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Physiology and immunity of the invasive giant African snail, *Achatina* (*Lissachatina*) *fulica*, intermediate host of *Angiostrongylus cantonensis*

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Abstract :

As one of the most successful invasive land snail species, *Achatina* (*Lissachatina*) *fulica* Bowdich, 1822 has achieved wide global distribution, particularly in (sub)tropical regions, with further dispersal likely due to climate change. This species of giant African snails (up to 17 cm shell length) is a pest that has extensive negative impact on agriculture and can serve as vector for several parasites, including *Angiostrongylus cantonensis*, a nematode parasite that causes (human) eosinophilic meningitis, an emergent disease. Investigation showed that *A. cantonensis* infection negatively impacts the metabolism of *A. fulica* by depleting polysaccharide stores of the intermediate host, compromising the energy balance of the snail. A review of the literature indicates that *A. fulica* possesses potent innate type immune defenses to counter infection, including phagocytic hemocytes capable of deploying reactive oxygen species and lectins for non-self recognition, a serine protease-dependent coagulation response (not observed in other taxa of gastropods), as well as antimicrobial proteins including achacin, an antimicrobial protein. A recent chromosome level genome assembly will facilitate progressively detailed characterization of these immune features of *A. fulica*. We strongly encourage further immunological studies of *A. fulica*, ranging from organismal level to molecular biology to gain better understanding of the *A. fulica* internal defense response to nematode pathogens like *A. cantonensis* and the contribution of immune function to the invasiveness of (snail) species. Characterization of immunity of *A. fulica*, representing the understudied Stylommatophora (panpulmonate landsnails) will also broaden the comparative immunology of Gastropoda.

Highlights

► The giant African snail *Achatina fulica,* the most widely distributed invasive pest land snail, will likely disperse further with climate change. ► *Achatina fulica* is intermediate host for the nematode *Angiostrongylus cantonensis* that causes eosinophilic meningitis. ► Study of metabolic and immunological aspects of this parasite host provide better understanding of epidemiology and inform on comparative immunology of gastropods.

Keywords : *Achatina fulica*, immunology, physiology, nematodes, *Angiostrongylus cantonensis*

1- Introduction

Gastropoda comprises the largest Class of Mollusca, occurring in a wide variety of aquatic and terrestrial habitats, showing a great diversity in anatomical structure. Accordingly, gastropods have contended with many diverse pathogens, including opportunists and several phylogenetic lineages of specialized eukaryotic parasites such as the digenetic trematodes (Loker, 2018; Arkitips et al., 2008). Considering the framework of great species richness and diversity of lifestyles, gastropod immunology is likely highly diverse due to lineage-specific evolution of novel features or modification and/or loss of ancestral aspects of immune function (e.g. Gorbushin, 2019). To date, however, immune function has been studied consistently in only a modest number of gastropod species (e.g. Schultz and Adema, 2017) that does not validly represent the phylogenetic diversity of gastropods.

1.1. *Achatina fulica*, a prominent invasive pest.

Importantly, comprehensive understanding of immunology is especially lacking for air breathing terrestrial slugs and snails that belong to the order Stylommatophora within the clade of Eupulmonata (Gastropoda, Euthyneura, Heterobranchia). The stylommatophorans comprise of Scolodontidae (a small basal group) and two large clades of non-achatinoid and achatinoid snails (e.g. Wade et al., 2006, Saadi and Wade, 2019). The latter clade includes the family Achatinidae of medium to large sized tropical land snails, native to the African continent, that contains the subject of this review. *Achatina* (*Lissachatina*) *fulica* Bowdich, 1822. Currently *A. fulica* is included within the subgenus *Lissachatina* and some but not all recent publications (e.g. Borrero et al., 2009; Guo et al., 2019) have treated *Lissachatina* as a genus; however, at present, no published

taxonomic works are available to sustain this treatment. This review will employ the binomial *Achatina fulica*. This species, commonly named the giant east African snail grows to large adult size (shell length up to 17cm). Like other giant African snails, *A. fulica* is considered a food source and a pet animal, and it may be applied in traditional medicine for antibacterial activity of secreted snail mucus (Etm et al., 2016). A few (or even single) *A. fulica* are capable of colonize new habitats; they are hermaphrodites that can self-fertilize and have a high reproductive output. It is known to consume more than 50 species of tropical plants, agricultural and horticultural crops, modify habitats and outcompete native snails in newly colonized non-native ecosystems (Raut and Barker, 2002), by predation and herbivory. It is of note that *A. fulica* impacts humans severely as a general nuisance, since snails populations may reach very high densities (Mead, 1961; Thiengo et al., 2007; Hoxha et al., 2019).

