Developmental and Comparative Immunology 104 (2020) 103557

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Biomphalaria glabrata immunity: Post-genome advances

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A B S T R A C T

The freshwater snail, *Biomphalaria glabrata*, is an important intermediate host in the life cycle for the human parasite *Schistosoma mansoni*, the causative agent of schistosomiasis. Current treatment and prevention strategies have not led to a significant decrease in disease transmission. However, the genome of *B. glabrata* was recently sequenced to provide additional resources to further our understanding of snail biology. This review presents an overview of recently published, post-genome studies related to the topic of snail immunity. Many of these reports expand on findings originated from the genome characterization. These novel studies include a complementary gene linkage map, analysis of the genome of the *B. glabrata* embryonic (Bge) cell line, as well as transcriptomic and proteomic studies looking at snail-parasite interactions and innate immune memory responses towards schistosomes. Also included are biochemical investigations on snail pheromones, neuropeptides, and attractants, as well as studies investigating the frontiers of molluscan epigenetics and cell signaling were also included. Findings support the current hypotheses on snail-parasite strain compatibility, and that snail host resistance to schistosome infection is dependent not only on genetics and expression, but on the ability to form multimeric molecular complexes in a timely and tissue-specific manner. The relevance of cell immunity is reinforced, while the importance of humoral factors, especially for secondary infections, is supported. Overall, these studies reflect an improved understanding on the diversity, specificity, and complexity of molluscan immune systems.

1. Introduction

1.1. Snails and their importance

Snails (phylum Mollusca, class Gastropoda) are a diverse group of molluscs that remains understudied despite their important roles in community ecosystems (reviewed in Wallace and Webster, 1996; Dillon, 2000; Sturrock, 2001; Gutiérrez et al., 2003), and disease transmission (Bayne and Loker, 2018). Snails are of medical, veterinary, and economic importance as they transmit diseases that affect many animals for instance birds, reptiles, amphibians, and mammals including humans (Loker and Mkoji, 2005). Notably, around the turn of the 20th century, freshwater snails were recognized as key players in the transmission of schistosomiasis, an important chronic parasitic disease of humans caused by trematode infections in tropical and subtropical regions around the world (reviewed in Sturrock, 2001; Miyairi and Suzuki, 1914; Leiper and Atkinson, 1915; Cook, 2007). Since then, these gastropods have been included among other medically relevant invertebrates such as mosquitos and ticks, as essential factors for control of parasitic diseases that affect humans.

The World Health Organization (WHO) acknowledges that snails serve as intermediary hosts in the parasitic zoonoses: schistosomiasis, trematodiasis, and angiostrongyliasis, 3 of 18 neglected tropical diseases (NTDs) (WHO, 2019). Snails are considered a target and integral component in long-term measures for controlling transmission of these aforementioned diseases. Despite challenges and transient setbacks in the efforts to control NTDs, progress has been made (Touré et al., 2008; Savioli et al., 2015). In recent years these endeavors have been re-energized worldwide, in part catalyzed by the London Declaration of 2012 and the World Health Organization's 2020 Roadmap that aim at, beyond controlling morbidity, the elimination of NTDs including schistosomiasis (Wang et al., 2012a; WHO, 2012). Special interest is given to snail-related research, specifically, studies focused on the immune system of vector snails and the role invertebrate immunity plays in defense against schistosome parasites. One goal of continuing the characterization of the snail immune system (e.g. of *Biomphalaria glabrata*) is to find biological targets that may lead to the development of complementary strategies to block or prevent schistosome infection in snails to prevent parasite transmission to the human host. Such investigations are anticipated to further advance the discovery of determinants of host susceptibility (permissive to infection and transmission) or resistance (refractory to infection, no transmission) to the parasite that can later be selected (Mendelian genetics) or genetically modified to reduce or prevent snail-to-human transmission.

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https://doi.org/10.1016/j.dci.2019.103557
Received 3 August 2019; Received in revised form 11 November 2019; Accepted 16 November 2019
Available online 21 November 2019

Furthermore, a better understanding of snails' genetics and physiology can also reveal unique characteristics in these organisms toward the identification of new targets for biological snail control in the field, or the development of novel chemicals that may be used as targeted molluscicides.

Thanks to advances in molecular and analytical tools, we are at a point where abundant genomic, transcriptomic, proteomic, and metabolomic data are available that provide information related to the immunobiome of invertebrates and disease-vectors, including gastropod snails such as those that transmit schistosomiasis (Hanelt et al., 2008; Adema et al., 2010; Ittiprasert et al., 2010; Lockyer et al., 2012; Zahoor et al., 2014; Buddenborg et al., 2017). The recent publication of the genome of the snail *Biomphalaria glabrata* (Adema et al., 2017), added a much-needed level of overarching genetic resources that have, and will continue to improve our understanding of the mechanistic basis of gastropod immunity by building further on the knowledge generated by previous studies. This is reflected by several recent post-genome studies that expand and advance insights into the diversity and function of *B. glabrata*'s immunity, most with special emphasis on the snail response to larval schistosome parasites and their secreted products. Considering that many recent investigations focus on a specific snail tissue or the interaction with either miracidia or sporocysts, specific schistosome developmental stages, this review is organized according to these host-parasite interfaces. First we describe an overview of the intramolluscan schistosome larval stages and their specific association with snail intermediate hosts. Subsequent sections discuss novel scientific contributions and insights to snail immunity and how these associate with specific host-parasite interfaces. The final section identifies several current challenges and areas of research that may advance our understanding of snail-schistosome immune interactions.

2. *Biomphalaria glabrata*: A molluscan model

The three most important freshwater snails transmitting schistosome parasites to humans are gastropods in the families Planorbidae and Pomatiopsidae, with subclasses Pulmonata and Prosobranchiata respectively. *Schistosoma mansoni* and *Schistosoma haematobium* are transmitted by multiple species of *Biomphalaria* and by *Bulinus* snails respectively; while *Schistosoma japonicum* utilizes *Oncomelania* as vector snails (reviewed in Adema and Loker, 2015). In this review, we will focus on the most studied snail vector model, *Biomphalaria glabrata* that transmits *S. mansoni* in South America, the Caribbean, and in some regions of North Africa.

Globally, nine species of *Biomphalaria* have been found naturally infected with *S. mansoni*: *Biomphalaria glabrata* (Say, 1818), *Biomphalaria pfeifferi* (Krauss, 1848), *Biomphalaria alexandrina* (Engels et al., 2002), *Biomphalaria sudanica* (Martens, 1870), *Biomphalaria tenagophila* (Orbigny, 1835), *Biomphalaria straminea* (Dunker, 1848), *Biomphalaria choanomphala* (Martens, 1879), *Biomphalaria prona* (Martens, 1873), and *Biomphalaria camerunensis* (Boettger, 1941) (Carvalho et al., 2008; Caldeira et al., 2016). However, many other *Biomphalaria* species are susceptible to infection if experimentally exposed in the laboratory. Of all these species, research emphasis has historically been placed on *B. glabrata* as a laboratory research model to study snail-trematode interactions, culminating in selection of a Brazilian strain of this species to study the genome of *B. glabrata* (BB02) as intermediate host of *S. mansoni* (Adema et al., 2017).

3. Schistosomiasis

3.1. Disease transmission, prevention, treatment, and control

Schistosomiasis is a chronic parasitic disease that has been reported in 78 countries around the world (WHO, 2019), with more than 220.8 million people requiring preventive treatment in 2017, and 770 million people at risk of infection (Steinmann et al., 2006; Hotez et al., 2014; WHO, 2017WHO, 2019). In 2016, the global burden of schistosomiasis was estimated at over 1.9 million disability adjusted life years (DALYs) (GBD, 2016 DALYs and HALE Colley et al., 2017). Until recently, the WHO's morbidity reduction and eradication strategies relied heavily on chemotherapy administration as a preventive and therapeutic measure (WHO, 2002; Wilson et al., 2008). For many years control has entailed the use of just a single drug, praziquantel, the only drug effective against the adult stage of all *Schistosoma* species, although resistance has been described in the laboratory (Fallon et al., 1996, 1997; William et al., 2001; Botros et al., 2005) and suspected in the field (Ismail et al., 1999; Melman et al., 2009; Wang et al., 2012b). The low efficacy of drug-centered strategies stresses the need to incorporate complementary approaches for disease control, including implementation of sanitary and hygiene measures, and intermediate host suppression (Engels et al., 2002; Colley et al., 2017; Toor et al., 2018). In addition to the risk of spreading or shifting schistosomiasis to new areas through human migration (Boissier et al., 2015; Kincaid-Smith et al., 2017), there is the impending threat that climate change may alter the distribution and natural susceptibility of vector snails (Stensgaard et al., 2019). Vertebrate definite hosts are infected through contact with contaminated water that contains cercariae of *Schistosoma* parasites (e; Fig. 1). Several key aspects contribute to the reduced efficacy of human-based interventions to control schistosomiasis infections, including that a vaccine is not yet available, and that treatment with drugs does not prevent (rapid) reinfection, especially when life-sustaining activities of endemic populations challenge avoidance of contaminated waters. Thus, populations at risk are usually exposed to multiple infections throughout their lives causing long-term and permanent developmental and physiological problems, additional to chronic abdominal pain, diarrhea, anemia and malnutrition as prominent pathologies (King et al., 2015). With transmission driven not only by biological but also by ecological, sociocultural, economic, and political factors the complex nature of this disease require multifaceted strategies must be adopted to control, and eventually eradicate schistosomiasis (Krauth et al., 2019).

In the past, several methods to control snail populations in the field have been implemented with various degrees of success (reviewed in Sokolow et al., 2016). However, efficiency of such methods was highly dependent on the specific locality and environment, making their implementation on a global scale challenging (Clennon et al., 2006; Gurarie and Seto, 2009; Mari et al., 2017; Gurarie et al., 2018). These strategies included the use of chemical and biological molluscicides, snail competitors/predators, and environmental management (McCullough et al., 1980; McCullough, 1981; Pieri and Thomas, 1987; Lardans and Dissous, 1998; Mkoji et al., 1999; King et al., 2015). Similar to other vector organisms of human disease (Wang and Jacobs-Lorena, 2013; Kyrou et al., 2018), snails are considered as target for genetic modification to enhance or improve natural traits by which they resist infection or prevent the intramolluscan development of schistosome parasites. In addition, it is expected that novel transgenic techniques such as CRISPR-Cas9 will be valuable in functional genomic studies of important immune candidate genes as the methods continue to be adapted to molluscan systems (Abe and Kuroda, 2019; McVeigh and Maule, 2019). An obvious group of potential targets for genetic modification are immune-related genes and the molecules associated with expression and modulation of such genes. Thus, it is critical to understand the snail response and immunity to the parasite.

3.2. Snails as obligate intermediate hosts

A characteristic of schistosome parasite life cycles is the involvement of two animal hosts; in addition to the vertebrate definitive host, freshwater snails are required as (obligate) intermediate host. The snail species suitable as host varies depending on the schistosome species, usually with a strict parasite-host compatibility at the level of species and strain. The development of *S. mansoni* in the intermediate host *B.*

