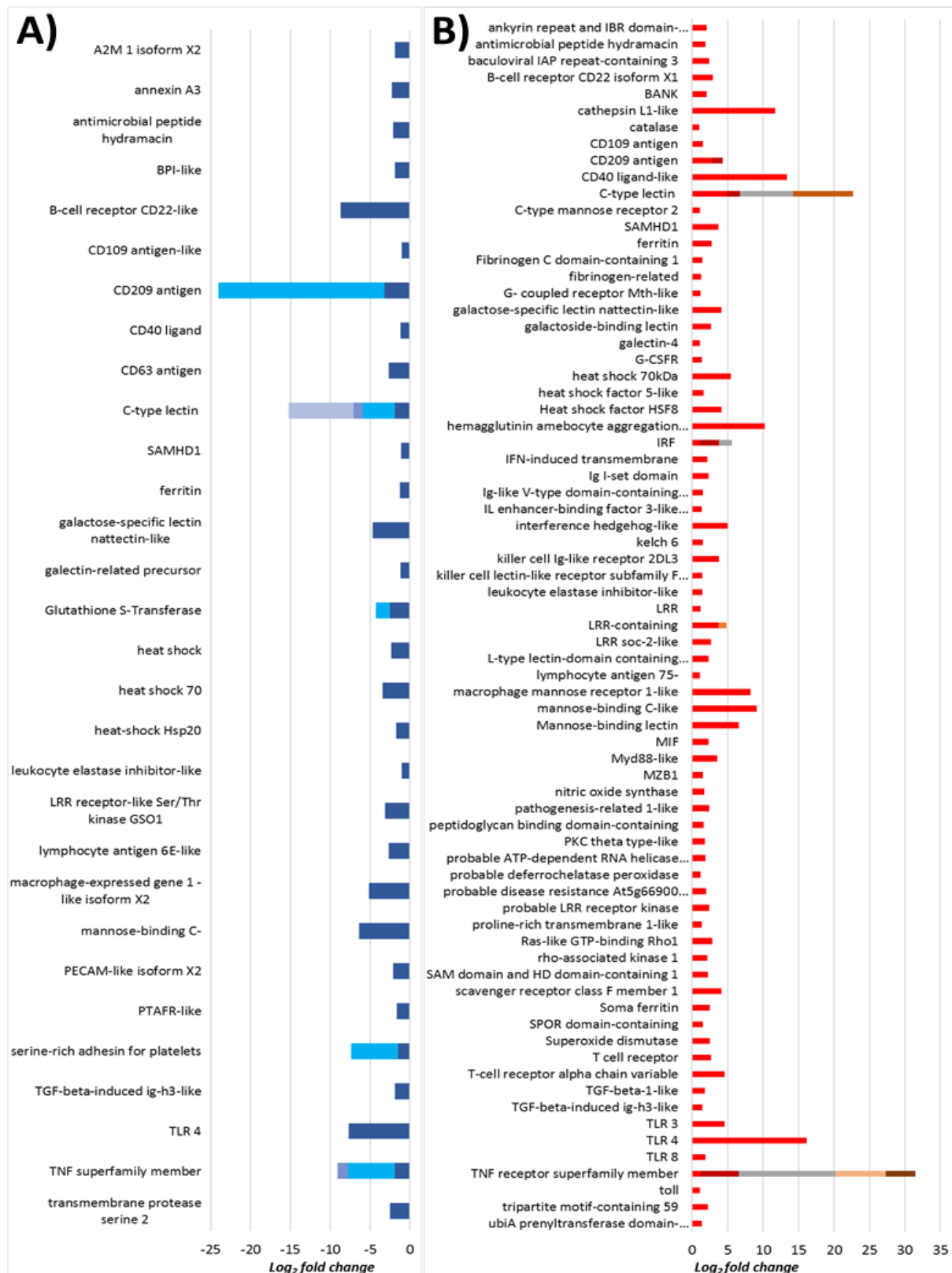
Differentially-expressed transcripts related with defense response in *P. columella* are shown in Figure 3. An overall over-representation of several pathogen recognition receptor/adhesion molecules such as several lectins (*e.g.* C-type lectin, mannose and galactose binding molecules), CD109, molecules with peptidoglycan binding domains were observed in naive resistant snails (Figure 3b). In addition, higher abundance and diversity of transcripts with regulatory roles in immunity like several molecules with leucine-rich repeat domains (LRR), macrophage migration inhibitory factor (MIF), granulocyte colony stimulatory factor (G-CSFR), transforming growth factor 1-beta (TGF1B) and lymphocyte antigen 75 were recorded in the resistant isolate. In this sense, it is also noteworthy the

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over-representation of transcripts related with signaling pathways involved in the activation and orchestration of a defense responses such as several Toll-like receptors (TLR) and the myeloid differentiation primary response (Myd88), different members of the superfamily of tumoral necrosis factor (TNF) receptors, protein C kinase (PKC) theta, L-type lectin-domain containing receptor kinase-like and interferon regulatory factors (IRF). Anti-parasitic defense like ferritin and nitric oxide synthase were also significantly abundant in resistant snails (Figure 3b). Higher diversity of other overexpressed immune-relevant transcripts related with leukocyte, adhesion, rolling and tethering (*i.e.* interference hedgehog-like, Ras-like GTP-binding Rho1, rho-associated kinase 1), wound healing (*i.e.*: Ras-like GTP-binding Rho1) and antioxidant molecules (*i.e.*: catalase, superoxide dismutase (SOD) and probable deferrochelatase peroxidase) were also significantly abundant in resistant snails (Figure 3b).

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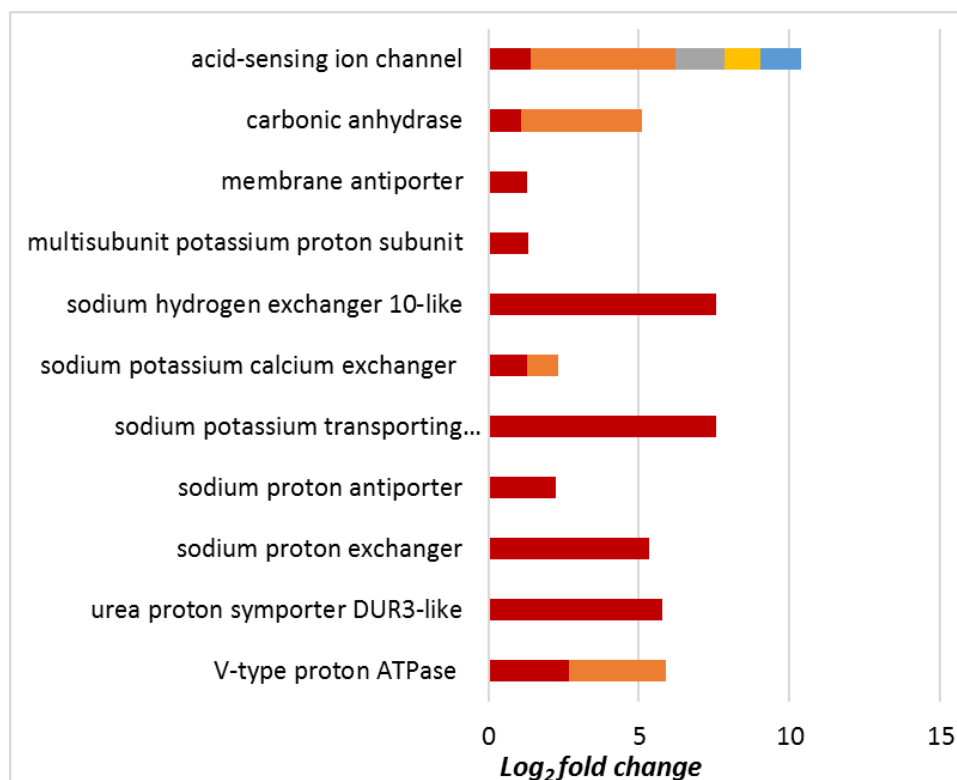
**Figure 3.** Immune-related under- (blue, left) and over- (red, right) represented transcripts in resistant *P. columella*. Different colors within the same bar account for different variants within the same family of transcripts. Only transcripts with known homologs are shown. A2M:  $\alpha$ -2-macroglobulin; BANK: B-cell scaffold with ankyrin repeats-like isoform X1; BPI:

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bactericidal/permeability increasing protein; G-CSFR: granulocyte colony stimulatory factor; Ig: immunoglobulin; IL: interleukin; IFN: interferon; IRF: interferon regulatory factors; LRR: leucine rich repeats; MIF: macrophage migratory inhibitory factor; Myd88: myeloid differentiation primary response; MZB1: marginal zone B- and B1-cell-specific -like; PKC: protein kinase C; PTAFR: platelet-activating factor receptor-like; PECAM: platelet endothelial cell adhesion molecule; SAMHD1: deoxynucleoside triphosphate triphosphohydrolase SAMHD1; TGF: transforming growth factor; TLR: toll-like receptor; TNF: tumor necrosis factor.

Interestingly, a thorough look at some major ion regulations, and other transporters that contribute to the pH and osmotic regulation of the organisms were found to be highly overrepresented in resistant snails. In addition, carbonic anhydrase transcripts were also found highly abundant in this isolate (Figure 4).

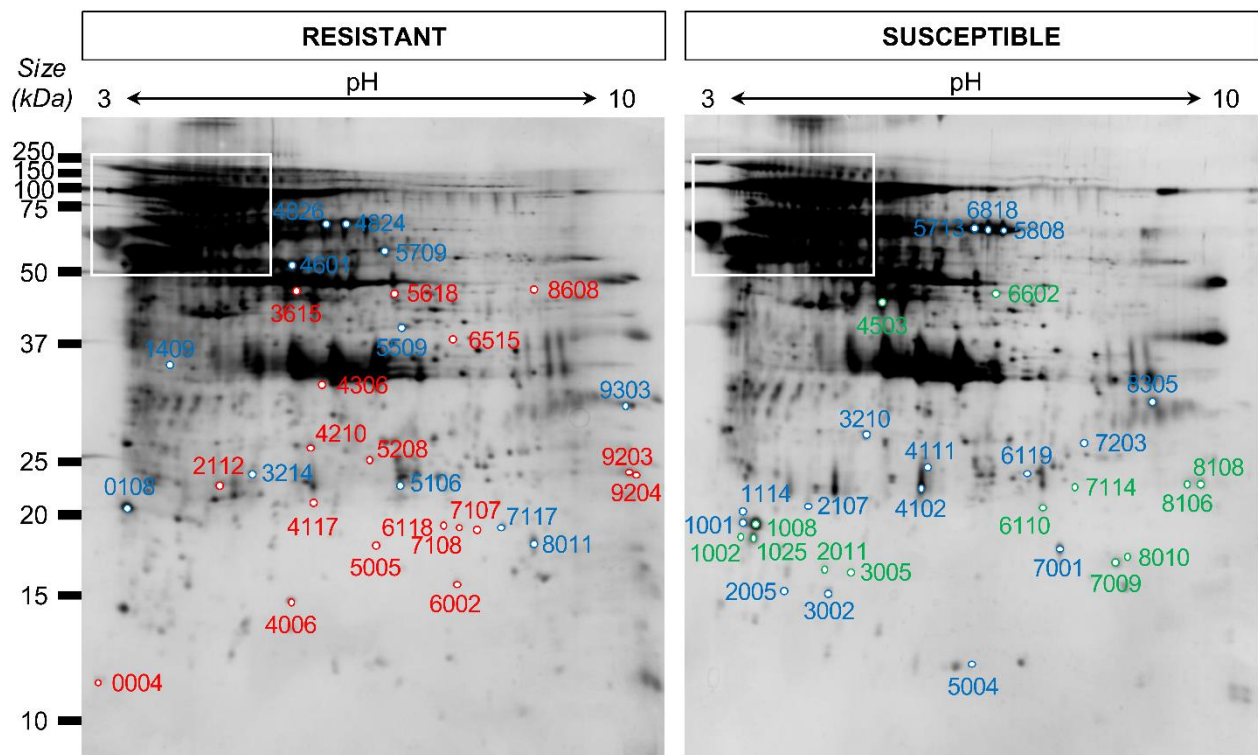


**Figure 4.** Overrepresented transcripts related with acid-base regulation in resistant *P. columella*. Different colors within the same bar account for different variants within the same family of transcripts. Only transcripts with known homologs are shown.

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### 2D-electrophoretic profiles of the albumen gland: qualitative and quantitative differences between susceptible and resistant *P. columella* phenotypes

Albumen glands of *P. columella* exhibited complex profiles, notably consisting of highly abundant proteins of high molecular weight and acidic pI (upper left part of the gels in Figure 5). Such highly abundant proteins with several isoforms can mask many nearby proteins and impede the quantitative analysis and proper protein identification. Therefore, this part of the gel identified as a white rectangle in Figure 5 was excluded from the analysis. A total of 554 spots were identified and included in the analysis, from which the majority of the spots (more than 80%) were found conserved and similarly abundant in both isolates.



**Figure 5.** Two-dimensional-electrophoretic profiles of the albumen gland of *Pseudosuccinea columella* resistant (gel on the left) and susceptible (gel on the right) to *Fasciola hepatica* infection. Spots identified only in the resistant or susceptible strains are indicated in red and green, respectively. Spots with significant four-fold difference between the two strains are indicated in blue, only on the gel corresponding to the strain in which they are overrepresented. Next to each spot is written the four-digit identification code used in the Table 1 and Supplementary File 3. The square within the gels represents an area of highly

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abundant proteins with several isoforms left aside of the differential analysis as its complexity impede the quantitative analysis and proper protein identification.

However, 18 spots were uniquely present in the profiles of resistant individuals while 13 were uniquely observed in susceptible individuals' ones (Supplementary File 3; Figure 5). Twenty-eight spots were significantly ( $P < 0.01$ , Mann-Whitney U-test) four-fold differentially abundant between the resistant and the susceptible strains (Supplementary File 3; Figure 5). Among them, 12 and 16 spots were over- and under-represented in the resistant strain compared to the susceptible strain respectively, possibly due to a differential expression/consumption of these molecules in each phenotype (Supplementary File 3; Figure 5). The resistant/susceptible quantity ratio ranged from up to 26.11 (spot # 7117) to 0.24 (spot # 2107) which account for high differences in differentially represented proteins between phenotypes.

***Protein identification of the differentially abundant/present spots between susceptible and resistant *P. columella****

Forty-nine proteins and several isoforms were successfully identified from the 59 previously selected spots (Table 1; Supplementary File 3). No identification was possible from spots 7107, 7108 and 6515 while spot 6002 contained two isoforms of an uncharacterized protein with 67% sequence similarity ( $2e-67$ ) to an uncharacterized one from *Biomphalaria glabrata* snails (LOC106073623) predicted from genome analysis (Supplementary File 3). The presence of more than one protein or of different isoforms of the same protein in the same gel plug was expected due to the complexity of the electrophoretic profile. Nevertheless, it remained limited due to the restriction in the gel zone analyzed (Figure 5).

Table 1 summarizes the identified proteins (from one to up to three hits found per spot) from the 59 excised spots. An overall overrepresentation of defense/stress and metabolism-related molecules is observed in resistant snails which could be directly related to its phenotype towards *F. hepatica* infection. Particularly, G-type lysozyme and lipopolysaccharide-binding protein/bactericidal permeability-increasing protein (LBP/BPI) were only observed in resistant snails (Table 1).



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**Table 1.** Identified proteins from the albumen gland qualitatively (underlined spot #) or quantitatively differing between resistant and susceptible *Pseudosuccinea columella* strains.

Primary Function	Protein Annotation	Present/abundant (spot #)	
		Susceptible	Resistant
Defense / stress	G-type lysozyme	-	<u>6118</u> ; 7117
	Glutathione S-transferase	2107	-
	Lipopolysaccharide-binding protein/bactericidal permeability-increasing protein (LBP/BPI)	-	<u>3615</u>
	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	-	<u>3615</u>
	Matrix / adhesion	Cartilage matrix protein-like	<u>7114</u>
Dermatopontin 2		<u>1025</u>	-
Filaggrin-like/ cartilage acidic protein 1-like		<u>4503</u> ; <u>1025</u> ; 1001; 1114	<u>5208</u> ; 7117; 1409; 8011
Matrilin/Cartilage matrix protein		6119	-
TGF- $\beta$ -induced protein ig-h3		<u>3005</u> ; 2107; 8301; 8305	<u>4306</u> ; 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	-	<u>4306</u>
	Cytochrome b-c1 complex subunit 2 mitochondrial-like	-	<u>8608</u>
	Citrate synthase mitochondrial like	<u>6602</u>	<u>5618</u>
	L-amino acid oxidase –like	2005; 5004; 8301; 8305; 7203	<u>3615</u> ; 4826; 4824; 5709
Purine nucleoside phosphorylase-like	-	<u>4306</u>	
Protein synthesis / assembly / folding	40S ribosomal protein S2	8301; 8305	-
	40S ribosomal protein S7-like	<u>6110</u>	-
	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	<u>6602</u>	<u>5618</u>
	Peptidyl-prolyl-cis-trans isomerase 5-like	1114	-
	Peptidyl-prolyl-cis-trans isomerase B-like	<u>8108</u> ; <u>8106</u>	<u>9203</u> ; <u>9204</u>
Protease and protease inhibitors	Serine peptidase 2/ fibrinolytic enzyme, isozyme C-like	3210	<u>4210</u>
	Serpin B-like protein 2	-	<u>5005</u> ; 3214; 5106
	Serpin B3-like	<u>6602</u>	<u>5618</u>
	Serpin Z2B-like	<u>4503</u> ; <u>7114</u> ; <u>2011</u> ; 2005; 1114; 6818;	108; 1409; 4601

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		2107; 6119; 5808; 5713	
	Dopamine $\beta$ -hydrolase (DBH)-like monooxygenase protein 1	6818; 5808; 5713	-
Signaling process and transduction	Guanylate kinase like	-	<u>9203</u>
	Neurogenic locus Notch protein	4102; <u>7009</u> , <u>7001</u> ; <u>6110</u> ; 4111; 2107	<u>2112</u> ; 108
	Tyramine-beta hydroxylase like	-	4824; 4826
	Endo-beta-1,4 glucanase	<u>7009</u> ; 8305	-
	Glutamyl tRNA (Gln) amidotransferase subunit A-mitochondrial like	-	1409
	Hemocyanin-like	6818; 5808; 5713	-
Other functions	Histone H4	-	<u>4117</u>
	Lamin B1-like	-	4826
	Probable ATP-dependant RNA helicase DDX 43	3210	-
	Ribonuclease UK-144-like	3002	<u>4006</u>
	Protein DGCR14-like	7203	-
	WD repeat-containing protein 36-like	<u>2011</u>	-

## Discussion

### Overview on the omics approaches on *P. columella*: a baseline to understand the phenotypic features of resistant snails

Resistance to *F. hepatica* in *P. columella* has been associated with an active encapsulation of the parasite shortly after its penetration that leads to the death of the larvae and the resolution of the infection [4]. According to these authors, such immune response can be fully observed as soon as 24h post-exposure [4]. It could thus be reasonable to think, that some features involved in the susceptibility/resistance to the parasite are constitutively expressed in each phenotype.

Here, we assembled the first transcriptome of *P. columella* and studied the constitutive differentially-represented transcripts and enriched functions associated with the resistant phenotype at whole snail level. From this, we observed that biological processes related with stress/defense responses, and a number of overrepresented immune-related transcripts in resistant compared to susceptible snails. The latter points at a higher allocation of resources to increase the responsiveness to stressors, either pathogens or other stimuli, since both processes are physiologically linked by several molecular functions, effectors and signalling pathways [25, 26]. Similar results are also reported from resistant *Biomphalaria glabrata* snails to *Echinostoma caproni* and *Schistosoma mansoni* [27, 28]. Interestingly, the same

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pattern of abundance of transcripts involved in defense/stress responses in resistant snails was also patent at the proteomic level when we analyzed the differentially expressed molecules of the albumen gland. Particularly, such enrichment in immune response processes and molecules observed in resistant snails could explain, at least in part, the phenotypic differences between susceptible and resistant *P. columella* towards *F. hepatica*, as it could imply an advantage for resistant snails to “better” recognize, orchestrate and regulate defences when infected. Interestingly, it has been proposed a similar pattern in which constitutive molecular processes were the base for refractoriness of the latter to the parasite's immunosuppressive factors in experimentally selected resistant *B. glabrata* strains to *E. caproni* [29]. It is also worth mentioning that in *B. glabrata* – *E. caproni* model, significant differences in the representation of plasma proteins between susceptible and resistant snails were reported and linked to a differential regulation of several genes expressed in the albumen gland [30].

Beyond the immunological features, it was particularly interesting to find an overall significant abundance on response to stress in resistant snails since a higher tolerance for lower pH and total hardness levels is also associated to resistant *P. columella* populations [7]. Therefore, mechanisms for restoring acid-base balance could be expected to be globally enhanced in these snails. Here, we found an over-representation, in resistant snails, of several carbonic anhydrase isoforms, whether at the transcriptomic (RNAseq on whole snails) or at the proteomic levels (2D electrophoresis on the albumen gland). This points at an overall higher abundance of this enzyme on the resistant strain and concomitantly, at a higher potential to compensate pH variations in these snails [31]. In addition, as for aquatic invertebrates, water acidification negatively affected osmotic regulation [31, 32], it is likely that snail populations that tolerate better low pH/TH freshwater environments also rely on an increase activity/abundance of major ion regulator transporters (*e.g.* Na<sup>+</sup>/K<sup>+</sup>-ATPase, H<sup>+</sup>-ATPase, Na<sup>+</sup>/H<sup>+</sup>-exchanger) to stabilize the pH of the hemolymph. As it could be expected based on the previous observation, herein, we also recorded a constitutive over-representation of different ion transporters related with acid-base and osmotic balance in resistant compared to susceptible snails (see Figure 4). The latter could figure among the molecular bases related to the increased tolerance of resistant *P. columella* to changes in the pH of the water previously informed [7].

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In this sense, it is also interesting to note the enrichment in molecular functions related to  $\text{Ca}^{2+}$  binding. This ion is essential for shell formation but it is recruited to the hemolymph during infection and pH regulation, which can result in shell hypocalcification [33-35]. Therefore, it could also be hypothesized that the resistance to *F. hepatica* and the high tolerance to low pH and total hardness observed in *P. columella* is also related with an enhanced fitness in calcium balance and might be somehow linked to the characteristic pigmentation features displayed in the mantle of resistant snails.

Energy budget constraints determine a trade-off in such constitutive allocation of resources to defense/stress against other biological processes. Enhanced immune responsiveness can reconfigure intermediate metabolism towards a relative increase of the respiration rate, glycolysis, proteolysis and lipolysis [36]. Similarly, lower energy reserves (protein and glycogen content) and higher metabolic rates can be also found, supposedly in response to the increase of the defences against stress (*e.g.* antioxidative enzymes; [31]). At the transcriptomic level, enrichment analysis showed a negative association of biological processes and molecular functions related with protein biosynthesis (*e.g.* ribosome biogenesis, peptide biosynthetic process, translation) with the resistant phenotype (Figure 2). At the proteomic level, we also found a lower abundance of protein synthesis/folding-related molecules in resistant snails, which contrasted with an over-representation of other metabolism-related molecules in resistant snails (see Table 1).

Particularly, in the albumen gland, energy production through the respiratory chain and energy storage (synthesis of fatty acid), could be significant in resistant snails, as suggested by the abundance of cytochrome b-c1 complex subunit 2 mitochondrial-like isoforms and of 3-oxoacyl- reductase FabG-like, which catalyzes the first step of the fatty acid synthesis, respectively (see Table 1). In this sense, over-representation of 4-hydroxyphenylpyruvate dioxygenase-like might indicate that the former process could be funneled by the catabolism of aromatic amino acids, whose intermediates enter into the Krebs's cycle. These might constrain the production of the proteinaceous components contained in the perivitelline fluid as the latter constitute the primary function of the albumen gland and it is directly involved in the sustainability of the embryos [9, 10]. Consequently, the reproductive outputs of resistant snails might be affected, especially if the cellular machinery is also devoted to the synthesis of proteins involved in other functions like immunity, as has been observed

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here (see Table 1). In this sense, these differences endorse the preference of resistant snails for self-maintenance as a strategy that results in a trade-off against reproduction, particularly egg production (Stearns, 1992). Thus, our results could help to explain, at a molecular level, the lower fecundity rate observed in resistant (La Coca) snails compared to susceptible snails (Aurora and Negrines; [7]) while providing insights into the potential molecular bases involved.

Regarding signaling processes we found an overall enrichment on signaling pathways associated to the resistant strain, possibly related with its enhanced responsiveness toward stressors and the regulation of hemostasis. Particularly, in the albumen gland interesting qualitative differences between isolates: in resistant snails, abundance of tyramine  $\beta$ -hydrolase like (TBH), an enzyme that catalyzes the conversion of tyramine into octopamine, contrast with the underrepresentation of dopamine  $\beta$ -hydrolase (DBH)-like monooxygenase compared with susceptible snails (Table 1). The presence of TBH in resistant snails suggests a particular involvement of tyramine and, mainly, octopamine in the neuroendocrine control of the albumen gland in this strain. Both molecules are analogs of norepinephrine/epinephrine transmitters in invertebrates, even though they signal by different G protein-coupled receptors [37]. Particularly, octopamine has been associated with the regulation of a number of functions in invertebrates (*e.g.* insects and nematodes) including metabolism, ovulation and egg laying [38-40]. However, little is known about its functional role regarding reproduction in mollusks with only a few approximations on bivalves [41], while a number of studies refer to a potential involvement in regulation of feeding and locomotion in gastropods [42-44]. Interestingly, the increased of TBH concentration has been suggested as a stress marker in insects [45], and in free living nematodes a rise in octopamine levels allows metabolic rearrangements to maintain energy homeostasis during stress induced by starvation [40]. Similarly, a significant increase of octopamine has been observed in the central nervous system of severely food-deprived *Lymnaea stagnalis* snails [46]. We could speculate that a preferential signaling through octopamine can be associated with the differential metabolic planning and allocation of resources, at the albumen gland level, between resistant and susceptible *P. columella* proposed here. In this sense, the presence of a guanylate kinase like protein, a molecule possibly involved in cGMP signaling was also found uniquely present in resistant snails.

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Nevertheless, the lack of information regarding representation of neurotransmitters and their receptors in the albumen gland of *P. columella* certainly limits our definitive interpretation and further studies will be needed to fully elucidate this matter.