These factors, along with trends of cross-border migration and trading, have likely contributed greatly to transport and introduction of *A. fulica* outside traditional native distribution ranges, allowing this snail to emerge as an invasive alien species (Knobler et al., 2006). While fortunately, it seems that only a modest proportion of introduced organisms possesses the phenotypic plasticity of relevant traits for behavior and fitness needed to become an invasive species (Richardson et al., 2000; 2006), several species of terrestrial gastropods have been able to invade and establish in a wide variety of new ranges, including natural zones like the Amazon Rainforest and large urban agglomerations worldwide (Ruiz and Carlton, 2003; Roda et al., 2016; Hoxha et al., 2019). Classified among the 100 worst invasive pests in the world (Lowe et al., 2004), *A. fulica* is considered the most widely introduced and invasive land snail species pest with particular impact in tropical and subtropical regions. This may be exacerbated by climate change as this may lead to range expansion of invasive species and interact synergistically with invaders to further worsen the impact on local ecosystems (Rekha Sarma et al., 2015; ; Bellard et al., 2012; Lowe et al., 2000; Raut and Barker, 2002). Concerning the invasive colonization of the Americas, *A. fulica* was initially introduced from Hawaii (where it persists to date) to Florida, mainland of the USA in 1966 (Robinson, 1999; Kim et al., 2014). In the Caribbean, *A. fulica* was established by 1984 on the island of Guadeloupe, by 1988 on Martinique and subsequently on Barbados and Saint Lucia according to Raut and Baker (2002). More recently, *A. fulica* was reported from Jamaica and Haiti

by Lindo et al., (2002) and Raccurt et al., (2003), respectively. Since initial reports from the 1980s, *A. fulica* has been reported from many countries in South America including Argentina (Gutiérrez Gregoric et al., 2011), Colombia (De la Ossa-Lacayo et al., 2012); Ecuador (Correoso Rodriguéz, 2006), Paraguay (Vogler et al., 2013), Peru (Borrero et al., 2009) and Venezuela (Martinez-Escarbassiere and Martinez, 1997). In Brazil, *A. fulica* was first introduced deliberately in 1988 in the southern state of Paraná (Teles and Fontes, 2002), where snails (probably from Indonesia) were sold at an agricultural fair as food resource for human consumption. Nowadays, *A. fulica* occurs in 25 out of the 26 states and the Federal District of Brazil (Morassutti et al., 2014; Thiengo and Fernandez, 2013; 2016).

Achatina fulica is very difficult to control once established; eradication has proven virtually impossible, especially in developing countries. In excess of $1 million dollars were spent for (ongoing) counter measures against *A. fulica* during the initial five years after this invasive snail species established in Florida (USA), (see e.g. Mead, 1961; Prociv et al., 2000; Raut and Barker, 2002; Thiengo et al., 2010). To date, it is illegal to import or keep *A. fulica* in the USA (see https://www.aphis.usda.gov/aphis/ourfocus/planthealth/plant-pest-and-disease-programs/pests-and-diseases/giant-african-snail/gas) and control efforts include chemical phytosanitary treatment of agricultural products to be shipped out of quarantined areas. Molluscicide treatments of *A. fulica* habitats can significantly inhibit snail development and reduce transmission of the parasites that these snails harbor (Gomot-de Vaufleury and Bispo, 2000). Unfortunately, the efficacy of molluscicides varies (Hallman 2011; 2016), and intensive use has a negative environmental impact as it leads to chronic soil contamination (Jansch et al., 2006; Arias-Estévez et al., 2008; Thiengo et al., 2007).

2 – *Achatina fulica,* vector of parasitic nematodes.

Another negative aspect of invasion of new ranges by *A. fulica* is that this snail may transmit or even introduce pathogens with undesirable health and socioeconomic effects. Like several other species of land snails that harbor parasites, e.g the widely distributed brown garden snail *Cornu aspersum (*Müller, 1774; formerly *Helix aspersa*), vector of the nematodes *Angiostrongylus vasorum* and *Aelurostrongylus abstrusus* (Druart et al., 2011; Di Cesare et al., 2015); *Theba pisana* (Müller 1774), the white garden snail present in Australia, Israel,

and USA), intermediate host for the nematode lungworm *Muellerius capillaris* and the trematode *Brachylaima cribbi* (Baker, 1986; Butcher and Grove, 2001; Grewal et al., 2003), also *A. fulica* snails may spread plant pathogens and they are intermediate host of several parasitic nematodes, such as *Aelurostrongylus abstrusus*, *Phasmarhabditis hermaphrodita*, *Rhabditis* sp, *Strongyluris* sp (Turner, 1967; Alicata, 1991; Zanol et al., 2010; Williams and Rae, 2015; Oliveira and Santos, 2018). Importantly, *A. fulica* is also vector of two zoonotic nematodes: *Angiostrongylus costaricensis*, causing abdominal angiostrongyliasis and the congeneric rat lungworm, *Angiostrongylus cantonensis*, which causes eosinophilic meningoencephalitis (or the rat lungworm disease) in humans (Alicata, 1991; Prociv et al., 2000; Carvalho et al., 2003; 2012). Abdominal angiostrongyliasis appears to be restricted to the Americas, with reports ranging from the southern United States to northern Argentina (Graeff-Teixeira, 2007). Originally endemic in Southeast Asia and Pacific Islands, to date, eosinophilic meningitis has been reported from about 30 countries of all continents with exception of Europe and Antarctica (Cowie, 2013a). In Hawaii, *A. fulica* has been associated with transmission of eosinophilic meningoencephalitis (Kim et al., 2014).

The life cycle of *A. cantonensis* involves rodents as definitive hosts and several species of terrestrial and freshwater snails that may serve as intermediate hosts. Gastropods become infected by ingestion of rodent feces that contain *A. cantonensis* larvae (L_1 stage). Inside the snail, the parasite molts twice over about 15 days. The resulting L_3 larvae are directly infective for the definitive host (Cheng and Alicata, 1965; Maldonado et al., 2010) but an optional role for paratenic (carrier) hosts such as land crabs, freshwater prawns, fish, frogs and planarians increases the chances for transmission of the parasite. *Angiostrongylus costaricensis* has a similar life cycle but does not employ paratenic hosts (Morera, 1973; Spratt, 2015).