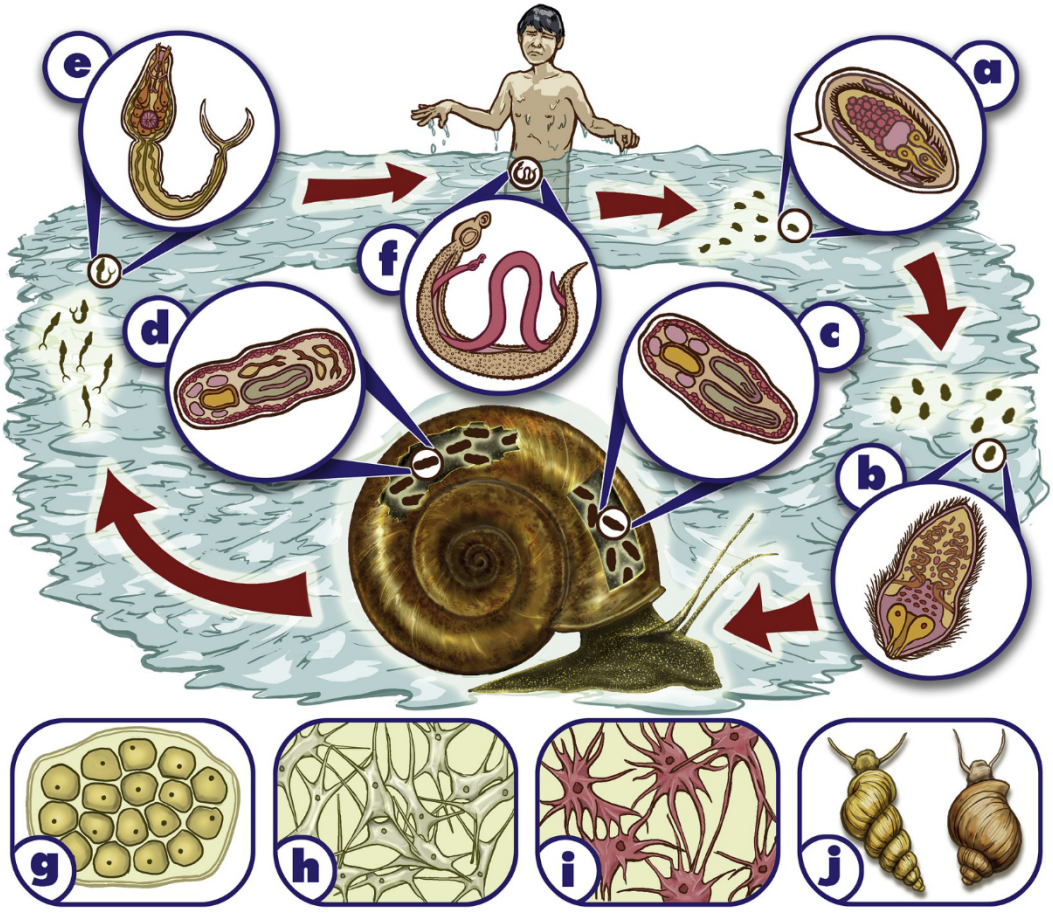

Fig. 1. *Schistosoma mansoni* intramolluscan larval stages in *Biomphalaria glabrata* and biological tools used in host-parasite studies. The life cycle of the trematode *Schistosoma mansoni* uses the planorbid snail *Biomphalaria glabrata* as an obligatory intermediate host. Infection of snails is initiated when **eggs (a)** produced by adult *S. mansoni* worms found in the human host are released into the environment. Upon contact with fresh water, the eggs hatch into **miracidia (b)**. The miracidium is a free-living and motile larval stage of the parasite that after locating a snail host, it will penetrate its exposed epithelia. Inside snail tissues, miracidia transform into the **primary (mother) sporocyst (c)**, the first intramolluscan larval stage. After 24–48 h post-infection, primary sporocysts start migrating to other snail tissues, preferentially the digestive gland. In these internal tissues, germinal cells within primary sporocysts develop into **secondary (daughter) sporocysts (d),** which on their own will produce more sporocysts. Alternatively, secondary sporocysts will also produce **cercariae (e)**, the last intramolluscan larval stage, that once matured will break out of snail tissues and swim in the aquatic environment in search of a new vertebrate host. Cercariae can penetrate the skin of humans and start a new cycle of infection when **adult worms (f)** develop, pair and start producing eggs. Studies in snail immunity utilize a variety of biological tools including various developmental stages of snails including **embryos and juveniles (g),** the ***B. glabrata* embryonic (Bge) cell line (h)**, cellular components such as **hemocytes (i)**, and other snail species for example ***Bulinus* spp. and *Oncomelania spp.* (j)**.

glabrata (described below) is characteristic for the complex intramolluscan biology and intimate, long term interactions with the snail host of various schistosome species.

With excreta, vertebrate hosts release fully embryonated **eggs** into aquatic habitats of snails (a; Fig. 1), where the changes in osmolarity, light, and temperature (15–30 °C) stimulate the hatching of ciliated **miracidia** (Upatham, 1972) (b; Fig. 1), the schistosome larval stage that is infective to the snail intermediate host. **Miracidia** are free-living, highly motile swimmers due to multiple rows of ciliated plates that cover their body surface. Miracidia locate snail hosts by employing chemical signals that are as yet not well understood (see section 5.3, Wang et al., 2019). After attaching with the apical papilla by suction or adhesion to snail surface epithelia (usually on the exposed headfoot area; reviewed in Whittington and Cribb, 2001; LoVerde, 1975), miracidia penetrate into the snail soft tissue, aided by secretions from the penetration glands and possibly the primordial gut (Kinoti, 1971; Pan, 1980). One of these secretory components may be *S. mansoni* venom allergen-like 9 (SmVAL9), proposed to also aid in egg release from the mammalian host, infection of the snail host, and intra-molluscan migration and development (Yoshino et al., 2014). Upon penetration into snail tissues, a **miracidium** sheds its ciliated plates exposing a tegument with microvilli (Basch and DiConza, 1974; Bayne et al., 1980). At this stage, the parasite has transformed in to a **primary sporocyst** and will remain close to the infection site for at least the next 48 h (c; Fig. 1). This developmental stage of the parasite is considered the main target of the snail's immune response (see Snail immunity, section 4). The surface of primary sporocysts is covered by a continuous tegument or syncytium (Smith and Chernin, 1974) that is about 0.5 μm thick and contains multiple vesicles (Basch and DiConza, 1974). The schistosome syncytium is thought to be an important antigenic target and a challenge to the host's immune system, as it changes in composition throughout the life stages of the parasite (Simpson et al., 1984; Robijn et al., 2005; Braschi et al., 2006). If the **primary sporocyst** is in a compatible snail host that permits development, the larval parasite will survive and grow. In this regards, a recent study identified a sporocyst secreted metalloprotease, *S. mansoni* leishmanolysin (Hambrook et al., 2018), that interferes with snail hemocyte migration, and thus encapsulation of sporocysts. *In vivo* studies describe that primary sporocysts actively migrate inside host tissues, possibly following open sinuses towards the mantle, leading to concentrating parasites in the snail hepatopancreas (digestive gland) (Jourdane and Théron, 1987). At this time, primary sporocysts contain multiple **secondary**

Table 1
Functionally characterized immune molecules in *Biomphalaria glabrata*.

Molecules	Tissue Location	Type/Function	Target	Reference
Biomphalysin	Hemocytes, Bge cells, secretions	Opsonin/Porin?	Sporocysts	Galinier et al. (2013)
Cu/Zn SOD	Hemocytes cytoplasm	ROS Enzyme	Sporocyst	Goodall et al. (2004), 2006; Bender et al. (2007)
FREP2	Hemocytes, Bge cells, plasma	PRR, Opsonization	Miracidia, LTPs	Adema et al. (1997); Pinaud et al. (2016)
FREP3	Hemocytes, plasma	PRR, Opsonization	Sporocysts, LTPs, bacteria, fungi	Hanington et al. (2010b); Pinaud et al. (2016)
FREP4	Hemocytes, plasma	PRR, Opsonization	Sporocysts	Adema et al. (1997); Zhang et al. (2008); Moné et al. (2010); Pinaud et al. (2016)
Galectin	Hemocytes	Opsonization	Sporocysts	Yoshino et al. (2008)
Granulin	Unknown	Growth Factor	Hemocytes	Pila et al. (2016b)
Grctm6	Hemocytes	PRR	Sporocysts	Allan et al. (2017)
Hydrogen peroxide	Hemocytes secretions	ROS/Oxidative damage	Sporocysts	Adema et al., 1997; Hahn et al., 2000
LAPD2	CNS	Regulator/Hormone?	Miracidia	Wang et al. (2016)
MIF	Hemocytes, Bge & other cells, secretions	Cytokine/Proliferation, Encapsulation	LTPs, Sporocysts	Baeza Garcia et al., 2010
Nitric Oxide	Hemocytes secretions	NOS/Oxidative damage	Sporocysts	Hahn et al., 2001b
TEP1	Secretions, hemolymph	Opsonin/PRR	LTPs, SmPoMucs	Moné et al. (2010)
TLR	Hemocytes	PRR	Sporocyst	Pila et al. (2016a)

Note: To be classified as "characterized", listed molecules have been reported to: (1) have complete CDS available (predicted genes are excluded), and (2) a direct role in defense or resistance to parasite has been demonstrated for example, through gene knockdown, or direct activity/binding to *S. mansoni* parasites or secreted/released products (ESPs/LTPs).

(also called **daughter**) **sporocysts** (d; Fig. 1). As described by Smith and Chernin (1974), 6–13 days after infecting the snail host, a primary sporocyst is "a sack filled with daughter sporocysts in various developmental stages". **Daughter sporocysts** originate from germinal cells and mature in about 8–10 days with observed densities of 20–25 per primary sporocyst (Hansen, 1975). Mature secondary sporocysts are released from the mother sporocyst and migrate towards the snail ovotestis, while germinal cells inside the daughter sporocysts produce additional generations of (secondary) sporocysts. Alternatively, numerous **cercariae** (the last intramolluscan larval stage) will develop inside a proportion of these secondary sporocysts (d, e; Fig. 1). Cercarial output per miracidium ranges from hundreds to thousands depending on the schistosome species, but is also influenced by the snail age, size, nutrition status, other parasite coinfections, and the level of snail-schistosome compatibility (reviewed in Sturrock, 2001). **Cercariae** are the infective stage for the (vertebrate) definite host (e; Fig. 1). Cercariae exit the secondary sporocyst and migrate towards the snail's surface epithelia and emerge from the snail host into their aquatic habitat and swim in search of a mammalian host to continue the transmission cycle. Cercariae are small (~150 μm in length), almost invisible to the naked eye (Salter et al., 2000). Infection occurs by means of skin penetration, a rapid (few minutes) process that is not detected by the definitive host. Initial adhesion to and penetration of human skin is aided physically via the cercarial oral sucker, and chemically by adhesive secretions and proteolytic enzymes from the pre-acetabular and post-acetabular glands of the parasite (reviewed in Whittington and Cribb, 2001; Haas et al., 1997a,b). During skin penetration of the vertebrate host, a cercaria loses its tail and transforms to a schistosomulum, the last and only larval stage of the parasite in the definite host. After about 72–96 h the schistosomula leave the epidermis and migrate through the vascular and lymphatic systems to reach the liver where they mature (Wilson, 1987). In 4–5 weeks, male and female adult worms pair up (f; Fig. 1) and using their ventral suckers migrate via the portal system to their final residency, the mesenteric vessels. Once there, adult worms remain paired for the rest of their lives while producing eggs that will be released in the environment for the life cycle to continue.

4. Snail immunity

Starting at the moment a *Schistosoma* miracidium penetrates the surface epithelium of a compatible snail, signals released from damaged tissue and parasite-derived molecules (released or secreted) are detected by the host. If the snail is from a resistant strain, these signals will initiate an immune response to prevent the parasite from establishing and continuing development to sporocyst. This is not true for susceptible snails, where the response is somewhat inadequate and the parasite finds a favorable environment to grow and develop. Technically, it has been difficult to study snail host-parasite *in situ* interactions, due mainly to the small size of the parasites, their non-homogenous distribution in snail tissues, and the variability among individual snails in terms of resistance and susceptibility to infection. Recognizing the pivotal role in the transmission of the disease to humans, many of the longstanding studies of schistosomiasis have focused on understanding the physiology and biology of the snail. Research focusing on the snail immune response has taken multiple approaches to elucidate what defines host suitability for schistosome development. Recent transcriptome studies are examples of investigations of systemic reactions in whole snail tissues (Lockyer et al., 2007, 2008; Hanington et al., 2010a; Dheilly et al., 2014; Buddenborg et al., 2017; Mansour et al., 2017; Portet et al., 2017). Following discovery of the importance of hemocytes (Cheng, 1975) (i; Fig. 1) in the snail antiparasitic response (Harris, 1975; Harris and Cheng, 1975; Bayne et al., 1980; Loker et al., 1982), these cells have been the focus of much research, as well as the soluble components found in the plasma (blood fluid) that hemocytes are bathed in, and the tissues associated with hemocyte proliferation such as the amebocyte producing organ (Joky et al., 1985; Sullivan and Spence, 1994; Sullivan et al., 1995). A different approach to study snail immunity has been to examine tissues relevant to specific stages of schistosome infection, such as the headfoot region, which is penetrated during early infection by primary sporocysts (c; Fig. 1), or the hepatopancreas region where secondary sporocysts develop (d; Fig. 1). In addition to *in vivo* studies, *in vitro* experiments using snail secretions, hemolymph, or the *B. glabrata* embryonic (Bge) cell line (h; Fig. 1) in co-culture with parasite larval stages have also provided important insights into the molecular aspects of host-parasite interactions. Finally, studies on the interactions of schistosomes with other snail developmental stages (embryos, juveniles; g; Fig. 1) and snail species (*Bulinus spp.*, *Oncomelania spp.*) (j; Fig. 1) are also extremely valuable. Table 1 lists a summary of molecules for which direct activity has been assessed and linked to the *B. glabrata* immune response to *S. mansoni*. Many more molecules have been associated with the response or resistance to schistosomes, especially through transcriptome and proteomic studies, however, the specific functions of these molecules in defense require further characterization. There is still much to learn about the mechanisms and molecular pathways of *B. glabrata* anti-schistosome

responses, and about the complexities of species and strain compatibilities that are observed in the field. Several recent reviews provide comprehensive cover of the major aspects of cellular and humoral immunity in snails (Bayne, 2009; Yoshino and Coustau, 2011; Knight et al., 2014; Adema and Loker, 2015; Coustau et al., 2015; Pila et al., 2017; Famakinde, 2018; Loker and Bayne, 2018; Schultz et al., 2018). In the present review we focus on the advances in snail immunology and related topics that have been made since the publication of the *B. glabrata* genome (Adema et al., 2017). In addition, two sections include new insights in relation to immune receptors, cytokines, and signaling pathways that were only made possible through the analysis of genomic data. Finally, and with a look toward the future, we discuss areas of scientific exploration that likely will advance our understanding of snail immune processes and that may be utilized in the pursuit of eradicating schistosomiasis. Whenever possible, information is organized in reference to the snail-schistosome interfaces defined by the parasite developmental stages (miracidia, sporocysts, and cercariae).