### ***Overview to constitutively over-represented immune related molecules in resistant snails***

A thoroughly comparative look into the differentially expressed immune-related genes between phenotypes showed an overall immune enhanced capacity in resistant *P. columella* compared to naïve susceptible snails concerning the arsenal of overexpressed pathogen recognition/interacting molecules, particularly of C-type lectins and mannose and galactose binding molecules (see Figure 3b). Given that the first step of any defense response is for the host to identify the injury, the variety of up-regulated transcripts displayed by resistant snails might constitute an advantage. C-type lectins, in particular, are known for a broad selectivity of carbohydrate-binding site and even, of classical pathogen-associated molecules like LPS and peptidoglycan. In addition, they have roles in microbe agglutination and opsonization, may trigger signaling pathways leading to induction of phagocytosis and encapsulation and in humans, they have been particularly associated with parasite recognition (Vázquez-Mendoza et al. 2013). C-type lectin molecules from *B. glabrata* have been found to interact with antigens of *S. mansoni* sporocyst [47] and are overexpressed in host resistance model towards *E. caproni* [11]. An investigation of the surface of *F. hepatica* sporocyst showed that this larva is mainly covered with N-acetyl-D-glucosamine and  $\alpha$ -mannose while galactose and N-acetyl D-galactosamine are present in lesser proportions [48]. Therefore, the particular abundance of mannose binding molecules might increase the chances for the snails to interact with the parasite. Recorded overexpression of CD109, a thioester-containing protein family member, might also contribute to recognition of the parasite by resistant *P. columella* since its homolog in *B. glabrata* is known to interact with larval proteins of *S. mansoni* [47]. Interestingly, a strong immune modulation/suppression of the vertebrate host is induced by *F. hepatica* infection, which is particularly associated with stimulation of different lectins (e.g. Dectin-1 [49-51]; DC-SIGN in dendritic cells [52]; macrophage Gal/GalNAc lectin; [53]). However, the immune modulatory mechanism of tegumental parasitic antigens has been recently pointed to be independent of their interaction with the mannose receptor [54].

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The abundance of immune-relevant transcripts related with leukocyte adhesion, rolling and tethering (*i.e.* interference hedgehog-like, Ras-like GTP-binding Rho1, rho-associated kinase 1), recognition and wound healing (*i.e.*: Ras-like GTP-binding Rho1) points at an increased immune surveillance as an additional feature related to the resistant phenotype. In addition, overexpression of cytokine transcripts such as G-CSFR, TGF1B and MIF in resistant snails might be related to the effective cellular response triggered in this phenotype through their involvement in the proliferation, recruitment and activation of immune cells. In vertebrates, the cell-surface receptor G-CSFR have being related to a diversity of roles including production, survival and activation of neutrophil, development and migration of other myeloid cells and hematopoietic stem cell mobilization [55]. TGF1B is conserved among different phyla and affects immune functions in invertebrates such as phagocytosis, chemotaxis and cell motility while having important roles in stress response and wound healing [56]. On the other hand, MIF are present in hemocytes of *B. glabrata* and promotes an aggregative behavior of snail embryonic cell line stimulated with *S. mansoni in vitro* while the *in vivo* knockdown of MIF expression impairs the changes in circulating hemocytes in response to parasite challenge, and was significantly associated to an increase of parasite burden [57]. Additionally, recombinant MIF induced cell proliferation and inhibited NO-dependent p53-mediated apoptosis in *B. glabrata* embryonic cells [57]. In *Oncomelania hupensis*, MIF is associated with immune defenses against *S. japonicum*: its expression increased after parasite challenge and the knockdown of MIF decreased the proportion of phagocytic circulating hemocytes but stimulated the recruitment of blood cells from the host to the site of infection [58].

Particularly significant was the abundance, in resistant snails, of transcripts related with pro-inflammatory signaling pathways (*e.g.* TLR/Myd88 and PCK) and with differences related with regulatory activities (*e.g.* LRR and IRF), which endorse the higher immune responsiveness associated to the resistant phenotype. Toll-like receptors are widely studied primary mediators of innate immunity in different phyla and in mollusks they can be activated and up-regulated after septic injury to promote hemocyte activation (see [59, 60] for review). Concerning snail-trematode interaction, the functional characterization of a *B. glabrata* TLR was recently reported demonstrating to be an important snail immune receptor that is capable of influencing infection outcome following *S. mansoni* challenge

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[61]. According to these authors, significantly higher over-representation of the TLR was found in hemocytes from a *S. mansoni*-resistant strain and knockdown of its expression by siRNA oligonucleotides resulted in a significant reversion of the resistant phenotype when challenged with the digenean parasite [61]. *Fasciola hepatica* excretion-secretion antigens are supposed to indistinctively activate or block, *in vitro*, different TLR of human monocytes depending on the parasitic protein specifically interacting with the receptors (*i.e.* cathepsin, serpins and endopin can activate TLR2, TLR4, TLR8 and likely TLR5 and TLR6 while fatty acid-binding protein strongly suppressed the stimulation induced by various TLR ligands; [62]). However, while little is known about the secretome of *F. hepatica* during its intramolluscan development, we also found ultimate downstream effectors of this signaling pathways (*i.e.* Myd88 and NF- $\kappa$ B) significantly abundant in resistant snails (see Figures 1 and 3).

On the other hand, LRR domains are evolutionarily conserved and provide an important structural framework required for molecular interactions, some of which are associated with immune regulation of the host [63], while IRF regulates a plethora of process involving immunity, metabolism and response to stress [64]. In addition, PKC is associated with NO and H<sub>2</sub>O<sub>2</sub> production in snail hemocytes [65-67] and with cell spreading in the embryonic cell line from *B. glabrata* [68]. Involvement of PKC and ERK pathways and focal adhesion kinase in cell adhesion, spreading and formation of lamellipodia necessary for phagocytosis in *Lymnaea stagnalis* has been also described [69].

Anti-parasitic defense like ferritin and nitric oxide synthase and antioxidant enzymes were also significantly abundant in resistant snails and could also contribute to the orchestration of the effective encapsulation response that eventually leads to parasite death in resistant *P. columella*. On the one hand, hemocytes of resistant *B. glabrata* strain to *S. mansoni* were found to over express ferritin prior to parasite infection in a previous study [28]. Ferritin, a crucial protein involved in the regulation of iron distribution, participates in iron sequestration depriving infectious microorganisms from iron acquisition and inhibiting their multiplication [70]. The expression of this acute phase protein is up-regulated after microbial challenge, acts as antibacterial agent in mollusks [71, 72] and, particularly in *B. glabrata*, a certain immune-specificity in the induction of its different ferritin isoforms has been found depending on the type of pathogen [73]. It has been hypothesized that ferritin could affect the cellular balance of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl free radical (OH $\cdot$ ) and



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therefore, influence the parasite killing through reactive oxygen species in *B. glabrata* – *S. mansoni* [28]. On the other hand, nitric oxide synthase catalyzes the production of NO in vertebrates and invertebrates, it is strongly up-regulated following infection [74, 75] and it is involved in destroying invading microorganisms [76]. In *Drosophila*, it has been proposed that NO mediates an early step of the signal transduction pathway, inducing the innate immune response upon natural infection with gram-negative bacteria [74]. Particularly in snail-trematode interaction, the production of NO, together with H<sub>2</sub>O<sub>2</sub>, is significant for *S. mansoni* killing by *B. glabrata* hemocytes [77, 78]. Similarly, while up-regulation of catalase was essential to maintain redox balance and to resist bacterial infection in the clam *Meretrix meretrix* [79], high constitutive levels of Cu/Zn SOD has been positively related to anti-parasite resistance in *B. glabrata* snails as this molecule participates in the production of oxygen reactive species like superoxide radicals [28, 80]. In the case of resistant *P. columella*, higher constitutive antioxidant potential prior to parasite infection might be particularly beneficial since *F. hepatica* infection on definitive hosts has been associated to an increase of oxidative reactions and reduced antioxidant defense [81].

From the comparative proteomic approached on the albumen gland, we observed an overall abundance of two immune-effectors: LBP/BPI and G-type lysozyme. The G-type lysozymes are ubiquitously distributed lytic proteins with potent activities against microbial pathogens, mainly bacteria, that has been also reported from several mollusk species where they function as immune effectors and can be induced after infectious challenged [82-84]. In terms of snail-trematode interaction, a significant increase on the expression of three G-type lysozymes from *Oncomelania hupensis* was observed in *Schistosoma japonicum*-infected individuals suggesting their involvement in the defense against this pathogen [84]. Within the resistant strain of *P. columella*, the identification of LBP/BPI is also of interest as this molecule has been associated with parental investment on immune protection of snail offspring [85]. Particularly, an isoform of LBP/BPI that is produced by the albumen gland and can be found in *B. glabrata* laid egg masses [12] and is related with protection against oomycete infections [85]. Thus, the abundance of both immune effectors in the albumen gland of *P. columella*, if part of the perivitelline fluid, could induce an enhanced protection for the laid egg masses of resistant snails that might compensate the constrained production/laying of eggs per snail observed [7, 8].

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## Conclusions

The high throughput RNAseq performed in this study allowed the generation of the first transcriptome of *P. columella*, a globally distributed and highly invasive species and one of the main intermediate hosts of the veterinary and medically-important trematode *F. hepatica* [1]. It also brought forward the first overview to the albumen gland on this species. Through the inclusion of comparative approaches between resistant and susceptible strains to *F. hepatica*, this study provides a baseline for deciphering the molecular bases behind the different phenotypical and ecological features related to the resistant phenotype.

Particularly, the similar pattern observed in resistant snails at the transcriptomic and the proteomic levels on whole snail and albumen gland respectively (*i.e.* abundance of biological process/transcripts/proteins involved in immune and stress responses and under-representation of functions/molecules involved in anabolic metabolisms related with protein synthesis), endorse an overall allocation of resources towards survival and self-maintenance in these snails. In addition, these results offer a direct link between parasite resistance in *P. columella* and between the increased tolerance to pH and total hardness variations observed in resistant populations with the enhanced immune and stress responsiveness found at the molecular level. On the other hand, the differences in metabolic planning in detriment of protein synthesis/folding could be particularly significant, in the case of the albumen gland, as to contribute to reproductive trade-offs and could explain, at least in part, the fitness cost regarding resistance.

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### Supplementary materials (upon request to author)

**Supplementary file 1:** Excel files showing the detailed DESeq2- information and annotation description (Blast2GO information) of the 6876 transcripts of *Pseudosuccinea columella* showing more than 2-fold differential-expression and *P* adjusted < 0.05 between resistant and susceptible phenotypes to *Fasciola hepatica* infection. In addition, separate lists relating the differentially-expressed transcripts identified as 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) are also included.

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

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

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## CHAPTER 3

### Phenotypic features of *P. columella* resistant and susceptible to *F. hepatica*: from experimental infections to the dynamic of the immune response

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Each organism has a defense system that has evolved mainly under two different and equally important selective pressures: fight back, in a regulated and effective manner, the plethora of infective or harmful agents while remaining tolerant to host self (including self-antigens and microbiota). However, determinants of specificity might vary with host-parasite combinations since different digenean species might have distinctive interactions with snail defenses (Sapp & Loker, 1991). In fact, host – parasite interactions can be highly specific and examples varies from molluscan hosts that can be infected with and also resist specific parasite genotypes (*e.g.* Théron et al. 2014), as well as exhibit variation in these traits (Osnas & Lively, 2005; Vázquez et al. 2014). Thus, we aim to deeply characterize the resistance and susceptibility of *P. columella* snails described in Cuba to understand the extent of each phenotype through two different approaches that will be presented in this chapter: (1) performing experimental exposures to various isolates of *F. hepatica* (article 3) and to another digenean parasite to assess infection outcomes, and (2) exploring the dynamic of cellular immune response during *F. hepatica* challenge (article 4).

Experimental infections are a valuable approach to investigate differences in compatibility at a phenotypical level since they allow to manage and control variables, *e.g.* the combination of snail and parasite to test, the maturity of the snail, the infective parasite dose and the housing conditions, all of which could significantly influence the infection outcome (Sorensen & Minchella, 2001). While it remains difficult to predict the result when novel host-parasite combinations are tested, through the use of experimental infections overall patterns can be observed concerning compatibility polymorphism (*e.g.* Théron et al. 2014; Vázquez et al. 2014), host-parasite interaction (*e.g.* Rondelaud et al. 2013; Ittiprasert & Knight, 2012), and even life history outcomes after infection (see Sorensen & Minchella, 2001), all of which can ultimately shed light on the possibility of transmission. Thus, through



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the first approach, we will firstly observe the effect of increasing *F. hepatica* infective dose, either by single or serial exposures, and of allopatric parasites from distant origins (Europe and the Caribbean) on different infection traits on resistant and susceptible *P. columella* phenotypes. These factors can significantly affect the infection outcome (Osnas & Lively, 2005; Gasnier et al. 2000; Vázquez et al. 2014) and will be tested for the first time in this model. Secondly, we will challenge both *P. columella* phenotypes with *Trichobilharzia* sp. to explore the parasitic outcome on these snails when exposed to another digenean species.

Additionally, we were interested in gaining insights into the features supporting the differential response to *F. hepatica* in susceptible and resistant *P. columella* since only one attempt (from Gutiérrez et al. 2003a) was carried out to explore the immunobiology of this interaction. Since Gutiérrez et al. (2003a) associated the resistant phenotype to the occurrence of an immunological reaction towards *F. hepatica* characterized by the encapsulation of the sporocysts, we will be reviewing, using comparative approaches, the dynamic of the immune response (*i.e.* morphology of hemocytes, proliferative and functional activity, cytokine expression) triggered on susceptible and resistant snails when challenged with *F. hepatica*. Most of the attention has been focused on the hemocytes as ultimate effectors of the differential phenotype observed in resistant *P. columella* (parasite encapsulation).

The results presented in this chapter address significant questions related to *P. columella* phenotypes and are building steps for improving efforts in deciphering the factors affecting parasite transmission and understanding the immunobiology of *P. columella* – *F. hepatica* interactions and the mechanistic bases behind the resistance.

### **3.1 Effect of increasing parasite doses, successive exposures and parasite geographic origin on the infection outcome of *F. hepatica* in *P. columella***

**Article 3 (Parasites & Vectors. 2018; 11 : 559; doi: 10.1186/s13071-018-3155-3)**

***Fasciola hepatica* – *Pseudosuccinea columella* interaction: effect of increasing parasite doses, successive exposures and geographic origin on the infection outcome of susceptible and naturally-resistant snails from Cuba**

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Parasites &amp; Vectors

RESEARCH

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# *Fasciola hepatica*-*Pseudosuccinea columella* interaction: effect of increasing parasite doses, successive exposures and geographical origin on the infection outcome of susceptible and naturally-resistant snails from Cuba



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## Abstract

**Background:** *Pseudosuccinea columella* is one of the most widespread vectors of *Fasciola hepatica*, a globally distributed trematode that affects humans, livestock and wildlife. The exclusive occurrence in Cuba of susceptible and naturally-resistant populations to *F. hepatica* within this snail species, offers a fascinating model for evolutionary biology, health sciences and vector control strategies. In particular, resistance in *P. columella* is characterized by the encapsulation of the parasite by host's immune cells and has been experimentally tested using different Cuban *F. hepatica* isolates with no records of successful infection. Here, we aimed to explore for the first time, the effect of different parasite doses, successive exposures and different parasite origins on the infection outcomes of the two phenotypes of *P. columella* occurring in Cuba.

**Methods:** To increase the chances for *F. hepatica* to establish, we challenged Cuban *P. columella* with increasing single parasite doses of 5, 15 or 30 miracidia and serial exposures (three-times) of 5 miracidia using a sympatric *F. hepatica* isolate from Cuba, previously characterized by microsatellite markers. Additionally, we exposed the snails to *F. hepatica* from different geographical origins (i.e. Dominican Republic and France). Parasite prevalence, redial burden and survival of snails were recorded at 25 days post-exposure.

**Results:** No parasite development was noted in snails from the resistant populations independent of the experimental approach. Contrastingly, an overall increase in prevalence and redial burden was observed in susceptible snails when infected with high miracidia doses and after serial exposures. Significant differences in redial burden between single 15 miracidia and serial 3 × 5 miracidia infected snails suggest that immune priming potentially occurs in susceptible *P. columella*. Compatibility differences of allopatric (Caribbean vs European) *F. hepatica* with susceptible snails were related to the geographical scale of the combinations.

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**Conclusions:** Here, the effectiveness of *P. columella* resistance to *F. hepatica* does not decline with increasing parasite doses, successive infection or different geographical origins of parasite isolates, while presenting new evidence for specificity for infection in susceptible *P. columella* snails. Understanding the peculiarities of the *P. columella*-*F. hepatica* interaction and the extent of the resistant phenotype is crucial for an effective parasite control and for developing alternatives to tackle fasciolosis transmission.

**Keywords:** Snail-trematode interaction, Lymnaeidae, Liver fluke, Experimental infection, Immune priming, Compatibility, Allopatric parasites

## Background

The liver fluke *Fasciola hepatica* Linnaeus, 1758 is the main causative agent of fasciolosis, a snail-borne parasitic disease that affects humans, livestock and wildlife [1]. The occurrence of this trematode in all continents except in Antarctica has been largely explained by the introduction of infected livestock and susceptible snails into new areas, with *F. hepatica* being able to parasitize a wide range of host species [1, 2]. The arrival of the liver fluke to the Americas presumably occurred during the early events of colonization of the New World by Europeans, with several native freshwater lymnaeid snails transmitting the parasite today [1].

*Pseudosuccinea columella* (Say, 1817), considered native from North America [3], can be also cited among the intermediate host species of *F. hepatica* in South America and the Caribbean [4–6]. In addition, it is a globally invasive freshwater snail that has been largely introduced out of its native range [3] with reports of established populations from Europe [7], Africa [8], Australia [9] and the Pacific islands [10, 11]. The global spread of some invasive genotypes of *P. columella* might complicate the epidemiological scenario of fasciolosis transmission [3].

Interestingly, in Cuba, *P. columella* displays two different phenotypes regarding *F. hepatica* infection with natural populations being either susceptible [5] or resistant to the liver fluke [12]. Notably, the resistant phenotype in field-occurring *P. columella* is characterized by the encapsulation of the parasite by the host's immune cells [12]. These populations have been extensively tested for infection using different Cuban isolates of *F. hepatica* but no successful parasite development has ever been recorded (see [12–14] for details). From a genetic perspective, studies exploring the existence of polymorphism in *P. columella* have shown that resistant snails cluster separately from susceptible populations in Cuba [13] and other regions of the world [3].

The challenging of resistant individuals of *P. columella* has been always carried out using a constant standard dose of five miracidia (infective larva for the snails). Therefore, we wanted to explore the effect of higher infective doses of the parasite, either by single or serial

exposure trials on both susceptible and resistant *P. columella* using a known polymorphic *F. hepatica* isolate from Cuba (La Palma; [15]). With these approaches we aimed at tipping the scales in favour of the infection success by (i) increasing the probability of encounter of compatible host-parasite genotypes, and (ii) by circumventing or hijacking the effectiveness of host immune defences with large miracidia numbers and/or enhanced genetic diversity of the parasite at which each snail is confronted [16, 17].

In another experiment, given that resistant individuals had always been challenged with Cuban isolates of *F. hepatica*, we exposed for the first time, susceptible and resistant *P. columella* from Cuba to two allopatric liver fluke isolates from the Caribbean (short distance) and Europe (large distance), and compared their infection outcomes with those of the sympatric Cuban isolate. The theory of local adaptation predicts that parasites perform better on their local (sympatric) hosts rather than foreign (allopatric) hosts [18]. However, exceptions exist (e.g. [14, 19]), thus a differential exposure of a host population to an “unknown” entity (i.e. allopatric parasites) might result in differential outcomes and can test for different patterns of susceptibility or even resistance. Given the geographical isolation between the parasite isolates used, genetic differences are expected and should account for variations in compatibility with the snail host, particularly between the Cuban and the European isolates. With this experimental approach, it is likely that a higher infection success from exposing susceptible *P. columella* to sympatric *F. hepatica* would be observed, while the resulting outcome with resistant populations could give clues concerning the specificity of their resistance. If no infection occurs in resistant *P. columella* then we might hypothesize that this phenotypic response is not restricted only to local (Cuban) parasites but it has a broader or even global scale.

Here, we gain new insights on the susceptible *P. columella*-*F. hepatica* interaction, presenting evidence related to differences in compatibility and immune priming as factors affecting the infection outcomes (e.g. prevalence, redial burden, host survival) in this model. Moreover, we demonstrate that resistant populations described from

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Cuba remain resistant to a high *F. hepatica* miracidial dose, after serial exposures or challenged with allopatric *F. hepatica* isolates. This highly resistant phenotype opened new perspectives of applications for fasciolosis control.

## Methods

### *Fasciola hepatica* isolates and laboratory-reared susceptible and resistant *P. columella*

We collected adults of *F. hepatica* in local abattoirs from eastern Cuba (sympatric isolate: La Palma, Pinar del Río Province), north-western Dominican Republic (allopatric isolate, narrow scale: Dajabón Province) and southern France (allopatric isolate, large scale: Camargue region). The genetic structure of eight *F. hepatica* isolates from Cuba (including parasites from La Palma and other regions within its vicinity) had been previously characterized [15]; this study demonstrated the existence of high polymorphism and genetic diversity within Cuban *F. hepatica* with no clear genetic differentiation among isolates due to a high genetic flow within the island and a preferential out-crossing as reproduction strategy. In particular, the La Palma isolate (local isolate used in the present study) showed over 75% prevalence in sacrificed bovines and a mean of five alleles per analysed microsatellite locus with an observed heterozygosity of 0.511 [15]. Unfortunately, no previous data on genetic diversity is available for the Dominican and French isolates used, but we can expect differences with Cuban flukes given the geographical isolation.

Flukes were collected from the liver of infected cattle and kept alive for 6 h in a solution of 0.85% NaCl (saline solution) and 5% glucose (Sigma-Aldrich, St. Quentin Fallavier, France) for egg laying. Eggs were preserved at 4 °C in the dark and in saline solution supplemented with gentamicin (Sigma-Aldrich, 40 mg/ml) until use.

We used two susceptible *P. columella* populations, Negrines (Havana Province) and Aurora (Mayabeque Province), and two resistant populations, La Coca (Havana Province) and La Palma (Pinar del Río Province). Snails were reared in the Laboratory of Malacology of the Institute of Tropical Medicine “Pedro Kouri”, Cuba, for two to three generations and derived from field-collected populations of *P. columella*. Snails were cultured in Petri dishes with growing algae, in 26 °C de-chlorinated water complemented with crushed shells as a carbonate supplement as previously described [20]. The snails were fed on the algae *ad libitum* and routinely changed to other Petri dishes with growing algae to avoid starvation.

### Experimental exposure of *P. columella* snails to *F. hepatica*

Experimental exposure of 2-weeks-old laboratory-reared *P. columella* from the four populations (two resistant and two susceptible) were carried out using freshly

hatched miracidia, according to the methodology described by Vázquez et al. [14]. Briefly, eggs of *F. hepatica* were incubated in distilled water in total darkness at 28 °C for 15 days to complete maturation. Miracidia hatching was induced by direct exposure of eggs to light. For each assay, we always used 30 individuals of *P. columella* at varying conditions (see Fig. 1) as described below.

#### A. Exposure to different doses of *F. hepatica* miracidia

We exposed each *P. columella* population separately to single doses of 5, 15 and 30 miracidia (M) from the sympatric isolate of La Palma (Cuba) to increase the probability of exposing host populations to different parasite genotypes.

#### B. Serial exposure to *F. hepatica* miracidia

We explored the effect of serial infections with *F. hepatica* by exposing each *P. columella* population three times to the standard dose of 5 M [14] of the sympatric *F. hepatica* isolate of La Palma (Cuba; 3 × 5 M). Each exposure was performed at a three-day interval, after which each snail received a serially-delivered 15 M dose. The selection of the re-infection interval was based on the timing at which the immune response occurs (0 to 3–4 days post-exposure with patent parasite encapsulation as early as 24 h post-exposure; see Gutiérrez et al. [12]), which is also accompanied by possible “consumption” of defence related resources in the snail.

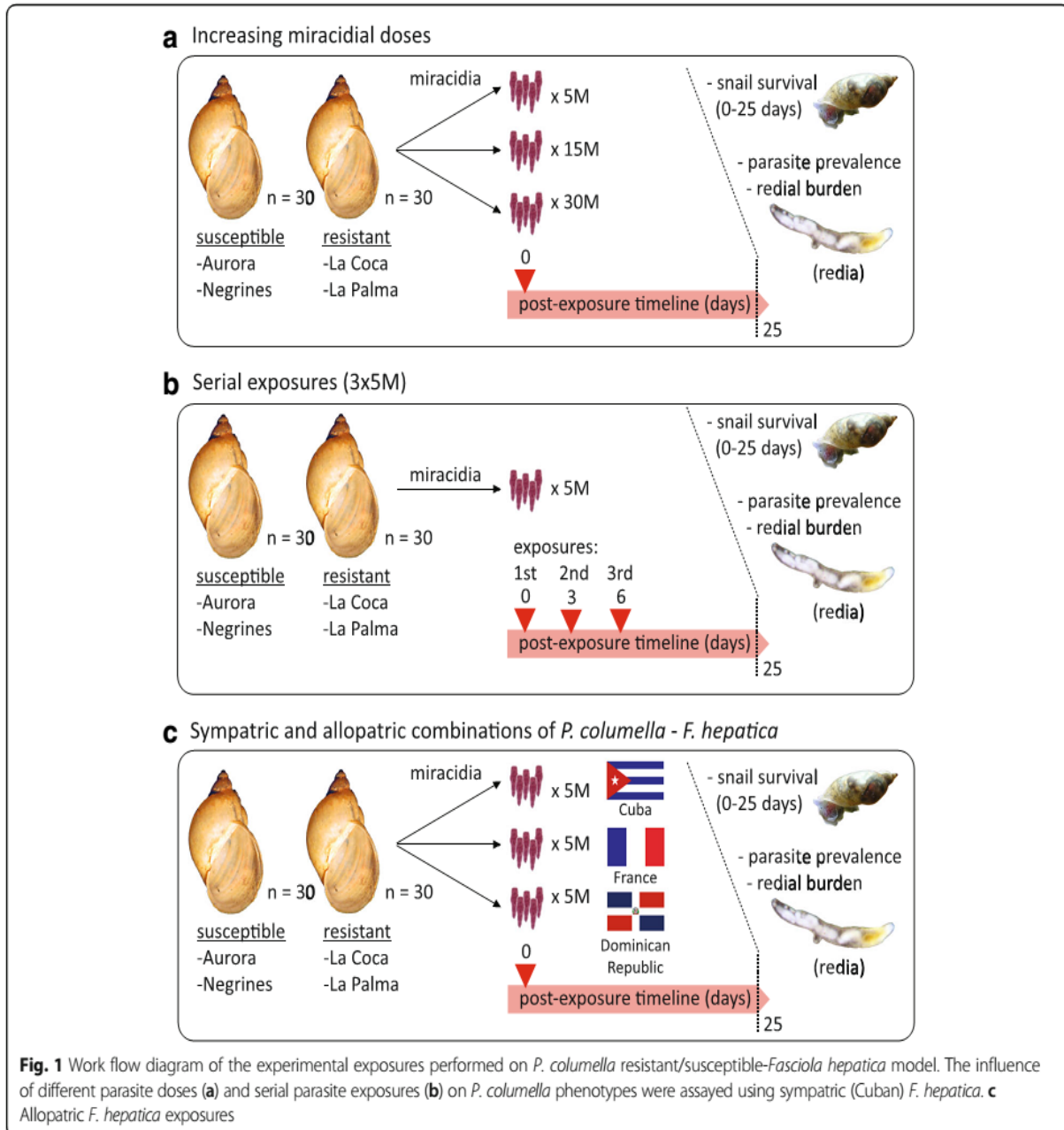
#### C. Exposure to sympatric and allopatric *F. hepatica*

We exposed each *P. columella* population separately to the standard dose of 5 M [14] of each allopatric *F. hepatica* isolate (Camargue, France and Dajabón, Dominican Republic). In order to save biological material, we used the results of the 5 M dose (see A above), for comparison against sympatric interactions.