Human *Angiostongylus* infections result from ingestion of raw gastropods or paratenic hosts (in case of *A. cantonensis*) that harbor L_3 nematode parasites, or by food that was contaminated with L_3 larvae from decayed, disrupted snail bodies or perhaps shed from infected snails (Graeff-Teixeira et al., 2009). Because humans are not natural definitive hosts, neither *Angiostrongylus* species is able to complete their life cycle; parasite eggs or larvae are not released into the feces and this makes diagnosis difficult. Although the parasites do not survive long in humans, infection causes uncomfortable symptoms including nausea, vomiting, neck

stiffness, fever and headaches. Occasionally, patients develop complications that lead to intestinal obstruction and perforation (*A. costaricensis*) or neurological damage (*A. cantonensis*) and even death.

Both *A. costaricensis* and *A. cantonensis* show low specificity for intermediate hosts; many different species of terrestrial and aquatic (fresh water) gastropods with natural *Angiostrongylus* infection have been observed around the world (Wallace and Rosen, 1969a; Malek and Cheng, 1974; Carvalho et al., 2012; Thiengo et al., 2010; Kim et al., 2014). The introduced snail species *A. fulica* and South American ampullariid *Pomacea canaliculata* (Lamarck, 1822) are the main intermediate hosts of *A. cantonensis* in the south of China (Lv et al., 2011), and an extensive list of molluscs transmits angiostrongyliasis on the Hawaian Islands (Kim et al., 2014). From Brazil, reports of natural intermediate host molluscs (Morassutti et al., 2014; Thiengo and Fernandez, 2016) include various terrestrial pulmonates (Stylommatophora): *Belocaulus willibaldoi* Ohlweiler, Mota and Gomes, 2009, *Bradybaena similaris* (Férussac, 1821), *Sarasinula linguaeformis* (Semper, 1885) (=*Sarasinula marginata* according to Thomé, 1989), *Subulina octona* (Bruguière, 1792) and *A. fulica*. Transmission of *A. cantonensis* was also reported for another ampullariid *Pomacea lineata* (Spix in Wagner, 1827), in northeast Brazil (Thiengo et al., 2010).

In the laboratory, the life cycle of both *A. cantonensis* and *A. costaricensis* has been maintained through experimental infection of *Biomphalaria glabrata* (Say, 1818) a freshwater pulmonate snail (Richards and Merritts, 1967; Tunholi-Alves et al., 2011; Bonfim et al., 2018). Taking in account the epidemiology of eosinophilic meningitis transmission, reports of *A. fulica* infected with *A. cantonensis* in 33 municipalities from 11 different Brazilian states (Thiengo and Fernandez, 2016; Cognato et al., 2016), and that the widely distributed *A. fulica* snails usually occur in dense populations which favors the contact of people with the parasite, *A. fulica* is considered the main vector for *Angiostrongylus*-associated eosinophilic meningitis in Brazil. Study of snail physiology, parasitology and immunity will likely provide inroads for improved or novel control strategies to extend the options to mediate the overall impact of invasive *A. fulica*

3 – Impact of nematode infection on *Achatina fulica* physiology.

Study of the physiology of infected snails revealed the burden of *A. cantonensis* infection upon *A. fulica*. Regulation of energy metabolism is essential for biological functions and the maintenance of life (Fraga et al., 2013). In pulmonate gastropods, including *A. fulica*, this regulation centers around availability of carbohydrates, deposited as glycogen and galactogen in special storage cells that are located in the mantle, among the digestive gland acini, the gonads, and also the muscular tissue of the cephalopedal mass (Joosse, 1988; Joosse and van Elk, 1986; Tunholi-Alves et al., 2014; Tunholi et al., 2013; Pinheiro and Amato, 1994). During physiological stress conditions, such as caused by parasite infection, the carbohydrate reserves are mobilized to make glucose available as substrate to oxidative metabolism for production of ATP. This involves a complex activation process, including glycogenolysis and components of oxidative respiration; glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (Tunholi-Alves et al., 2014; Massa et al., 2007; Bezerra et al., 1997).

Alterations in this carbohydrate metabolism have been recorded from infected snails using different snail host-larval parasite systems, and glycemia is generally reduced significantly (Becker, 1980; El-Ansary et al., 2000; Lima et al., 2016, 2017; Martins et al., 2018). Larval helminths absorb glucose from snail hemolymph as substrate for ATP generation in support of the parasite metabolism and the development of intramolluscan larvae (Becker, 1980).

Demonstration of glycolytic enzyme activities provided evidence that oxidative pathways exist in larval *A. cantonensis* (Shih and Chen, 1982). The competition by the parasite for nutrients causes a physiological state in the snail hosts that is similar to starvation (Pinheiro et al., 2009). Brockelman and Sithithavorn (1980) first described changes in the carbohydrate metabolism of *A. fulica* snails following experimental infection with *A. cantonensis*. Initially, the glucose content of the snail hemolymph was significantly reduced during the pre-patent infection, returning to normal values after one week. Tunholi-Alves et al., (2018) showed that reduction of glucose levels was associated with depletion of glycogen deposits in the digestive gland and cephalopedal mass of *A. cantonensis*-infected *A. fulica*. Along with similar observations from another parasitized snail species, *B. glabrata* harboring *A. cantonensis* (Tunholi-Alves et al., 2014), this demonstrates the existence of

homeostatic mechanisms that rely on mobilization of tissue stores of carbohydrate (glycogen and galactogen) to recover and maintain glycemia levels in snail hemolymph.