As invertebrates, the *B. glabrata*'s defense system consists only of innate immune components, with representatives of the major common groups of innate immune molecules. These include pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and lipopolysaccharide-binding proteins and bactericidal/permeability-increasing proteins (LBPs/BPIs) that recognize and bind to pathogen-associated molecular patterns (PAMPs). Snails also possess soluble immune molecules such as antimicrobial peptides, lectins, and complement-related molecules as part of their humoral response capabilities (see Table 1). Some of these immune constituents are expressed in a generalized (non-specific) manner in response to immune challenge (Coustau et al., 2015). Others are more pathogen specific, as reported in transcriptome analyses (Hanelt et al., 2008; Adema et al., 2010 and Deleury et al., 2012) and with the expression of selective molecules such as fibrinogen-related proteins (FREPs) (Zhang et al., 2008). A prominent aspect of the detectable response in snail tissues during a primary *S. mansoni* infection is contributed by the hemocytes (Bender et al., 2005; reviewed in Loker, 2010). These defense cells circulate in the hemolymph and are found throughout snail tissues. Hemocytes migrate to sites where schistosome sporocysts are located, bind to the sporocysts' tegument and form a multi-layered cellular capsule around these parasites (Loker et al., 1982). Hemocytes within these encapsulating layers secrete toxic compounds, mainly reactive oxygen species (ROS) that will eventually kill the invading parasite (Hahn et al., 2001a). Direct hemocyte-mediated cytotoxicity is not the only means to killing *S. mansoni* larvae; in resistant snails, some primary sporocysts are killed without any evidence of a cellular encapsulation or the presence of hemocytes in the surrounding areas. This suggests that humoral components can also contribute effective anti-schistosome reactions (Galinier et al., 2013). In addition, recent publications report the protective characteristics of humoral components and their involvement in antiparasitic responses during secondary infections (see section 5.4.3).

5. Post-genome advances

5.1. B. glabrata genome

The *B. glabrata* genome (Adema et al., 2017) plus accompanying analyses and datasets represent valuable resources for a variety of research topics including host-parasite interactions, invertebrate biology, and innate immunity. Furthermore, it also represents a good source of information for those interested in other molluscan and invertebrate organisms for which transcriptome data exists but no genomic resources are currently available. In addition, for *Biomphalaria*, access to the genome data offers an opportunity to study gene families for which only transcriptomic and proteomic data was previously available, and the prospect to correlate previous experimental observations with genetic data. This report offered quantitative information regarding the specifics of *B. glabrata* genomics that can be used in comparative analyses with other molluscs. For example, comparative analyses revealed a relatively small number of genes coding for antimicrobial peptides in *B. glabrata* compared to other invertebrates, and *B. glabrata* has uncommon biosynthetic pathways lacking several enzymes that are commonly used in invertebrates to make steroids from cholesterol. The genome publication also highlights several areas that could be explored to aid in the efforts to control snail populations in the field. To illustrate, the presence of the *Capsaspora* snail symbiont was confirmed (Hertel et al., 2002), and a previously unknown mycoplasma endosymbiont was discovered; both symbionts could potentially be used as vehicles for introducing genetic modifications to the snail host. In addition, genes involved in biomineralization, detoxification mechanisms, and snail chemosensory attraction, were identified as potential targets to be used in developing snail control methods.

The innate immune system of *B. glabrata* was a key topic in the genome report. Important to note is that many of the stress- and immune-related molecules previously reported to be important for the snail, particularly in response to schistosome parasites, were not present as single coding genes, but instead were members of multigene families. This important information may in part explain the previous difficulties in determining differences in gene expression between resistant and susceptible strains of *B. glabrata*, especially if gene identification was based on short or shared sequences. The genomic data now offers the opportunity to investigate whether the differentially expressed gene variants are associated with resistance traits. This is not a new idea, as previous research has demonstrated the complexity of *B. glabrata's* immune response exemplified with the fibrinogen related proteins (FREPs) (Gordy et al., 2015). This diverse family of proteins is coded by about 20 genes that can be somatically mutated differently in each individual snail (Zhang and Loker, 2004). Another molecule that has raised interest is biomphalysin, a putative pore-forming protein associated with innate immune memory (Pinaud et al., 2016, 2019). Sequencing of the genome revealed that these proteins are actually encoded by a family of 21 different genes. It would be useful to determine if all of these gene products share similar functions, or if expression of unique genes or alleles associates with specific tissues and immune responses to various pathogens. Interestingly, the *B. glabrata* genome also contains multigene families of pattern recognition receptors such as TLRs, PGRPs, and LBPs/BPIs; with 56 genes (27 coding complete proteins), eight, and five respectively. Furthermore, the genome analysis confirmed the presence of genes important for stress responses, such as heat shock proteins (HSPs), and proteins associated with production of reactive oxygen and nitrogen species (ROS/NOS). The snail genome also revealed a role for epigenetic regulation, harboring enzymes involved in chromatin modification, including methyltransferases, demethylases, deacetylases and acetyltransferases, as well as transcript regulation by microRNAs. Exploration of cytokines and signaling pathways involved in cellular communication and activation resulted in finding homologs of IL-17, TNF, and components of the NF-κB pathway. This is just the “tip of the iceberg”, and an example of yet additional data that can be extracted from the genome; sections 5.5.4 and 5.7 below present new insights into the diversity and signaling associated with thioester-containing proteins and cytokines, and their potential transcription binding sites.

The post-genomic publications reviewed here present a genome for the Bge cells (Wheeler et al., 2018), proteomic studies on parasite-host interactions using *in vitro* assays (Dinguirard et al., 2018; Wu et al., 2017), innate memory and antiparasitic responses (Gourbal et al., 2018; Portet et al., 2018; Pinaud et al., 2019), the genetic basis of resistance (Tennessen et al., 2017; Allan et al., 2017; Allan et al., 2018a, b), snail pheromones and neuropeptides (Pila et al., 2017; Wang et al., 2019), attractants for miracidia (Wang et al., 2019), and epigenetics (Geyer et al., 2017; Queiroz et al., 2017).

5.2. Bge cell line genome

The availability of an immortal cell line from *B. glabrata*, established by Hansen (1976), has always been considered a useful tool to investigate the molecular and genomic aspects of snail biology (Coustau and Yoshino, 2000; Yoshino et al., 2013b). The Bge cell line was instrumental for the development of an *in vitro* culture system for schistosome and echinostome larval stages (DiConza and Basch, 1974; Basch and DiConza, 1974; Yoshino and Laursen, 1995; Yoshino and Laursen, 1995; Coustau et al., 1997; Ataev et al., 1998; Bixler et al., 2001), and for the study of cell-to-cell interactions between snails and parasites (Yoshino et al., 1999; Vermeire and Yoshino, 2007; Humphries and Yoshino, 2006; Castillo et al., 2007; Geyer et al., 2017; Wright et al., 2017). The Bge cell line originated from a trypsinized 4-5-day-old embryo of an albino strain *B. glabrata* that was susceptible to *S. mansoni* infection (Hansen, 1976). Although the precise cell type that gave rise to Bge cells is not known, the morphological and behavioral characteristics are very similar to those of snail hemocytes, and as a result Bge cells have been extensively used to study host-parasite interactions and encapsulation reactions. In recent years, interest in Bge cells has grown because of potential uses as a tool in the development of gene-modifying protocols such as gene editing via CRISPR, or transcript control modification with miRNA (see Epigenetics section 5.6).

As a resource for the use of the Bge cell line, Wheeler et al. (2018) sequenced the genome of one of the three Bge cell line subcultures available (Bge3). This effort addressed several key questions to answer were: how similar/dissimilar are the genomes of these Bge cells compared to the whole organism (B. glabrata)? And, can research findings from Bge cells be easily translated with confidence to the *in vivo* system? Such concerns deserve significant consideration, especially in light of previously report of extensive mutations and aneuploidy in two other Bge subcultures (Odoemelam et al., 2009). Wheeler et al. (2018) reported mapping 98.6% of the Bge3 cell genome reads to the *B. glabrata* reference genome and confirmed aneuploidy in Bge cells by determining that the read coverage varied among the 18 linkage groups previously published by Tennessen et al. (2017). The genomic differences between Bge3 and the reference genome were further evident from analysis that identified 10,031,395 single nucleotide variants (SNPs) that after filtering, were determined to affect 3,277 transcripts. These gene products contained "high impact" variants due to mutations with a high theoretical possibility of affecting protein structure and/or function, that is, mutations predicted to occur in exon/intron splice regions, or that would result in the gain or loss of a start/stop codon. These Gene ontology (GO) analysis showed that the majority of the affected transcripts were in categories involved in binding activity (cell-substrate or cell-matrix), and many were cytoskeletal components. It was concluded that some of these mutations are characteristic of immortalized cell lines, likely resulting from selection pressures that favor the capacity to adhere to a substrate during culture conditions. Verification by PCR and Sanger sequencing confirmed mutations in 88% of 151 *in-silico* predicted transcript variants. Data from the variant analysis are accessible through VectorBase (https://www.vectorbase.org) in support of research employing the Bge cell line.

The above findings suggest that many years of active culture and passaging of Bge cells in laboratories have resulted in mixed, non-clonal cell populations showing considerable aneuploidy from one another, giving rise to the need to verify sequences of interest before engaging in experimental procedures. The original cell line had a diploid chromosome number of 36, as snails do (Hansen, 1976; Bayne et al., 1978), while the population of Bge3 cells studied had an average of 62 chromosomes, similar to a previous study that showed two other Bge subcultures to be a mixture of cells with 63 and 67 chromosomes each (Odoemelam et al., 2009).

Regardless, the Bge cell line is still the only molluscan immortal cell line; its ease of use and maintenance makes it a valuable resource and tool that is considered in the development and design of experimental procedures difficult to perform on whole snails, especially gene editing and transgenics.

5.3. Attractants and pheromones: Miracidia interface

It remains a basic question as to how *S. mansoni* miracidia locate snail hosts in the water. The parasite is thought to employ chemosensory receptors to follow bioactive molecules released or secreted by their invertebrate hosts. Previous reports have shown that miracidia swim following a chemical gradient from snail secretions (Kalbe et al., 1997), including those of *S. mansoni* (Chernin, 1970; Roberts et al., 1979, 1980). If the chemicals responsible for attracting newly hatched miracidia were identified, then miracidial migration could be possibly manipulated to prevent infection of new snail hosts. Recently Wang et al. (2019) followed this notion, with the development of an assay to observe and measure the movements of *S. mansoni* miracidia in an aqueous environment to test and record the effects of whole and fractionated snail secretions, or synthetic peptides on miracidial swimming behavior (velocity, angularity, and tortuosity). Mass spectrometry allowed the identification of a single fraction from snail secretions containing multiple peptides that attracted miracidia. Within that fraction, one peptide designated P12, was responsible for the activity. P12 was identified as a 13 amino acid long peptide containing alpha-helix secondary structure and derived from a larger, water soluble precursor. The precursor was found in various tissues including the central nervous system. This study is the first to specifically identify a short compound of snail origin that attracts *S. mansoni*. Previous studies have only identified small chemical molecules (Macinnis, 1965) or a mixture of snail mucus glycoconjugates (Haberl and Haas, 1992; Kalbe et al., 1996; Haberl and Haas, 1992). Wang et al. (2019) suggest that P12 peptides may be used to lure miracidia away from snails in natural environments or to identify the receptor in the parasite in order to block it and reduce snail infections.