In all trials and control groups, snails were individually allocated in 96-well plates and individually exposed overnight to *F. hepatica* miracidia. Each well was checked to record the penetration of all miracidia into the snails used for each experiment. Snails were maintained at 28 °C and monitored daily. Day-to-day mortality was recorded and exposed snails found dead were carefully dissected [21] to assess infection. Between days 7–10 post-exposure, infection was clearly patent by the presence of rediae in dissected individuals. The number of rediae per infected snail was counted to estimate the intensity of *F. hepatica* infection (redial burden) [14] per experimental group, always 25 days post-exposure or post-first-exposure in the case of serial exposure trials. Non-exposed snails from the same breeding batch were reared in the laboratory and subjected to the same conditions to serve as control of survival for the infection assays.

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**Data analysis**

The prevalence of *F. hepatica* in each experimental group was expressed as the percentage of infected snails of all those initially exposed ( $n = 30$ ); confidence limits were calculated at the 95% confidence level by the Wilson score interval. Differences in prevalence between groups were checked by Fisher's exact test. Survival curves of exposed snails [from 0–25 days post-(first-)exposure] were constructed based on the number of live snails at each time point divided by the number of exposed individuals and expressed as a percentage. We performed log-rank tests of Kaplan-Meier curves to assess the statistical differences on survivorship data. Data of *F. hepatica* redial counting per experimental group was checked for normality and variance homogeneity

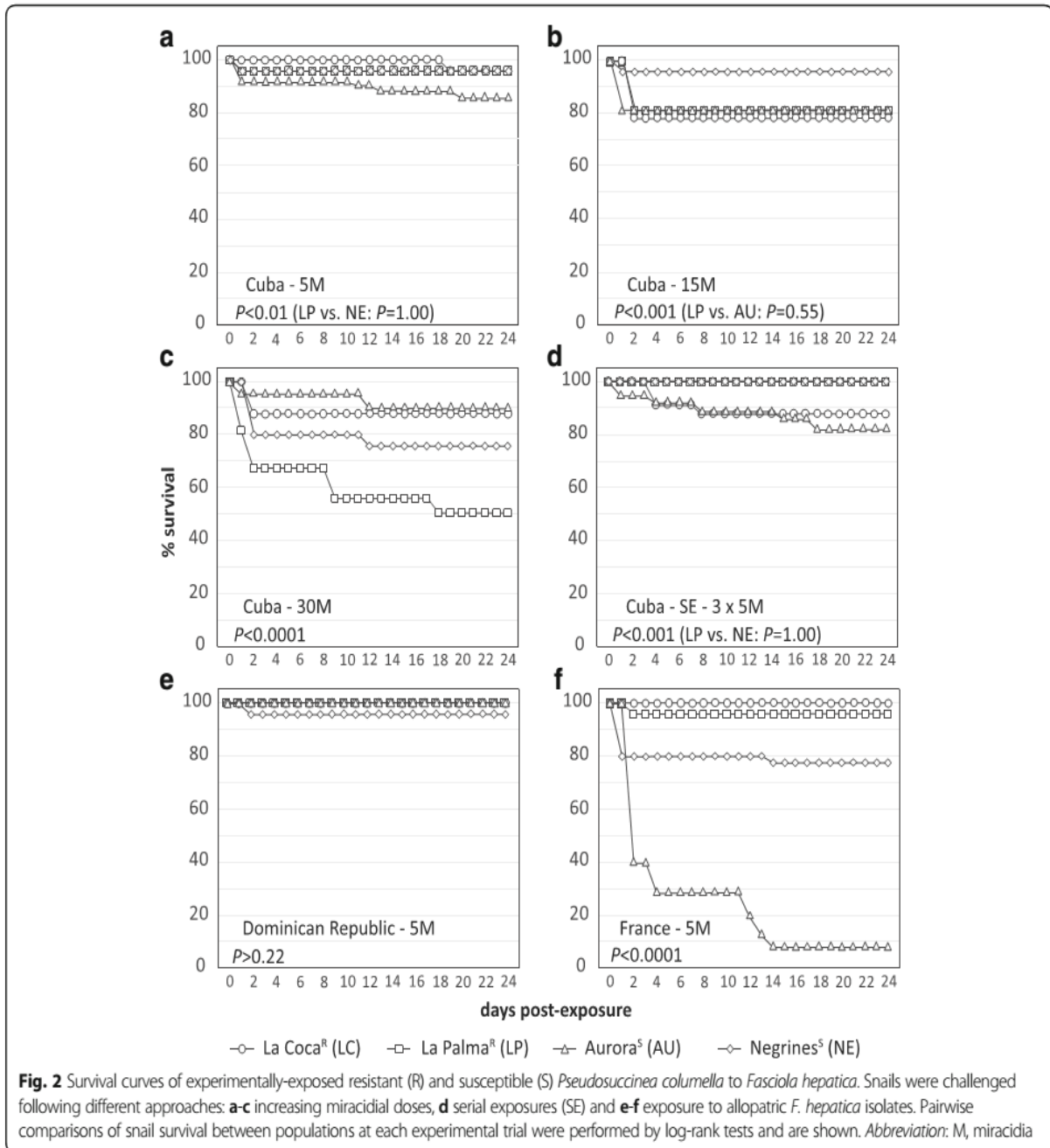
using Shapiro-Wilk and Levene tests, respectively. Factorial ANOVAs followed by a *post-hoc* multiple comparison Tukey test were carried out to assess statistical significance of the effect of (i) different miracidial doses and serial exposures within host populations, and (ii) allopatric and sympatric (data of experimental infection with Cuban *F. hepatica* at a 5 M dose) in redial burden. All calculations were performed in Statistica v.12 (StatSoft. Inc., Tulsa, OK, USA) and differences were always considered significant at values of  $P < 0.05$ .

**Results**

Results of host survival in the exposed groups are shown in Fig. 2. Overall, we observed a mortality peak on exposed snails occurring at 24–48 h post-exposure and a

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significant decrease of snail survival with increasing miracidia dose (Fig. 2a-d; log-rank tests:  $P < 0.05$ ; Aurora, 5 M vs 30 M,  $P = 0.24$ ; Negrines, 5 M vs 15 M,  $P = 1$ ). No mortality was observed within the studied time frame in the control group (non-exposed snails; data not shown).

#### Infection outcome in resistant *P. columella*

As expected, while susceptible *P. columella* became infected after exposure to *F. hepatica*, resistant snails from La Palma and La Coca failed to develop larval stages and no sign of infection was observed following each of the experimental approaches tested (Table 1). Notably, resistant populations showed higher overall survival than susceptible *P. columella* (Fig. 2).

#### Infection outcome in susceptible *P. columella*, increasing miracidial dose and serial exposures

An overall increase of both prevalence (Fisher's exact test: Aurora, 5 M vs 30 M,  $P = 0.021$ ; Negrines, 5 M vs 15 M / 3 x 5 M,  $P < 0.03$ ; Table 1) and redial burden (Fig. 3a) was noted in susceptible individuals with the increase of the miracidial dose, either by single or serial exposure events. However, no significant differences in prevalence and redial burden were observed when snails were infected with 15 M or 30 M (Fisher's exact test: Aurora, 15 M vs 30 M,  $P = 0.423$ ; Negrines, 15 M vs 30,  $P = 0.472$ ; Fig. 3a).

An interesting result regarding redial burden was observed when comparing infection outcomes after

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**Table 1** Prevalence (95% confidence interval) of *Fasciola hepatica* in resistant and susceptible *Pseudosuccinea columella* using different experimental exposure approaches

Experimental infections	Experimental groups	<i>Pseudosuccinea columella</i> populations (%)			
		La Coca (n = 30) <sup>a</sup>	La Palma (n = 30) <sup>a</sup>	Aurora (n = 30) <sup>b</sup>	Negrines (n = 30) <sup>b</sup>
Increasing infective dose: sympatric <i>F. hepatica</i>	5 M	0	0	66.7 (48.1–85.2)	63.3 (44.4–82.2)
	15 M	0	0	83.0 (66.4–92.6)	90.0 (74.4–96.5)
	30 M	0	0	93.3 (78.7–98.2)	80.0 (62.7–90.5)
Serial exposure: sympatric <i>F. hepatica</i>	3 × 5 M	0	0	90.0 (74.4–96.5)	100 (88.7–100)
Exposure to allopatric <i>F. hepatica</i>	Dominican Republic 5 M	0	0	50.0 (33.1–66.9)	66.7 (48.8–80.8)
	France 5 M	0	0	13.3 (4.2–29.9)	60.0 (40.7–76.6)

Abbreviation: M miracidia

<sup>a</sup>Resistant populations<sup>b</sup>Susceptible populations

exposures to 15 miracidia, either singly (15 M) or serially-delivered (3 × 5 M; Fig. 3a): it was significantly lower in serially-exposed susceptible snails (Aurora,  $P = 0.0145$ ; Negrines,  $P = 0.0326$ ; Fig. 3a). Contrastingly, a similar parasite prevalence was attained with both conditions (Fisher's exact test: Aurora,  $P = 0.7065$ ; Negrines,  $P = 0.2372$ ).

#### Infection outcome in susceptible *P. columella*, comparing exposure to sympatric and allopatric *F. hepatica* isolates

Infection with a 5 M dose of either Cuban and Dominican isolates produced similar prevalence and redial burden in the two susceptible *P. columella* populations (Table 1, Fig. 3b). However, compared to the Dominican isolate, a significant decrease on snail survivorship was observed with the Cuban parasite (Fig. 2a, e; log-rank tests:  $P < 0.01$ ).

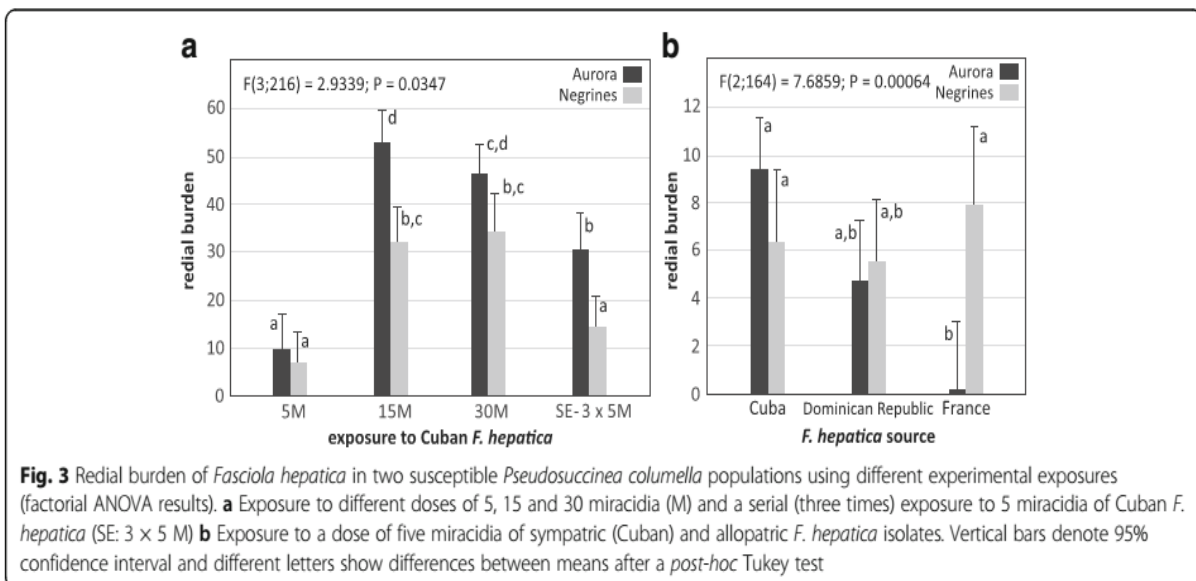
On the other hand, we found significant differences in compatibility between susceptible *P. columella* and the French *F. hepatica* isolate and this variability depended

upon the snail-parasite combination (Table 1, Fig. 3b). A poor performance of French *F. hepatica*, in terms of prevalence and redial burden, was recorded when infecting Aurora snails but it appears as compatible as the Cuban isolate with Negrines (Table 1, Fig. 3b). It is noteworthy that an overall impairment of snail survivorship on susceptible populations was observed with the French parasite isolate when compared with the sympatric *F. hepatica* (Fig. 2f; log-rank tests:  $P < 0.001$ ).

#### Discussion

##### *P. columella* resistance to *F. hepatica* remains after high parasite doses, serial exposures and parasites from different geographical origins

*Fasciola hepatica* infection of its snail host is a dynamic and complex process in which several stages (i.e. snail-finding, penetration, migration, establishment and larval development) and requirements (e.g. biochemical, physiological and immunological), accounting for host-parasite compatibility, must be met to ensure



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parasite success [22]. In the present study, the experimental exposure of *P. columella* snails to *F. hepatica* was followed by the successful penetration of all miracidia into the snails in every trial. After this initial interaction, the first 24–48 h are crucial to the final outcome of the infection. In this time frame, a series of fundamental events occurs inside the host: (i) complete transformation of miracidia into sporocysts [22]; (ii) migration within the digestive tract and first colonization attempts [23]; and (iii) orchestration of snail defence response, whether protective or not [12]. Either compatible *F. hepatica* larva survive and become established (the host becomes infected), or no further parasite development occurs and exposed snails are free from infection. Given the complexity of the initial interaction, it is not surprising that a peak of mortality in *P. columella* occurred during the first 24–48 h post-exposure, even in individuals from resistant populations (see Fig. 2).

As mentioned before, exposure to a large number of miracidia and/or to serial infections could differentially modify the parasite/host possibilities to infect or to be infected, most likely in favour of the parasite [17]. Our results on susceptible *P. columella* coincide with other investigations that have also rendered higher infection rates, mainly in sympatric snail-trematode combinations, after experimental infections with large numbers of parasite or serial miracidial exposures [17, 24, 25]. This strongly points at the pertinence of the first two experimental schemes used for tipping the scales in favour of parasite success. However, in this sense, the effectiveness of the resistant phenotype was not affected even after being challenged with a large number of miracidia and an enhanced genetic diversity of the parasite (either singly or serially delivered) with which each snail was confronted (*F. hepatica* isolate from La Palma isolate is known to be polymorphic [15]). Thus, host involvement in defence contributing to parasite elimination in resistant *P. columella* appears to be enough to protect the snails even from high infective doses without reversion of the resistant phenotype.

In addition, we investigated if resistant *P. columella* snails could resist other parasite isolates beyond the local (Cuban) isolates, challenging them with geographically distant *F. hepatica*, in an attempt to increase the chances of exposing the snails to parasites with different genetic diversity. Although there are no data on the genetic population structure of the allopatric *F. hepatica* isolates used, this species is known to be highly polymorphic with a preferential out-crossing reproduction [26]. Moreover, we should expect genetic differences among the isolates used given the geographical isolation, particularly between the Cuban and the European flukes. In this sense, the differences in *F. hepatica* performance between allopatric and sympatric isolates when infecting

susceptible *P. columella* suggest significant differences in compatibility depending on host-parasite combination (as seen elsewhere [14]). These differences, most likely related with the assumed genetic differences, were highly marked between *F. hepatica* from Cuba and France. However, no infection developed in resistant snails exposed to either allopatric *F. hepatica* used, suggesting that the defence mechanisms involved in the resistant phenotype (protective immune response towards the parasite) might not be tightly restricted to a local genotype-genotype type interaction.

The underlying mechanism mediating the encapsulation of *F. hepatica* observed in resistant *P. columella* 24 h post-exposure [12] remains to be fully elucidated. However, mounting an efficient immune response against *F. hepatica*, or any other parasites, is expected to be costly [27] and would certainly result in trade-off against significant energy and resources of the host. However, host investment in defence might result all-too-costly for some individuals, particularly when parasites are highly pathogenic or virulent (as seen for the French *F. hepatica*-Aurora snails interaction), if the infective dose is high, or even if the regulatory mechanisms of the host response are not entirely effective. This may explain the decreased survival of resistant snails when exposed to a high miracidia dose, an effect that was also observed by Gutierrez et al. [12]. In any case, and considering both the individual and population levels, resistant *P. columella* populations failed to develop the infection.

#### Redial production in relation to high miracidial doses and successive exposure approaches

As for parasite prevalence, redial burden had an overall increase in susceptible snails when infected with doses higher than 5 M. However, the method of delivering the infective parasite dose influenced the parasite count (more rediae in a single 15 M dose compared to serially-exposed snails). This result could be linked to an increased ability to control parasite development on serially-exposed snails driven by boosting defences with the first exposure, a phenomenon commonly known as immune priming. Immune priming is acknowledged to occur in invertebrates and has been previously reported in the system *Biomphalaria glabrata*-*Schistosoma mansoni*, where enhanced protection of the host (ranging from decreased prevalence and parasite intensity to complete protection) may appear after subsequent parasite encounters [28–30]. According to these authors, the extent of such acquired protection increases towards genetically similar challenges and when re-exposure occurs 10 days after the primary infection. The demonstration of an immunological memory process in susceptible *P. columella*-*F. hepatica* deserves further investigations.

On the other hand, no significant variation was observed in infected snails exposed to 15 or 30 M which



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could be related to intraspecific competition of parasite larvae for host resources. Rondelaud et al. [31] referred that multiple miracidial infections could impact *F. hepatica* productivity in its intermediate host by limiting redial numbers on second and third generations.

#### Performance of allopatric *F. hepatica* with susceptible *P. columella* snails

In a previous study involving experimental infections with several Cuban *F. hepatica*-lymnaeid snail combination, Vázquez et al. [14] proposed the existence of a polymorphism of compatibility in this interaction when presented with a variety of outcomes in terms of snail survival and the parasite's prevalence and redial burden in susceptible snails. From our results, we also observed differences in compatibility between the local and allopatric *F. hepatica*-*P. columella* systems that varied depending on the host-parasite combinations.

In this sense, the significantly lower performance of French *F. hepatica* in susceptible *P. columella* from Cuba contrasted with what was observed with the Dominican allopatric isolate. The latter displayed a similar prevalence and redial burden to the local Cuban isolate. These results could be related with the peculiarities of the epidemiological scenario from each geographical area (Caribbean and Europe), especially concerning the differences on intermediate host species and environmental conditions. In fact, lymnaeid vectors of fasciolosis in Cuba (*Galba cubensis* and *P. columella*) are the same occurring in the Dominican Republic and in most of the Caribbean region [32, 33]. Thus, even when local adaptation of Dominican parasites to Cuban snail populations is improbable due to geographical isolation (both are separated islands), lower differences can be expected between allopatric parasites evolving under similar conditions (e.g. the same host species) and could explain the similar performances recorded between Cuban and Dominican *F. hepatica*. Conversely, *F. hepatica* transmission in France is mainly related to other snails (e.g. *Galba truncatula* and *Omphiscola glabra*) [34] which could lead to a divergent evolution of the European parasite and may explain its lower compatibility with Cuban *P. columella*. Rondelaud et al. [35] also observed differential redial development and cercarial production when parasites from Argentina and France were tested against a European *G. truncatula*. Thus, different vector species from different geographical regions could be particularly important when analysing compatibility of allopatric and sympatric *F. hepatica* isolates and might bring a deeper understanding on parasite evolution.

#### Conclusions

We found that resistance in *P. columella* to *F. hepatica* infection remains independent of the parasite dose, serial

parasite exposures or the geographical origin of the parasite. Conversely, the *F. hepatica*-susceptible *P. columella* interaction seems specific for infection and is favoured by high parasite doses. Finally, our results endorse the potential use of resistant *P. columella* snails as an alternative for the control of parasite transmission.

#### Abbreviations

M: Miracidia; R: Resistant; S: Susceptible

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#### Availability of data and materials

All data and materials presenting in this research paper are available upon request by contacting AA or BG.

#### Authors' contributions

AA designed, performed and analysed the experiments and drafted the manuscript. AV, DD, SH and BG participated in design of the experiments, analysis and the reviewing process. JS, ML and ES participated in the experiments and reviewing process. HH participated in the reviewing process. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

French laboratory holds permit #A66040 for experiments on animals, which was obtained from the French Ministry of Agriculture and Fisheries and the French Ministry of National Education, Research and Technology. The housing, breeding and care of the utilized animals followed the ethical requirements of France. The experimenter possesses an official certificate for animal experimentation from both of the above-listed French ministries (Decree #87-848, October 19, 1987). The various protocols used in this study have been approved by the French Veterinary Agency of the DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de l'Agriculture et de la Forêt), Montpellier, France (authorization # 007083). The Cuban laboratory of Malacology holds permit for experiments on animals obtained from the Cuban government. The housing, breeding and care of the utilized animals followed the ethical requirements of Cuba. The experimenter possesses an official certificate for animal experimentation from the Cuban government.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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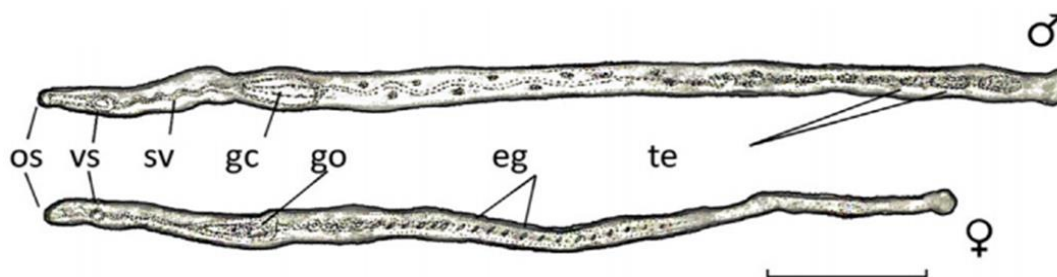
### 3.2 Experimental exposure to *Trichobilharzia* sp. of *P. columella* resistant and susceptible to *F. hepatica*

#### Introduction

The role of *Pseudosuccinea columella* as intermediate host of several digenean parasites besides *F. hepatica* is acknowledged (Grabner et al. 2014; Zimmermann et al. 2014). However, while natural infections are difficult to be observed in snails, the experimental screening for snail susceptibility to parasites is limited by the rearing of the snails on the laboratory and the availability of parasites, particularly of their infective stage if the life cycle is not occurring under controlled conditions. Here, we collect in the field adult parasites of a bird schistosome belonging to the *Trichobilharzia* genus to obtain the eggs and then, the miracidia. With these larvae, we performed experimental infections on susceptible and resistant *P. columella* to challenge both phenotypes against a different trematode species, aiming at depicting the specificity of the resistant phenotype.

#### Material and methods

*Trichobilharzia* parasites (see Figure 21) were obtained from blue-winged teals (*Spatula discors*) captured by licensed sportive hunters when the ducks were arriving to western Cuban shores (September) and kindly donated for scientific purposes. Teals were kept in ice to be quickly transported to our facilities at the Institute "Pedro Kourí" of Tropical Medicine for parasite screening (see Sánchez et al. 2018 for details).



**Figure 21.** Schematic drawing made on mounted *Trichobilharzia* adults (male and female) retrieved from the Blue-winged Teal. Morphological details allowing identification are signaled; eg: eggs; gc: gynecophoric canal; go: genital opening; os: oral sucker; te: testes; vs: ventral sucker, sv: seminal vesicle. Bar = 1 mm.

We found that *Trichobilharzia* sp. was highly prevalent and found in the intestines, the liver, the lungs and nasal sinus of the sampled teals. To obtain the eggs, capillary vessels annexed to the nasal sinus, where adult female parasites occurred, were carefully

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checked for layered eggs. Eggs were washed with sterilized tap water which was followed by miracidial hatching. Immediately, these larvae were used for experimental infections of laboratory-reared snails.

Fourteen species within the genus *Trichobilharzia* are described in North America and are transmitted by snails from the Lymnaeidae or the Physidae families (Hórák et al. 2002). Thus, we included in the experimental infection scheme snails from both taxa: *P. columella* (including resistant and susceptible snails) and *Physa acuta* (Physidae). Briefly, three-weeks-old *P. columella* snails from La Coca (resistant) and from Aurora (susceptible) populations (n = 30) were used for experimental infections. Thirty individuals from *P. acuta* were also independently exposed to the parasite. Snails were individually placed in 96-well plates and each one exposed for 6 hours to 5 freshly-hatched *Trichobilharzia* miracidia. Exposed snails were maintained at 28°C following Sánchez et al. (1995) and monitored daily to assess mortality.

Infection status was verified by cercarial shedding and the prevalence of *Trichobilharzia* sp. in each *P. columella* population was expressed as percentage of infected snails of all initially exposed (n = 30). Confidence limits were calculated at 95% confidence level by Wilson score interval. Differences in infection rates between resistant and susceptible snails were checked by Fisher's Exact Test. Survival curves of exposed snails (from day 0 to the end of cercaria shedding) were constructed based on the number of alive snails at each time point divided by the number of exposed individuals and expressed in percentage. A log-rank test of Kaplan-Meier curves was performed to assess the statistical differences on survivorship data. Statistica v.12 (StatSoft. Inc., Tulsa, OK, USA 2014) was used for calculations and differences were always considered significant at values of  $P < 0.05$ .