Parasite load (intensity of infection) influences the extent to which the oxidative metabolism of *A. fulica* is altered by *A. cantonensis*, as revealed by different degrees of impact on activities of hexokinase (HK) and lactate dehydrogenase (LDH), catalytic enzymes that are linked the oxidative metabolism of the snail host. The activity of HK, catalyst of the first step of glycolysis, increased significantly (relative to controls) when *A. fulica* was infected with $\geq$ 2,500 (L_1) *A. cantonensis* larva. Accompanied by reduced glucose levels, heightened HK activity reflects an increased energy (ATP) demand in the *A. fulica* host, warranted by the need to maintain basic vital processes in the presence of developing larval *A. cantonensis* (Tunholi-Alves et al., 2014). Depending on parasite load, *A. cantonensis* causes yet additional subsequent changes in the oxidative metabolism of *A. fulica.*

Snails infected with fewer than 10,000 L_1 larvae develop significant reductions of pyruvate and oxaloacetate. These changes may be due to either (I) accelerated redox reactions of the TCA cycle to support increased demand for ATP that challenge snail metabolism, causing a shortfall in the adequate supply of critical TCA intermediates, like oxaloacetate (substrate for the first reaction of the TCA cycle) or (II) the shunting of oxaloacetate away from the TCA cycle to drive gluconeogenesis for production of glucose using non-glycemic substrates in an attempt to restore normoglycemia (Tunholi-Alves et al., 2014). Furthermore, *A. fulica* snails that received high infective doses (10,000 and 15,000 L_1 larvae) of *A. cantonensis* (Tunholi-Alves et al., 2018) showed significant increases in activity of LDH (key enzyme for anaerobic respiration), along with lactate accumulation and decreased levels of pyruvate in the hemolymph, indicating a transition of the oxidative metabolism from aerobic to anaerobic during infection, similar to results obtained with chromatographic and spectrophotometric methods for *Biomphalaria* harboring *A. cantonensis* (Lima et al., 2016). The activation of anaerobic metabolism (LDH), enables continued production of energy (ATP), avoids inhibition of the glycolysis pathway by increased pyruvate levels, facilitates availability of NAD+ (oxidized electron carrier), and supports formation of intermediate metabolites that are needed for the survival of the host and for parasite development (Lima et al., 2016).

Ultimately, prolonged infection (especially with high parasite load), leads to depleted carbohydrate deposits that cause the snail metabolism to use alternative substrates as energy source, leading to breakdown of lipids (Tunholi-Alves et al., 2011a) and proteins (Tunholi-Alves et al., 2012). Direct absorption of amino acids by developing larval *A. cantonensis* may further reduce protein levels; higher infection loads led to increasingly lower protein concentrations in *A. fulica* (Tunholi-Alves et al., 2015). The carbon backbone of proteins supplies intermediate compounds for neoglucogenesis but continued protein degradation not only reduces protein concentration but also leads to elevated levels of nitrogen compounds, altering the chemical composition of snail host hemolymph (Pinheiro et al., 2009). The excretion as urea, uric acid or ammonia, of amino groups that result from deamination, challenge the water balance of *A. fulica*, a terrestrial gastropod (Tunholi-Alves et al., 2014). While specific data from *A. fulica* are not available, *Angiostrongylus* infections may reduce the lifespan of snail hosts (Wallace and Rosen, 1969c).

4 – Immunity of *Achatina fulica*

Despite considerable initial investigative attention from comparative immunologists, general aspects of the immune function in *A. fulica* are interpreted largely by considering findings from several other gastropod species, as presented in section 4.1. This is with the realization that some aspects of immune defense will be lineage-specific, however, the sharing of several immune features across animal evolution and the commonalities evident among immune defenses of phylogenetically diverse gastropods motivate reasonable confidence that this approach is generally informative. For example, a limited diversity of types of AMPs characterizes gastropods of both the Planorbidae and Physidae (sister families within the Hygrophila, Panpulmonata), and caenogastropods share particular categories of immune lectins with panpulmonate snails (Loker et al., 2004; Gorbushin and Borishova, 2015; Gorbushin, 2019; Schultz et al., 2018). Likewise, the available insights specifically regarding *A. fulica* immunity (4.2) include antimicrobial proteins found throughout the gastropod clade, as well as a capacity for hemolymph coagulation not found in hygrophilid panpulmonates. It is of note that immunological studies of *A. fulica* have employed mostly cell biology and biochemical-type analyses that did not yield a detailed characterization of the factors involved as is now

common in the "omics"-era. In light of resurging interest in *A. fulica*, also for the reasons provided in this review (Penagos-Tabares et al., 2018), such data may be anticipated in the near future.

4.1 – General consideration of gastropod host-pathogen immune interactions.

Historically, studies of gastropod immunity have largely focused toward immune interactions between aquatic vector snails and digenetic trematodes, such as the medically relevant schistosomes, with occasional consideration of other (microbial) pathogens (Adema et al., 2009; DeLeury et al., 2012; Loker and Bayne, 2018).