An alternative approach to control transmission involves removing snails from areas where people and other animals risk exposure to parasites. This may be effected by using an attractant for the snail host. An example of such candidate attractant is a recently reported temptin-like protein produced by *B. glabrata* snails (Pila et al., 2017). Temptin was originally identified as a pheromone released by *Aplysia californica* (Sea Hare, Euopistobranchiae, Cummins et al., 2004, 2005) along with other proteins during mating aggregations (Cummins and Bowie, 2012). Based on a partial EST with homology to temptin (Adema et al., 2006), Pila et al. (2017) a complete coding sequence from the *B. glabrata* genome was obtained and subsequently used as a recombinantly-expressed protein in maze experiments to quantitatively test attractiveness of temptin. Results showed that *B. glabrata* snails move towards temptin in a specific and concentration-dependent manner. The authors indicated that it remains to be tested whether snails infected with *S. mansoni* respond in a similar manner to this pheromone, or if infection changes the behavior of the snail.

5.4. Sporocyst interface

The following section summarize new findings from either the use of sporocyst stages or that focused on interaction between snail host and the sporocyst stage.

5.4.1. Guadaloupe resistance complex

Tennessen et al. (2015a) selected laboratory resistant and susceptible snails out of a field population from the island of Guadaloupe. After 10 generations of in-breeding, they identified a region of the *B. glabrata* genome associated with snail resistance to *S. mansoni* infection containing 15 genes. This genomic region was named the Guadeloupe Resistance Complex (GRC), with three phenotype forms: one resistant and two susceptible. Additionally, the resistant haplotype was dominant. The 15 genes in the GRC had no known homologs, but seven

genes contained domains characteristic of transmembrane proteins, and therefore could serve as cell surface receptors. Interestingly, there was no significant difference in expression of GCR-genes between resistant and susceptible individuals when exposed to the parasite. This led the authors to note that the correlation between the GRC genes and snail phenotype (resistant or susceptible) was not due to different gene expression levels, but instead most likely due to the inherent amino acid divergence of the products from the various alleles. Tennessen et al. (2015a) described the natural *B. glabrata* population in Guadaloupe to be naturally resistant at a level of 50–60%; after selection, this resistance increased to about 80–90% in homozygous RR snails, while homozygous susceptible snails were infected at a rate of about 80% (Tennessen et al., 2015b; Allan et al., 2018). Most of the protein divergence among phenotypes was located on the extracellular domains of the putative transmembrane proteins. The authors hypothesized that these proteins could recognize PAMPs such as *S. mansoni* surface glycoproteins (SmPoMucs) (Roger et al., 2008a, b) in a similar fashion to FREPs (Adema et al., 1997; Moné et al., 2010; Mitta et al., 2012), and that resistant snails have more efficient receptors than susceptible individuals.

Subsequent studies further characterized two of the GRC transmembrane-coding genes (*grctm5* and *grctm6*). These two genes deserved focus because comparison of resistant and susceptible alleles showed non-synonymous amino acid substitutions (Allan et al., 2017). *grctm5* encoded for a fibrinogen type-III protein similar to chitinase, while *grctm6* encoded a single-pass transmembrane protein with structural similarity to TLRs and Fc receptors. The Grctm6 transcript was found in all tissues tested but expression was more abundant in the headfoot area. However, the GRC story is complicated; one of the susceptible GRC-genotypes was found to have about two-times higher constitutive expression levels of *grctm6* than the resistant phenotype. This further support the notion that it is more plausible that resistance correlates with the actual amino acid differences among GRC alleles and the function of the proteins *in vivo*. At protein level, Grctm6 could only be detected in whole hemolymph, but not in hemocytes or cell-free plasma alone. Furthermore, the immune role of Grctm6 was confirmed when RNA interference knocking down led to an increased the cercarial release, by 3–4 times in resistant snails challenged with *S. mansoni* miracidia when Grctm6 protein was low (day three post RNAi-treatment). This led to the conclusion that Grctm6 may control the number of miracidia infecting a snail, or alternatively, is part of the mechanism that regulates parasite development into cercariae and/or their release. Once genetic manipulation is available, genes in the GRC such as *grctm6*, could be manipulated in *B. glabrata* field populations to reduce host suitability and susceptibility to schistosome infection. Finally, Allan et al. (2017) noted that not only is the study of the gene products associated with the GRC valuable, but that identification of the antigenic targets of these snail receptors could be used to make the parasites easier to recognize and more susceptible to attack by snail defense responses.

Two additional studies furthered the analyses of the GRC phenotypes (Allan et al., 2018a, b). To test the effect of the genes in the GRC during the early stages of infection, snails with resistant or susceptible haplotypes were exposed to *S. mansoni*. Histological studies disclosed no difference in the composition or structure of the headfoot's surface epithelia among snails, nor in the number of invading miracidia, or the number of developing sporocysts within the first 24–48 h. However, snails carrying the susceptible alleles had more unencapsulated sporocysts after 24 h exposure than snails with the resistant haplotypes. In addition, resistance could be transferred to snails with susceptible GRC genotypes by injecting whole hemolymph from resistant snails. Furthermore, this resistance was not transferable when injecting cell-free plasma, suggesting the importance of hemocytes in the early stages of antiparasitic response. These results differ from other studies that report the transfer of resistance to susceptible snails through cell free hemolymph (Pereira et al., 2008; Pinaud et al., 2016). An explanation for such different results could be that genes not coded in the GRC can confer resistance to snails, also Pereira et al. (2008) used different species and strains of snails (*B. tenagophila*) and parasites, and the Pinaud group (2016) was testing the immune properties of plasma on snails with secondary *S. mansoni* infections. To investigate the possible differences in cytotoxic capabilities among snails from various GRC haplotypes, Allan and Blouin (2018b) tested the release of hydrogen peroxide (H_2O_2) by snail hemocytes after exposure to various PAMPs. Hemocytes from the three GRC haplotypes demonstrated similar responses to 6 of the 8 PAMPs used. There was no difference in hemocyte capacity to produce H_2O_2, this indicates that the cellular machinery to produce ROS in a defensive manner is not affected by the genetic variance in GRC alleles. Only one of the susceptible GRC haplotypes had ~60% reduction in H_2O_2 production compared to the other two genotypes when exposed to BSA-galactose. Based on these results, it was concluded that one or more of the GRC products act as lectin-like receptors with a binding specificity for galactose-containing glycoconjugates. Because none of the GRC genes was directly linked to the recognition of galactose, an alternative explanation could be that a different gene (not in the GRC) is responsible for the lectin activity, or that one of the genes in the GRC may be working in conjunction with another gene not accounted for in the genotype testing.

In a separate but related publication (Tennessen et al., 2017), a new linkage map for the *B. glabrata* genome was generated using targeted capture methods. This work was performed in order to reduce the number of more than 300,000 scaffolds in the original genome assembly (Adema et al., 2017). The updated approach mapped ~75% of the genes in the *B. glabrata* genome to 18,613 scaffolds. Eighteen of these scaffolds were large enough (> 10 Mb) to theoretically represent the snail haploid chromosomes. As an example of the accuracy of the new linkage map, three genes (*actin*, *ferritin*, and *hsp70*) that were previously localized in different chromosomes (Adema et al., 2017), were assigned to separate linkage groups. This linkage map was used for associating gene loci of interest with nearby genes and control regions. With this strategy, it was confirmed that genes known to be important for the response to schistosome parasites are organized into what could be considered immunity loci or clusters. For instance, the newly discovered GRC was localized in cluster IX along with the previously identified genes for *sod1*, *prx4*, *cat*, and biomphalysin (Blouin et al., 2013; Tennessen et al., 2015b), as well as with genes for a TLR and spondin-1 (Mitta et al., 2005; Pila et al., 2016a).

5.4.2. Proteomics

Soluble snail proteins are thought to be important in the recognition and killing of sporocysts. Nonetheless, the molecules that are specifically involved in binding to the parasite surface, and how this binding contributes to effective defense, remain to be fully characterized. Facilitated by the genome sequence data, proteomics approaches were utilized to help answer these queries by investigating the interaction between *S. mansoni* sporocysts and plasma components of *B. glabrata*; Wu et al. (2017) used BS90 and NMRI snail strains, while Tetreau et al. (2017) used BRE *B. glabrata*. Both studies employed *in vitro*-transformed *S. mansoni* sporocysts for mass spectrometry analysis after incubation with *B. glabrata* plasma (cell-free hemolymph) from unexposed snails. Proteins identified in plasma from unexposed snails comprised the constitutively expressed *B. glabrata* plasma-proteome. Any snail proteins found bound to sporocysts represented components that could be associating with the parasite during the first hours of infection *in vivo*. These two studies corroborated each other in revealing similar cohorts of snail proteins that bind to sporocysts, including: biomphalysin, dermatopontin, collagen, hemoglobin, acetylcholine binding protein, a-disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and alpha amylase (Tetreau et al., 2017; Wu et al., 2017). However, the results of the two studies also contrasted; for instance, Tetreau et al. (2017) did not detect the binding to sporocysts of plasma lectins, such as FREPs. In contrast, Wu et al. (2017) identified the

presence of FREPs, CREPS, and GREPs proteins associated with sporocysts. Perhaps the lack of lectin binding in Tetreau et al.'s study is due to methodological differences. Furthermore, Wu et al. (2017) utilized two different snail strains, allowing the comparison of the plasma of binding properties of resistant (BS90) and susceptible (NMRI) snails. Interestingly, the latter study showed that multiple immune factors are constitutively expressed in both strains prior to infection: LPS-BPI, TEP, CD109, HSP 70 and 60, biomphalysin, dermatopontin, FREPs, CREPs and GREPs. Between the two strains, however, protein expression patterns clearly change following parasite exposure, in agreement with previous reports of the differential expression of some of these proteins (Hertel et al., 2005; Hanington et al., 2010b; Portet et al., 2018). Although the plasma of NMRI and BS90 snails shared the presence of several sporocyst-binding proteins, strain specific differences were also apparent. ADAMTS binding to sporocysts was detected from the NMRI plasma only. In addition, a new GREP was identified with different isoforms in BS90 (GREP1.1) and NMRI (GREP1.2). Interestingly, GREP1.1 expression was detected in all 10 BS90 snails whereas GREP1.2 transcripts were detected in just 4 of 10 NMRI snails. It is possible that these strain-specific differences contribute to the resistant and susceptible phenotypes of BS90 and NMRI snails, respectively.

Many of the plasma proteins that bind to *S. mansoni* sporocysts as recorded by proteomics analyses have previously been linked with the *B. glabrata* immune system (Cu/Zn SOD1: Goodall et al., 2004, 2006; Bender et al., 2007; FREPs: Hanington et al., 2010b; HSP70, 90: Zahoor et al., 2010; MIF: Baeza-Garcia et al., 2010; Biomphalysin: Galinier et al., 2013; TLR: Pila et al., 2016a; Granulin: Pila et al., 2016b; TEP: Portet et al., 2018). However proteomics also identified sporocyst-binding proteins that are not typically associated with immunity and defense, such as actin, collagen, hemoglobin, GAPDH, apolipophorin, and histone 4. The independent corroboration suggests that these unexpected plasma protein-sporocyst interactions are specific, and the functional significance of such interactions warrants further examination. Future research into the binding of such "non-immune" proteins may indeed indicate a yet undescribed role in immunity. To illustrate, recent research suggests that apolipophorin in the greater wax moth, *Galleria mellonella* (Whitten et al., 2004) and histone 4 can function in immune responses in various organisms (reviewed in Hoeksema et al., 2016).

The interaction between plasma and sporocysts is followed by hemocyte-sporocyst interactions, including hemocyte defense effector functions. A proteomic profiling of hemocytes of BS90 and NMRI snails, when encapsulating *S. mansoni* sporocysts *in vitro* (Dinguirard et al., 2018) provides insight into such hemocytes-sporocysts interactions within the first 24 h of infection and/or encapsulation. In accordance with previous gene expression studies, different proteomic profiles were evident from NMRI and BS90 hemocytes. Compared to the BS90 hemocyte encapsulation, there was a greater number of down-regulated proteins in the NMRI hemocyte encapsulation, and many of these proteins were associated with protein synthesis or trafficking, metabolism, cell signaling, and redox reactions. In addition, some down-regulated proteins may play a role in defense such as those reported for LPS-BPI, superoxide dismutase, and apolipophorin. Interestingly, just a few proteins in encapsulating NMRI hemocytes were substantially increased in abundance, such as IRAK, argonaute 18, arginase 1 and bacterial permeability protein (BPI). Of these, IRAK, argonaute 18 and BPI may function in defense. However, arginase can compete for arginine, the substrate required for NO synthesis, so increased arginase may lead to a decrease in NO production and therefore weaken the immune response. NO was previously found to be directly involved in the cytotoxic killing of sporocysts in resistant (13-16-R1) snails (Hahn et al., 2001b). In contrast to NMRI (susceptible) hemocytes, BS90 hemocytes encapsulating sporocysts demonstrated increased expression of protein synthesis machinery constituents and anti-apoptotic factors (compared to control BS90 hemocytes). Likewise, factors associated with immune defense, such as those involved in ROS production (dual oxidase), autophagy and the proteasome, were increased in encapsulating hemocytes from BS90 (Dinguirard et al., 2018).