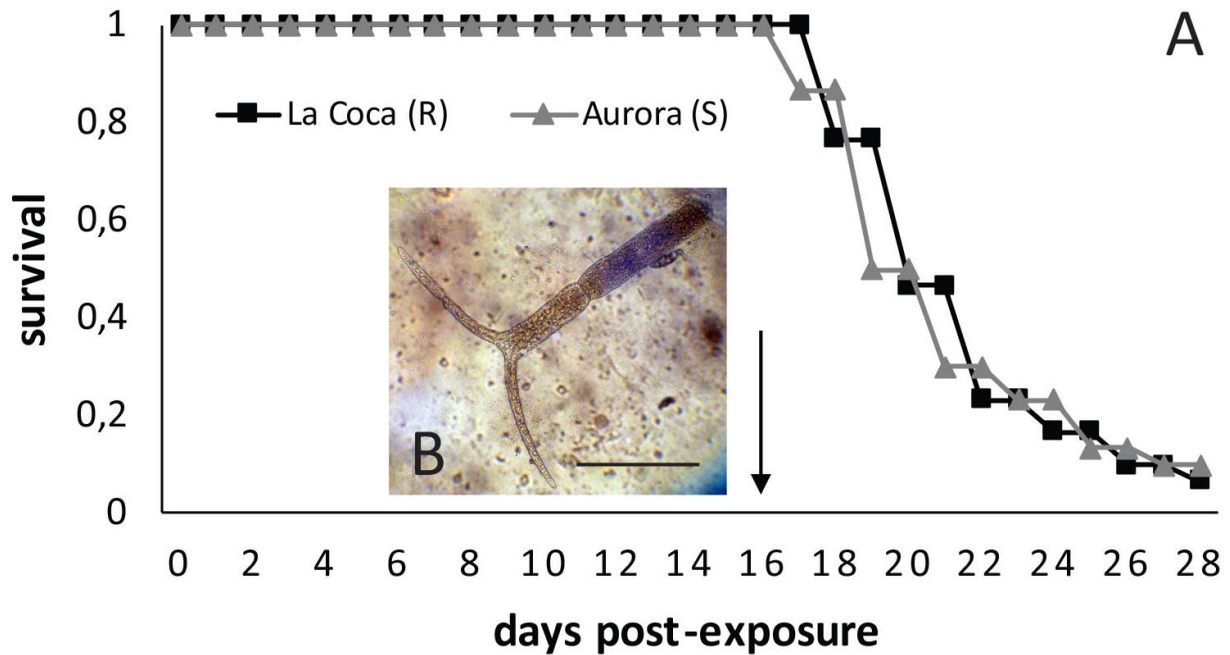
## Results

No infection was developed on *Trichobilharzia*-exposed *P. acuta* individuals. Infection of *Trichobilharzia* sp. successfully developed on both *P. columella* phenotypes and it was characterized by high parasite prevalence (La Coca – 93.8% (69.8 - 99.8%); Aurora - 90.9% (70.8 – 98.9%)) and high snail survival during the prepatent period (before cercarial shedding; Figure 22A). Interestingly, no significant differences ( $P = 1.000$ ) regarding *Trichobilharzia* prevalence was found between resistant and susceptible *P.*

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*columella* and in both populations shedding of cercaria (Figure 22B) was clearly patent for 8-9 days and began at day 16 post-infection (Figure 22A). Likewise, similar survival curves (Log-rank Test,  $P = 0.86$ ) were observed on Aurora and La Coca after infection, with a mortality peak that start prior the beginning of the cercarial shedding and concluded with the death of all infected individuals (Figure 22A).



**Figure 22.** A) Survival rate of resistant (La Coca) and susceptible (Aurora) populations of *Pseudosuccinea columella* to *F. hepatica* after experimental infection with *Trichobilharzia* sp. The arrow indicates the beginning of the patent infection. B) Photo of one of the emitted *Trichobilharzia* cercariae during the patent infection. Scale bar = 50 µm.

## Discussion

Here, morphological features of the adult flukes obtained in the field are consistent with those of the North American species: *T. querquedulae*, *T. physelae* and *T. elvae* (see Sánchez et al. 2018). However, while *T. querquedulae* and *T. physelae* are transmitted by snails from the Physidae family, *T. elvae* are compatible only with lymnaeid snails (Hórák et al. 2002). The results presented here strongly suggest that the recovered flukes belong to the latter species since infection was solely developed in *P. columella*. However, molecular tools are needed to achieve confident discrimination (Brant & Loker, 2009).

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Interestingly, we found no differences on the infection traits between susceptible and resistant *P. columella* which support the idea of a certain specificity of the resistant phenotype towards *F. hepatica*. The latter might be related with the selection of the feature by the *F. hepatica* itself or by other similar digenean species. However, it needs to be considered that *F. hepatica* and *Trichobilharzia* are different parasites and further experimental challenges and approaches with other trematodes would be mandatory to validate this hypothesis. Beyond the phylogenetic distance, the intramolluscan development should be also considered for future investigations. Overall differences in the survival curves between *F. hepatica* and *Trichobilharzia*-exposed snails (higher mortality during prepatent vs. patent infection) are likely related with the fact that *F. hepatica* is a redia-produced parasite while in *Trichobilharzia* sp. cercaria develops from daughter sporocyst. It has been stated that the type of intramolluscan stage determines the host life history outcome concerning the timing of mortality since rediae infections tend to reduce host survival earlier in the infection than sporocyst infections do (Sorensen & Minchella, 2001).

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### 3.3 Comparative dynamic of the immune response between resistant and susceptible *P. columella* following *F. hepatica* challenge: hemocytes in the spotlight

#### Article 4 (in preparation)

Unravelling the immunobiological patterns of *Pseudosuccinea columella* resistant/susceptible snails - *Fasciola hepatica* trematode interaction: hemocytes in the spotlight

### Unravelling the immunobiological patterns of *Pseudosuccinea columella* resistant/susceptible snails – *Fasciola hepatica* trematode interaction: hemocytes in the spotlight

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## Abstract

The *Fasciola hepatica*/*Pseudosuccinea columella* interaction in Cuba displayed a unique pattern of compatibility phenotypes within field-occurring snail populations: most are susceptible to *F. hepatica*, but a few populations actively resist its infection (parasite encapsulation by snail's hemocytes occurs). Thus here, we focus our attention on the hemocytes of *P. columella* resistant and susceptible and investigated their morphology, abundance, proliferation and *in vitro* encapsulation activity following *F. hepatica* infection. Our results on parasite challenged-resistant snails, showed a significant increase of circulating cells and a certain aggregative behavior on large adherent hemocytes. Significant proliferation of circulating blast-like hemocytes was recorded *in vitro* in 24h-exposed resistant snails which was also consistent with the enhanced expression of the cytokine granulins in resistant snails following infection, demonstrated by RT-qPCR. *In vitro* assays showed a strong association of hemocytes from resistant snails with high encapsulation grades of *F. hepatica* sporocyst, compared to susceptible snails and a concomitant increase on their encapsulation activity following exposure. Additionally, pre-incubation of naïve *P. columella* hemocytes with different monosaccharides was associated with lower encapsulation activity. This observation suggests the involvement of saccharides (particularly mannose and fucose)/glycan-sensing molecules in this host-parasite interaction in association with hemocyte activation and/or binding to *F. hepatica*.

**Keywords:** host-parasite interaction, hemocyte, proliferation, parasite encapsulation, glycan, granulins



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## 1.1 Introduction

*Fasciola hepatica*, a worldwide and long-established parasite in many countries, produces an important liver disease known as fasciolosis, affecting 17 million people and causing severe burden in livestock (particularly bovine and ovine) (Mas-Coma et al., 2009). This trematode is transmitted by a number of small freshwater snails of the family Lymnaeidae which are key actors in the successful maintenance of its life cycle (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.

Particularly, the *Fasciola hepatica/Pseudosuccinea columella* system in Cuba is a fascinating model to study the immunobiology within a trematode/snail interaction since two phenotypes can be distinguished in Cuban snails: while most *P. columella* populations are susceptible to the parasite infection and thus, further transmission is possible, some are resistant to *F. hepatica* (Gutiérrez et al., 2003b). To our knowledge, this is the only lymnaeid snails/*F. hepatica* system in which field-occurring resistance to the parasite exists within the same snail species (Gutiérrez et al., 2003b, Gutiérrez et al., 2003a) and remains effective irrespective 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 are able to encapsulate *F. hepatica* larvae shortly after penetration (Gutiérrez et al., 2003b). According to these authors, the observed cellular immune response mediates parasite killing and resolution of infection, and has been associated with the resistant phenotype.

The hemocytes figure among the main arsenal of the innate immune system within invertebrates, playing crucial roles in both cellular and humoral defenses (Hillyer, 2016, Pila et al., 2016b). Being the leading actors of the cellular immune response, hemocyte functions in mollusks go beyond surveillance, phagocytosis (Cueto et al., 2015, Ataev et al., 2016) and encapsulation (Gutiérrez et al., 2003b), reaching cytotoxic reactions (Humphries and Yoshino, 2008) and the production of a plethora of molecules involved in signal transduction, immune defense regulation or immune effector activities (Baeza-Garcia et al., 2010, Galinier et al., 2013, Pila et al., 2016a). Thus, the production, differentiation and activation of these cells are essential, and are guaranteed and regulated by molecular signaling pathways in

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relation to different stimuli which are still to be well elucidated (Pila et al., 2016b, Zhang et al., 2016). Particularly, in digenean – snail interactions, encapsulation of the parasite by snail hemocytes is an important defense response that involves the isolation of the parasite from host signals and nutrients, and 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 reports can be cited in which an increase in the number of circulating cells has been associated with lower parasite susceptibility (Gorbushin and Iakovleva, 2008, Larson et al., 2014, Oliveira et al., 2010) or where parasite killing is mediated by hemocytes (Sullivan and Yeung, 2011, Gutiérrez et al., 2003b, Coustau et al., 2015). Therefore, the study of hemocytes is mandatory to fully understand snail – trematode interactions.

Here, we report the first attempt to explore the hemocytes of resistant and susceptible *P. columella*. We used comparative approaches aiming at describing their morphology, total numbers, proliferative activity, and *in vitro* encapsulation capacity in each phenotype on naïve snails and after parasite exposure. This study provides novel insights into the immunobiology of *P. columella* – *F. hepatica* interaction.

### 1.2 Material and methods

#### 1.2.1 Laboratory-reared susceptible and resistant *P. columella* snails

All snails used were obtained in the Laboratory of Malacology of the Institute of Tropical Medicine “Pedro Kourí”, Cuba. Susceptible snail cultures were established with parental *P. columella* sampled from two field populations, Negrines and Aurora, located in Havana and Mayabeque province, respectively. The resistant snail cultures were established with snails originating from La Coca locality (Havana province) and, from La Palma, (Pinar del Rio province), which displayed the phenotypic mantle marker associated with resistant populations (Gutiérrez et al., 2003b). All snails were reared following Sánchez et al. (1995) recommendations, in Petri dishes growing algae in 26°C de-chlorinated water supplemented with crushed shells as carbonate resources. The snails were fed on the algae *ad libitum* and changed to other Petri dishes growing algae once the food got scarce.

#### 1.2.2 Experimental infections of snails with *F. hepatica* miracidia

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*Fasciola hepatica* flukes from Jaruco (Mayabeque province) were obtained from a local cattle abattoir 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.

Eggs of *F. hepatica* were incubated in distilled water, in total darkness, at 28°C, for 15 days for complete maturation. At day 15, miracidia were obtained after the induction of egg hatching by exposure to light. Experimental infections of snails were carried out using freshly hatched miracidia, with five miracidia per snail, according to Vázquez et al. (2014) to test for resistant and susceptibility. As expected, no infection was developed on resistant snails from La Coca and La Palma while Aurora and Negrines populations showed prevalence of *F. hepatica* infection of 76.3% and 54.67%, respectively.

### 1.2.3 Hemolymph sampling

Five-to-six weeks old *P. columella* lab-reared snails were used to bleed. Snail shells were cleaned with de-chlorinated water and cotton swabs to remove any surface debris and mucus, and wiped dry. Cardiac puncture (Bayne et al., 1980) was used to obtain hemolymph, which was collected using sterile pipette tips. Hemolymph that pooled on the shell was also sampled.

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

Flow cytometry: Hemocytes from naïve snails were explored in a MACS Flow Cytometer equipment (Miltenyl Biotech), previously calibrated according to the manufacturer's protocol, using the MACS Quantity™ v. 2.5. (2003) software. Every acquisition was performed at a flow rate of 1 µL/s and 10 000 events were analyzed. We make pools of four snails (5 µL of freshly collected hemolymph per snail, see section 1.2.3) per strain to 180 µL of saline snail buffer (SSB: 5 mM HEPES, 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM MgCl<sub>2</sub>, 4 mM CaCl<sub>2</sub>; pH 7.9). Samples were placed in conical skirted polypropylene tubes and placed on ice until measurement to minimize cellular adhesion. A blank sample containing only SSB was always included and five replicates were made 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.

Confocal Fluorescence microscopy: Collected hemolymph from naive and 24h-exposed snails were applied onto polystyrene slides and incubated at room temperature for 45 min in a

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humid chamber to allow for cells adherence and spreading. 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 Na<sub>2</sub>HPO<sub>4</sub>, 0,2 g/L KH<sub>2</sub>PO<sub>4</sub>, pH 7,2) for 30 s. Afterwards, 500 µL of PBS-diluted Alexa Flour® 488 Phalloidin (Invitrogen), 1/40, v/v, was added to each sample for actin labelling and incubated for 30 min. The excess of phalloidin was removed with PBS for 30 s and 500 µL of 4',6-diamidino-2-phenylindole (DAPI: Invitrogen) diluted 1/1000 in PBS was added to the slides for DNA (nucleus) staining and incubated for 2 min. Samples were washed in PBS for 30 s, let to dry and mounted in fluorescence mounting medium (Dako). Observation of stained hemocytes was carried out on a Zeiss LSM 700 confocal microscope. The associated software Zen (2008) enabled the adjustment of acquisition parameters. The images were treated with ImageJ 1.41o. Based on this staining, the overall percentage of the different morphotypes was estimated at naïve and at 24h-post exposure in each strain.

### **1.2.5 Counting of *P. columella* hemocytes at early time points post-exposure to *F. hepatica***

The hemolymph was obtained (see section 1.2.3) and mixed 1:5 with PBS, loaded on a Neubauer Improved hemocytometer and left the cells to settle for 1 min. Total cells were counted in five 1 mm squares at 200X magnification and cell numbers were calculated as cells/µL of hemolymph. Counts were done at 6 designated time points: 0 h (naïve snails), 6 h, 12 h, 24 h, 48 h and 96 h after exposing the snails to *F. hepatica* miracidia. Samples of hemolymph of nine snails per strain were individually counted at each time point. The experiment was repeated twice as independent experimental settings.

### **1.2.6 Assessment of *in vitro* hemocyte proliferation**

The proliferation activity of circulating hemocytes from resistant and susceptible *P. columella* was estimated by *in vitro* assays performed to measure DNA synthesis with Click-iT® EdU Imaging kit (Invitrogen) according to manufacturer's guidance. Briefly, 5 µL of freshly collected hemolymph from naïve and 24h-exposed snails (6 snails per time point per strain) were applied onto polystyrene slides (Evergreen) and gently diluted 1:2 with SSB. EdU solution was added to each sample to a final concentration of 0.1 mM and incubated for 3h at room temperature in a humid chamber. Afterwards, cells were fixed with 4%

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formaldehyde (100  $\mu$ L) for 15 min, washed twice with 3% bovine serum albumin in PBS (PBS-BSA) and permeabilized with 100  $\mu$ L of 0.5% Triton X-100 for 20 min. After washing with PBS-BSA, samples were incubated for 30 min in the dark on freshly prepared Click-iT solution. For nucleus counterstaining, DAPI (diluted 1/1000 in PBS) was added after washing with PBS-BSA and incubated for 2 min in the dark. Samples were washed in PBS-BSA twice for 30 s, left to dry and mounted in fluorescence mounting medium (Dako). Observation was performed in an epifluorescence microscope. Phase-contrast was also used to characterize morphology of the proliferative-marked cells. The overall number of cells (Dapi-stained nucleus) and of those in proliferation (EdU-marked nucleus) were recorded for each snail strain ( $n = 4$ ) at 0h and at 24h post-exposure.

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

A non-annotated transcriptome of *P. columella*, from-pair-end RNAseq-de novo assembly, sequenced from triplicate samples of susceptible and resistant snails (Alba et al. in preparation; transcriptome available on laboratory database: <http://ihpe.univ-perp.fr/acces-aux-donnees/>), was used as template for RT-qPCR primers design. Briefly, the sequence of the proliferation-related cytokine granulin (HQ661843.1) from *Biomphalaria glabrata* snails was retrieved from Genbank database and used to identify the granulin ortholog gene in *P. columella* transcriptome (TR27784|c1\_g1/4\_i1). The ortholog was recovered by using a Blastn research against the assembled transcriptome and the best Blast hit (E value =  $10^{-56}$ ) was further verified through nucleotide or primary protein sequence analysis and functional domains characterization with Blastx, Expsy and CDD servers. The Primer3Plus software was used to design different set of primers for RT-qPCR from the identified granulin sequence of *P. columella* and primers' parameters were double checked with OligoProperties Calculator (Kibbe, 2007). Only the set of primers rendering a single qPCR product with an efficiency higher than 1.9 was considered for granulin expression quantification (F: 5' TGTCTGCCCTTGCTTACATTCA, R: 5' GCAAGGGTATTTACCTTTGATTC). The nuclear 18S ribosomal gene of *P. columella* retrieved from Genbank was used as standard for relative quantification (F: 5' AGGGACGAGTGGCGTTTAGC, R: 5'CTTCGTGCTAGGGATTGGGG).

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**RNA extraction:** Five-weeks-old susceptible and resistant *P. columella* snails were used to assess the variation of the relative gene expression of proliferative signals at 5 designated time points: 0 h (naive individuals), 6 h, 12 h, 24 h and 96 h after exposing the snails to *F. hepatica* miracidia. Thirty snails from each population per time points were carefully separated from the shell and immediately placed in individual vials containing RNA*later*<sup>®</sup> (Ambion) solution at a ratio of 1 mg of snail: 10 µL of RNA*later*<sup>®</sup>, w/v. Samples were kept overnight at 4°C and then stored at -20°C until use. RNA*later*<sup>®</sup> was discarded, whole snail tissues were pooled into three biological replicates, each of 10 individuals per time points, immediately frozen in liquid nitrogen and homogenized on a laboratory mixer mill MM400 (Retsch). RNA extraction was performed with TRIzol<sup>®</sup> Reagent (Ambion) following manufacturer's guidelines. Extracted RNA was digested with Turbo DNA-free<sup>™</sup> kit (Ambion) and quantity was estimated by spectrophotometric measurements.

**RT-qPCR:** RNA (0.5 µg) of each biological replicate were used to obtain the cDNA by reverse transcription with random primers using Thermo Scientific<sup>™</sup> Maxima<sup>™</sup> H Minus First Strand cDNA Synthesis kit (Thermo Fisher). RT negative controls (RT-) for each time point and snail strain were simultaneously included and kept, afterwards, at -20°C until further used as controls in qPCR. Amplification and detection of qPCR were performed in a LightCycler<sup>®</sup> 480 Instrument II system (Roche). The amplification mix per reaction was normalized to: 1X of SYBR Green Master Mix Takyon<sup>®</sup> qPCR kit (Eurogentec), 0.5 µM of each primer, 2 µL of ten-times diluted cDNA and molecular grade water up to a final volume of 10 µL. Standard curves of cDNA from naive snails (two-fold diluted from 1/10 to 1/640) were included to calculate the primer efficiencies. Negative controls in which water or RT-products were added instead of cDNA templates were also included. Three technical replicates for each biological replicate were done. 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 granulin was estimated against the housekeeping gene (18S) for each strain at each time points using the LightCycler<sup>®</sup> 480 Instrument II software v. 1.5.0 (Roche), considering an efficiency of 2. The resulted RQ of each time point post-exposure were normalized against the RQ calculated at 0h for each strain.

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

Hemolymph from naïve and 24h-challenged resistant/susceptible snails, were obtained by cardiac puncture as previously described (section 1.2.3) to assess the encapsulation grade of *F. hepatica* sporocyst by snail hemocytes *in vitro*. The functional activity of the cells toward parasitic larvae was tested in two conditions: (1) naïve hemocytes were pre-incubated with monosaccharides, to assess the effect of specific glycans on the encapsulation activity of the cells, and (2) hemocytes were collected 24h post-*F. hepatica* exposure to evaluate variations on the performance of the cells after parasitic challenge. Naïve hemocytes were used as baseline for comparing the results from the two experimental conditions. Six snails/strains were used in each case and results were independently recorded for the different assays and *P. columella* strains.

Briefly, extracted hemolymph from naïve and 24h-challenged snails was individually added to 96-well culture plates previously filled with sterile SSB (110  $\mu$ L/well) up to 130  $\mu$ L. The plates were kept in ice to avoid attachment of the hemocytes until filling up all wells. The counting of alive cells was performed in a Neubauer chamber and cell viability was assessed with Trypan blue from aliquots taken from 2 random wells/conditions. Naïve pre-incubated-with-monosaccharides hemocytes resulted from separately incubated the cells with each of the following monosaccharides: D-mannose, D-glucose, L-fucose and D-galactose (from Sigma), at a final concentration of 150 mM (20  $\mu$ L of 1M stock), for 30 min, in agitation (100 rpm) at 27°C. A volume of 20  $\mu$ L of SSB were added to the remaining wells containing naïve or 24h-challenged hemocytes to standardize the volumes. Afterwards, one newly-*in-vitro*-transformed *F. hepatica* sporocyst was located in each well with the help of a stereoscope, and plates were then incubated at 27°C, for 2h, in agitation (100 rpm). Transformation of *F. hepatica* miracidia into sporocysts was performed as previously described (Gourbal et al., 2008) after an overnight incubation, at 26°C, of freshly hatched miracidia in sterile Chernin's balanced salt solution and 1X antibiotic-antimycotic cocktail (Sigma).

Afterwards, encapsulation activity was checked in each condition. For comparison, we categorized, after pilot studies, the thicknesses of the capsules around the parasite larvae into five grades (from 1 to 5: from almost none to complete encapsulation) to express the observed differences concerning encapsulation in a semi-quantitative scale, as done elsewhere (Kryukova et al., 2014).

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### **1.2.9 Data analysis**

We plotted means and standard deviations 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 1.2.5). 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 1.2.4 and 1.2.6). A Kruskal-Wallis Test and Duncan's Tests were used to estimate statistical differences between RQs of granulin at each time point in each strain (section 1.2.7). The encapsulation grade showed by the hemocytes from each strain was associated to the assayed conditions (preincubation with monosaccharides or parasitic challenge) by means of a multivariate correspondence analysis using the previously established qualitative scale (see section 1.2.8). All analyses were performed in Statistica v.12 (StatSoft. Inc., Tulsa, OK, USA 2014). Differences were considered statistically significant for values of  $P < 0.05$ .

## **1.3 Results**

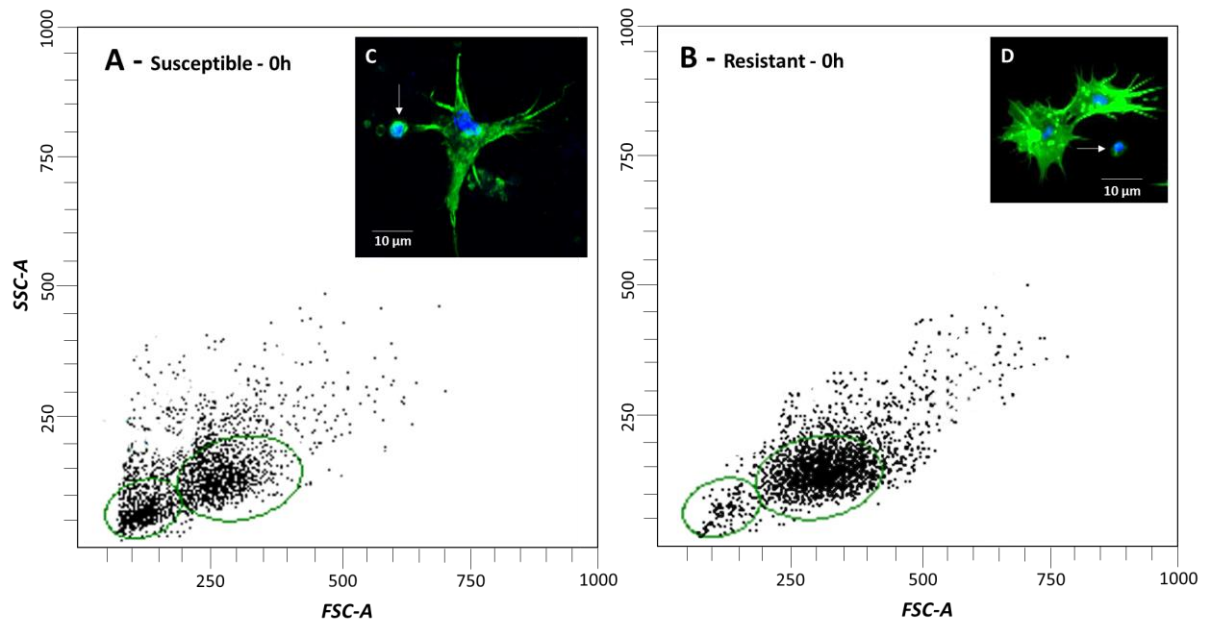
### **1.3.1 Morphological observations of *P. columella* hemocytes**

At least two well-defined morphotypes of circulating cells, differing in size/granulocytic content and size/spreading capacity, were found in the hemolymph of both susceptible and resistant strains of *P. columella*. Flow cytometry analysis performed on 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 1a,b). Consistently, observation from hemocytes-stained preparations also showed two morphotypes (i) large adherent cells (7 to 15  $\mu\text{m}$ ) displaying pseudopodia and (ii) roundly-shaped small cells (3 to 5  $\mu\text{m}$ ), with high nucleus/cytoplasm ratio and low cytoplasmic complexity, similarly to blast-like cells, in both phenotypes (Figure 1c,d).



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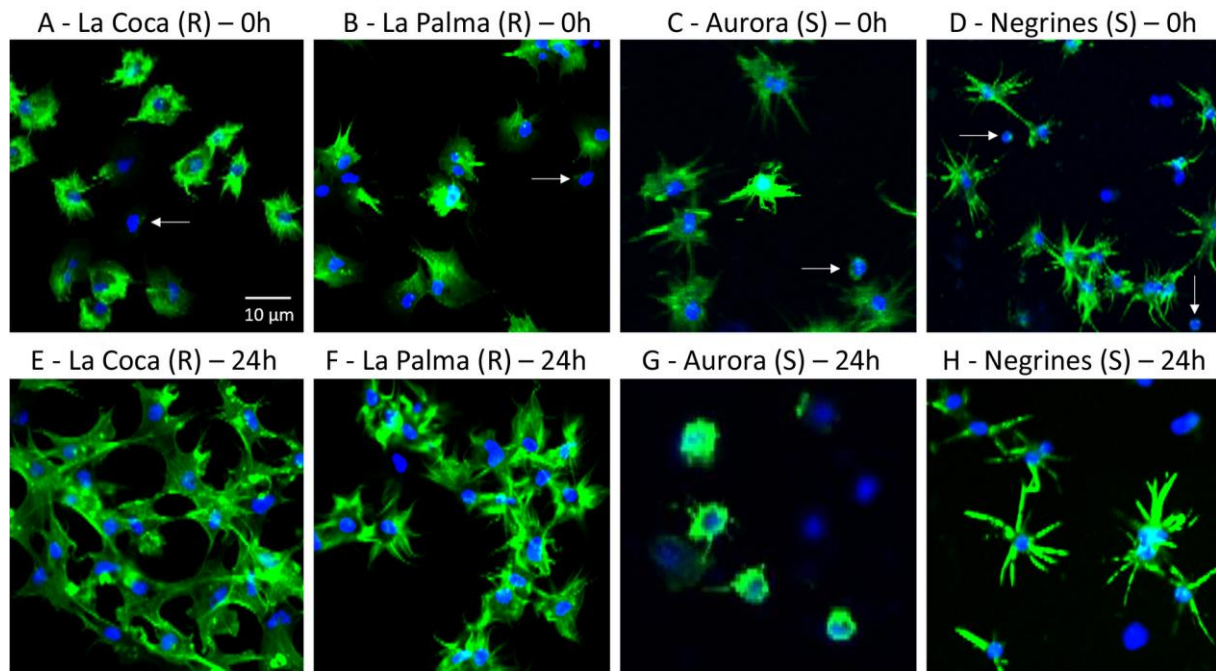


**Figure 1.** Dot plots (A-B; pooled hemolymph from 4 snails/strain) and Dapi/Phalloidin stained (C-D) naïve hemocytes from *Pseudosuccinea columella* susceptible and resistant to *Fasciola hepatica* where two main morphotypes of cells, segregated by size and cytoplasmic complexity can be observed Phalloidin (actin labelling, green) and Dapi (nucleus labelling, blue). The arrows indicate the roundly-shaped small cells.