Snail immune systems, like those of other invertebrates (Murthy and Ram, 2015) do not include vertebrate-type acquired immunology but comprise highly effective innate immune mechanisms. Immune function can be selective, allowing mutualistic interactions between gastropods and microbiota; commensal communities of bacteria and fungi can be instrumental in adaptation and evolution by affecting multiple fitness parameters of the host (Soen, 2014), even viruses can modulate host-parasite interactions in invertebrates (Galinier et al., 2017). Normally, however, (opportunistic) pathogens are countered by potent internal defenses. Actual immune-elimination by snails of metazoan parasites such as digenetic trematodes depends on complex interactions between determinants of compatibility. First, snail immune receptors must be able to recognize the antigens of the invader in order to activate host defenses. Intra-individual polymorphisms of such receptors and antigens determine matched versus mismatched status of host and parasite phenotypes, resulting in either survival or immune-elimination of the parasite (Mitta et al., 2017). Secondly, host immune systems may be negated by anti-effector strategies of the parasite (Lie, 1982).

These interactions play out in a continuous arms race that drives evolution of (mechanisms that generate) diversity and polymorphism of the factors that determine the outcome of the host–parasite interplay (Combes, 2000; Gourbal et al., 2015). For instance, antigenic variation is a common strategy of eukaryote pathogens to evade a host immune response (Finlay and McFadden, 2006; Perrin et al., 2013).

Absent the vertebrate-specific rearranging genes that yield specific immune receptor mechanisms (Di Noia and Neuberger, 2007; Tonegawa, 1983), the remarkable effectiveness of invertebrate immune function is

underscored by the evolutionary success of invertebrates (Pinaud et al., 2016). Evidence has mounted that also invertebrates, and gastropods specifically, are capable of considerable diversification of innate-type immune receptors and possess forms of immune memory to effectively counter diverse pathogens (Zhang et al., 2004, Ghosh et al., 2010; Müller et al., 2017; Netea et al., 2011; Wang et al., 2013; Pinaud et al., 2016).

Snail immune defenses include the body surface as external physical barriers, chemically fortified by (mucus) secretions containing antimicrobial factors. Pathogens that penetrate through these defenses encounter lytic factors, antimicrobial proteins and peptides (AMPs) in tissues and hemolymph. The antimicrobial bacterial pathogen-associated molecular patterns (PAMPs; also referred to as microbe-associated molecular patterns: MAMPs) or damage-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs). The PRRs are highly diverse, comprising secreted molecules, cell surface- and cytoplasmic receptors (Schmitt et al., 2011; Escoubas et al., 2015). Among soluble receptors are Gram-negative bacteria-binding protein (GNBP), peptidoglycan recognition protein (PGRP) and a variety of lectins, including fibrinogen-related proteins (FREPs) (Escoubas et al., 2015, Gorbushin, 2019). Cell surface receptors like Toll-like receptors (TLRs) and scavengers receptors (SRs) are highly conserved evolutionarily (Peiser et al., 2002; Sarrais et al., 2004), just like cytoplasmic receptors from the RIG-I-like helicase (RLH) family and nucleotide-binding oligomerization domain (NOD)-like receptors (Sirrad et al., 2007; Yoneyama and Fujita, 2007; Barber, 2011). PPR-binding leads to coagulation, (direct) opsonization, triggering of proteolytic enzyme cascades that produce toxic compounds, or initiates intracellular signaling to activate immune defense cells to exert cytotoxicity (van der Knaap et al., 1983; Janeway, 1994; Lacchini et al., 2006, Hanington et al., 2010; Le Clec'h et al., 2016).

The cellular mediators of gastropod immunity are circulating macrophage-like phagocytic immune cells, named hemocytes, capable of production of cytolytic molecules including reactive intermediates of oxygen and nitrogen, as well as of phagocytosis and encapsulation of pathogens (Levine and Strand, 2002; Zanker, 2010). Hemocytes are the main effectors of the defense system but they are also involved in wound repair, nutrient transport, shell calcification, digestion and excretion processes (Cheng, 1996). Based on morphology, hemocytes are commonly classified into three main cell types: granulocytes, hyalinocytes and blast-like cells (Allam et al., 2015; Mahilim and Rajendran, 2008; Cavalcanti et al., 2012). Hemocytes bear yet

additional surface receptors like integrins, SRs, and C-type lectins (Liu et al., 2011; Humphries and Yoshino, 2003) and will internalize pathogens (bacteria, fungi, and experimental particles like latex beads) through phagocytosis, larger targets such as larval helminth are encapsulated by multiple hemocytes. Little is known about the molecular processes involved in encapsulation (Ray et al., 2013; Queiroga et al., 2013; Francisco et al., 2010; Laruelle et al., 2002; Mladineo et al., 2012; Batista et al., 2009; Kawasaki et al., 2013; Wootton et al., 2006; Wang et al., 2012).

Efficiency of cellular immune defense, and perhaps also the ability to effectively counter parasites may increase with the intrinsic number of circulating hemocytes (Larson et al., 2014). The number of hemocytes in hemolymph varies between and within species (Adema et al.,1992), because of age, infection and under influence of physiological and environmental factors (Livingston and de Zwann, 1983). For the actual killing of pathogens, hemocytes produce and release lysosomal enzymes, antimicrobial peptides, reactive species hydrogen peroxidase, ROS and RNS (Loker, 2010; Schimitt et al., 2011). Continued research may broaden the perspective of general immune defenses, for instance an ancient and evolutionarily conserved host defense mechanism was revealed by the recent finding that animal immune cells may deploy their DNA as extracellular traps (ETs) to capture pathogens (Poirier et al., 2014; Robb et al., 2014). Relatively little is known about anti-nematode immunity in gastropods, however, literature indicates that snail species do not indiscriminately support the development of any nematode species that they may encounter (Dróźdź et al., 1971), however, several species of nematodes attract but most often do not appear to be eliminated by snail host immunity (Bayne, 1983). Further investigations may help clarify to what extent immune function is a codeterminant of vector suitability of a snail like *A. fulica* for *A. cantonensis.*

4.2 – Immune features of *Achatina fulica*.