Cumulatively, these studies highlight the down regulation and the up regulation of immune responses in susceptible and resistant *B. glabrata* strains, respectively. However, despite numerous comparative studies, the factor(s) determining the contrasting gene expression changes in resistant and susceptible strains remain unclear, as do their origin (host or parasite) and the mechanism(s) that cause such modulations. One possibility is that differences in the plasma lectin profile between snail strains is responsible. One can imagine a scenario where parasite products with the ability to interfere with snail immune responses are mopped up in a complex comprising lectins, and other associating molecules (TEPs, etc.) in resistant snails, and therefore down regulation does not occur. Whereas in susceptible snails, the specific lectins required to neutralize these parasite molecules are absent, are expressed at a low level, or different alleles of the lectins are expressed that are not as efficient as the resistant forms. Furthermore, the signaling pathways and transcription factors that facilitate the strain-specific changes have yet to be identified as well as the possible influence of these entities on resistance to infection.

5.4.3. Immune memory

Immune memory was previously considered an exclusive characteristic of the adaptive immune system. That paradigm, however, is shifting as evidence suggests that in both invertebrates and vertebrates, the innate immune system may produce an enhanced secondary response to a pathogen (Gourbal et al., 2018). The first indication of *B. glabrata* induced resistance to *S mansoni* was reported over 30 years ago by exposing snails to irradiated miracidia (Lie et al., 1983); and the first report of immune memory to *S. mansoni* infection was reported 20 years ago, where schistosome-susceptible *B. glabrata* infected with a single miracidium, demonstrated resistance to reinfection two weeks following the initial infection (Sire et al., 1998). Furthermore, the second infection elicited a humoral response and there was no evidence of cellular encapsulation (Sire et al., 1998). A more recent study has likewise reported immune memory in *B. glabrata*; 10 days following a primary infection, snails were resistant to additional (secondary) infection by the same strain of *S. mansoni* (Portela et al., 2013). It was demonstrated that the initial immune response is primarily cellular (hemocyte-associated), whereas the second or memory immune response is humoral (Pinaud et al., 2016). This last molecular-level study implicated FREPs 2, 3 and 4 in the generation of immune memory. In addition, the pore-forming factor biomphalysin was identified as a component of the humoral, second response (Pinaud et al., 2016). More recently, Pinaud et al. (2019) addressed invertebrate adaptive immune responses by using a variety of tools and techniques (transcriptome, proteomics, snail "vaccination", and *in vitro* sporocyst-toxicity assays) to identify the molecules and possible mechanism responsible for the observed immunological memory, with special emphasis on the differences associated diverse parasite genotypes. Major conclusions from this study are: (i) *B. glabrata*'s immune memory to the parasite is strain-specific and long lasting (for the life span of a snail); (ii) Of the molecules that were associated with immune memory, some were modulated irrespective of the parasite strain used in the immune challenge, and included several transcript isoforms; (iii) The response to immune challenge was stronger (greater abundance) and more efficient (protective) when the parasite strain used as immune challenge was more genetically related (homologous) to that of the initial infection; (iv) The three main types of immune molecules represented in the immune memory response were: immune receptors, immune effectors, and immune modulators/activators. The most abundant protein found in the components of the hemolymph responsible for immune memory was biomphalysin, but also present were C-type lectins, FREPs, galectins, LBP/BPI, as well as proteins characteristic of the extracellular matrix; (v) Vaccination experiments showed that the primary sporocyst stage is the most immunogenic to snails, but is not equivalent to a natural

infection, and that vaccination efficacy also depends on the genetic background of the parasite used in the challenge; (vi) Cell-free plasma from infected snails also provided some protection (up to ~50%), but only if taken from the donor at one day after challenge and used to protect against homologous parasite infections. In addition to providing protection against challenge infection, this plasma was also effective in killing homologous strains of parasites in *in vitro* assays.

Considered together, these studies support early observations that suggested a molecular mechanism for innate immune memory in invertebrates like snails. In addition, the protective effect of plasma is observed when the challenge parasite is genetically identical or similar to that of the initial infection. As expected, the humoral response included a complex mixture of proteins with a variety of isoforms (or alleles) being expressed, and that these variations depended on the parasite strain used as challenge. The authors suggest that rather then one or a few molecules being responsible for the observed immune protection, a variety of proteins is required and these may need to be used in a specific manner sequentially, or simultaneously to form complex multimers to enable efficient recognition of the pathogen challenge and respond appropriately. Still, there are many more aspects yet to be investigated in relation to snails immune memory; for example, does immune memory offer any advantage to snails that already are carrying an infection? does innate memory provide any fitness to the snail host or their offspring? what is the mechanism involved in immune memory production? are hemocytes major player in this process as has been found in other invertebrates (Tassetto et al., 2017)? As a final note, the parasite does not necessarily have a passive role, rather there is the likely possibility that some of the differences and lack of complete protection in vaccination experiments and plasma injections are due to molecules secreted by the schistosomes (Lodes and Yoshino, 1990; Guillou et al., 2007; Yoshino et al., 2014; Nowacki et al., 2015) that prevent or block recognition, similar to those operating in antigenic or molecular mimicry (Yoshino and Bayne, 1983; Damian, 1997; Yoshino et al., 2013a).

5.4.4. Thioester proteins

Among the newly recognized snail immune molecules that interact with *S. mansoni* sporocysts is the thioester-containing protein (BgTEP), for which continued attention provided an update (Portet et al., 2018) on a previously reported study (Moné et al., 2010). This molecule was originally identified in 2008 by Yoshino and coworkers (GenBank: FJ480411.1) and later characterized by Moné et al. (2010). BgTEP is a plasma protein from snails that forms a heterocomplex with FREPs and interacts with *S. mansoni* sporocysts polymorphic surface mucins (SmPoMucs). Moné et al. (2010) used antibodies against sporocyst mucins to co-immunoprecipitate several snail proteins for identification with mass spectrophotometry. Among these proteins were several FREPs (2, 12, and 13), a galectin, cell adhesion proteins (peroxinctin-like protein and Dec-1/Matrilin-like protein), a protease inhibitor cystatin-like molecule, a homolog to the pore-forming toxin aerolysin, and AIF (allograph inflammatory factor, a pro-inflammatory protein). The detection of FREPs was in line with previous reports that these are important players in immune recognition against schistosomes (Adema et al., 1997; Zhang and Loker, 2004; Zhang et al., 2004a; 2008; Hanington et al., 2010b). FREPs are highly diversified molecules that show expression variations of single genes even among individuals of the same snail strain (Zhang and Loker, 2004), and the authors speculated that FREPs are a logical host immune component to recognize and bind to the polymorphic components found on parasite mucins (Roger et al., 2008a, b), resembling the capacity of antibodies in vertebrate immune systems to recognize highly diverse antigenic epitopes. It is of note however, that Moné et al. (2010) found that sporocyst mucins were bound by a complex that included two snail plasma proteins, FREP2 and a fragment of BgTEP, suggesting a possible link between immune recognition and immune activation.

Thioester-containing proteins (TEPs) are well recognized as part of the battery of pattern recognition molecules that are employed by invertebrates to differentiate and defend against potential pathogens. TEPs are involved in innate immunity, and originally were defined as containing a characteristic thioester domain (TED) (Dodds and Law, 1998) that contains a β-cysteinyl-γ-glutamyl bond that is highly reactive and allows TEPs to form covalent bonds with target molecules (Law et al., 1980; Tack et al., 1980); The binding to the surfaces of microorganisms by invertebrates TEPs has been associated with immune functions, (Levashina et al., 2001; Blandin et al., 2004, 2008; Kopacek et al., 2012; Li et al., 2012; Urbanová et al., 2015). TEPs are organized into three major groups based on their structural domains: the alpha-2-macroglobulin (A2M) group, the complement C3-related (C3) group, and the classical thioester-containing proteins (TEPs) (Blandin and Levashina, 2004; Fujito et al., 2010; Sekiguchi et al., 2012). Each group is further divided into subgroups depending on structural or functional characteristics. For example, the A2M group includes proteins such as CPAMD8 (C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8) and PZP (pregnancy-zone protein); the C3 group is further divided into the complement C3, C4, and C5 subgroups; and the TEP group is subdivided into classical TEPs or insect TEPs (iTEPs), and CD109 (Blandin and Levashina, 2004; Urbanová et al., 2015). The application of next generation sequencing methods has identified multiple members of the TEP family in all major groups of metazoan (deuterostomes and protostomes). Although the mechanism of action is not well understood, and functional data is limited for many of them, most invertebrate TEPs have been associated with immune-related functions. For example, complement factors and activity have been identified in invertebrates (reviewed in Smith et al., 1996; Blandin and Levashina, 2004; Pinto et al., 2007; Armstrong, 2010; Nonaka, 2014), including early ancestral species such as cnidarians, molluscs, arthropods, and echinoderms (Spycher et al., 1987; Iwaki et al., 1996; Iwanaga et al., 1998; Blandin et al., 2004; Nonaka and Kimura, 2006; Zhang et al., 2007; Obbard et al., 2008; Castillo et al., 2009; Fujito et al., 2010; Nonaka and Satake., 2010; Bou Aoun et al., 2011; Buresová et al., 2011; Sekiguchi et al., 2012; Urbanová et al., 2015; Yazzie et al., 2015; Gorbushin, 2018; and many others). In the snail *B. glabrata*, the only known representative of the TEP superfamily (referred to as TEP1 in this review), recorded by Moné et al. (2010), was studied in greater detail by Portet et al. (2018). Computational characterizations showed that TEP1 had all the major structural domains and motifs characteristic of TEPs proteins, and phylogenetic analysis placed this molecule among the insect TEPs and invertebrate TEP/CD109 subgroup. TEP1 was differentially expressed among snail tissues, with the highest abundance in ovotestis, headfoot, and circulating non-phagocytic hemocytes. TEP1 expression was differentially modulated in a time-dependent manner when snails were exposed to microbes (Gram+ and Gram– bacteria, and yeast). Immuoblotting with anti-TEP1 antibody identified three different-sized protein bands in snail plasma, correlating with proteolytic cleavage that commonly leads to activation of many TEPs. *In vitro* binding and western blot analysis also revealed that TEP1 from snail plasma bound to the surface of all microorganisms tested, including *S. mansoni* miracidia and sporocysts, with a greater affinity to sporocysts. Treatment with the inhibitor methylamine resulted in loss of activity indicating that this binding was mediated via the TED motif. To investigate opsonic properties following previous reports of similar activity in mosquitos (Blandin et al., 2008), the role of TEP1 in encapsulation of *S. mansoni* was tested. Immunocytochemical labeling of snail tissue, sectioned 24 h post-exposure to miracidia, revealed a diffuse positive signal surrounding the parasite and into the hemocytic capsule, as well as in the hemocytes in close proximity to encapsulations, suggesting these cells were responsible for TEP1 secretion. Still, the mechanics of TEP1 binding remains to be characterized. Although it was proposed that this TEP1 is the same molecule that was previously characterized biochemically and termed alpha-2-macroglobulin by Bender and Bayne (1996) and Fryer et al. (1996), Portet et al. (2018) conclusion was not

Table 2
Biomphalaria glabrata BB02 TEPs and closest homologs.