Thorough observations of large adherent hemocytes, indicate that these are the main morphotype and that they display a sheet-like conformation on snails from both phenotypes on naïve conditions (Figure 2A-D). However, some qualitative differences can be observed on large hemocytes recovered from susceptible and resistant snails at 24h-post exposure (Figure 2E-H). Particularly, in challenged resistant snails, large adherent hemocytes of resistant snails tend to aggregate, to increase the length of its broad-based cytoplasmic projections which extends into a clearly sheet-like spreading pattern (Figure 2E,F). Contrastingly, in 24h-exposed susceptible snails, large adherent cells appear more scattered without a clear tendency to aggregated and showing a more discrete spreading pattern with even shorter pseudopodia as in the case of Aurora (Figure 2G,H). In this sense, intra and inter-strain variability regarding large hemocytes morphology were more noticeable in susceptible snails (Figure 2). The percentage of large adherent hemocytes was always higher in resistant (ranging from 89 to 97%) compared to susceptible snails (62 to 78%), irrespective of the conditions.

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**Figure 2.** Confocal microscopy images of *Pseudosuccinea columella* hemocytes from naïve (upper panel, 0h) and *F. hepatica*-challenged (lower panel, 24h post-exposure) resistant and susceptible snails recovered from four different populations. Cell staining was performed with Phalloidin (actin labelling, green) and Dapi (nucleus labelling, blue).

Concerning blast-like cells, overall, this morphotype was less represented compared to large adherent hemocytes (ranging from 3 to 36% of the total cell counts in naïve *P. columella*; Figure 2A-D). Interestingly, in naïve conditions, significantly higher percentages of blast-like cells were recorded in susceptible snails (Aurora - 26.3%, Negrines - 36.5%) compared to resistant strains (La Coca - 3.7%, La Palma - 10.1%; Fisher's Exact Tests  $P < 0.05$ ). However, at 24h post-exposure, an overall slight increase of blast-like cells was observed regardless of the phenotype (La Coca - 11 %; La Palma - 10.8%, Aurora - 22.5%, Negrines - 37.6%; Fisher's Exact Tests  $P > 0.05$ ).

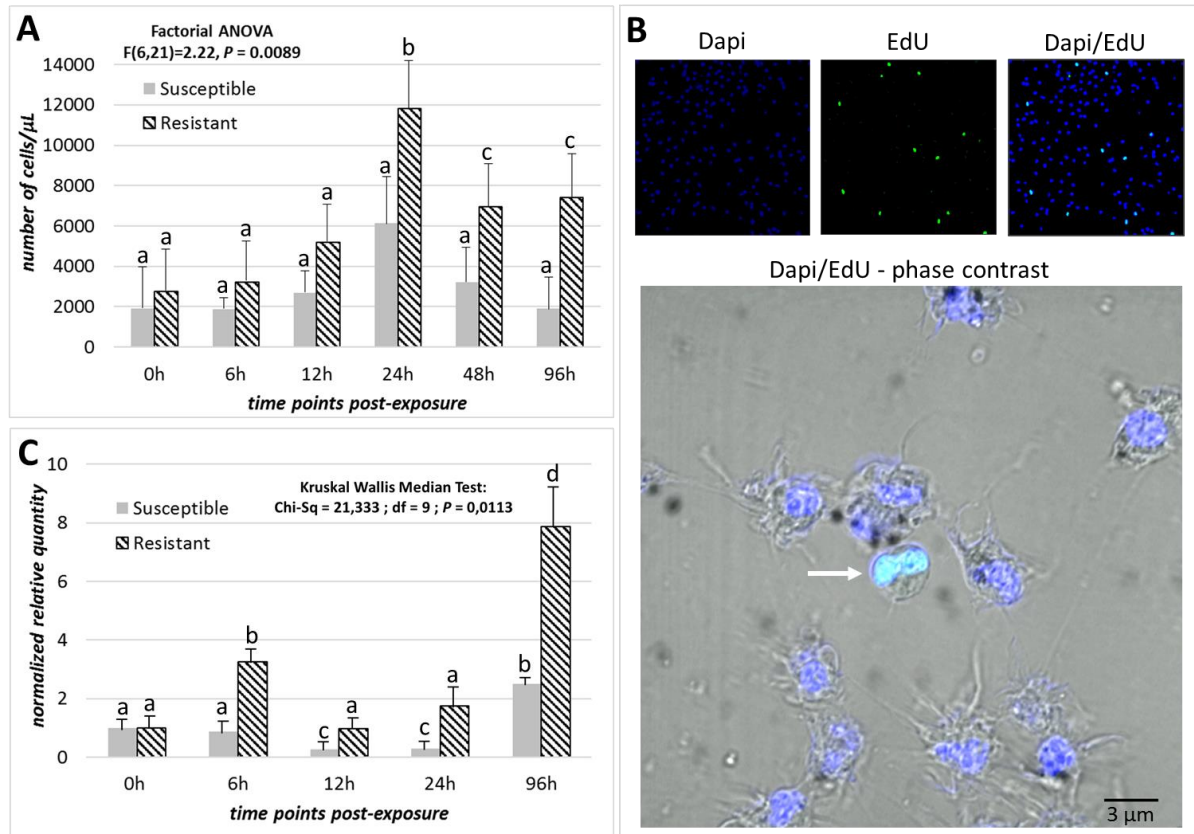
### 1.3.2 Hemocyte counts, proliferation rate and granulin expression following exposure to *F. hepatica*

The cellular response of susceptible and resistant *P. columella* to *F. hepatica* at early times post-exposure displayed basically the same tendency, characterized by an eventual and gradual increased on the amounts of hemocytes during the first hours that reached its maximum, in both strains, at 24h time point and begin to decrease afterwards (Figure 3A). Nevertheless, the overall number of hemocytes was significantly higher on resistant snails

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compared to the susceptible isolates, with an almost two-fold increase of cell counts at 24h post-exposure ( $P < 0.001$ ; Figure 3A). In addition, at 48 and 96h post-exposure the number of circulating hemocytes in challenged-resistant snails was still significantly higher compared to naïve snails (Figure 3A). The experiment was repeated twice and similar results were observed regarding both phenotypes.



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

Interestingly, EdU staining of hemocytes, a specific marking for DNA synthesis, was positive in 24h-challenged resistant snails (La Coca - 6.25%; La Palma - 7.14%,  $P = 0.8496$ ) which suggests the occurrence of cellular proliferation within circulating cells (Figure 3B).

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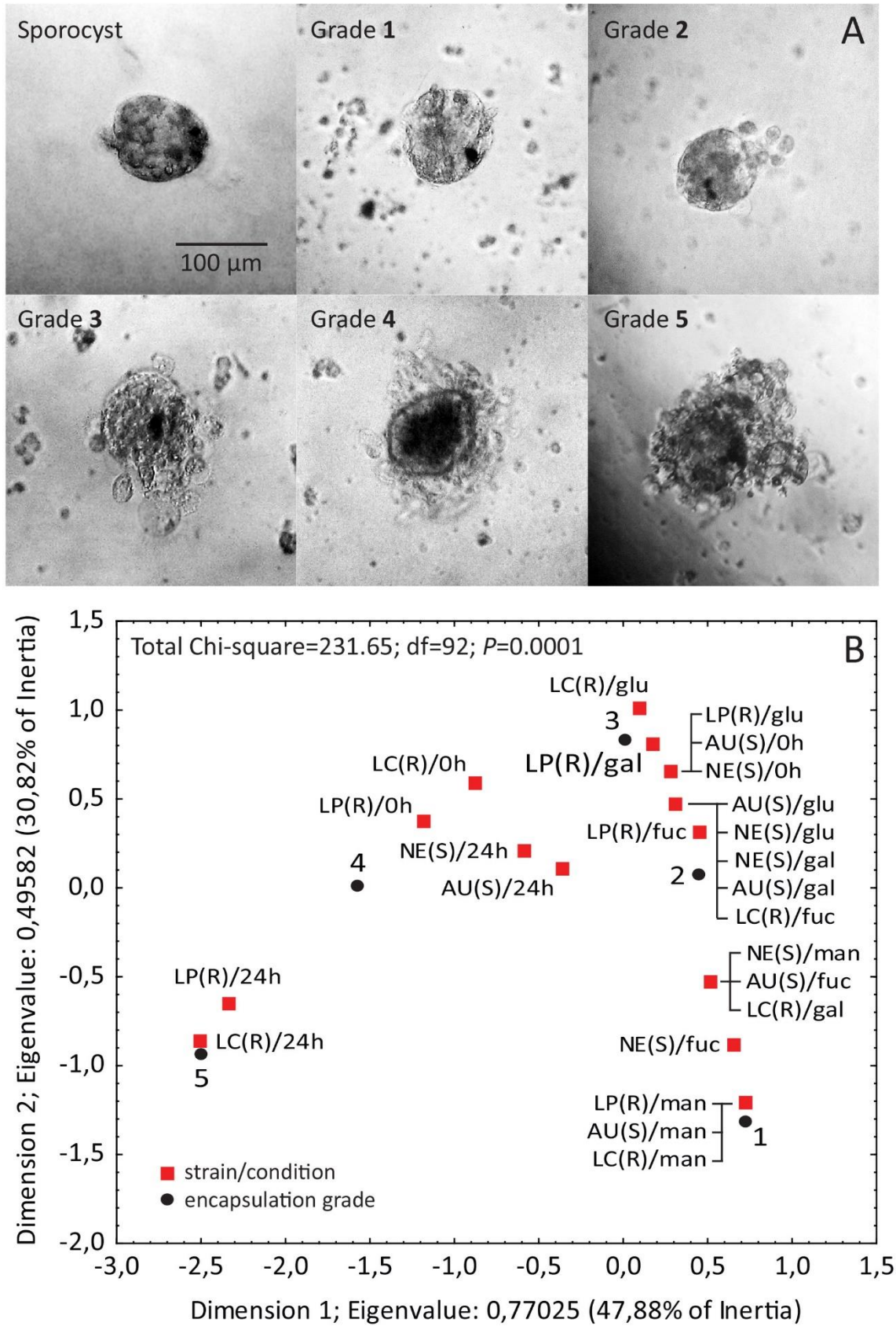
Conversely, no proliferation was observed in Aurora snails in either condition (naïve and 24h post-exposure). Only in one 24h-challenged individual from Negrines (susceptible strain) a moderate proliferation was observed (1.96%), however remaining significantly lower ( $P < 0.05$ ) than the proliferative rate observed on resistant *P. columella* hemocytes. Noteworthy, EdU marked-cells always corresponded to small and roundly-shape hemocytes (Figure 3B, lower panel).

In addition to the previous results, a significant increase of the relative expression of the cytokine granulin was recorded only in resistant *P. columella* very early after exposure (6h;  $P = 0.041490$ ). Contrastingly, at 12h and 24h time points, a decrease on granulin's expression was observed in susceptible snails ( $P < 0.05$ ; Figure 3C). This differences between phenotypes accounted for an almost 3-fold differences, at 24h post-exposure, when relative expression of granulin is compared in resistant against susceptible snails. Additionally, an increase of expression was recorded on both strains four days after parasite exposure, however significant differences were also observed between phenotypes at this time point (Figure 3C).

**1.3.3 *In vitro* encapsulation assays**

The quantitative scale established in the present study, according to observations from pilot *in vitro* assays (Figure 5A), was used to associate the encapsulation grade of *F. hepatica* sporocysts by *P. columella* hemocytes with different conditions (preincubation of naïve hemocytes with monosaccharides or *F. hepatica* challenge) in a multivariate correspondence analysis (Figure 4B). Encapsulation activity of naïve hemocytes from resistant snails was higher (strong association with Grade 4) than those from 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 to hemocytes from 24h-challenged resistant snails (Figure 4B). Interestingly, naïve hemocytes from susceptible *P. columella* were strongly associated with an encapsulation grade 3 but no clear pattern, particularly on Aurora snails, were observed on 24h-challenged hemocytes (encapsulation ranged between grades 4 and 2 (Figure 4B)).

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**Figure 4.** A) Quantitative scale used to represent the observed differences (encapsulation grade: 1 to 5) on the *in vitro* encapsulation of *Fasciola hepatica* sporocyst by *Pseudosuccinea*

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*columella* hemocytes. B) Association, through a multivariate correspondence analysis, of the encapsulation grade of hemocytes from resistant (R) and susceptible (S) *P. columella* with the assayed conditions (preincubation of naïve hemocytes with monosaccharides or *F. hepatica* challenge). *P. columella* populations: LP: La Palma, LC: La Coca, AU: Aurora, NE: Negrines; Monosaccharides: man: mannose, fuc: fucose, gal: galactose, glu: Glucose; Time-points of experimental infection: 0h: naïve hemocytes, 24h: 24h-challenged hemocytes.

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

## 1.4 Discussion

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

No classification consensus exists regarding hemocytes lineages and subtypes in mollusks due to the use of diverse criteria to differentiate them and to the lack of biological markers (see (Pila et al., 2016b)). As to illustrate the complexity of this topic, no difference on hemocyte types were observed in the gastropods *Viviparus ater* (Franchini and Ottaviani, 1990), *Megathura crenulata* and *Aplysia californica* (Martin et al., 2007), while in the abalone *Haliotis tuberculata* and the planorbid *Biomphalaria glabrata* three main subsets are generally described (Travers et al., 2008, Cavalcanti et al., 2012, Portet et al., 2018c). In the lymnaeid snails *Lymnaea stagnalis*, hemocytes were morphologically differentiated following segregation by centrifugation gradient as: small cells with high nucleus/cytoplasm ratio, medium cells displaying some pseudopodia and large hemocytes in which the acid phosphatase activity was weak or absent (Adema et al., 1994). However, in a recent study on the same species, the initial size/appearance based-segregation was observed in flow cytometry but light microscopy made difficult to discriminate granulocytes from hyalinocytes and further ultrastructural analysis showed only one cell population (Boisseaux et al., 2016). From this, these authors endorse the hypothesis of just one cell lineage of hemocyte in *L. stagnalis* showing a differentiation/maturation continuum.

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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, are believed to be the mature effectors in phagocytosis and encapsulation processes and constitute the majority (if not all) of hemocyte populations, and (b) cells that do not spread or spread minimally when placed on artificial surfaces, often called as round or blast-like cells (Loker, 2010). In the present study, we based our morphological interpretation of *P. columella* hemocytes on the previous description looking at differences in size, cytoplasmic complexity and spreading properties, and observed the same two main morphotypes: i) non-adherent small, roundly-shaped hemocytes with low cytoplasmic complexity that shared the characteristic features described for blast-like cells and ii) adherent cells which might be further segregated into granulocytes and hyalinocytes by thoroughly looking into their granularity, as occurred in *Biomphalaria* spp. and *Lymnaea palustris* (Russo and Lagadic, 2000, Cavalcanti et al., 2012). However, for insights on the latter, other staining and functional approaches would be needed.

In any case, distinct morphological patterns on snails hemocytes have been related to certain functional differences (whether they represent different subtypes of cells or different developmental stages of a single type of hemocytes; Matricon-Gondran and Letocart (1999)). The occurrence of high numbers of large adherent (spread) cells in the hemolymph has been associated with lower susceptibility to *Schistosoma mansoni* in *B. glabrata* snails and is suggested as a good predictor of resistance (Larson et al., 2014). Here, large adherent cells were highly predominant in resistant snails. In addition, we observed a higher grade of encapsulation activity of *F. hepatica* by naïve hemocytes from resistant *P. columella* compared to both susceptible strains. This suggests that the circulating hemocytes of resistant snails are constitutively more competent against the pathogen than those from susceptible *P. columella* (Figure 5).

Interestingly, in both *P. columella* phenotypes, the involvement of molecules with different carbohydrate specificities appears to be fundamental in the functionality of these cells, as lower encapsulation grades were generally associated to monosaccharide-pre-incubated hemocytes (see Figure 4B). The lowest encapsulation grade of *F. hepatica* sporocyst was observed when naïve hemocytes were pre-incubated with mannose regardless of the population used, and with L-fucose particularly, in both susceptible populations. Similarly,

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inhibition of *B. glabrata* embryonic cell line (Bge cells) adhesion to *S. mansoni* sporocyst by carbohydrate compounds, including mannose-6-phosphate, heparin, dextran sulfate, and mainly, fucoidan, has been reported (Castillo and Yoshino, 2002).

Lectins, and their concomitant 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* hemocytes, stimulation with galactose, mannose and fucose conjugated to bovine serum albumin promotes the generation of reactive oxygen species (Hahn et al., 2000). Particularly, fucosylated glycans have been pointed out as crucial in the interaction of *S. mansoni* sporocyst with surface membrane proteins of *B. glabrata* embryogenic cells (Castillo et al., 2007), while fucoidan has been found to promote cell division in the amebocyte-producing organ of this snail species (Sullivan et al., 2014). Interestingly, the mitogenic activity of this carbohydrate was mainly associated to the presence of sulfate groups (Sullivan et al., 2014). Contrastingly, a recent study concerning the evaluation of the effect of different immunogenic antigens (*i.e.* LPS, peptidoglycan and fucoidan) on *B. glabrata* amebocyte producing organ informed that in fucoidan-exposed snails, the number of downregulated genes exceeded those upregulated (even compared to LPS or peptidoglycan-stimulated snails), some of which are directly involved in immune response (Zhang et al., 2016). In addition, fucosylated-motives on *S. mansoni* have been among the carbohydrates proposed to participate on molecular evasion of the *B. glabrata* immune system through glycan mimicry (Lehr et al., 2008, Yoshino et al., 2013).

In any case, while tegumental antigens of *S. mansoni* sporocyst are highly fucosylated (Johnston and Yoshino, 1996, Peterson et al., 2009), the surface of *F. hepatica* sporocysts showed no fucose motives when screened with UEA-I lectin (Georgieva et al., 2016). According to these authors, this larval stage mainly displays N-acetyl-D-glucosamine and  $\alpha$ -mannose at their surface, with galactose and N-acetyl-D-galactosamine in minor proportions (Georgieva et al. 2016). It is worth to mention, that a thorough screening of carbohydrate motives in the tegument of *F. hepatica* juveniles has also reported a predominance of mannosidic but also of core-fucosylated truncated N-glycans (Garcia-Campos et al., 2016). Thus, it is also possible that *F. hepatica* sporocyst present other fucosylated glycans without



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a terminal fucose--galactose-motif. In any case, the significant diminishing of the encapsulation activity observed here with both mannose and fucose could be related with two non-exclusive hypotheses: the saturation of glycan-binding molecules able to sense exogenous organism as parasite through carbohydrate motifs (non-self recognition) or with the over-activation of particular glycan-binding molecule involved in the regulation of hemocyte activity. It should be noted that these carbohydrates have been also associated with *F. hepatica*-driven immune modulation/suppression of the immune response of definitive host (Rodríguez et al., 2017, Rodríguez et al., 2015, Guasconi et al., 2011). However, given the significant presence of mannose glycans at the surface of *F. hepatica* sporocyst, it is likely that this glycan could be particularly important for parasite recognition/binding by snail hemocytes.

Further studies are needed to fully elucidate which is the particular contribution of the different glycan / lectin combinations in shaping *P. columella* - *F. hepatica* interaction. However, differential intrapopulation patterns encounter after hemocyte pre-incubation with galactose and glucose but, particularly with L-fucose (strongly associated with low encapsulation grades on susceptible individuals; Figure 4b) point at the occurrence of qualitative/quantitative differences concerning the molecular surface of naïve hemocytes between *P. columella* strains and phenotypes. This variability of molecular determinants on the membrane of immune cells has been also reported for susceptible and resistant *B. glabrata* snails, suggesting that this could contribute to determine the ultimate phenotypic response after parasite infection (Agner and Granath, 1995, Yoshino et al., 2013). These potential differences occurring on hemocytes surface could be related to the higher encapsulation grade observed in naïve hemocytes from both resistant populations. Adding this result to the previous line of thought, an enriched surface with, for example, glycan-related receptors mediating parasite recognition (*e.g.* through mannose) and further interaction on hemocytes from resistant snails could increase the chances for cell-parasite interaction to occur. Contrastingly, enriched hemocytes on receptors with regulatory activities are more likely to be driven towards a less responsive phenotype by parasites' immune modulatory mechanism.

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

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An overall increase of hemocytes count during the first 24h after immune challenge was observed on *P. columella* suggesting an induced response very early after parasite exposure. Similar results have been reported also for *B. glabrata* infected with *S. mansoni* and *Echinostoma* spp. (Mounkassa and Jourdane, 1990, Loker et al., 1987). In this sense, an increase in hematopoiesis rate has been observed in amoebocyte-producing organ of *B. glabrata* after stimulation with parasite-specific antigens (Noda, 1992, Sullivan et al., 2004). However, infection of *L. stagnalis* with *Trichobilhariza ocellata* results only in a net elevated level of activity of circulating hemocytes (Amen et al., 1991) while exposure to *Plagiorchis mutationis* causes a severe immune suppression of the cellular response (Kryukova et al., 2014), illustrating the complexity of snail-trematode interactions.

Particularly, Ribeiro et al. (2017) compared the hemocyte dynamics between naïve and *F. hepatica*-infected susceptible *P. columella* and found a rapid decrease on hemocyte numbers in exposed snails 30 minutes after infection related with the mobilization of the cells towards the site of infection. However, even when this was followed by a significant increase of cell counts at 24 h post-exposure compared to naïve snails, it did not lead to protection since redial development and cercarial shedding was observed in infected snails (Ribeiro et al., 2017). Interestingly, herein, the increase of cells counts in susceptible snails did not reached significant values while an almost 2-fold increase on circulating cell counts were recorded in 24h-challenged resistant *P. columella*. To highlight, this is the same time point at which hemocyte encapsulation of *F. hepatica* sporocyst has been observed in resistant snails (see Gutiérrez et al. (2003b)).

In this sense, even when hematopoiesis it is generally believed to occurs in a primary hematopoietic site (Pila et al., 2016b), in the present study we succeed to record proliferative blast-like hemocytes in the hemolymph of challenged resistant snails. This strongly suggests that exposure to *F. hepatica* induced mitotic events on immune cells in resistant snails, a differential effect that could contribute to this particular phenotype. In addition, this increase of hemocyte proliferation could be directly related with the significant increase of cell counts on resistant *P. columella* during infection. It is also worth mentioning, that while the role of blast-like cells is still under debate our results provides additional clues of their potential stem-cell capacity. Particularly, blast-like cells are mainly considered as an immature stem-like developmental stage (Gorbushin and Iakovleva, 2008, Wang et al.,

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2012). However, they 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., 2018a).

Since cellular immunity is responsible, at least in part, of solving *F. hepatica* infection in resistant *P. columella* (Gutiérrez et al., 2003b), the production of significantly higher numbers of cells compared to susceptible snails could contribute to the orchestration and maintenance of the protective response. Similar results have been reported in *B. glabrata* – *S. mansoni* model in which lesser number of hemocytes were associated with increased susceptibility to the parasite infection (Oliveira et al., 2010, Larson et al., 2014) and an increased proliferation rate of circulating hemocyte was observed in incompatible snails (Portet et al., 2018b). Contrastingly, in *Littorina littorea*, an increase of the proliferation rate of circulating hemocytes was observed in *Himasthla elongate*-infected snails but this was not associated with increased resistance due to parasite induced-shift of hemogram towards more immature and less competent cells (Gorbushin and Iakovleva, 2008).

Therefore, for encapsulation to occurs, hemocytes must recognize and adhere to the surface of non-self particles and to each other, and significantly spread to form layers of overlapping cells, which surround and isolate the parasite (Loker et al., 1982). Here, at 24h-post exposure, we observed a strong tendency on large adherent hemocytes from resistant snails to form a sheet-like spreading pattern through an increase of the number and length of their broad-base pseudopods and to display a more cohesive aggregation (see Figure 2). Thus, it is likely that this tendency to spreading and aggregate could contribute to the success of the cellular immune response against *F. hepatica* in the resistant *P. columella* strains. Interestingly, ultrastructural studies in *B. glabrata* have demonstrated that hemocytes responsible for the consistent destruction of *S. mansoni* sporocysts typically formed extensive pseudopodia and cytoplasmic projections and display enhanced phagocytic capacity (Loker et al., 1982). Remarkably, the highest encapsulation grade was strongly associated to hemocytes from resistant snails at 24h post-exposure suggesting that all the changes observed on immune cells from resistant snails following parasitic challenge ultimately promotes its effector activity (Figure 4B). The latter provides a functional validation that endorse the differential phenotypic immune response previously observed in resistant snails towards *F. hepatica* (Gutiérrez et al., 2003b).

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Contrastingly, large hemocytes from challenged-susceptible snails remained mostly scattered, displaying slender or shorter pseudopodia and even smaller size compared to naïve cells, while no significant proliferative activity was recorded in blast-like cells. The observed variability between conditions is likely related with a more discrete activation of the immune response in the susceptible strain either driven by the host itself after parasite infection or directly modulated by the parasite. In this sense, it is significant that no clear pattern regarding encapsulation activity, particularly on Aurora snails, were observed on challenged hemocytes with encapsulation grades in between 4 and 2. This could be explained by differences in compatibility within snails of the same populations, from which different infection outcomes might result depending on each single host/parasite combinations, driven by particular genotype/genotype interactions. In this sense, while both Aurora and Negrines populations are susceptible to *F. hepatica*, 20% and 45% of snails respectively do not developed infection. This polymorphism of compatibility has been suggested to occur in susceptible *P. columella* – *F. hepatica* system (Vázquez et al., 2014, Alba et al., 2018) and it is used to explain compatibility in the *B. glabrata*/*Schistosoma mansoni* model (see Theron and Coustau (2005)).

In addition to the previous observations so far discussed, the significant increase on the expression of host proliferative cytokines, *i.e.* granulins, recorded in *P. columella* after *F. hepatica* infection appeared as another interesting fact. This evolutionary-conserved molecule is known to promote hematopoiesis and to modulate immune response and inflammation (Jian et al., 2013). Particularly, the expression and activation of this cytokine in *B. glabrata* not only promotes hemocytes proliferation but also drives differentiation of these cells towards a more adherent highly phagocytic phenotype, more prompt to cellular response that have been associated with an increased immune response to *S. mansoni* infection (Pila et al., 2016a). In this sense, it should be noted that, a significant decrease on the expression of this cytokine compared to the levels recorded on naïve snails was observed solely in susceptible snails at 12 and 24h post-infection, most likely related to an infection-driven effect conditioned by host parasite-compatibility. Multiple determinants and molecular signals from both host and parasite are probably related to the different profiles recorded in challenged *P. columella* and thus, remain to be elucidated. However, granulins might be a factor to consider in *P. columella* – *F. hepatica* interaction if we linked

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the phenotypical and functional changes on resistant hemocytes (Figure 2-4), to the wide influence of granulin on hemocyte proliferation, differentiation and activation following parasitic challenge (Pila et al., 2016a) and to the overall higher granulin expression noticed in resistant snails all along the firsts 24h after challenge (Figure 3C).