The following aspects of immunity have been shown specifically from *A. fulica.* Included are immune features that are distributed across a wide phylogenetic range of gastropods, like achacin representing a category

312 of conserved antimicrobial proteins and that are more restricted like ability for haemolymph coagulation, that
313 has not been observed from e.g. aquatic panpulmonate gastropods.

314 **4.2.1 – Shell encapsulation of nematodes.**

315 Exploration of the use of nematode infection as control measure against snail pests revealed that
316 *A. fulica* was remarkably resistant to the parasitic nematode *Phasmarhabditis hermaphrodita*, a lethal
317 pathogen that is effectively against several other gastropod species. Starting at 3 days post exposure,
318 inspection of experimental snails evidenced an immune response that involved the *A. fulica* shell.
319 Infective *P. hermaphrodita* larvae are encapsulated, killed and remain permanently encased in the shell
320 (Williams and Rae, 2015; 2016). This immune mechanism is effected by cells on the inner layer of the
321 shell that adhere to the nematode cuticle, spread over the worm body to then fuse and encase the
322 nematode to the inside of the shell. Remarkably, DNA extracted from shell-encased dead worms allows
323 for PCR and sequence-based identification of the infecting nematodes. Follow-up investigation with
324 experimentally exposed and field collected snails showed that this novel anti-nematode immune
325 mechanism is shared by snail species of both achatinoid and non-achatinoid clades of the
326 Stylommatophora (Rae, 2017).
327

328 **4.2.2 – Achacin**

329 In 1985, Kubota et al., isolated a protein with an antibacterial activity, composed of 70-80kDa
330 subunits from the body surface mucus of *A. fulica*. Further characterization of this protein designated as
331 Achacin yielded the first cDNA sequence from *A. fulica* ever deposited in GenBank (Obara et al., 1992).
332 Achacin (purified or recombinantly expressed) inhibited growth of Gram-negative and Gram-positive
333 bacteria, and it was shown to attack the surface membrane of bacteria but not directly cause lysis bacterial
334 cells (Otsuka-Fuchino et al., 1992; 1993; Ogawa et al., 1999). The mode of antibacterial action of
335 Achacinin was first indicated by similarities in amino acid sequence with *l*-amino acid oxidases (LAAOs)

from toxins in snake venoms and experimentally demonstrated to result from oxidative deamination of amino acids, producing ketoacids, ammonia (NH_3) and hydrogen peroxide (H_2O_2). Additional to generating toxic metabolites, also depletion of amino acids leading to reduction of available nutrients was proposed to contribute to the microbicidal activities of Achacin (Ehara et al., 2002). It is of note that Achacin presents a landmark discovery of comparative immunology of gastropods and molluscs. Starting with a report of Aplysianin from *Aplysia kurodai* (sea hare) an euopisthobranch gastropoda (Kamiya et al., 1986; Takamatsu et al., 1995), many additional LAAOs been recovered from several gastropod and other molluscs. Phylogenetic analyses distinguish molluscan LAAOs as a major subfamily separate from other animal LAAOs, such independent evolution indicates an important reliance of molluscs on LAOOs for innate immune function (Hughes, 2010; Suwannapan et al., 2018).

4.2.3 – Mytimacin and other cytoloytic peptides from mucus.

The body surface mucus of *A. fulica* contains multiple components with lytic activity additional to Achacin. Zhong et al., (2013) purified Mytimacin-AF, a cysteine-rich antimicrobial peptide (AMP) from the macin family. The cDNA sequence contains an open reading frame that encodes for an 80 amino acid-long peptide that has antimicrobial activity against fungi, Gram-negative and Gram-positive bacteria, particularly *Staphylococcus aureus*. A search for potential anticancer peptides motivated a broad survey of novel lytic factors present in *A. fulica* mucus. Bioinformatics analyses of mass spectrometry data obtained from two HPLC-fractions of mucus that displayed lytic activity toward *in vitro* cultured primate epithelial cells and (human) cancer cells, predicted another 16 lytic peptides *in silico* (E-Kobon et al., 2015). The lack of sequence similarity of predicted lytic peptides with existing entries in public sequence databases, suggest that these mucus-derived lytic peptides have not been encountered previously, but was also considered to reflect the limited availability of sequence data from mollusc, especially the stylommatophoran land snails, especially (E-Kobon et al., 2015).

4.2.4 – Lectins

Considering current insights into the lectin diversity among gastropods (e.g Gorbushin and Borushova, 2015), it was not surprising that initial searches for agglutinins as mediators of non-self recognition in *A. fulica* led to identification of several (mucus and) hemolymph lectins, each with different carbohydrate specificity (Basu et al., 1986; Mitra and Sarkar, 1988; Sarkar et al., 1984; Mitra et al., 1988). Relative levels of lectin-mediated hemagglutinating activity increase with age of *A. fulica* snails (Baskakov et al., 2000), indicating a maturation of immune function from juvenile to adult *A. fulica.* Befitting the technological capabilities at the time of these pioneering studies, these lectins were identified only by carbohydrate specificity and the molecular weight of biochemically purified (subunits of) proteins, sequence characterization has remained pending, to date. Functional aspects, however, have been studied in greater detail for two lectins.