Name	Accession	Complete	BGLB ID	NCBI predicted	Closest Homolog
A2M-1	MK573558	Yes	016521-RB	XP_013081252.1 (96% identity)	A2M *L. littorea* AIC31934.1 (45%)
A2M-2	MK576002	No	3′ end- 022655-RA	XP_013084477.1 (100%)	CD109 *L. littorea* AVP12647.1 (55%)
C3-1	MK583200	Yes	018444-RA	XP_013068508.1 (99% identity)	C3 *L. littorea* AVP12644.1 (54%)
C3-2	MK583201	Yes	5′ end- 030610-RA 3′ end - 020436	XP_013086914.1 (97% identity) XP_013087010.1 (100% identity)	C3 *L. littorea* AVP12645.1 (33%)
C3-3	MK583202	No	3′ end- 025256-RA	XP_013064315.1 (98% identity)	C3 *L. littorea* AVP12645.1 (32%)
CD109-1	MK576003	Yes	5′ end- 021085-RA 3′ end- 031746-RA	XP_013094127.1 (98% identity) XP_013094132.1 (100% identity) XP_013076313.1 (100% identity)	TEP *Epanerchodus* BAR45598.1 (29%)
CPAMD8-1	MK576004	No	3′ end- 035268-RA	XP_013061675.1 (100% identity)	CD109 *L. littorea* AVP12647.1 (62%)
TEP-1	MK583203	Yes	5′ end- 021162-RA 3′ end- 035158-RA	ADE45332.1 (98% identity)	CD109 *L. littorea* AVP12646.1 (38%)
TEP-2	MK583204	Yes	5′ end- 000155-RA 3′ end- 032760-RA	XP_013065920.1 (93% identity)	CD109 *M. yessoensis* OWF38485.1 (32%)
TEP-3	MK583205	No	5′ end- 000023-RB	XP_013091771.1 (100% identity)	TEP *E. tau* BAE44110.1 (53%)
TEP-4	MK583206	Yes	021854-RA	XP_013071291.1 (98% identity)	TEP *E. tau* BAE44110.1 (69%)
TEP-5	MK583207	No	5′ end- 021062-RA	XP_013075528.1 (100% identity)	C3 *L. littorea* AVP12645.1 (41%)

confirmed by phylogenetic analysis, neither proteinase activity tested for this protein. Aa an alternative explanation, the molecule responsible for the proteinase activity in *B. glabrata* could be another yet unidentified member of the TEP family in the snail.

In continuation of investigating this topic, we are presenting new data that was obtained by searching the *B. glabrata* genome for additional members of the TEP superfamily. It was hypothesized that the *B. glabrata* genome contains a variety of TEP factors, and that further characterization of these molecules will improve our understanding of snail immunity. Furthermore, localization of such genes provided upstream genome sequences to help predict potential transcription factor-binding sites, and thus, associate the expression of TEPs with particular stimuli and identify the specific signaling pathway involved. For detailed methods please refer to supplemental file 1.

Screening the genome assembly and associated RNAseq databases yielded no less than 11 novel (partial) TEP-like sequences in *B. glabrata*, that did not correspond to the sequence or genome locus for the previously identified *B. glabrata* TEP1 (NCBI accession No. MK583203, Moné et al., 2010). Sequences were improved by mapping RNA-Seq reads to the *B. glabrata* genome (Table 2). Accordingly, *B. glabrata* has at least 12 members of the TEP family. Based on sequence domain composition and BLAST results, lacking functional data and full-length data for all sequences, these TEPs are tentatively classified as three members of the A2M group (A2M-1, A2M-2, CPAMD8-1); three complement C3-like molecules (C3-1, C3-2, C3-3), and six classical TEP-related proteins (TEP-1, TEP-2, TEP-3, TEP-4, TEP-5, and CD109-1) (Fig. 2) (Table 2). Since the publication of the genome (Adema et al., 2017), ongoing automated annotation has resulted in ID assignments to several of these TEPs by VectorBase, and most can also be found as predicted proteins in the NCBI database, see Table 2 for accession numbers of verified TEPs.

To further this new TEP diversity contributions, we examined whether the expression of *B. glabrata* TEP genes may be under the regulation of NF-κB transcription factors, the genomic sequences upstream of coding regions were analyzed following methods described in Humphries and Deneckere (2018) and supplemental file 1. Putative binding sites for NF-κB (κB) were predicted upstream of all genes examined except for TEPs 1 and 2 (Table 3). TEP-4 was excluded from the analyses, as the upstream genomic sequence was not found. It is not yet known whether *B. glabrata* TEPs function downstream of binding to a target and if they interact with a signaling pathway(s). Moita et al. (2005) implicated two transmembrane receptors in TEP signaling pathways in *Anopheles* mosquitoes: a low density lipoprotein (LDL)-receptor related protein, LRP1, and an integrin β subunit, BINT. Interestingly, an integrin β subunit was previously identified in *B. glabrata* (Davids et al., 1999), and an LRP1 homolog was predicted from the *B. glabrata* genome assembly (XP_013085247.1). In addition, Moita et al. (2005) implicated intracellular proteins, CED-2, CED-5 and CED-6 in TEP signaling, and a *ced*-6 like gene was detected in the *B. glabrata* genome (XP_013075601.1). As components of these pathways appear to be present in *B. glabrata*, perhaps they function in TEP signaling pathways in snails as well. It is also possible that there is an interaction between TEPs and TLR signaling in *B. glabrata*, similar as has been reported in insects (Shokal and Eleftherianos, 2017; Dostálová et al., 2017). For example, in *Drosophila*, inactivation of TEP4 led to increased activity in Toll and IMD signaling pathways but a reduction in Jak/STAT and Jnk pathway activity (Shokal and Eleftherianos, 2017). In contrast, Dostálová et al. (2017) demonstrated that *Drosophila* TEPs contribute to activating a Toll signaling pathway in response to Gram + bacterial and fungal infections. Perhaps the TLR signaling pathway can induce TEP expression through NF-κB in *B. glabrata*, as putative κB binding sites were predicted upstream of several TEP genes (Table 3). The feasibility of hypothetical regulation of *B. glabrata* TEPs by a TLR–NF–κB signaling pathway is supported by recent demonstration that in the crustacean, *Littopeneaus vannamei*, TEP expression is dependent on NF-κB and activator protein 1 (AP1) (Li et al., 2012).

5.5. Epigenetics-methylation and non-coding small RNA

Epigenetics is a topic that has generated considerable interest lately. This is mainly, because understanding the underlying mechanisms could facilitate the development of methods to genetically manipulate snails in ways that may modulate immune function and general gene expression in snails to possibly interfere with the transmission of schistosomiasis.

DNA methylation processes have not been well studied in invertebrates. However studies with the pacific oyster *Crassostrea gigas* (Gavery and Roberts, 2010) and the scallop *Chlamys farreri* (Sun et al., 2014) have identified common targets of the methylation machinery in molluscs. In *B. glabrata*, searches of the genome assembly identified core components of methylation pathways including DNMT1 (a maintenance DNA methyltransferase), DNMT2 (a DNA/tRNA methyltransferase), and MDB2/3 (a methyl-CpG-binding domain protein) (Geyer et al., 2017). Additionally, the finding that BgDNMT1 and BgMBD2/3 transcripts were upregulated in snail gonads and in Bge cells after exposure to *S. mansoni* larval transformation products (LTPs

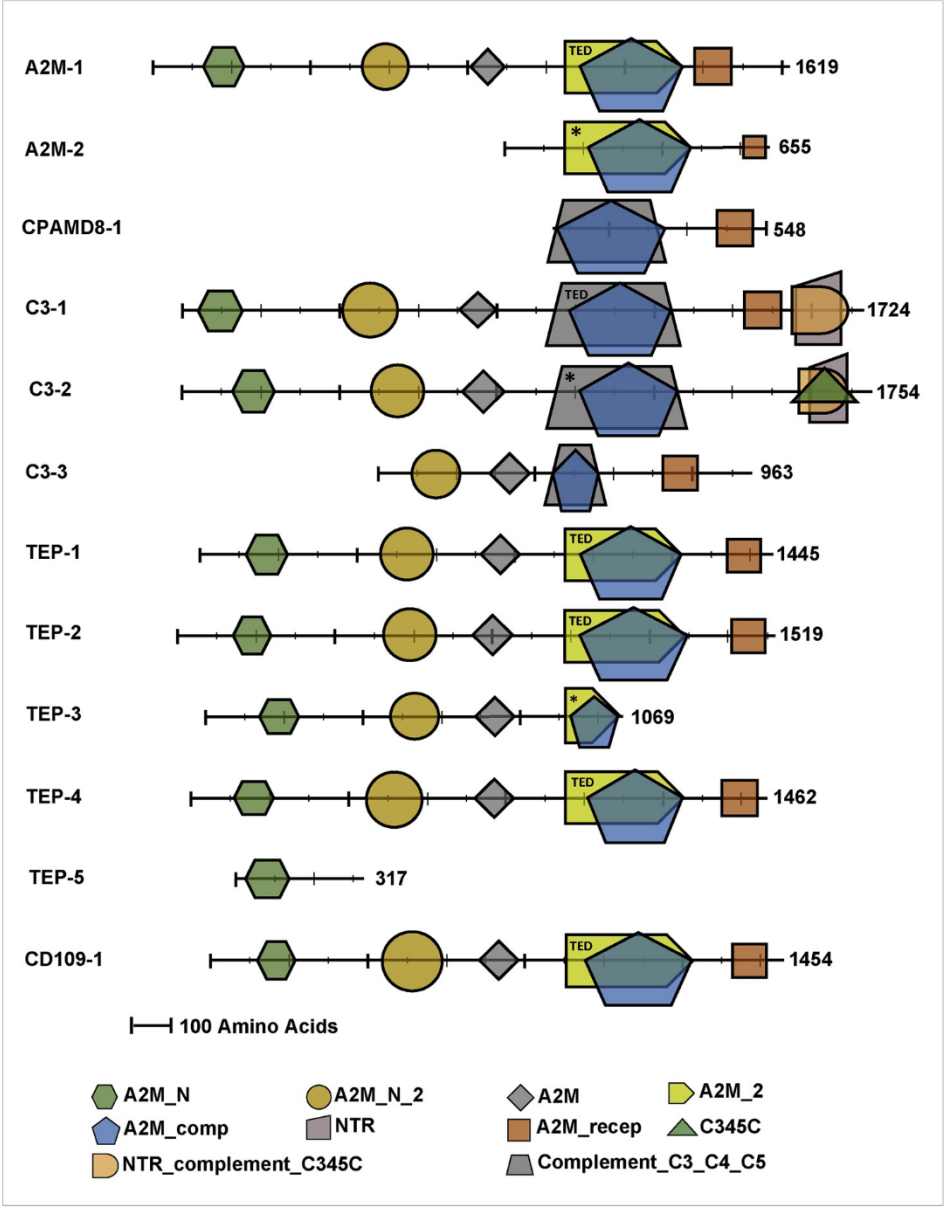

Fig. 2. Protein domains in *B. glabrata* TEP family. Graphical representation of the putative conserved domains identified in the *B. glabrata* BB02 TEP sequences. The conserved domains found in all *B. glabrata* TEP-sequences have similar content and organization as those reported in other invertebrate and vertebrate TEPs. The National Center for Biotechnology Information (NCBI) Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/cdd) was utilized for TEP domain nomenclature and abbreviations. A2M_N = MG2 (macroglobulin) domain of alpha-2-macroglobulin; A2M_N_2 = Alpha-2-macroglobulin family N-terminal region; A2M = Alpha-2-macroglobulin family, includes the C-terminal region of the alpha-2-macroglobulin family; Complement_C3_C4_C5 = Proteins similar to C3, C4 and C5 of vertebrate complement, thioester bond located within the structure of C3 and C4; A2M_2 = Proteins similar to alpha2-macroglobulin (alpha (2)-M). This group also contains the pregnancy zone protein (PZP); A2M_comp = Complement component region of the alpha-2-macroglobulin family; A2M_recep = Receptor domain region of the alpha-2-macroglobulin family; NTR_complement_C345C = NTR/C345C domain, NTR domains found in the C-termini of complement C3, C4 and C5; NTR = UNC-6/NTR/C345C module, sequence similarity between netrin UNC-6 and C345C complement protein family members; C345C = Netrin C-terminal Domain. The characteristic GCGEQ thioester domain is labeled with "TED" and regions labeled with an asterisk do not completely align with the representative GCGEQ residues, having one or more dissimilar amino acids for the thioester domain sequence. No label signifies that no thioester domain was identified. The intervals of each domain and the length of each sequence are illustrated to scale with the most up-to-date amino acid length labeled at the 3′-end.

are molecules released by parasites during the *in vitro* transformation of miracidia to sporocyst stages; Wu et al., 2009) suggest that epigenetics play an important role in snail reproduction and defense against parasites. This concords with previous findings, including methylation of cytidine residues of the *hsp70* gene in heat-stressed or *S. mansoni*-exposed snails, possibly leading to increased gene expression, and the observation of a "mosaic DNA methylation" pattern in the *B. glabrata* genome (Fneich et al., 2013; Adema et al., 2017), described as consisting of regions with high methylation in "house-keeping" genes, interspaced by low- or non-methylated (encoding inducible genes) DNA segments. Moreover like in other molluscan species studied, DNA methylation in *B. glabrata* appears to be restricted to CpG sites (Geyer et al., 2017).