### 1.5 Conclusions

Here, we used comparative approaches to explore the immunobiological response behind resistant and susceptible *P. columella* in interaction with *F. hepatica*. A decrease of encapsulation of *F. hepatica* sporocyst by naïve hemocytes pre-incubated with different monosaccharides, regardless of the phenotype suggests the involvement of carbohydrate binding molecules in the functionality of these cells towards the parasite, and points at future investigations aiming at deeply exploring the lectin – ligand interactome behind the hemocytic response in this model.

On the other hand, our results showed significant phenotypical and functional differences related with the main actors of the cellular immune response in invertebrates, *i.e.* the hemocytes, promoted after parasitic challenged, which could endorse that differential phenotypes observed in *P. columella* towards *F. hepatica* infections. Particularly, in resistant *P. columella*, while resistant hemocytes showed higher encapsulation grades of *F. hepatica* sporocyst, exposure to the parasite boosted the activation of the hemocytes possibly promoting the associated immune-phenotype observed in these snails. The latter is, so far, characterized by an overall significant increase of hemocyte counts, enhanced proliferation of blast-like cells, morphological changes of large adherent cells and a greater encapsulation activity *in vitro* (observed herein) and *in vivo* (Gutiérrez et al., 2003b). In addition, a significant increase of production of granulin, a cytokine involved in proliferative, activation and differentiation of hemocytes, also features the dynamic of the immune response to *F. hepatica* in resistant *P. columella*.

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# GENERAL DISCUSSION

## New hypotheses, conclusions and perspectives

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### I. On the possible origins and mechanistic bases behind the resistance in *P. columella*

One of the most interesting biological models in which we can frequently stumble upon is that of snail-trematode interactions, most of which ultimately result in the transmission of several important diseases, particularly in the tropics. In this thesis we have approached a trematode – snail system in which certain populations of *P. columella* (a common host species) are naturally-resistant to *F. hepatica*, in association with an effective encapsulation of the parasite by host hemocytes (Gutiérrez et al. 2003a). Herein, we found two additional phenotypic features associated with resistant *P. columella* populations: they could tolerate slightly acid and soft waters better than susceptible snails and they experienced certain reproduction constrains compared to susceptible populations (see Chapter 1). In this sense, both, a protective immune response to *F. hepatica* infection and an enhanced tolerance to pH/TH variations, involved a higher competence to respond to stressors (biotic or abiotic). However, the impairment of reproductive traits is likely a consequence of energetic trade-offs among potentially competing physiological processes (*i.e.* self-maintenance /survival vs. reproduction; Stearns, 1992). Interestingly, we found a significant allocation of resources to molecular functions/processes related with stress/defence response and to the maintenance and regulation of homeostasis associated to resistant *P. columella* snails (see Chapter 2). All these could explain, at the molecular and phenotypical levels, the restricted distribution of resistant *P. columella* populations in nature to sites where the mentioned features could be considered an advantage (sites with low pH/TH or high parasitic pressures) thus, compensating for their low competitive capacities.

However, there is still the question of the origins of the primary feature of resistant snails (*i.e.* the protective immune response to *F. hepatica* infection) and its association with the higher tolerance to pH/TH variations also observed in these populations. Noteworthy, both immune

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response and acid-base/osmotic regulation, are physiologically linked through several molecular functions, effectors and signalling pathways (Demas et al. 2011; Matozzo et al. 2012). Therefore, it could be hypothesized that either the higher pH/TH tolerance is a “by-product” resulting from parasite-driven selection of resistance, or in turn the resistant phenotype is merely a “side effect” of an environmental-driven selection related with an enhanced potential to compensate osmotic and acid-base variations.

On the one hand, changes on environmental and internal pH could lead to certain enhanced immune-related features such as an accelerated haematopoiesis (Meseck et al. 2016), increased phagocytic capacities of the hemocytes (Matozzo et al. 2012; Meseck et al. 2016), increased acid-balance potential and antioxidative defences (Freitas et al. 2016), and higher calcium recruitment and hypercalcemia (Tunholi-Alves et al. 2014; Tunholi et al. 2017). Due to the fundamental roles in immune surveillance and orchestration of defence of the mentioned features (see Connors et al. 1995; Goodall et al. 2004; Nikapitiya et al. 2010; Larson et al. 2014 for some examples), such snails could be more prone to efficiently respond to infections and to be “better prepared” to recognize, phagocyte/encapsulate and eliminate intruders when infected. In this sense, it is worth mentioning that we observed a constitutive abundance of transcripts/proteins particularly associated with activation of immune response on resistant compared to susceptible snails (Chapter 2).

On the other hand, an enhanced immune responsiveness can reconfigure intermediate metabolism towards a relative increase of the respiration rate, glycolysis, proteolysis and/or lipolysis (Lochmiller & Deerenberg, 2000). This could lead to a concomitant increase of protons, ROS and CO<sub>2</sub> levels in the internal milieu and, therefore, to an enrichment of other related functions, such as antioxidative enzymes, carbonic anhydrase and ion transporters (*e.g.* Na<sup>+</sup>/K<sup>+</sup>-ATPase, H<sup>+</sup>-ATPase, Na<sup>+</sup>/H<sup>+</sup>-exchanger), to cope with the membrane energetic requirements under this biochemical setting (Epstein et al. 2014; Biller-Takahashi et al. 2015). The latter could be particularly associated to the enhanced pH/TH tolerance observed in resistant snails since in aquatic animals acid-base balance and regulation of osmolarity are intimately linked due to the coupled movement of H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> with that of Na<sup>+</sup> through ion transporters (Matthews, 2017). In this sense, it is interesting to note that we observed an enrichment on major ion regulators and of carbonic anhydrase in association with the

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resistant phenotype (see Chapter 2), which endorse the particular fitness of these snails to tolerate pH and osmotic variations.

Unfortunately, information is still inconclusive as to confidently retain one hypothesis over the other. An environmental driven selection could, perhaps, explain the broad resistance towards *F. hepatica*, irrespective of the infective strain (see Chapter 3.1; Calienes et al. 2004; Vázquez et al. 2014), through an overall enhancement of multiple immune functions. In this sense, a more “global” competence to fight any infection could be also expected. However, it is interesting to note that resistant and susceptible *P. columella* are equally sensitive to *Trichobilharzia* infection (Chapter 3.2). In addition, we presented evidences of an induced immune response following *F. hepatica* challenge that is significantly different between *P. columella* phenotypes in terms of hemocyte morphology, proliferation and encapsulation activities, and of granulins expression (Chapter 3.3). These results suggest that there is a certain specificity of this “enhanced immune competence” towards *F. hepatica* that allows to recognize and to “better” respond to its challenge, and fit better with a parasite-driven selection hypothesis. In this sense and as it was presented here, resistant *P. columella* populations occurred mostly in western Cuba, particularly in Pinar del Rio province (Chapter 1). This region is known to be an important focus of *F. hepatica* transmission. High prevalence of infection in lymnaeid snails (Alba et al. 2016) and livestock (Vázquez et al. 2016), and even human cases and outbreaks (Rojas et al. 2010; Diaz et al. 2011) have been reported in Pinar del Rio, where parasite circulation is presumed to occur all year-round (Rojas et al. 2010).

As future perspectives, experimental infections on stressed-snails (*e.g.* food-deprived, heat-stressed or acid-stressed) could be worth testing in this model. Environmental factors such as heat waves and starvation have been reported to affect snail – parasite interaction by decreasing immune capabilities of the host (Seppälä & Jokela, 2010; Leicht et al. 2013), increasing their susceptibility to digenean infections (Leicht & Seppälä, 2014) and, even, reverting certain resistant phenotypes (Ittiprasert & Knigh, 2012; Knight et al. 2016). In the case of resistant *P. columella*, exposure to heat waves, low pH/TH and low food income prior infection could negatively affect the energetic budget necessary for the sustainability of its enhanced immune competence. Meanwhile, it would be worth to explore if housing susceptible snails in low pH/TH conditions could decrease its sensitivity to *F. hepatica* as it

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could strengthen the argument towards an environmental-mediated resistance to the parasite.

In addition, experimental evolution of parasite resistance on susceptible *P. columella* by selectively pressing with *F. hepatica* or with low pH/TH housing conditions might shed lights on the mechanistic bases behind the phenotypic features recorded in resistant populations. In parallel, it could be also interesting to evaluate the sensibility of resistant and susceptible *P. columella* populations to other parasites, mostly those more similar to *F. hepatica* and compatible with *P. columella* (e.g. *F. gigantica* and *F. magna*; Flowers, 1996; Grabner et al. 2014). In this sense, results of experimental infections with *F. magna* could be particularly motivating because: (1) as *P. columella*, it is native from North America (Juhászová et al. 2016), (2) a high field transmission rate is believed to have existed in this region (Malcicka, 2015), and (3) it is particularly virulent when infecting *P. columella* snails (Pankrác et al. 2016). The outcomes of challenging resistant *P. columella* populations with *F. magna* might offer insights into the parasite-driven hypothesis on the origin of resistance including an alternative option on the trematode species.

## **II. On the immunobiology of *P. columella* resistant / susceptible – *F. hepatica* interaction**

The Red Queen hypothesis predicts that while trematode parasites should evolve towards a certain degree of genetic matching with its snail hosts to increase its chances to infect, the high selective pressure exerted by some parasite populations should favour the selection of rare host genotypes, as an adaptation to parasitism (Lively and Dybdahl, 2000; Osnas & Lively, 2005). Thus, some host populations usually display variations in compatibility traits depending on the genotypes of the infecting parasites (Osnas & Lively, 2005; Théron et al. 2014; Vázquez et al. 2014). However, certain rare host genotypes favored by parasite selection within a given population could become more than “locally rare” and, rather than just be less susceptible genotypes, result in resistance (Lively & Dybdahl, 2000; Osnas & Lively, 2005). These very rare genotypes are usually less abundant due to a fitness cost of such resistance in the absence of parasitism (Lively & Dybdahl, 2000). Results showed here (Chapter 1) and elsewhere (Calienes et al. 2004; Lounnas et al. 2017) demonstrate that resistant populations clustered separately from most *P. columella* populations from Cuba and from the rest of the world, which support the notion of a genetic determinism of the resistance. In addition, the segregation of the

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resistant populations indicates that they could have been selected from a different pool than the ones (susceptible) that have spread everywhere, and that *P. columella* snails grouped outside of the “resistant” clusters could be primarily thought as susceptible to *F. hepatica*. In these susceptible snails, *F. hepatica* infection is characterized by a significant fecundity reduction (Gutiérrez et al. 2002; Salazar et al. 2006), higher redial and metacercariae productivity compared to *G. 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).

In the widely studied model of *B. glabrata* – *S. mansoni*, parasite encapsulation by host hemocytes is observed when incompatible host-parasite combinations occur following a matching alleles model (Théron & Coustau, 2005). Such infectivity mosaic has its molecular base on the interaction of the highly diversified host FREPs with the highly polymorphic SmPoMucs, as one of the major determinants for infection (Portet et al. 2017). Although little is known about the molecules involved in *F. hepatica* – lymnaeid host interaction, similar variations in infection outcomes, depending on the parasite isolate – snail population combination, have been also observed on susceptible *P. columella*, possibly due to the existence of a polymorphism of compatibility (Vázquez et al. 2014; Chapter 3.1). Contrastingly, resistant *P. columella* populations always succeeded in fighting back *F. hepatica* infection regardless of the Cuban (*e.g.* Calienes et al. 2004; Vázquez et al. 2014) or the geographically-distant (see Chapter 3.1) parasitic strain used in the challenge. Interestingly, while *F. hepatica* is recognized as a genetically diverse parasite (*e.g.* Vázquez et al. 2016; Cwilinsky et al. 2015), our results suggest that resistance in *P. columella* is not tightly restricted to a host genotype – parasite genotype interaction. From these results it could be hypothesized that there is a major factor dictating *P. columella* – *F. hepatica* interaction (*i.e.* determining infection/non-infection status after *F. hepatica* penetration if absent or scarce/present or abundant), possibly featured by a single or few low-diversified loci. This major host factor would be proximately or ultimately involved in the immunobiological interaction with a single or few highly conserved loci of the parasite resulting crucial for its establishment within snails (as to be displayed by any or, at least, most *F. hepatica* strains). In addition, the extent of the infection is ultimately dictated by other molecular determinants, possibly coded by different loci with several allelic variants. This is, though theoretical, a plausible explanation since several steps ranging from snail finding, parasite establishment and development, and

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molecular determinants mediate compatibility in any host-parasite interaction (Chen et al. 1968; Combes, 2001; Tennenssen et al. 2015a; Portet et al. 2017). Therefore, in this sense, while resistant populations are likely enriched on the above mentioned major immunobiological factor, susceptibility in *P. columella* appears to be determined by the lack of the resistant genotype.

The scarce information concerning the molecular and physiological dialogue between *F. hepatica* and its lymnaeid hosts gives little room to deeper speculations on which particular molecules/functions are proximately and ultimately related with infection outcomes and, hence, with resistance or susceptibility to the parasite. However, certain *F. hepatica*-driven immune modulation/suppression could be presumed to occur in its intermediate hosts. Direct immune modulation figures in the list of trematode countermeasures to snail immune system and reports exist for several models, e.g. *S. mansoni*/*E. paraensei* – *B. glabrata* (Lodes & Yoshino, 1990; DeGaffeé & Loker, 1998; Connors et al. 1991; Loker et al. 1992; Zahoor et al. 2008; 2009), *Himasthla elongata* – *Littorina littorea* (Iakovleva et al. 2006), *Trichobilharzia regent* – *Radix lagotis* (Skála et al. 2014) and *Plagiorchis mutationis* – *L. stagnalis* (Kryukova et al. 2014). Particularly, the establishment of *F. hepatica* infection in the definitive host is mediated by a potent immune suppression/modulation induced by parasite antigens (see Prowse et al. 2002; Gironènes et al. 2007; Hamilton et al. 2009; Dowling et al. 2010 for some examples). In addition, the significant decrease on the expression of the cytokine granulins and the differences in the morphological and functional features between naïve and *F. hepatica*-challenged hemocytes from susceptible snails recorded here (Chapter 3.3), are preliminary evidences of a certain *F. hepatica* interference on immune functions of *P. columella*. Recently, it has been suggested that coevolution of parasites with specific immune components (receptors for detection/interaction or effectors for elimination) shifts the host's optimal allocation from induced towards constitutive immunity (Kamiya et al. 2016). According to these authors, if co-evolutionary feedback between parasite and host populations determined less efficient induced immune responses due to parasite counter-evolution upon host receptors, then the host will be driven to invest more heavily in constitutive immunity (Kamiya et al. 2016). In this sense, the likely *F. hepatica* driven-immune modulation/suppression during parasite migration and establishment within the snail would certainly impose a challenge difficult to overcome for host defenses if these mainly rely on induced immunity. In this sense,

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an enhanced constitutive immune responsiveness could give the host an advantage to fight back the parasite before it can start to settle and to display its arsenal of immune modulatory mechanisms. Interestingly, results from the “omic” approaches applied here to naïve snails informed that a significant allocation of resources to immune defense, featured by an overall abundance of pathogen interacting molecules, pro-inflammatory signaling pathways, cytokines, antimicrobial and anti-oxidant molecules, occurs in resistant *P. columella* (Chapter 2). In addition, the higher encapsulation grade of *F. hepatica* sporocysts observed for naïve hemocytes from resistant populations (Chapter 3.3) constitutes a functional evidence of the enhanced constitutive immune responsiveness associated to the resistant phenotype. Similarly, the existence of constitutive differences between susceptible and experimentally-selected resistant *B. glabrata* strains when infected with *E. caproni* has been proposed as the base for refractoriness of the latter to the parasite's immunosuppressive factor (Humbert & Coustau, 2001). This might suggest that the major determinant involved in *P. columella* resistance is primarily associated with the constitutive upregulation of the immune competence that most likely leads to the activation of specific immune pathways resulting in parasite elimination.

It also worth mentioning that the increase of the effective number of infective larvae (from 5 to 30 miracidia/snail), through single or serial exposures, do not prevent resistant *P. columella* from resisting *F. hepatica* infection (Chapter 3.1). This endorses the assumption of a significant allocation of resources to defence also occurring in these snails following infection. Results obtained during hemocyte characterization after *F. hepatica* challenge (*i.e.* high adherence and aggregation, higher proliferation and encapsulation activities of the hemocytes, higher granulin expression; Chapter 3.3) also endorse the inherent ability of resistant snails to orchestrate a powerful and protective response after *F. hepatica* infection. In insects for example, phenoloxidase (PO) is a defence effector molecule that is constitutively present but can be further up-regulated upon induction through the pro-phenoloxidase (PPO)-cascade (Lemaitre & Hoffmann, 2007). In *B. glabrata*, certain FREPs like FREP 4 exist in the hemolymph of naive snails but are upregulated after infection with *E. paraensei* (Zhang et al. 2008).

Future studies aiming at comparing the transcriptomic dynamics during *F. hepatica* – *P. columella* resistant/susceptible interactions following challenge experiments can help focus further investigations. In addition, deciphering the differential interactome between *F.*



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*hepatica* with susceptible and resistant *P. columella* and the characterization of the identified molecules can be proposed to shed light on the molecular determinants mediating each phenotype. In all these studies, the inclusion of snails from several susceptible and resistant populations might help in the search and in the proposition of global mechanisms.

### **III. On the applicability of the results: considerations for the control of *F. hepatica* transmission**

*Fasciola hepatica* is known to be a highly diverse parasite, with high non-synonymous polymorphism in genes shared with different parasitic taxa and refined anti-oxidative and detoxification pathways (Cwiklinsky et al. 2015). With such biological traits is no wonder that the anti-flukicide therapy consists of only a few drugs of variable efficacy with the increase of resistant strains (Fairweather, 2011) and that *F. hepatica* has a variety of intermediate (more than 20 lymnaeid species, Vázquez et al. 2018) and definitive host species (six different orders within Mammalia, Mas-Coma et al. 2009a). In addition to the high genetic polymorphism of *F. hepatica*, its refined immune suppressive/modulatory strategies are among the significant factors contributing to the lack of available vaccines (see Molina-Hernández et al. 2015 for review). Beyond its high parasitic fitness and evolutionary potential for rapid adaptation, transmission of this species is also facilitated by human activities (e.g. livestock production, habitat transformation; Sauborin et al. 2018) and even climate change (Mas-Coma et al. 2009b), all of which have contributed to its re-emergence. Contrastingly, the generally low mortality and morbidity of *F. hepatica* infection related to its “elegant” parasitic strategies, concomitantly with the fact that the highest human prevalences are informed in developing countries, bias its significance in the debates concerning public health, and impose its anonymity for decision makers, funding sources, scientists and “*le grand public*” (WHO, 2007). Meanwhile, high economic losses and even increasing prevalence in humans and livestock are reported (WHO, 2013; Menhood et al. 2017). With all these been said, the control of *F. hepatica* transmission is both a necessity and a challenge.

The transmission of *F. hepatica* strictly depends on the presence of the intermediate host, thus like other vector/snail-borne diseases, the accurate prediction, suitable prevention and sustainability and effectiveness of its control is inextricably linked to the study and control of the transmitting snails (Caron et al. 2011; Adema et al. 2012; Knubben-Schweizer & Torgerson, 2015). These could ultimately allow to interrupt the parasite’s life cycle, preventing the

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infection to reach (and harm) new definitive hosts, while reducing and optimizing the administration of flukicides (Knubben-Schweizer & Torgerson, 2015). In fact, anthelmintic treatments are not always cost-effective, threaten to increase drug resistance and do not confer long-term protection against reinfections (Fairweather, 2011). However, while the epidemiological surveillance of lymnaeid populations and their infection status is an adequate approach to monitor and manage endemic areas (Arias et al. 2010; Caron et al. 2014; Alba et al. 2016), the application of appropriate strategies to control lymnaeid populations is far from being easy and straight-forward, for multiple reasons, from biological to practical.

The ecological features of lymnaeid snails (*e.g.* wide habitat preferences, high selfing and reproduction rates, high amphibious nature of certain species; Goumghar et al. 2004; Vázquez et al. 2009; Pointier et al. 2009; Meunier et al. 2004; Lounnas et al. 2018) imposes significant challenges for the success of traditional physical, chemical and biological control approaches. In addition, physical and biological -related strategies could be time-consuming and require a certain level of expertise (Rondelaud et al. 2006; Vazquez et al. 2009; Knubben-Sweizer & Torgerson, 2015). The molluscicides currently available have low selectivity and need to be applied on freshwaters, most of which are usually used for livestock watering or culture irrigation (Khan et al. 2013). In this sense, gathering ecological, phenotypical and molecular information related with trematode-snail interaction (*e.g.* environmental factors affecting snail establishment and parasite transmission, genetic markers of compatibility and transmission regulators), could be essential to understand and predict the dynamic of parasite transmission, and to design integrative and comprehensive strategies for its control (*e.g.* ecologically-friendly control methods; Adema et al. 2012).

Here we have approached one of the main fasciolosis-transmitting snails, *P. columella*, and its particular interaction with *F. hepatica* at different levels (*i.e.* ecological, molecular and phenotypical), only to marvel with their complexity. From a theoretical point of view, we offer a new perspective on the history and colonization of *P. columella* in Cuba and on its pattern of ecological preferences. We also present ecological evidences of the cost of resistance, both in the field and the laboratory (restricted distribution in nature, reproductive impairments), gained insights into other phenotypic features associated with resistance in *P. columella* (*i.e.* higher tolerance to pH/TH variations; Chapter 1) and provide an overall molecular and functional landscape, prior to infection, that endorse the observations concerning the

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differential phenotypes of *P. columella* (Chapter 2; Chapter 3). In addition, we increased our knowledge on the immune-phenotype of *P. columella* particularly on the functional dynamics of the hemocytes following parasite challenge but, also, on the extent (parasite dose, local vs. allopatric *F. hepatica* strains) and specificity of the resistant phenotype (Chapter 3).

From a practical point of view, we reported the second *F. hepatica* naturally-infected population of *P. columella* for Cuba and the Caribbean and the ongoing spreading of two highly invasive genotypes that are compatible with the parasite, all of which alert on the increasing complexity of the epidemiological scenario of fasciolosis in Cuba. We unravelled the ecological patterns associated with the differential occurrence of resistant and susceptible *P. columella* snails and characterized the life-history traits of different populations under experimental settings consistent with the different ecological requirements observed in the field (Chapter 1). We provided the first assembled and annotated transcriptome of this lymnaeid species, and the first insights on the differential transcriptome, at the whole snail level, and proteome, at the albumen gland level, of resistant and susceptible *P. columella* (Chapter 2). Though not providing proof of function, these results offer a baseline platform of molecular data concerning the processes/functions/molecules particularly involved in the resistant phenotype to be used for subsequent studies onto promising targets (Greenwood et al. 2016). We also present evidences of the stability of the resistant phenotype irrespective of the infective dose, serial exposures and geographic origins of the parasite and of its reliance on differential cellular immune response after *F. hepatica* challenge, which could be useful for the prospective application of this system on parasite control (Chapter 3). In addition, the rise of new questions and hypothesis (see previous sections I and II for details), and of new perspectives and experimental approaches to answer them, can be referred as one of the most significant contributions of the present thesis.

However, as promising as it could be, the utilization of *P. columella* resistance for the control of *F. hepatica* transmission, at the moment, remains a vision. The introduction of resistant snails in particular high-transmission focus (*e.g.* livestock farms or crop cultures) could be applicable as a traditional biological control approach (see Perera et al. 1990; Rondelaud et al. 2006 for other examples). Although in these sites, the cost of resistance might be countered by the selective advantage of resistance over *F. hepatica*-parasitized snails (see Gutiérrez et al. 2002), this should be an issue to consider. A previous study of the site would be possibly

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needed before introducing resistant snails in order to overcome the competition impairments associated to this phenotype. Additionally, physical or chemical control strategies prior introduction could diminish the snail species richness and aid to the establishment of the resistant population afterwards. In any case, this approach will require a thorough planning, execution and further monitoring. Nevertheless, even if the introduced population eventually disappear, it is likely that during its occurrence a decrease or even the interruption of transmission for the time being could be registered (see Rondelaud et al. 2006 for an example).

A more ambitious goal would be to drive resistance genes into *P. columella* susceptible populations and achieve a very competitive resistant strain which could then be introduced in endemic areas as a means of biological control (Gutiérrez et al. 2005). In this sense, while out-crossing is unlikely to function, given the high self-fertilization rate of the species observed here (Chapter 1) and elsewhere (Lounnas et al. 2017), genetic manipulation and controlled selection of resistant/susceptible features are promising alternatives (Tennenssen et al. 2015b). In this sense, the development of novel techniques like the CRISPR/Cas9-based genome editing tools provide new opportunities for generating trans-genetic vectors and has been successfully assayed in the vector mosquito *Anopheles gambiae* for controlling *Plasmodium falciparum* infection (Dong et al. 2018). In addition, inducing the constitutive expression of resistant-mediated mechanisms in wild vector populations could be the goal of future research (Tennenssen et al. 2015b). Seeking how to manipulate snails such that this signal cascade leading to resistance is upregulated regardless of the genotype or infection status, similar as to what has been proposed here to occur in resistant *P. columella* snails (see section II), might be a suitable approach. Concomitantly, the identification of the matching ligands and molecules of the parasite interacting with resistant genes could also lead to the development of new therapeutic drug targets or vaccine candidates to be used on definitive hosts (Tennenssen et al. 2015b).

Nonetheless, for achieving significant advances on any of the mentioned variants, much remains to be learned concerning the molecular pathways of this particular snail-parasite interaction. In this sense, the incorporation of additional research efforts, new competences and technology, and funding is as challenging as it is essential to accomplish any of these goals. The scarce resources and interest devoted to tackle snails and the diseases they transmit

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should be always considered, as it is a major limitation for exploring related scientific and practical opportunities (Adema et al. 2012). In any case, while the discussion of feasible ways to exploit *P. columella* resistance to disengage *F. hepatica* transmission remains open, integrative studies like the one presented in this thesis are building steps onto the road that lies ahead.

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Alba A.