The first is an abundant hemolymph lectin of *A. fulica*, designated as C-reactive protein (CRP) based on cross-reactivity with antisera to CRP protein of the horse shoe crab *Limulus polyphemus* (Agrawal et al., 1990) and specific binding of phosphoryl choline, a trait shared with CRPs (members of the pentraxins), innate immune proteins from both invertebrate and vertebrate animals, (Mandal et al., 1991). The aminoacid content was determined and *A. fulica* CRP (a multimeric protein of 400kDa) was found to be heavily glycosylated (30-40% carbohydate), but no nuclueotide sequence is currently available. Agglutinating activity was enhanced in the presence of calcium-ions (Mandal et al., 1991). The CRP of *A. fulica* is multifunctional, with bacteriostatic activity for Gram (-) bacteria and bactericidal activity for Gram (+) bacteria, likely through ROS production and induction of apoptosis, and it may serve to protect the snail against toxicity from heavy metals (Mukherjee et al., 2014; Bose and Bhattacharya, 2000).

Secondly, research has also focused on Achatinin (Basu et al., 1986). This (9-*O*-AcSA)-specific hemolymph lectin (242 kDa, subunits of 15kDa) is expressed in the albumen gland (Sen et al., 1992) and in hemocytes (Biswas et al., 2000), binds sialic acid-epitopes on LPS in a calcium-dependent manner and is bacteriostatic toward Gram-negative *Escherichia coli.* Remarkably, Achatinin is also multifunctional,

386 additional to antimicrobial activity, LPS binding of this PRR also activates other aspects of *A. fulica*
387 immunity as is described in the next section.

388 **4.2.5 – LPS-mediated coagulation.**

389 Bacterial LPS activates a defense response in *A. fulica* hemocytes that involves the release of
390 several interactive proteins into the hemolymph that orchestrate a coagulation reaction; gel formation to
391 aggregate and sequester bacteria. This response is highly reminiscent of well-characterized endotoxin-
392 dependent plasma coagulation from the horseshoe crab *Limulus polyphemus*, resulting from an enzyme
393 cascade that employs zymogens and serine proteases released by amboecytes that degranuate in response
394 to detection of LPS.

395 The coagulation reaction of *A. fulica* can be affected *in vitro* with the protein lysate of snail
396 hemocytes (Biswas and Mandel, 1999). The name of this preparation, "Achatina amboecyte lysate
397 (AAL)" may seem less intuitive because it employs the alternative designation of amoebocyte for
398 hemocyte. Protein biochemical analysis implicated Achatinin as major, important component of AAL.
399 Depletion of Achatinin reduced the coagulation activity of AAL by 77%. Additional to lectin function
400 (LPS binding), Achatinin also has enzymatic serine protease activity that is critical for the coagulation, as
401 shown by sensitivity of this reaction to protease inhibitors (Biswas et al., 2000).

402 Another protein component, endotoxin-senstive factor (ESF), was purified from AAL using
403 heparin affinity chromatography. ESF differs from Achatinin by a native molecular weight of 140 kDa
404 (consisting of two non-covalenty bound 70 kDa subunits), and although both of these immune factors
405 bind LPS, ESF is activated by alpha-chymotrypsin instead of endotoxin. Again, the use of specific
406 inhibitors showed that active ESF functions a serine protease (Biswas et al., 2000). It is of note that the
407 endotoxin-dependent coagulation reaction of *A.fulica* may be unique to Achatinoid gastropods (e.g.
408 *Archachatina marginata*, Salawu et al. 2011) or to (larger taxonomic subsets of) the Stylommatophora; it
409 has not been reported fresh water pulmonate snails (Hygophila) like the well studied *B. glabrata* a main
410 model for gastropod immunity. Along with characterization of all AAL factors and the mechanism of the

protein cascade needed for coagulation, the phylogenetic distribution of this immate immune response merits further investigation.

4.2.5 – Hemocytes

The hemolymph of *A. fulica* can easily be sampled by heart puncture, a procedure that can be survived by the snail. Microscopical observation delineated two categories of cell types among glass-adherent hemocytes; granulocytes and agranulocytes (Adema et al., 1992), generally corresponding with hemocyte types recognized in other gastropods (Yoshino, 1976; Barraco et al., 1993). Karuthapandi (2010) revealed that the total hemocyte count of active *A. fulica* snails was higher than that of the snails that aestivated. The granulocytes of *A. fulica* sub categorized into granulocyte I, granulocyte II and granulocyte III. Only few round small cells with high nucleus to cytoplasm ratio were observed. Overall, agranulocytes and granulocytes were proportioned at an 80%/20% ratio in the hemolymph of *A. fulica*, without evidence of sesonal variation. Differential hemocyte counts revealed only modest variation in the percentage of agranulocytes, granulocyte I and granulocyte III of active versus aestivated snails, whereas the percentage of granulocyte II was higher in aestivated *A. fulica* relative to active snails (Karuthapandi, 2010), perhaps due to the difference in metabolic state, especially food uptake.