Gene regulation may also be influenced by small non-coding RNAs (ncRNAs), including microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) (Hirose et al., 2014; Morris and Mattick, 2014). Analyses of the *B. glabrata* genome led to the identification of 95 conserved pre-miRNAs, and the prediction of a further 107 novel pre-miRNAS. In addition, homologs of 9 genes associated with the processing of small ncRNA processing genes, were also identified in the genome (Adema et al., 2017). Queiroz et al. (2017) have since investigated the potential role of miRNAs and piRNAs in *B. glabrata* development and in *S. mansoni* infection, by determining the expression profiles of the ncRNA processing genes Argonaute, Piwi, Drosha, Exportin-5, and Tudor. Although the expression profiles of these genes differed, overall the data suggest that all genes except Tudor, play a role in the first 40 days of development. Furthermore, the potential role of small ncRNAs in the interaction between *B. glabrata* and *S. mansoni* was probed at several time points post-infection. Though the expression profiles of the 5 genes were not uniform, all were downregulated at 4 h and 30 days post-infection, and most were also downregulated at various time points in between. The results suggest that downregulation of these genes in *B. glabrata* facilitates infection by the parasite. However, this study was performed on a single, schistosome-compatible strain of *B. glabrata*, so it will be important to examine whether small ncRNAs play a role in defense against infection in a non-compatible snail strain. In addition, the possible manipulation of *B. glabrata* gene expression by schistosome-produced small ncRNAs should be considered and deserves examination.

Table 3
Prediction of NF-κB binding sites upstream of complement-related genes. Genomic sequences upstream of the coding region for complement-related genes were surveyed for putative NF-κB binding sites (κB) using LASAGNA 2.0. Based on a previously generated consensus *B. glabrata* κB, only sequences containing a G in position 2 and a C at position 10 in the predicted κB were included (Humphries and Deneckere, 2018). Positions represent the approximate locations of the predicted binding sites in nucleotides upstream of ATG start codon. The 2000bp upstream of TEP-4 were not available in the genome.

Gene	Scaffold region	Predicted binding site	Position
C3-1	LG48i_random_Scaffold305: 282201:284201	GGAATTTCTC	−48
		GGGGACGTTC	−1479
C3-2	LG7_random_Scaffold444: 159247:161247	GGGAAATCCC	−920
		AGGATTTCCC	−131
C3-3	LGUN_random_Scaffold16274: 7177:9177	GGTAATCTAC	−1794
		TGGGGACTTC	−1954
CD109-1	LGUN_random_Scaffold569: 102601:104601	GGGGTGTTCC	−1405
TEP-1	LGUN_random_Scaffold4524: 25148:27148	–	–
TEP-2	LG21_random_Scaffold104: 146283:148283	–	–
TEP-3	LG9_random_Scaffold563: 19966:21979	GGAAATTCAC	−493
TEP-5	LGUN_random_Scaffold15662: 110:2110	TGGAATTTTC	−385
		GGGAAGACCC	−757
CPAMD8-1	LGUN_random_Scaffold2480: 5066:7066	GGAAATCTCC	−1349
		GGAAATCTCC	−1399

5.6. Signaling

5.6.1. Cytokine signaling

Whereas pregenomics studies suggested the presence of an interleukin 1(IL-1)-like factor and a tumor necrosis factor (TNF) functioning in regulation of the immune system of *B. glabrata* (Granath et al., 1994; Ouwe-Missi-Oukem-Boyer et al., 1994), specific analyses on the evolution of cytokines so far suggest that homologs of IL-17, TNF, transforming growth factor β (TGFβ) and AIF (apoptosis inducing factor) are the only vertebrate-like cytokines in molluscs (DeFilippo and Beck, 2018). This notion is supported by *B. glabrata*, as IL-17 and TNF homologs were predicted from the genome, but IL-1-like sequences were not (Adema et al., 2017). An additional survey of the genome provided evidence of a TGF signaling pathway, although we were unable to detect an AIF-like sequence.

Specifically, eleven TNF-like genes were predicted from the *B. glabrata* genome (supplementary data 17, Adema et al., 2017). In a new analysis, we further investigated the predicted genes, and considered sequences to be TNF homologs if they were supported by the RNA-Seq data and contained a TNF domain as indicated by SMART (http://smart.embl.de; Letunic and Bork, 2018). The annotations of eight TNF homologs were confirmed, though two of the transcripts were incomplete (lacking 5′ termini and 3' stop codons); in addition, BgTNF3 appears to have two variants (Table 4). Transmembrane domains, considered a feature of mammalian TNFs (MacEwan, 2002; Aggarwal et al., 2011), were predicted in all BgTNFs using TMHHM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM).

Subsequent survey of the genome assembly and RNA-Seq data identified three potential TNF receptors based on the presence of a TNFR superfamily domain, however the transcript for BgTNFR3 lacked a 5' start codon (Table 5). All BgTNFRs were classified as TNFR2 subtype because of a lack of a cytoplasmic death domain (DD) in the cytoplasmic tails, according to the nomenclature of mammalian BgTNFRs (MacEwan, 2002; Aggarwal et al., 2011).

The RNA-Seq data collected as part of the characterization of the *B. glabrata* genome not only allows for the confirmation and/or correction of predicted gene models but also provides information on specific tissue localization of transcripts, as mRNA sequences were obtained from 12 different snail tissues/organs. This information indicated that the various TNFs were not detected in all tissues, and in those in which they were, they were differentially expressed, such that: (i) No TNF transcripts were detected in salivary glands; (ii) TNF4 transcripts were detected in only 2 tissues; (iii) TNF2 and 3 transcripts were found in four to five tissues; (iv) TNF1 transcripts were detected in seven tissues; (v) Transcripts representing TNF7 were found in eight tissues; (vi) TNFs 6 and 8 were found in ten tissues; and (vii) TNF5 transcripts were found in eleven of the twelve tissues (Table 6). Similar expression patterns were recorded for TNF receptors: (i) No TNFR transcripts were detected in either the albumen gland or terminal genitalia; (ii) TNFR1 and 2 were found in seven and nine different tissues respectively; and (iii) TNFR3 was only detected in transcripts from the stomach; interestingly the stomach was the only tissue in which all BgTNFRs were found (Table 7). Apart from TNF4 and TNFR3, the *B. glabrata* TNFs and TNFRs demonstrated constitutive and ubiquitous tissue expression, similar to what has been reported for other molluscs (De Zoysa et al., 2008; Xiang et al., 2016; Xing et al., 2016). In addition, the number of TNF and TNFR transcripts, as well as tissue distribution patterns, suggest that each TNFR can bind more than one TNF.

In mammalians, the cytoplasmic DD of TNFR1 allows the receptor to interact with apoptosis-associated signaling proteins such as TRADD (TNF receptor-associated DD) and RIP (receptor-interacting protein), through DD to DD binding (MacEwan, 2002). It is notable that all three putative BgTNFRs lack a cytoplasmic DD and in addition, homologs to TRADD and RIP were not found in the *B. glabrata* genome. Likewise, transcriptomic study of embryonic *B. glabrata*, identified transcripts for TNF and TNFR whereas those for TRADD and RIP were absent (Kenny et al., 2016). These findings suggest that the BgTNFRs signal via direct interactions with a TRAF2 protein, similar to mammalian TNFR2 receptors (MacEwan, 2002). This is further supported by the recent identification of a TNF receptor associated factor 2 (TRAF2) homolog in *B. glabrata* (Adema et al., 2017; Humphries and Deneckere, 2018). Based on mammalian studies, ligand binding by BgTNFRs may lead to the activation of mitogen-activated protein kinase (MAPK), NF-κB and AP1 signaling pathways (Aggarwal et al., 2011, Fig. 3); components of all these pathways have been identified in *B. glabrata* (Humphries and Yoshino, 2006; Zhang and Coultas, 2011; Humphries and Harter, 2015; Adema et al., 2017).

The identification of a transmembrane domain in each of the BgTNFs, in conjunction with the presence of a putative TNF-α-converting enzyme (TACE) in the *B. glabrata* genome (XP_013083719.1), support the notion that *B. glabrata* TNFs are first expressed as membrane proteins that may be subsequently cleaved to a soluble form, similar to TNF processing in mammalians (MacEwan, 2002). Furthermore, both the membrane and soluble forms may be capable of inducing signaling in receptor-bearing cells through TNFR binding. Interestingly, the binding of membrane TNFs by a TNFR can cause signaling in the TNF-bearing cell; this is known as outside-inside or reverse signaling (Harashima et al., 2001; MacEwan, 2002; Aggarwal et al., 2011).

The identification of TNF and TNFR homologs in *B. glabrata* complements the discovery of similar homologs in other molluscs (De Zoysa et al., 2008; Zhang et al., 2015; Xiang et al., 2016; Xing et al., 2016). Pending functional confirmation, it is hypothesized that TNF signaling functions in *B. glabrata* immunity just as TNF signaling participates in mammalian immune regulation. Furthermore, studies of other molluscan species have demonstrated increased expression levels of TNF and TNFR genes in response to pathogen exposures, thus supporting a role for TNF signaling in the mollusca immune defenses (De Zoysa et al., 2008; Xiang et al., 2016; Xing et al., 2016).

As stated earlier, studies on the evolution of cytokines suggest that, like TNFs, homologs of IL-17 are among the categories of vertebrate-

Table 4
TNF homologs in *B. glabrata*. TNF homologs predicted in the *B. glabrata* genome are identified by their NCBI accession number. Predicted genes that were confirmed using RNA-Seq data are listed in the table. Transcripts for which both 5′ and 3′ stop codons were present are classified as complete, whereas incomplete transcripts lacked one or both stop codons. Signal peptides, transmembrane domains and the TNF domains were identified using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP-3.0), TMHMM server, v2.0 (http://www.cbs.dtu.dk/services/TMHMM), and SMART (http://smart.embl.de).

	NCBI Predicted	Complete	Size	Predicted kD	Signal peptide	Transmembrane domain	TNF domain
BgTNF1	XP_013067535	No	309	35		36–58	163–302
BgTNF2	XP_013067539	Yes	322	36.19		41–63	152–308
BgTNF3v1	XP_013094494	No	240	27.3	1–36	15–37	106–232
BgTNF3v2	XP_013094495	No	210	24	1–36	15–37	106–232
BgTNF4	XP_013076957	Yes	271	30	1–30	12–34	106–252
BgTNF5	XP_013082590	Yes	366	41		95–117	206–358
BgTNF6	XP_013086591	Yes	618	69		382–404	480–612
BgTNF7	XP_013087321	Yes	384	43		85–107	235–384
BgTNF8	XP_013061250	Yes	369	41		116–138	235–369

Table 5
TNF-receptor (TNFR) homologs in *B. glabrata*. The genomic and RNA-Seq data from *B. glabrata* were surveyed for the presence of BgTNFRs. Signal peptides, transmembrane domains, and TNF receptor domains were identified using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP-3.0), TMHMM server, v2.0 (http://www.cbs.dtu.dk/services/TMHMM), and SMART (http://smart.embl.de). The total amino acid length of each BgTNFR is given in column 1. The positions of signal peptides, regions outside the membrane, the transmembrane domains, and intracellular regions are given in columns 2–5 respectively.

	Length	Signal peptide	Outside	Transmembrane domain	Inside
BgTNFR1	362	1–19	1–271	272–94	295–362
BgTNFR2	225	1–42	1–181	182–204	205–225
BgTNFR3	186	1–20	1–159	160–182	183–186

like cytokines to be found in molluscs (DeFilippo and Beck, 2018). The *B. glabrata* genome project (supplementary data 17, Adema et al., 2017) yielded twelve predicted IL-17-like genes. Investigation using the tissue-derived RNA-Seq data led to recovery of four of the twelve predicted *B. glabrata* IL-17 homologs, as characterized by containing IL-17 domains, as expressed transcripts (http://smart.embl.de; Table 8). In contrast to the TNF expression patterns described above, IL-17-like transcripts were not ubiquitously expressed across tissues. Each of the four BgIL-17 transcript variants was found in only one or two tissues; two of the BgIL-17 homologs were represented in the terminal genitalia sequences and two were found in the stomach. It should be noted, however, that the RNA-Seq data represent constitutive expression, as the tissues were isolated from uninfected snails. Perhaps most IL-17s in *B. glabrata* are expressed in response to stimuli, whereas TNFs are expressed constitutively to be upregulated further during an immune response. With confirmation of BgIL-17 homologs, a survey of the genomic and RNA-Seq data identified a single putative *B. glabrata* IL-17 receptor sequence that shares similarity with both invertebrate and vertebrate IL-17 receptors entries in NCBI.