## ***Résumé du texte de la thèse***

Biologie comparative de *Pseudosuccinea columella* (Mollusca : Gastropoda) naturellement résistant/susceptible à l'infection par *Fasciola hepatica* (Trematoda) à Cuba : aspects écologiques, moléculaires et phénotypiques

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### **INTRODUCTION GENERALE**

Parmi les maladies transmises par les mollusques, une des plus importantes concernant les parasites digènes est celle appelée fasciolose. Cette maladie est causée par les espèces du genre *Fasciola*, et notamment *Fasciola hepatica* est particulièrement intéressante à cause de sa très grande distribution dans le monde, ainsi que pour l'énorme impact qu'elle provoque chez le bétail avec un considérable coût économique associé à l'infection (Khan et al. 2013). En plus, l'accroissement abrupt de cas humains au cours des 20 dernières années, particulièrement dans les pays du sud, est alarmant (Mas-Coma et al. 2009a). L'Organisation mondiale de la santé a demandée en 1992 la mise à jour du taux d'infection de la fasciolose dans la population humaine. Dans ce moment-là, une prévalence de 2594 personnes a été enregistrée, dont la plupart des cas venaient des pays européens dont la France, l'Espagne et le Portugal, mais aussi de l'île de Cuba dans les Caraïbes (Chen, 1990). En revanche, aujourd'hui les prévalences de fasciolose humaine sont estimées à environ 2,4 à 17 millions de personnes infectées dans le monde (WHO, 2013). Plusieurs facteurs existent pour expliquer cette augmentation significative, parmi lesquels, les initiatives pour étudier la charge parasitaire (WHO, 2007), l'existence d'une vigilance ainsi que de méthodes de diagnostic plus précises mises en œuvre contre la fasciolose (Mas-Coma et al. 2014), et l'effet du changement climatique sur la répartition de la douve et de ses hôtes intermédiaires (Mas-Coma et al. 2009b), sont les plus liées à cette émergence ou réémergence. Il faut ajouter à cette équation, la très haute plasticité écologique de *F. hepatica* qui lui a permis de s'adapter avec succès à une très large gamme de mollusques limnaeidés et d'hôtes définitifs, ainsi que le très haut risque mondial de transmission. Tous ces facteurs épidémiologiques font du contrôle de la douve une tâche impérative à consolider.



Alba A.

## I. Impact mondial, épidémiologie, aspects cliniques et agents étiologiques de la fasciolose

Les espèces *F. hepatica* et *F. gigantica* (Trematoda : Digenea : Fasciolidae) sont communément connues comme les douves du foie et sont les principaux agents étiologiques de la fasciolose au niveau mondial. Le caractère cosmopolite de *F. hepatica* fait de cette espèce la responsable de la charge parasitaire plus importante tandis que *F. gigantica* a une répartition plus limitée aux régions tropicales et subtropicales de l'Asie et de l'Afrique (Mas-Coma et al. 2009). Ces parasites ont un cycle de vie complexe à deux hôtes en infectant plus de 20 espèces de mollusques de la famille Lymnaeidae et une très large quantité de mammifères et les êtres humains, en tant qu'hôtes intermédiaires et définitifs respectivement (Mas-Coma et al. 2005). En bref, les douves sont transmises par des mollusques susceptibles suite à l'infection par une larve nageant librement après l'éclosion d'un œuf si les conditions sont favorables (température de plus de 13°C ; Andrews, 1999). Ce miracidium cherche activement ses mollusques compatibles premièrement par phototaxis et après, en suivant un gradient de certaines molécules espèce-spécifiques sécrétées par les hôtes, par chimiotaxie (Kalbe et al. 1997). Une fois dans le mollusque, une métamorphose est accomplie qui transforme le miracidium en sporocyste et finalement en rédie avec une très importante multiplication asexuée qui augmente le nombre de parasites chez les limnées (Andrews, 1999). Entre 30 et 86 jours après l'infection, selon la température et la compatibilité douve-mollusque, une larve appelée cercaire émerge activement de la limnée et s'enkyste dans la végétation aquatique ou semi-aquatique, pouvant aussi rester flottante dans l'eau, et forme la metacércaire (Mas-Coma et al. 2005 ; Morley, 2015). Si les conditions environnementales sont favorables, les metacércaires peuvent rester viables près d'un an jusqu'à être ingérées avec la végétation ou l'eau par des mammifères susceptibles (Mas-Coma et al. 2009). Les douves adultes se développent dans le foie, particulièrement dans les canaux biliaires des hôtes définitifs dont chaque individu peut produire des milliers d'œufs par jour qui sont concentrés dans la vésicule biliaire et qui finalement gagnent le milieu extérieur par l'intermédiaire des fèces (Andrews, 1999).

La fasciolose est une maladie liée à l'eau et les aliments et qui est présente dans tous les continents (à l'exception de l'Antarctique) entre les régions tropicales et tempérées grâce à la distribution aussi large de ses mollusques hôtes et l'adaptabilité du parasite. En fait, la

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fasciolose est considérée comme la maladie transmise par vecteurs la plus répandue latitudinalement et longitudinalement mais également altitudinalement dans le monde (Mas-Coma et al. 2005 ; WHO, 2013). La prévalence la plus haute de fasciolose humaine est reportée en Amérique du Sud plus particulièrement au Pérou et en Bolivie à plus de 4000 m d'altitude dans les Andes (Esteban et al. 1997 ; 1999 ; Espinoza et al. 2010) mais aussi dans la région du delta du Nil en Egypte (Dar et al. 2005). Toutefois malgré l'accroissement des chiffres en ce qui concerne la santé publique, la fasciolose reste encore une maladie négligée (WHO, 2007 ; 2013). Par ailleurs, son énorme prévalence chez les animaux domestique, particulièrement le bétail, entraîne de très importantes pertes économiques liée à la condamnation des foies aux abattoirs, la réduction de production de viande et du lait, la mort des animaux infectés, ainsi que la baisse de fertilité et les très hauts coûts des traitements antiparasitaires (Kaplan, 2001 ; Mezo et al. 2011).

Comme les animaux domestiques restent les plus importants hôtes définitifs de *F. hepatica* et *F. gigantica*, la transmission de la fasciolose est fortement liée à l'activité humaine (Mas-Coma et al. 2009 ; Sabourin et al. 2018). En revanche, une relation directe entre les hauts taux d'infection des communautés humaines et celles existant chez bétail n'est pas toujours reportée, principalement dû à des différences qui existent dans les habitudes alimentaires, socioéconomiques et de conditions sanitaires (Mas-Coma et al. 1999). En tout cas, le nombre réel des animaux domestiques et des êtres humains infectés par la fasciolose dans certains pays est très sous-estimé dû à : a) des infections asymptomatiques chroniques ; b) certaines méthodes de diagnostic utilisées peuvent présenter des problèmes de sensibilité, de reproductibilité et ainsi du coût ; et c) la coordination et la mise à jour de l'information concernant cette maladie est très peu abondante, surtout dans les pays du Sud (Toet et al. 2014).

La fasciolose progresse en quatre différentes phases chez l'Homme : 1) une phase d'incubation initiale qui va de l'ingestion de metacércaires jusqu'à l'apparition des premiers symptômes, cette période peut durer de quelques jours à trois mois en fonction du nombre de parasites ingérés et de l'immunité de l'hôte ; 2) une phase invasive (appelée aussi intense) qui peut entraîner de la fièvre, des malaises, des douleurs abdominales, des problèmes gastro-intestinaux, urticaire, anémie et jaunisse ; 3) une progression vers une phase latente avec en général moins de symptômes ; et finalement 4) une phase chronique, voire obstructive

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quelques mois ou années après (Behm & Sangster, 1999; Mas-Coma et al. 2009). La phase de fasciolose chronique entraîne une inflammation des canaux biliaires et de la vessie qui peut aboutir à une très forte fibrose du tissu hépatique (Behm & Sangster, 1999). Le nombre et l'intensité des symptômes sont en relation avec la quantité de parasites, le moment de l'infection ainsi que de l'espèce d'hôte et de son immunité (Mas-Coma et al. 2009). Le taux de mortalité est généralement élevé chez les ovins et les bovins, mais très rare chez les humains (Torgenson & Claxton, 1999).

## II. Scénario épidémiologique de la fasciolose à Cuba

Cuba est l'archipel le plus étendu de toute la Caraïbe, se trouvant entre les deux péninsules de la Floride (au nord) et Yucatán (au sud) en face du Golfe du Mexique (ce qui lui vaut le surnom de « Clé du Golfe du Mexique »). Essentiellement, il y a deux îles principales dans l'archipel : l'île de Cuba et l'île de la Jeunesse (Isla de la Juventud), ainsi que plus de 4000 îlots. Ils existent trois massifs montagneux à l'ouest, au centre et à l'est tandis que le 90% du territoire cubain est constitué de plaines avec des terrains facilement inondables utilisés pour l'élevage et très favorable au développement de populations de limnées.

Le scénario épidémiologique de la fasciolose à Cuba est contraint à une seule espèce de douve, *F. hepatica*, mais avec deux patterns très différents de ses hôtes définitifs. La fasciolose humaine est caractérisée par des épidémies répétées et de petites envergures dans lesquelles on compte au moins huit événements d'infection très importants depuis la première description de la maladie en 1937 ; et par plusieurs cas isolés pendant toute l'année (Rojas et al. 2010 ; Díaz et al. 2011). En revanche, la fasciolose reste un problème important pour la santé vétérinaire dont une hyper-endémie est constatée chez le bétail avec de très fortes prévalences et charges parasitaires (Brito et al. 2010 ; Rojas et al. 2010 ; Vázquez et al. 2016). Le nombre élevé des animaux infectés à Cuba entraîne un très haut risque d'infection pour l'Homme car le parasite reste circulant dans la nature, particulièrement liée à l'activité humaine, surtout l'élevage (Vázquez et al. 2016 ; Alba et al. 2016). En plus, la température moyenne ainsi que le régime des précipitations restent favorables toute l'année pour l'existence des deux seules espèces de limnées présentes : *Galba cubensis* et *Pseudosuccinea columella*, dont les deux sont reconnues hôtes intermédiaires de *F. hepatica* à Cuba (Gutiérrez et al. 2011 ; Alba et al. 2016).

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### III. Les limnées et la transmission de *F. hepatica* à Cuba : l'existence de populations naturelles susceptibles et résistantes de *P. columella*

La transmission de *F. hepatica* dépend de l'espèce de limnée hôte, de la région géographique et des processus d'adaptation locale entre les deux (Dar et al. 2013 ; Vázquez et al. 2014). Plus de 20 espèces de limnées sont reconnues comme hôtes intermédiaires de *F. hepatica* à l'échelle mondiale (Correa et al. 2011). En revanche, à Cuba il n'existe que deux espèces mais qui peuvent normalement transmettre la douve (Gutiérrez et al. 2011 ; Vázquez et al. 2015) : *Galba cubensis* (connue avant comme *Fossaria cubensis* ou *Lymnaea cubensis* et décrit à Cuba par Pfeiffer, 1839) et *P. columella* (connue avant comme *Lymnaea columella* et reportée pour la première fois à Cuba par Poey en 1858 comme *Lymnaea francisca*).

La large aire de répartition de *G. cubensis* à Cuba, de ses caractéristiques écologiques (très forte amphibiose et grande tolérance à différents types d'habitats, quelquefois très anthropisés) et une très haute compatibilité avec les souches circulantes de douves, font de cette espèce le principal mollusque vecteur de la maladie (Vázquez et al. 2009 ; 2014 ; 2015). En fait, *G. cubensis* a été trouvé infecté sur le terrain dans plusieurs échantillonnages avec des intensités parasitaires très fortes pouvant aller jusqu'à 76 rédies dans une seule limnée (Vázquez et al. 2015) et des prévalences jusqu'à 34,32% dans une seule localité (Alba et al. 2016). Dans le reste de la Caraïbe, comme à Cuba, *G. cubensis* est considéré comme le principal vecteur de la fasciolose (Gutiérrez et al. 2011). En revanche, dans quelques régions au sud des Etats-Unis (Kaplan et al. 1997) mais aussi en Amérique du Sud (Bargues et al. 2011 ; Medeiros et al. 2014 ; Alda et al. 2018), cette espèce partage le rôle de vecteur de la douve avec les autres limnées qui coexistent en compliquant le scénario de transmission en Amérique continentale.

Par contre, on peut observer une situation différente chez *P. columella* par rapport à *G. cubensis* à Cuba. Cette limnée a une distribution beaucoup plus limitée et absente de la région orientale, très abondante dans les foyers ruraux (Vázquez et al. 2009) pour lequel on a proposé un rôle secondaire dans la transmission (Vázquez et al. 2014). Jusqu'à aujourd'hui, des infections naturelles n'ont été observées dans seulement une population dans la province occidentale de Pinar del Río (Pilón) et elle été considérées comme le première reportée pour Cuba et la Caraïbe malgré la prévalence de 3% (Gutiérrez et al. 2011). En revanche, même si la compatibilité globale de *P. columella* avec la douve reste plus basse que celle de *G. cubensis*,

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plusieurs populations de cette espèce venant de différentes régions de Cuba ont été infectées expérimentalement avec succès au cours de dernières années en utilisant différents isolats de douves (Gutiérrez et al. 2002 ; 2003a ; Vázquez et al. 2014). Dans ces conditions expérimentales, la prévalence peut varier entre 0 et 100% selon la combinaison douve-limnée, ce qui suggère l'existence d'un polymorphisme de compatibilité dans ce système (Vázquez et al. 2014). Ce qui est intéressant à Cuba, c'est le fait qu'il existe certaines populations naturelles surtout dans la partie ouest du pays, plus précisément six populations (La Palma, El Azufre, Babiney, Guanahacabibes, La Coca and Candelaria) qui n'ont jamais pu être infecté au laboratoire suite à l'exposition à différentes souches de *F. hepatica* (Gutiérrez et al. 2002 ; 2003a ; Calienes et al. 2004 ; Vázquez et al. 2014). Il a été noté dans des coupes histologiques que la résistance observée est due à une encapsulation des miracidia par les hémocytes dans les 24h après leur pénétration chez la limnée (Gutiérrez et al. 2003a). Tous ces résultats témoignent de l'existence d'une activation immunologique et pas d'une simple mal-adaptation/« unsuitability » de ses limnées envers la douve. Résistance résultante semble-t-il d'une sélection in « natura » (Gutiérrez et al. 2003a ; Vazquez et al. 2014).

Notamment, toutes les limnées résistantes au sein de ces populations partagent les mêmes caractéristiques coquillère (petite spire, large ouverture et des micro sculptures dans le periostracum) ainsi qu'anatomique (Pointier, 2008 ; Vázquez & Sánchez, 2015 ; Correa et al. 2011). De plus, l'amplification et séquençage de l'ADNr ITS (1170 pb de la région 3' 18S, ITS-1, 5.8S, ITS-2 et de la région 5' of the 28S) chez les limnées susceptibles et résistantes ont montré seulement un polymorphisme de deux bases, une dans chacun des fragment ITS, ce qui représente seulement 0,17% de variation et n'est pas suffisant pour envisager l'existence d'un processus de spéciation entre les deux phénotypes (Gutiérrez et al. 2003b). Ces critères sont normalement utilisés pour la discrimination d'espèces lorsque les différences sont majeures et ont été en effet utilisés chez les lymnaeïdes (e.g. *P. columella* ; Bargues & Mas-Coma, 1997 ; Correa et al. 2011). La forte similarité trouvée entre les populations susceptibles et résistantes de *P. columella* a suggéré l'existence de deux phénotypes présentant des compatibilités différentes envers *F. hepatica* (Gutiérrez et al. 2003a) et constitue, à notre connaissance, le seul exemple d'une résistance/susceptibilité naturelle dans un système trematode-mollusque.

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En revanche, les deux phénotypes peuvent être différenciés l'un de l'autre par des différences phénotypiques dans la pigmentation du manteau : tous les individus résistants présentent une bande de petits spots clairs et très nette dans la région centrale du manteau avec de plus grands spots plus ou moins placés uniformément de chaque côté, tandis que les individus sensibles présentent un pattern de spots très diffus et éparse (Gutiérrez et al. 2003a). Les populations résistantes de *P. columella* peuvent également être différenciés des susceptibles par des marqueurs génétiques polymorphes (Gutiérrez et al. 2003b ; Lounnas et al. 2017). Gutiérrez et al. (2003b) a réalisé une étude de RAPD en utilisant des marqueurs ADN polymorphes entre la population résistante de La Palma et deux populations susceptibles à la Havane et a montré des profils différents pour 17 des 26 amorces testées. Cette méthode a été utilisée aussi dans 12 populations naturelles en incluant trois résistantes (La Palma, El Azufre and Babiney) et les mêmes deux patterns différents pour les individus sensibles et résistants ont été obtenus avec très peu de polymorphisme intra groupe, ce qui permet de regrouper les populations dans deux clusters bien différenciés par rapport à leur phénotype de résistance/susceptibilité (Calienes et al. 2004).

En ce qui concerne les traits d'histoire de vie, les infections expérimentales ont montré des résultats très peu communs (Gutiérrez et al. 2002). Bien que *F. hepatica* soit connue pour entrainer une castration de ses mollusques hôtes et augmenter la taille des limnées infectées (Wilson & Denison, 1980), les individus de *P. columella* infectés (susceptibles) ont montré une plus forte fécondité pendant la période prépatente (Gutiérrez et al. 2002). En revanche, les individus résistants exposés à la douve présentent une meilleure survie et taux de fécondité par rapport au groupe contrôle non-exposé et aux individus sensibles infectés. Ces résultats ont suggéré l'existence d'un coût physiologique de la résistance associée au phénotype résistant de *P. columella* (Gutiérrez et al. 2002).

Le transfert des marqueurs spécifiques identifiés pour la susceptibilité et la résistance de *P. columella* entre générations reste encore inconnue. Cependant, des expériences de croisement au labo sont probablement inutiles dû fait d'un très fort taux d'autofécondation chez *P. columella*, ce qui a été montré par des analyses génétiques en utilisant plusieurs marqueurs microsatellites (Nicot et al. 2008 ; Lounnas et al. 2017). Une très haut taux d'autofécondation est, en fait, une caractéristique très commune chez les limnées et a été

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renseigné pour plusieurs espèces comme *Galba truncatula*, le principal vecteur de *F. hepatica* dans le monde (Meunier et al. 2004).

#### IV. **Prévention et contrôle de la fasciolose : les stratégies de contrôles basées sur les limnées sont-elles envisageables ?**

Dans un scénario mondial dans lequel la fasciolose humaine réémerge et pour laquelle de très hautes prévalences sont recensés chez le bétail, le contrôle précis de la transmission parasitaire est exigé urgemment. En revanche, même si plusieurs approches et candidats moléculaires ont été envisagés pour le développement de vaccins, une formulation commerciale reste encore très loin d'être disponible (Molina-Hernández et al. 2015). Pour l'instant, les schémas de contrôle restent basés sur les traitements antihelminthiques tant pour la population humaine que pour les animaux domestiques. Toutefois, ça reste un réel problème du fait de l'apparition régulière de souches de *F. hepatica* résistantes aux traitement médicamenteux (Triclabendazole) et de l'absence du développement de nouvelles chimiothérapies efficaces (Fairweather, 2011). De plus, la capacité de *F. hepatica* d'infecter toute une gamme d'animaux sauvages, comme des rongeurs, lagomorphes, lamas, marsupiaux ; (Robinson & Dalton, 2009 ; Mas-Coma et al. 2009), combiné avec l'éventuel mauvaise gestion du bétail (en incluant l'absence de traitement antihelminthiques ; Arias et al. 2010), assure la persistance du parasite si les mollusques restent infectés. Ces derniers facteurs en association avec une production du bétail chaque jour plus élevée pour assurer la demande du marché mondial et l'effet du changement climatique sur les populations de limnées hôtes, empêche un contrôle de la fasciolose seulement fondé sur des stratégies chez les hôtes définitifs (Knubben-Schweizer & Torgerson, 2015 ; Khan et al. 2013).

Alternativement, comme les douves du foie utilisent obligatoirement les mollusques pour leur développement d'une partie de leurs phases larvaires, l'hôte mollusque pourrait être utilisé pour surmonter les difficultés mentionnées ci-dessus en établissant des stratégies pratiques sur le terrain contre les limnées (Knubben-Schweizer & Torgerson, 2015 ; Knubben-Schweizer et al. 2010). En revanche, la distribution mondiale de limnées (Correa et al. 2010) ainsi que leur résilience et plasticité écologique, facilitent la transmission et imposent de très forts défis pour les politiques de contrôle de mollusques. En tout cas, toutes les stratégies, pour qu'elles soient effectives, doivent s'adapter au scénario particulier de transmission et se combiner pour aboutir à un contrôle efficace. Dans ce sens, la recherche appliquée pour développer des

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méthodes écologiquement réalistes (Adema et al. 2012) et des méthodes d'échantillonnage des mollusques infectés afin de détecter les foyers de transmission (Alba et al. 2016) reste essentielle. D'un autre côté, l'étude de la composante écologique, immunobiologique, génétique et évolutive de l'interaction mollusque-trematode sont des facteurs clés pour parvenir à proposer des méthodes innovantes de contrôle de la maladie (Adema et al. 2012). Même si l'interaction *Fasciola* spp. – limnées n'a été que peu étudiée, l'identification de phénotypes résistants et sensibles sélectionnés *in natura* chez la même espèce de limnée constitue un modèle unique afin d'explorer les mécanismes sous-jacents.

Les capacités invasives et d'adaptation de *P. columella* (Lounnas et al. 2017) ont permis son établissement dans plusieurs environnements à l'échelle mondiale : depuis son aire native de répartition en Amérique du Nord (Cruz-Reyes & Malek, 1987 ; Correa et al. 2010) jusqu'au Caraïbe et l'Amérique du Sud (Cucher et al. 2006 ; Gutiérrez et al. 2011), et plus récemment en Afrique (Grabner et al. 2014), Europe (Pointier et al. 2007), Australie (Molloy & Anderson, 2006) et plusieurs îles de l'Océan Pacifique (Pointier & Marquet, 1990 ; Cowie, 2001). Dans ce sens, l'application potentielle des souches résistantes de *P. columella* comme un agent de contrôle biologique (introduction des individus résistants dans des foyers à haut-risque, ou bien en modifiant génétiquement les populations locales de *P. columella* sensibles) peuvent constituer des solutions envisageables. La première étape à suivre pour répondre à cet objectif est donc d'approfondir notre connaissance de ce système d'interaction particulier.

Dans cette thèse, nous avons réuni donc synthétisé l'ensemble des approches récentes que nous avons développées afin d'aller plus loin dans la caractérisation des populations de *P. columella* résistantes/sensibles à *F. hepatica* à Cuba en particulier sur les aspects écologiques, moléculaires et phénotypiques.



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## Objectifs spécifiques de la thèse :

Pour une meilleure compréhension des résultats, nous avons divisé les objectifs en trois axes principaux, à savoir :

### Distribution et écologie de *P. columella* à Cuba

1. Comparaison des patterns de distribution et préférences écologiques des populations résistantes et sensibles de *P. columella* à Cuba.
2. Estimation de l'effet de différents facteurs écologiques sur les traits d'histoire de vie des populations résistantes et sensibles testé en conditions expérimentales.

### Caractéristiques moléculaires constitutives des individus résistants et sensibles de *P. columella*

3. Identification par transcriptomique et protéomique comparatives des différences moléculaires constitutives entre les individus résistants et sensibles de *P. columella*.

### Phénotypes de *P. columella* en réponse à l'infection par *F. hepatica*

4. Détermination de l'effet de différentes doses, exposition sériée et l'origine de *F. hepatica* sur le résultat de l'infection entre des individus résistants et sensibles de *P. columella*.
5. Evaluation de la sensibilité des individus résistants et sensibles de *P. columella* à d'autres trématodes digènes.
6. Caractérisation quantitative et fonctionnelle de la réponse hématocytaire chez les individus résistants et sensibles de *P. columella* suite à l'infection par *F. hepatica*.

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## DISCUSSION GENERALE

### I. Les origines possibles et les bases mécanistiques de la résistance de *P. columella*

L'un des modèles biologiques les plus intéressants sur lequel nous développons nos approches expérimentales est celui des interactions mollusque - trématode, qui aboutissent souvent à la transmission de plusieurs maladies importantes, en particulier dans les tropiques. Dans cette thèse, nous avons abordé un système mollusque - trématode dans lequel certaines populations de *P. columella* sont naturellement résistantes à *F. hepatica*. Cette résistance passe par une encapsulation efficace du parasite par les hémocytes de l'hôte (Gutiérrez et al. 2003a). Ici, nous avons trouvé deux caractéristiques phénotypiques supplémentaires associées aux populations de *P. columella* résistantes : elles peuvent mieux tolérer, que les escargots sensibles, des eaux légèrement acides et avec une dureté très faible, et elles ont également connu certaines contraintes de reproduction par rapport aux populations sensibles (voir chapitre 1). Cependant, alors qu'une réponse immunitaire protectrice à l'infection par *F. hepatica* et une tolérance plus élevée aux variations de pH / TH impliquaient une plus grande capacité à réagir aux facteurs de stress (biotiques ou abiotiques), la modification des traits écologiques associés à la reproduction est probablement la conséquence de compromis énergétiques entre les processus physiologiques potentiellement concurrents (autosuffisance / survie vs. reproduction; Stearns, 1992). Il est intéressant de noter que nous avons constaté une importante allocation de ressources aux fonctions / processus moléculaires liés à la réponse au stress / défense, et au maintien de l'homéostasie associé aux souches de *P. columella* résistantes, même pour un organe principalement impliqué dans la reproduction (voir chapitre 2). Tous ces facteurs pourraient réduire le potentiel de compétition de *P. columella* résistante sur le terrain en l'absence de pressions parasitaires élevées ou d'eaux à pH / TH bas, beaucoup moins tolérés par d'autres espèces de mollusques. Par conséquent, ces résultats confirment, au niveau moléculaire, les caractéristiques phénotypiques observées dans ces populations.

Cependant, la question de l'origine de la réponse immunitaire protectrice chez les mollusques résistants et son association avec la tolérance plus élevée aux variations de pH / TH observée également dans ces populations reste posée. Il convient de noter que la réponse immunitaire et la régulation acido-basique / osmotique sont physiologiquement liées via plusieurs

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fonctions moléculaires, effecteurs et voies de signalisation (Demas et al. 2011; Matozzo et al. 2012). Par conséquent, on pourrait émettre l'hypothèse qu'une tolérance plus élevée aux pH / TH est un « sous-produit » résultant d'une sélection de résistance induite par un parasite, ou que le phénotype de résistance est simplement un « effet secondaire » d'une sélection induite par l'environnement liée à un potentiel accru de compensation des variations osmotiques et acido-basiques.

D'une part, des modifications du pH interne et environnemental pourraient entraîner certaines caractéristiques immunitaires renforcées telles qu'une hématopoïèse accélérée (Meseck et al. 2016), une augmentation des capacités phagocytaires des hémocytes (Matozzo et al. 2012; Meseck et al. 2016), une augmentation de l'acidité du milieu intérieur et des défenses anti-oxydantes (Freitas et al. 2016), ainsi qu'un recrutement de calcium et une hypercalcémie plus élevés (Tunholi-Alves et al. 2014; Tunholi et al. 2017). En raison des rôles fondamentaux joués par les processus mentionnés ci-dessus dans la surveillance immunitaire et l'orchestration de la défense (voir Connors et al. 1995; Goodall et al. 2004; Nikapitiya et al. 2010; Larson et al. 2014), de tels escargots pourraient être plus enclins à réagir efficacement aux infections et à être « mieux préparés » pour reconnaître, phagocyter / encapsuler et éliminer les agents pathogènes. En ce sens, il est à noter que nous avons observé une abondance constitutive de transcrits et de protéines particulièrement associées à l'activation de la réponse immunitaire chez les mollusques résistants par rapport aux mollusques sensibles (chapitre 2).