Basic immune functions of *A. fulica* hemocytes as snail defense cells were demonstrated *in vitro* by phagocytosis of zymosan particles and production of ROS (Adema et al., 1992). More recently, an *in vitro* co-culturing assay that was designed to investigate the cellular immune interactions of stylommatophoran snails with *A. cantonensis* shows that *A. fulica* hemocytes attach to nematode larvae and proceed to hemocyte clusters around the parasites (Penagos-Tabares et al., 2018). The same study and work from Lange et al. (2017) also observed that hemocytes from *A. fulica*, as well as *Arion lusitanicus* and *Limax maximus* (gastropod species commonly referred to as slugs) deploy DNA-rich extracellular aggregates refered to as invertebrate extracellular phagocyte traps (InEPTs) similar to the phagocyte-derived extracellular traps (ETs) described from vertebrates catching larvae and decreasing their motility. It was proposed that hemocyte InEPTosis immobilizes nematodes to hamper migration of the larvae through the tegument or body and further facilitate exposure of the entrapped

parasites to cell-mediated cytotoxicity of additional hemocytes. The long-term fate of the parasites remains to be determined.

5 – *Achatina fulica,* entry into the omics era.

As described above, study of *A. fulica* has not only provided some of the earliest insights into general aspects of gastropod immunity but also continues to contribute to a more comprehensive overview of innate immunity in invertebrates. A continued reliance, mostly on cellular and biochemical research approaches has provided a steady yet rather slow progress in characterization of immune function of *A. fulica*, and of stylommatophoran snails as a larger, important group within the Gastropoda. The lack of specific sequence data to define the factors that enable the fascinating immune processes recorded from *A. fulica* hinders comparative approaches that can reveal the phylogenetic distribution of unique and shared defense capabilities across gastropods. Increasingly, routine and accessible next generation sequencing (NGS) technologies are now available to explore the genome sequences and transcriptomes from non-model organisms. While it remains challenging to derive high quality assemblies, extensive genome sequence and RNAseq data can be analyzed using computational bioinformatics for (potential) immune genes to indicate presence of immune mechanisms within an organism relative to the phylogenetic position. The validity of this approach (Dheilly et al., 2014, Schultz and Adema, 2017) is underscored by a broader view of immune function in a specific gastropod like *B. glabrata* (Adema et al., 2017) and the modeling of shared lectin-based complement-like system that serves immunity across the Mollusca and other protostome invertebrates (Gorbushin, 2019). Molecular level approaches have more usually been applied for population biology (e.g. Ayyagari and Sreerama, 2017; Fontanilla et al., 2014), but recently, the genome of *A. fulica* has become accessible with the publication of both the 15,057bp mitochondrial genome (He et al., 2016) and the nuclear genome (Guo et al., 2019) the first to represent the stylommatophoran gastropods. The genome (estimated at 2.12 Gb) of a single *A. fulica* snail from the Guangxi region of China was sequenced to yield a publicly available high quality, chromosomal-level genome assembly of 1.85 Gb with a contig N50 length of 726 kb. The predicton of 23,726 protein-coding genes will be of great value to facilitate proteomics level studies. It is anticipated here that this significant data

resource will lead to sequence characterization of immune factors previously identified from *A. fulica* through biochemical parameters only, like Achatinin, ESF and others, also providing provide a wide-ranging insight into related and additional immune genes in this snail species.

6 – Conclusions and Future Prospects

By phylogenetic identity alone, *A. fulica* merits investigative attention due to the potential as representative of a major branch of the Stylommatophora, to inform broadly on gastropod immune function. Already, *A. fulica* research has indicated both unique- and fundamental, evolutionary conserved aspects of snail immunity. The great biological and evolutionary diversity within the class Gastropoda of the phylum Mollusca makes it highly probable that particular clades of snails have evolved unique immune features. Importantly, broadening studies beyond the main focus on a very small segment of gastropod diversity indicated the possibility of differences in the elaboration of immune function between gastropod families. Schultz et al., 2017 observed that *Physella acuta* (Physidae, sister family of the Planorbidae) snails did not equally employ FREPs, a category of PPRs that is instrumental for coordinating immune elimination of schistosome parasites in the planorbid snail *B. glabrata*, vector of schistosomiasis (Hanington et al., 2012).

Renewed interests to study *A. fulica* are driven by the recognition of multiple negative biological properties that render this snail a highly effective invasive species that is recalcitrant toward eradication, a pest that can destroy (crop)vegetation and outcompete native species, and a potential vector that may transmit several parasites including *A. cantonensis* causative nematode pathogen for eosinophilic meningitis, an emerging infectious disease in humans. Elucidation of the underlying traits that determine the range if negative biological impact opens possibilities to develop strategies for control of *A. fulica*, or to mitigate, modify, or at least predict the distribution of the snail and associated parasite transmission.

A modest but strong tradition of biochemically centered investigations provides a several entry points for more detailed characterization of *A. fulica* immunity, with great potential to benefit from the recently available genome sequence data. We thus strongly encourage further immunological studies of the invasive nature of *A. fulica*, and interaction of *A. fulica* with *A. cantonensis*. In the process such studies will yield exciing

new insights also in comparative immunology of terrestrial snails to reveal evolutionary patterns in the evolution of immune function of Gastropoda.

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Highlights

- The giant African snail *Achatina fulica*, the most widely distributed invasive pest land snail, will likely disperse further with climate change.
- *Achatina fulica* is intermediate host for the nematode *Angiostrongylus cantonensis* that causes eosinophilic meningitis.
- Study of metabolic and immunological aspects of this parasite host provide better understanding of epidemiology and inform on comparative immunology of gastropods