The identification of IL-17 and IL-17 receptor homologs in *B. glabrata* is corroborated by the identification of similar homologs in other molluscs, such as *Octopus vulgaris* and *Crassostrea gigas* (Castellanos-Martinez et al., 2014; Zhang et al., 2015). Changes in the expression of other molluscan IL-17 homologs following pathogen exposure (Zhang et al., 2015), support the concept that BgIL-17s may have an immune role. Mammalian IL-17 homologs play a pro-inflammatory role in the adaptive immune system and regulate innate immunity. In the latter,

Table 6
Tissue distribution of BgTNFs. Tissue-specific RNA-Seq data was surveyed for the presence of BgTNF transcripts. Represented tissues include: albumen gland (AG); buccal mass (BUC); central nervous system (CNS); digestive gland/hepatopancreas (DG/HP); muscular part of the headfoot (FOOT); heart including amebocyte producing organ (HAPO); kidney (KID); mantle edge (MAN); ovotestis (OVO); salivary gland (SAL); stomach (STO); terminal genitalia (TRG). A ✓ indicates the presence of a BgTNF transcript.2

	BgTNF1	BgTNF2	BgTNF3v1	BgTNF3v2	BgTNF4	BgTNF5	BgTNF6	BgTNF7	BgTNF8
AG						✓	✓		
BUC	✓					✓	✓	✓	✓
CNS	✓					✓	✓	✓	✓
DG		✓	✓		✓	✓	✓	✓	✓
FOOT	✓	✓		✓		✓	✓	✓	✓
HAPLO	✓		✓	✓	✓	✓	✓	✓	✓
KID	✓	✓				✓	✓	✓	✓
MAN	✓		✓	✓		✓	✓	✓	✓
OVO						✓		✓	✓
SAL									
STO	✓	✓	✓	✓		✓	✓	✓	✓
TRG			✓	✓		✓	✓		✓

Table 7
Tissue distribution of BgTNF receptors. Tissue-specific RNA-Seq data was surveyed for the presence of BgTNFR transcripts. Represented tissues include: albumen gland (AG); buccal mass (BUC); central nervous system (CNS); digestive gland/hepato-pancreas (DG/HP); muscular part of the headfoot (FOOT); heart including amebocyte producing organ (HAPO); kidney (KID); mantle edge (MAN); ovotestis (OVO); salivary gland (SAL); stomach (STO); terminal genitalia (TRG). A ✓ indicates the presence of a BgTNFR transcript.

	BgTNFR1	BgTNFR2	BgTNFR3
AG			
BUC	✓	✓	
CNS		✓	
DG		✓	
FOOT	✓	✓	
HAPLO	✓	✓	
KID	✓	✓	
MAN	✓	✓	
OVO		✓	
SAL	✓		
STO	✓	✓	✓
TRG			

IL-17 signaling pathways increase the expression of chemokines, antimicrobial molecules, and acute phase proteins for example (Gaffen, 2008). Based on this, BgIL-17 homologs are assumed to function in the snail immune system, perhaps by regulating the expression and release of humoral factors that play a role in immunity.

5.6.2. Toll-like receptor (TLR) signaling

Toll-like receptors (TLRs) are an ancient family of pattern recognition receptors (PRRs) that has been identified in numerous animal phyla ranging from sponges and cnidarians (Prebilateria) to protostomes and deuterostomes including the chordates (Gilmore and Wolenski, 2012; Brennan and Gilmore, 2018). Vertebrate TLRs have been studied the most extensively, and are known to function as dimers that collectively bind a diversity of PAMPs, including double stranded RNA, CpG DNA, and lipopolysaccharide (Takeda et al., 2003). Targeted searches of the *B. glabrata* genome assembly led to the identification of twenty-seven complete TLRs (Adema et al., 2017). Of these, only one TLR (designated TLR1, most similar to TLR5 as listed in Adema et al., 2017) has been studied at the functional level (Pila et al., 2016a). It should be noted that the numbering of *B. glabrata* TLRs does not imply functional homology with mammalian TLRs. In comparing the responses of two *B. glabrata* strains following exposure to *S. mansoni*, BgTLR1 expression levels increased in BS90 snails (resistant) at 12 and 24 h post infection, whereas no significant changes were reported for M-line snails (susceptible). Furthermore, RNAi knockdown of BgTLR1 led to a reduction in hemocyte phagocytosis of beads coated with *S. mansoni* excreted-secreted products (ESP, Johnston and Yoshino, 2001), and 43% of siRNA-treated BS90 snails (normally resistant) became susceptible to infection (Pila et al., 2016a). These findings indicate that BgTLR1 functions in defense against *S. mansoni* in resistant *B. glabrata* of the BS90 strains. The specific ligand for BgTLR1 has not yet been identified, but Aksoy et al. (2005) previously demonstrated that a mammalian TLR3 bound *S. mansoni* dsRNA. It is possible therefore, that BgTLR1 directly binds a schistosome molecule. It is of interest to determine whether other TLRs contribute to *B. glabrata* anti-schistosome defense and to identify the ligands for each of these receptors. In addition, it is yet to be resolved whether *B. glabrata* TLRs function as monomers or dimers, given that mammalian TLRs typically work as dimers. A comprehensive TLR signaling pathway was identified

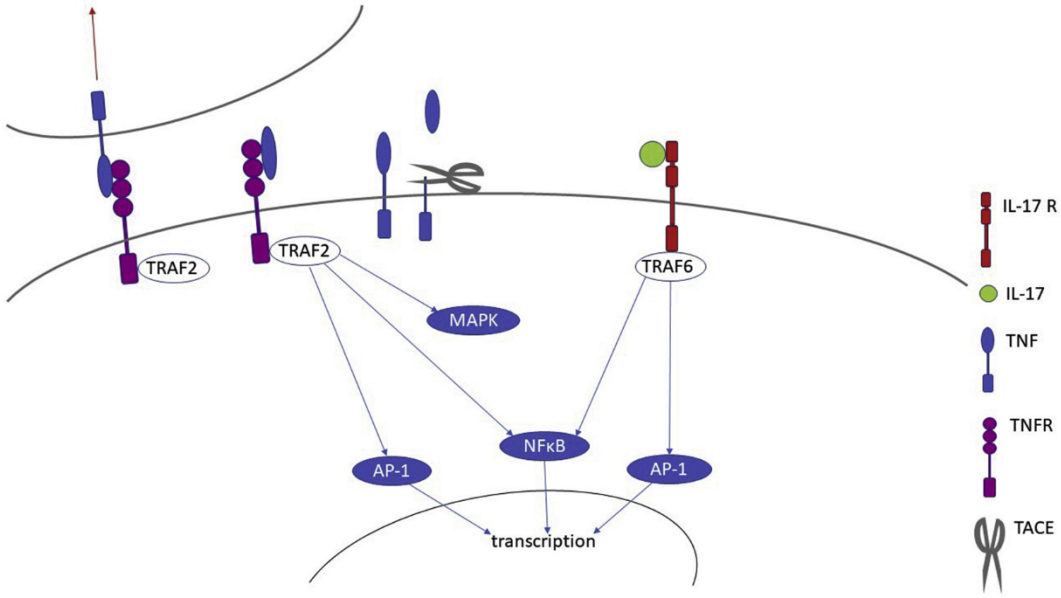

Fig. 3. Putative IL-17- and TNF-signaling pathways in *B. glabrata*. TRAF2 = TNF receptor associated factor 2; AP1 = activator protein 1; NF-κB = nuclear factor κB; TNF = tumor necrosis factor; TNFR = TNF receptor; TACE = TNF-α-converting enzyme; IL-17 = interleukin 17; IL-17R = IL-17 receptor; red arrow indicates outside-in signaling via a TNF receptor; blue arrows indicate outside-in signaling via IL-17 and TNF receptors. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 8
IL-17 homologs in *B. glabrata*. IL-17 homologs predicted in the *B. glabrata* genome are identified by their NCBI accession number. Predicted genes that were confirmed using RNA-Seq data are listed in the table. Transcripts for which both 5′ and 3' stop codons were present are classified as complete. The presence of IL-17 domains was confirmed through SMART (http://smart.embl.de).

	NCBI predicted	IL-17 domain	Complete
BgIL-17–1	XP_013065647	Yes	Yes
BgIL-17–2	XP_013090467	Yes	Yes
BgIL-17–3	XP_013080675	Yes	Yes
BgIL-17–4	XP_013085850	Yes	Yes
BgIL-17–5	XP_013085675	Yes	Yes

through the *B. glabrata* genome project (Adema et al., 2017; Humphries and Deneckere, 2018), and we hypothesize functions downstream of TLR-ligand binding. Possibly, activation of TLRs in *B. glabrata* leads to amplification of TLR signaling as indicated by the occurrence of kappa-binding DNA sites, and demonstrated by BgNF-κB being able to bind upstream of the genes for several members of the TLR signaling pathway (Humphries and Deneckere, 2018). As mentioned above (section 5.5.4), perhaps TEP- and TLR-mediated signaling intersect in *B. glabrata*, such that some TEPs can activate the TLR signaling pathway, which leads to increased expression of certain TEPs, while in contrast, other TEPs function to down regulate TLR signaling and the immune response.

6. Discussion

The sequencing of *B. glabrata*'s genome has transformed the field of snail biology, and will continue to do so. This is exemplified by recent post-genomic studies that utilize the genome resources to advance the understanding of immunity and general biology of this snail. This review examined several such reports that focused on snail-schistosome interactions and further develop previous findings aimed to better understating of snail-parasite (immune) interactions, potentially benefitting the control of human schistosomiasis. These studies included the sequencing of the Bge cell genome, the identification of a family of thioester proteins, analyses of TNF and IL-17 cytokines in *B. glabrata*, discovery of the potential roles for small nc-RNAs in snail development and during *S. mansoni* infection, and explorations of the immune memory in *B. glabrata*. These topics provide a preview of what promises to be a component-diverse molluscan immune system, and indicate a high complexity of snail-parasite interactions as well as yet to be discovered mechanisms underlying immune function in *B. glabrata*. With the now available *B. glabrata* genome and its associated data resources, newly gained insights can be more easily interpreted in connection to previously gained knowledge and observations to begin and facilitate development of more holistic hypotheses regarding the mechanisms of immunity in snails and invertebrates in general. What at this moment seems to constitute independently working, disconnected, pieces of a puzzle, may instead be parts of a larger, interconnected polygenic network. Are these pieces part of generalized immune reactions or unique to anti-helminthic responses in *B. glabrata*? Whereas it has become evident that snail- and parasite-derived molecules interact to form multimeric complexes, it remains to be clarified whether and how these complexes are part of the parasite pathogenic strategy, or perhaps play a role in effective snail immune defense against parasite infection. The *B. glabrata* genome and its associated data has allowed for the discovery of extended families of immune and immune related molecules as described here (e.g. TEPs). It is anticipated that more specific analyses of these resources could reveal novel immune targets to be characterized in *B. glabrata* as transcriptome explorations performed in the common periwinkle snail *Littorina littorea* transcriptome improving our understanding of molluscan lectin (Gorbushin and Borisova, 2015; Gorbushin, 2019) and complement-like (Gorbushin, 2018) families. Postgenomic investigations of B. glabrata can now begin to address diverse novel questions. What are the biological bases of the diverse levels of susceptibility of *B. glabrata* to schistosome infection seen in the wild? Are studies in the laboratory representative of what is happening in snail-parasite interactions in the field? Are parasite products being "mopped up" in multimeric complexes in resistant snails to prevent downregulation of snail's immunity or do these complexes facilitate immune recognition and activation to initiate downstream signaling (e.g., the TLR–NF–κB pathway) that leads to the synthesis and release of immune factors such as cytokines and immune effectors? Can snail immunity be artificially manipulated and improved? Fortunately, and despite that all these and many more questions remain to be answered, efforts are being made in related fields of study to further our knowledge and understanding of snail immunity and genomics; some of these include research on schistosome biology (Mickum et al., 2014; Yan et al., 2018), transgenics (Hagen et al., 2014; Ittiprasert et al., 2019), post-transcriptional regulation (Fiscon et al., 2018), vaccine and diagnostic tools (Sousa-Figueiredo et al., 2013; Prasanphanich et al., 2014; Sato et al., 2018), snail ecology (Perez-Saez et al., 2016; Civitello et al., 2018), computational modeling (Gurarie et al., 2018), and gene networks (Ruprecht et al., 2017) among others. Multidisciplinary and multifaceted approaches (reviewed in Colley et al., 2014; and McManus et al., 2018) will accelerate discovery and development of methods to decrease the burden of schistosomiasis in the future while at the same time expanding our understanding of immune function in *B. glabrata*.

Acknowledgements

The authors would like to acknowledge Ricardo Castillo for the art in this review and to Nathalie Dinguirard for helpful discussion of the manuscript. Portions of this research was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number 5SC2AI133645.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2019.103557.

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