D'autre part, une réactivité immunitaire accrue peut entraîner un changement du métabolisme en particulier une augmentation relative du taux de respiration, de la glycolyse, de la protéolyse et de la lipolyse (Lochmiller et Deerenberg, 2000). Cela pourrait entraîner une augmentation relative des niveaux de protons, de ROS et de CO<sub>2</sub> dans le milieu interne et, par conséquent, un enrichissement d'autres fonctions associées telles que les enzymes antioxydantes, l'anhydrase carbonique et les transporteurs d'ions (par exemple, Na<sup>+</sup> / K<sup>+</sup> - ATPase, H<sup>+</sup> - ATPase, échangeur Na<sup>+</sup> / H<sup>+</sup>), pour répondre aux besoins énergétiques de la membrane dans ce contexte biochimique (Epstein et al. 2014; Biller-Takahashi et al. 2015). Ces derniers pourraient être particulièrement associés à la tolérance accrue au pH / TH observée chez les mollusques résistants car, chez les animaux aquatiques, l'équilibre acido-basique et la régulation de l'osmolarité sont intimement liés en raison du mouvement couplé

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de  $H^+$  et de  $HCO_3^-$  avec celui de  $Na^+$  (Matthews, 2017). En ce sens, il est intéressant de noter que nous avons observé un enrichissement de molécules régulatrices de ions majeurs de l'organisme et en anhydrase carbonique en association avec le phénotype résistant (voir chapitre 2), ce qui explique potentiellement l'aptitude particulière de ces escargots à tolérer les variations osmotiques et de pH du milieu aquatique.

Toutefois, il est encore difficile de trancher entre ces deux hypothèses. Les pressions de sélection environnementales pourraient peut-être expliquer la résistance générale à *F. hepatica*, quelle que soit la souche infectante (voir Chapitre 3.1; Calienes et al. 2004; Vázquez et al. 2014), par le biais d'une amélioration globale des fonctions immunitaires. En ce sens, on pourrait également s'attendre à une compétence plus « globale » pour lutter contre tout type d'infection. Cependant, nous avons pu montrer que les *P. columella* résistants et sensibles à *F. hepatica* sont tous deux sensibles à l'infection par *Trichobilharzia* (chapitre 3.2). En outre, nous avons démontré l'existence d'une réponse immunitaire induite à la suite d'une infection par *F. hepatica*. Cette réponse est d'ailleurs totalement différente entre les phénotypes de *P. columella* en termes de morphologie des hémocytes, d'activités de prolifération et d'encapsulation et de production de la cytokine granuline (chapitre 3.3). Ces résultats suggèrent qu'il existe jusqu'à présent une certaine spécificité de cette « capacité immunitaire renforcée » envers *F. hepatica*, qui permet de supposer que la sélection de la résistance puisse être acquise en réponse à l'infection par le parasite. Dans ce sens, et tel qu'il a été présenté ici, la population de *P. columella* résistante se trouvait principalement dans l'ouest de Cuba, en particulier dans la province de Pinar del Rio (chapitre 1). Cette région est connue pour être un foyer important de transmission de *F. hepatica*. Une forte prévalence d'infection chez les limnées (Alba et al. 2016) et le bétail (Vázquez et al. 2016), et même des cas humains et des épidémies (Rojas et al. 2010; Diaz et al. 2011) ont été rapportés à Pinar del Rio, où la circulation du parasite est supposée stable tout au long de l'année (Rojas et al. 2010).

## **II. Sur l'immunobiologie de l'interaction résistant / sensible de *P. columella* pour *F. hepatica***

L'hypothèse de la Reine Rouge prédit que les parasites trématodes évoluent vers un certain degré de correspondance génétique avec leurs escargots hôtes afin d'augmenter leurs chances d'infection, réciproquement, la forte pression de sélection exercée par certaines populations de parasites doit favoriser la sélection de génotypes d'hôtes rares comme

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adaptation au parasitisme (Lively et Dybdahl, 2000 ; Osnas et Lively, 2005). Ainsi, certaines populations hôtes présentent généralement des variations des caractères de compatibilité en fonction des génotypes des parasites infectants (Osnas et Lively, 2005; Theron et al. 2014; Vázquez et al. 2014). Cependant, certains génotypes d'hôte rares, favorisés par la sélection du parasite au sein d'une population donnée, pourraient devenir plus que « localement rares » et, au lieu d'être simplement des génotypes moins susceptibles, entraîner une résistance (Lively et Dybdahl, 2000; Osnas et Lively, 2005). Ces génotypes très rares sont généralement moins abondants en raison d'un coût à la résistance « fitness cost » en l'absence de parasitisme (Lively et Dybdahl, 2000). Les résultats présentés ici (chapitre 1) et ailleurs (Calienes et al. 2004; Lounnas et al. 2017) démontrent que les populations résistantes se regroupent spécifiquement à part des *P. columella* de Cuba et du reste du monde, ce qui soutient la notion de déterminisme génétique de la résistance. En outre, la ségrégation des populations résistantes indique qu'elles auraient pu être sélectionnées dans un pool différent de celui (sensible) qui s'est répandu partout, et que les individus de *P. columella* regroupés à l'extérieur des grappes « résistantes » pourraient être considérés comme sensibles à *F. hepatica*. Chez ces escargots sensibles, l'infection par *F. hepatica* se caractérise par une réduction significative de la fécondité (Gutiérrez et al. 2002; Salazar et al. 2006), une productivité plus élevée de rédies et métacercaires par rapport à *G. truncatula* (Dar et al. 2014; Vignoles et al. 2015) et une plus grande survie par rapport aux espèces de *Galba* (Salazar et al. 2006; Vignoles et al. 2015).

Dans le modèle largement étudié de *B. glabrata* - *S. mansoni*, une encapsulation du parasite par des hémocytes hôtes est observée lorsque des combinaisons hôte-parasite incompatibles se produisent suivant un modèle d'allèles correspondant (matching phenotype model) (Théron et Coustau, 2005). Cette mosaïque d'infectivité a sa base moléculaire dans l'interaction des FREP de l'hôte hautement diversifié avec les mucines du parasite hautement polymorphes, en tant que facteur déterminant de l'infection (Portet et al. 2017). Bien que l'on connaisse mal les molécules impliquées dans l'interaction hôte *F. hepatica* - limnée, des variations similaires des résultats de l'infection ont été observées chez *P. columella* sensible, probablement en raison de l'existence d'un polymorphisme de compatibilité (Vázquez et al. 2014; chapitre 3.1). En revanche, les populations de *P. columella* résistantes ont toujours réussi à lutter contre l'infection par *F. hepatica*, indépendamment de la souche cubaine (par

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exemple Calienes et al. 2004; Vázquez et al. 2014) ou de la souche parasitaire provenant d'autres aires géographiques éloignées (voir le chapitre 3.1). Fait intéressant, bien que *F. hepatica* soit reconnu comme un parasite génétiquement diversifié (Vázquez et al. 2016; Cwilinsky et al. 2015), nos résultats suggèrent que la résistance à *P. columella* n'est pas étroitement limitée à une interaction génotype de l'hôte - génotype du parasite.

Les informations concernant le dialogue moléculaire et physiologique entre *F. hepatica* et les limnées sont très rares et laissent peu de place aux hypothèses sur les molécules / fonctions particulières liées de manière proximales ou ultimes aux résultats de l'infection chez les individus résistants et sensibles. Cependant, nous pouvons supposer que certaines modulations immunitaires ou immunosuppression induites par *F. hepatica* se produisent chez ses hôtes intermédiaires. La modulation immunitaire directe qui représente des contre-mesures du trématode pour contourner l'activation du système immunitaire de l'escargot a été vérifié dans plusieurs modèles (*i.e.* *S. mansoni* / *E. paraensei* - *B. glabrata* (Lodes et Yoshino, 1990 ; DeGaffeé et Loker, 1998 ; Connors et al. 1991 ; Loker et al. 1992 ; Zahoor et al. 2008 ; 2009), *Himasthla elongata* - *Littorina littorea* (Iakovleva et al. 2006). ), *Trichobilharzia regent* - *Radix lagotis* (Skála et al. 2014) et *Plagiorchis mutationis* - *L. stagnalis* (Kryukova et al. 2014). En particulier, l'établissement de l'infection par *F. hepatica* chez l'hôte définitif est induite par une puissante suppression / modulation immunitaire induite par des antigènes de parasite (voir Prowse et al. 2002; Gironès et al. 2007; Hamilton et al. 2009; Dowling et al. 2010). En outre, la diminution significative de l'expression de la granuline et les différences dans les caractéristiques morphologiques et fonctionnelles entre les hémocytes naïfs et ceux induits par *F. hepatica* chez des mollusques sensibles enregistrées ici (chapitre 3.3) constituent des preuves préliminaires d'une certaine interférence de *F. hepatica* sur les fonctions immunitaires de *P. columella*. Récemment, il a été suggéré que la coévolution de parasites avec des composants immunitaires spécifiques (récepteurs pour la détection / interaction ou effecteurs pour l'élimination) modifie l'allocation optimale de l'hôte de l'immunité induite à l'immunité constitutive (Kamiya et al. 2016). Selon ces auteurs, si la coévolution entre les populations de parasites et celle d'hôte déterminait des réponses immunitaires induites moins efficaces, en raison de la contre évolution des parasites sur les récepteurs de l'hôte, l'hôte serait alors amené à investir davantage dans l'immunité constitutive (Kamiya et al. 2016). En ce sens, la modulation / suppression immunitaire induite par *F. hepatica* au cours de la

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migration et de l'établissement du parasite poserait certainement un problème difficile à surmonter pour les défenses de l'hôte si celles-ci reposaient principalement sur l'immunité induite. En ce sens, une meilleure compétence immunitaire pourraient conférer un avantage significatif aux escargots pendant les premiers stades de l'infection parasitaire, ce qui pourrait donner lieu à une réaction immunitaire rapide et efficace et à son élimination. Les résultats des approches « omique » appliquées ici aux escargots naïfs ont révélé qu'une importante part des ressources était attribuée à la défense immunitaire chez les résistants par rapport aux individus sensibles, aux voies de signalisation pro-inflammatoires, aux cytokines, aux molécules antimicrobiens et aux facteurs antioxydants (chapitre 2). De plus, le niveau élevé d'encapsulation des sporocystes de *F. hepatica* observé dans des hémocytes naïfs provenant de populations résistantes (chapitre 3.3) constitue une preuve fonctionnelle de la réactivité immunitaire constitutive accrue associée au phénotype résistant. De même, il a pu être démontré dans un contexte de sélection expérimentale de *B. glabrata* résistants à *E. caproni* que les mollusques résistants pouvaient être capables de contourner les facteurs immunosuppresseurs du parasite (Humbert & Coustau, 2001). Cela pourrait suggérer que le principal facteur de la résistance chez *P. columella* soit associé à des capacités immunitaires constitutives accrues.

Il convient également de mentionner que l'augmentation du nombre de larves infectieuses (de 5 à 30 miracidia par escargot), par le biais d'expositions uniques ou sérielles, ne modifie pas la capacité de résistance de *P. columella* (chapitre 3.1). Cela confirme qu'un investissement important de ces escargots dans leur défense immunitaire se produirait également suite à l'infection. Les résultats obtenus concernant la caractérisation des hémocytes après l'exposition à *F. hepatica* (comportement élevé d'adhérence et d'agrégation, activités de prolifération et d'encapsulation supérieures des hémocytes, expression accrue de la granuline; chapitre 3.3) confirment également la capacité inhérente des escargots résistants à orchestrer une réponse immunitaire puissante et protectrice après l'infection par *F. hepatica*. Chez les insectes, par exemple, la phénoloxidase (PO) est une molécule effectrice de la défense qui est présente de manière constitutive, mais qui peut être régulée positivement lors d'une infection par le biais de la pro-phénoloxidase (PPO) (Lemaitre & Hoffmann, 2007). Chez *B. glabrata*, certaines FREP, telles que FREP 4, existent dans l'hémolymphe des individus naïfs, mais sont régulées positivement après une infection par *E. paraensei* (Zhang et al. 2008).

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### III. Application potentielle des résultats en termes de stratégie de contrôle de l'infection par *F. hepatica*

La transmission de *F. hepatica* dépend strictement de la présence de son hôte intermédiaire dans les foyers de transmission. Ainsi, à l'instar d'autres maladies transmises par des mollusques hôtes, la prévention appropriée ainsi que sa durabilité et son efficacité sont intimement liées à l'étude et au contrôle des escargots (Caron et al. 2011; Adema et al. 2012; Knubben-Schweizer et Torgerson, 2015). Celles-ci pourraient à terme permettre d'interrompre le cycle de vie du parasite, empêchant ainsi l'infection de nouveaux hôtes définitifs, tout en réduisant et en optimisant l'administration d'anthelminthiques (Knubben-Schweizer & Torgerson, 2015). En fait, les traitements anthelminthiques ne sont pas toujours rentables, menacent d'accroître les cas de résistance et ne confèrent pas de protection à long terme contre les réinfections (Fairweather, 2011). Cependant, si la surveillance épidémiologique des populations de limnées et de leur statut en matière d'infection constitue une approche adéquate pour surveiller et gérer les zones d'endémie (Arias et al. 2010; Caron et al. 2014; Alba et al. 2016), l'application de stratégies appropriées pour contrôler les populations de limnées sont loin d'être faciles et directes, pour des raisons biologiques et pratiques.

Les caractéristiques écologiques des limnées (par exemple, vastes préférences d'habitat, taux d'autofécondation et de reproduction élevés, caractère amphibie de certaines espèces ; Goumghar et al. 2004 ; Vázquez et al. 2009 ; Pointier et al. 2009 ; Meunier et al. 2004 ; Lounnas et al 2018) posent d'importants défis pour le succès des méthodes traditionnelles de lutte physique, chimique et biologique. En outre, alors que les stratégies physiques et biologiques pourraient prendre beaucoup de temps et nécessiter un certain niveau d'expertise (Rondelaud et al. 2006 ; Vázquez et al. 2009 ; Knubben-Sweizer & Torgenson, 2015), les molluscicides actuellement disponibles présentent une faible sélectivité et doivent être appliqués sur les eaux douces, la plupart utilisés habituellement pour abreuver le bétail ou bien pour l'irrigation des cultures (Khan et al. 2013). Dans ce sens, la collecte d'informations écologiques, phénotypiques et moléculaires liées à l'interaction trématode-mollusque (facteurs environnementaux affectant l'établissement des escargots et la transmission parasitaire, marqueurs génétiques de la compatibilité et des régulateurs de la transmission) pourraient être essentielles pour comprendre et prévoir la dynamique de la transmission



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parasitaire, et de concevoir des stratégies intégrales pour son contrôle (par exemple, des méthodes de contrôle respectueuses de l'environnement; Adema et al. 2012).

Dans cette thèse, nous avons porté un intérêt particulier aux escargots transmetteurs de fasciolose, *P. columella*, et à son interaction particulière avec *F. hepatica* à différents niveaux (écologique, moléculaire et phénotypique). D'un point de vue théorique, nous proposons une nouvelle perspective sur l'histoire et la colonisation de *P. columella* à Cuba et les modèles de préférences écologiques. Nous présentons également des preuves écologiques du coût de la résistance, à la fois sur le terrain et en laboratoire (distribution restreinte dans la nature, contraintes de la reproduction), nous avons également identifié d'autres caractéristiques phénotypiques associées à la résistance de *P. columella* (à savoir une plus grande tolérance au pH / TH ; Chapitre 1). L'ensemble de ces résultats nous donne une bonne vision du paysage immunitaire moléculaire et fonctionnel global des escargots avant l'infection, et nous donne des pistes permettant d'expliquer les phénotypes différentiels de *P. columella* (chapitre 2 ; chapitre 3). De plus, nous avons approfondi nos connaissances sur les caractéristiques phénotypiques de *P. columella* suite à l'infection par *F. hepatica* et sur la dynamique et les caractéristiques de la réponse immunitaire à la suite d'une infection parasitaire, ainsi que sur l'influence de l'origine géographique du parasite (*F. hepatica* sympatrique vs. allopatrique) et la spécificité du phénotype de résistance (chapitre 3).

D'un point de vue pratique, nous avons signalé la deuxième population de *P. columella* naturellement infectée par *F. hepatica* à Cuba et dans les Caraïbes, mais nous avons également démontré la propagation en cours de deux génotypes hautement invasifs et compatibles avec le parasite, qui alertent sur la complexité croissante du scénario épidémiologique de la fasciolose à Cuba. Nous avons analysé les schémas écologiques associés à l'occurrence différentielle de populations résistantes et susceptibles de *P. columella* et caractérisé les traits d'histoire de vie de différentes populations de *P. columella* dans deux contextes expérimentaux différents liés aux différentes exigences écologiques observées (chapitre 1). Nous avons fourni le premier transcriptome assemblé et annoté de cette espèce de limnée, ainsi que les premières informations sur l'analyse différentielle de ce transcriptome (au niveau de l'individu entier), et du protéome (au niveau de la glande de l'albumine) de *P. columella* résistant et sensible (chapitre 2). Ces résultats offrent des données moléculaires de base concernant les processus moléculaires particulièrement impliqués dans le phénotype

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résistant et qui pourraient être utilisés dans des études ultérieures afin d'identifier des cibles prometteuses (Greenwood et al. 2016). Nous présentons également des preuves de la stabilité du phénotype résistant quelles que soient la dose infectante, les expositions sérielles et l'origine géographique du parasite, ainsi que l'activation d'une réponse immunitaire cellulaire après l'infection par *F. hepatica*, ce qui pourrait être utile à l'identification de moyens de contrôle des parasites (chapitre 3). En outre, l'identification de nouvelles questions et hypothèses, ainsi que de nouvelles perspectives et approches expérimentales pour y répondre, peuvent être considérées comme l'une des contributions les plus significatives de la présente thèse.

Cependant, aussi prometteur que cela puisse paraître, l'utilisation de la résistance de *P. columella* pour contrôler la transmission de *F. hepatica* reste, pour le moment, une hypothèse de travail. L'introduction d'escargots résistants, en particulier dans les zones à forte transmission (fermes d'élevage ou cultures maraîchères, par exemple) pourrait s'appliquer en tant qu'approche traditionnelle de lutte biologique (voir Perera et al. 1990; Rondelaud et al. 2006). Cependant, le coût de la résistance décrit ici pourrait être un frein important à prendre en compte (Gutiérrez et al. 2002). Par conséquent, une étude préalable du site devrait être nécessaire avant d'introduire des escargots résistants afin de surmonter la faible aptitude écologique associée à ce phénotype. De plus, des stratégies de lutte physique ou chimique avant l'introduction pourraient diminuer la richesse en espèces d'escargots et aider à l'établissement de la population résistante par la suite. Quoi qu'il en soit, cette approche nécessitera une planification, une exécution et un suivi approfondis. Ainsi même si la population introduite finissait par disparaître, il est probable qu'une interruption de la transmission, pourrait être enregistrée (voir Rondelaud. et al 2006).

Un objectif beaucoup plus ambitieux consisterait à introduire des gènes de résistance dans les populations sensibles de *P. columella* et à obtenir une souche résistante très compétitive qui pourrait ensuite être introduite dans les zones d'endémie comme moyen de lutte biologique (Gutiérrez et al. 2005). En ce sens, bien que la fécondation croisée soit très rare étant donné le taux d'autofécondation élevé des espèces observé ici (chapitre 1) et ailleurs (Lounnas et al. 2017), la manipulation génétique et la sélection contrôlée des caractéristiques résistantes / susceptibles sont des alternatives prometteuses à prendre en considération (Tennenssen et al. 2015b). En ce sens, le développement de nouvelles techniques telles que les outils d'édition

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du génome basés sur CRISPR / Cas9 offre de nouvelles possibilités de générer des vecteurs génétiquement modifiés comme cela a été testé avec succès chez le moustique vecteur *Anopheles gambiae* pour le contrôle de l'infection par *Plasmodium falciparum* (Dong et al. 2018). En outre, arriver à induire une expression constitutive des mécanismes support de la résistance dans les populations de vecteurs sauvages pourrait être l'objectif de futures recherches. Parallèlement, l'identification des ligands et molécules cibles chez le parasite pourrait également ouvrir la voie à la mise au point de nouveaux médicaments, cibles thérapeutiques ou candidats de vaccins à utiliser sur les hôtes définitifs (Tennissen et al. 2015b).

Néanmoins, pour réaliser des progrès significatifs sur l'une des propositions mentionnées, il reste beaucoup à apprendre sur les voies moléculaires de cette interaction particulière entre escargot et parasite. Toutefois, les ressources et l'intérêt limités consacrés à la lutte contre les mollusques et les maladies qu'ils transmettent doivent toujours être pris en compte, car ils constituent une limite majeure pour l'exploration d'opportunités scientifiques et pratiques (Adema et al. 2012). En ce sens, l'incorporation d'efforts de recherche supplémentaires, de nouvelles compétences, de nouvelles technologies et de financement apparaissent essentiels afin d'atteindre les objectifs fixés. Quoi qu'il en soit, si la discussion sur les moyens qu'il est possibles d'exploiter concernant la résistance de *P. columella* à *F. hepatica* reste ouverte, des études intégratives comme celle présentée dans cette thèse ouvrent de nouvelles possibilités sur la voie à suivre dans le futur.

**ABSTRACT:** *Pseudosuccinea columella* is one of the main intermediate hosts of *Fasciola hepatica*, a cosmopolitan snail-borne trematode that affects humans, livestock and wildlife. The occurrence in Cuba of susceptible and naturally-resistant populations of this species to *F. hepatica* infection (host's hemocytes encapsulate the parasite upon penetration) offers an interesting Mollusca - Trematoda model with applications on evolutionary biology, health sciences and vector control strategies. Thus, here we explored different ecological, molecular and phenotypical aspects of this system to better understand *P. columella* resistance. We determined that while susceptible and resistant snails share similar ecological requirements, resistant populations occurred only at low pH (6.5-6.5) and total hardness (TH; 4°-10°d) waters, with low snail species richness, suggesting a high ecological cost of resistance. Comparison of life history traits between susceptible and resistant snails, experimentally-reared at low or common pH/TH conditions, showed that resistant populations display higher tolerance to pH/TH variations and a lower reproductive potential. To gain more insights on the molecular bases of the features associated to resistance in *P. columella*, we performed comparative "omics" approaches on naïve snails from both phenotypes at whole snail level (RNAseq) and at the albumen gland level (2D-electrophoresis). The molecular differences found were fundamentally associated with an enrichment on biological process/functions and a particular abundance of proteins/transcripts related with immune defense/stress response in resistant snails. These results indicate a differential allocation of resources, at the molecular level, to self-maintenance and survival on resistant individuals, that possibly trades-off against reproduction, and that could endorse the enhanced responsiveness against stressors (*F. hepatica* or pH/TH variations) and the ecological cost of resistance observed in resistant population. On the other hand, we explored the effect of high infective doses, successive parasite exposures and different geographic origin of *F. hepatica* (Europe and the Caribbean) on the infection outcomes on both *P. columella* phenotypes and observed no reversion of the resistance independent of the experimental approach tested. Additionally, we found both phenotypes equally susceptible to another digenean trematode, *Trichobilharzia* sp., indicating a certain specificity of *P. columella* resistance, so far, to *F. hepatica*. Finally, we performed comparative studies on the cellular immune response following *F. hepatica* exposure. The significant differences found in resistant (i.e. high adherence and aggregation, and higher proliferation and encapsulation capacity of the hemocytes, and higher cytokine granulin expression) compared to susceptible snails endorse the differential response towards *F. hepatica* that develops on both phenotypes. This thesis presents the latest efforts to broadly characterize this model, which constitute building steps for the comprehension of *P. columella* resistance and for its application to tackle parasite transmission.

**RÉSUMÉ :** *Pseudosuccinea columella* est un des principaux hôtes intermédiaires de *Fasciola hepatica*, un trématode cosmopolite infectant l'Homme et les animaux domestiques et sauvages, et transmis par des mollusques d'eau douce. L'existence exclusive à Cuba de populations de *P. columella* qui sont sensibles et naturellement résistantes (encapsulation hémocytaire du parasite) à l'infection par *F. hepatica* offre un modèle Mollusque-Trématode très intéressant en termes de biologie évolutive, de santé humaine et de stratégies de contrôle vectorielle. L'étude des aspects écologiques, moléculaires et phénotypiques est essentielle pour comprendre les particularités de ce système. Ainsi, nos résultats ont montré que les mollusques sensibles et résistants partagent des exigences écologiques similaires mais chez les résistants la distribution est limitée aux localités avec des eaux acides et peu dures (pH/dureté totale (DT), 6-6.5/4°-10°d) et une richesse des communautés de mollusques très faible, ceci semblant indiquer coût élevé à la résistance. La comparaison de traits d'histoire de vie en laboratoire a permis de confirmer une haute tolérance aux variations de pH/DT de l'eau chez les mollusques résistants mais a montré également une diminution du potentiel reproductif des souches résistantes par rapport aux sensibles. Sur la base de ces observations, des approches « omiques » comparatives ont été entreprises entre souches sensibles et résistantes. Ces approches se sont focalisées sur l'étude de (i) la glande de l'albumine par protéomique comparative et (ii) par une approche de transcriptomique massive sur mollusques entiers. Nous avons observé une grande richesse de processus et fonctions biologiques et une forte expression de molécules en lien avec l'immunité et la réponse au stress chez les individus résistants. Ces différences moléculaires identifiées entre les deux phénotypes indiquent un trade-off entre la survie et la reproduction chez les individus résistants et peuvent expliquer en partie leur haute réactivité vis à vis des agents de stress biotique et abiotique (*F. hepatica* ou variations de pH/DT) et le coût de la résistance observée. Ainsi, nous avons exploré l'effet de doses parasitaires croissantes, des infections sérielles et des souches allopatriques de *F. hepatica* (Europe et Caraïbe) sur la compatibilité, sans modification du statut de résistance des mollusques. De plus, les deux phénotypes sont sensibles à un autre trématode (*Trichobilharzia* sp.), ce qui indique que la résistance chez *P. columella* est dirigée et ou spécifique de *F. hepatica*. Enfin, nous avons observé des différences significatives chez les souches résistantes concernant la réponse immunitaire cellulaire (i.e. haute adhérence et agrégation des hémocytes, haute activité proliférative et d'encapsulation et expression de la cytokine granuline) pour rapport aux individus sensibles. Ces résultats peuvent expliquer, en partie, la réponse immunitaire différentielle à *F. hepatica* qui se développe chez les deux phénotypes. Ce travail de thèse nous a aidé à comprendre les particularités de ce modèle hôte-parasite, mais présente également une avancée en termes d'utilisation de la résistance de *P. columella* à des fins de contrôle de la transmission parasitaire.

