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HEMOCYANIN-LIKE 1 PROTEIN, UNIQUE TO PLANORBID SNAILS, SUPPORTS REPRODUCTIVE OUTPUT OF BIOMPHALARIA GLABRATA, VECTOR OF SCHISTOSOMIASIS

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SUPPORTS REPRODUCTIVE OUTPUT OF *BIOMPHALARIA GLABRATA*,

VECTOR OF SCHISTOSOMIASIS

By

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B.S., Microbiology, University of Texas at El Paso, 2009

DISSERTATION

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DEDICATION

Para mis hijos y mi querido esposo que caminaron conmigo en este trayecto. Es gracias a ustedes que tengo la fuerza y el valor de seguir adelante durante momentos oscuros de la vida. Sepan que este trabajo es tanto de ustedes como mio.

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PLANORBID HEMOCYANIN-LIKE 1 PROTEIN INFLUENCES REPRODUCTIVE OUTPUT OF *BIOMPHALARIA GLABRATA,* SNAIL VECTOR OF SCHISTOSOMIASIS

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ABSTRACT OF DISSERTATION

The Neotropical freshwater snail, *Biomphalaria glabrata* is a major intermediate host of the parasite *Schistosoma mansoni*, causative agent of human intestinal schistosomiasis. Disease control efforts consist mainly of mass drug treatment but it is recognized that targeting of the snail vector is essential to prevent parasite transmission that leads to rapid (re)infections. This dissertation combines the knowledge of basic snail biology with modern molecular techniques including protein analysis, next-generation sequencing, proteomics, qRT-PCR and RNA interference to: (1) characterize hemocyanin-like genes in *B. glabrata,* in particular with further description of the egg mass fluid (EMF) protein hemocyanin-like 1 (Hcl-1), (2) investigate phylogenetic distribution and evolutionary origin of Hcl-1 among snails of the family Planorbidae and close sister-taxa and (3) provide insight into the function Hcl-1, as an EMF protein, plays in *B. glabrata* eggs. Results indicate there are two hemocyanin-like genes (Hcl-1 and Hcl-2) in *B. glabrata* with Hcl-1 being present in all subgroups of the family Planorbidae tested, but not outside of this family. We report unique structural features of Hcl-1 when compared to respiratory gastropod hemocyanins and determine that Hcl-1 is important for

sucessful reproductive output of *B. glabrata* snails. Here, we provide an insight into unique planorbid snail biology that with further investigation has the potential of being exploited to control planorbid snails, which include several species that serve as intermediate host for human infecting schistosomes.

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CHAPTER 1: INTRODUCTION

Human schistosomiasis is a debilitating disease caused by parasitic flatworms of

the genus *Schistosoma*. Of the approximate 208 million people that suffer from this

disease globally, an estimated 10 million people live in South America (Gonzalez *et al.*,

2012; Bruun, Aagaard-Hansen & Watts, 2008). The Neotropical freshwater snail,

Biomphalaria glabrata is a major intermediate host that transmits *Schistosoma mansoni,*

one of the causative agents of human intestinal schistosomiasis. People acquire the

disease upon contact with water of lakes, ponds, streams, etc. that contains cercariae

(human-infective stage) released by *Biomphalaria* snails. Later, the infected person will

pass worm eggs in the feces. These eggs hatch in fresh water to release miracidia (snail-

infective stage), starting the life cycle again (Fig. 1). The World Health
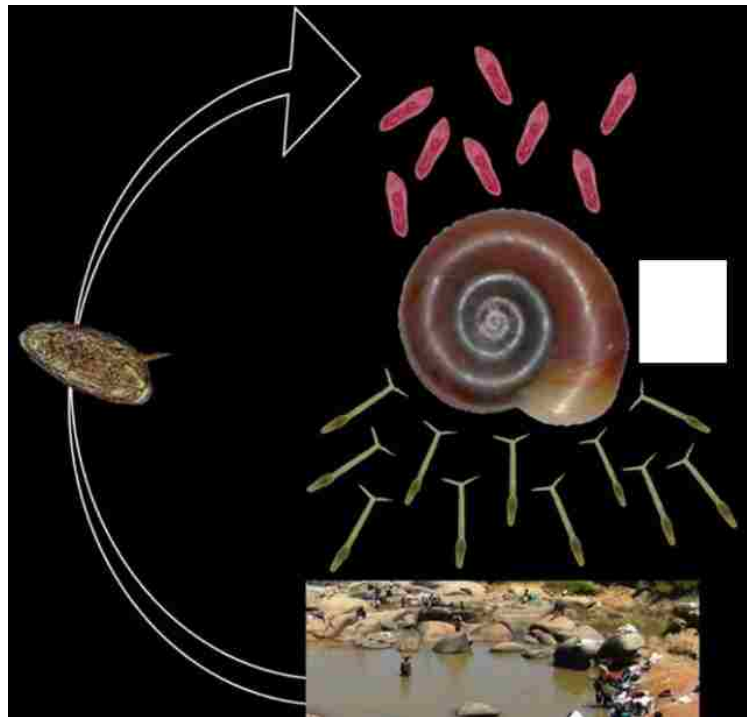
Figure 1. Life cycle of the parasite *Schistosoma mansoni.* A snail intermediate host, like *Biomphalaria glabrata*, is needed for parasite development toward release of cercariae, the human infective stage. Infected humans release eggs that hatch to produce miracidia (top) that again infect snails. Without snails, the parasite lifecycle is interrupted.

Organization (WHO) has set a goal for the global elimination of schistosomiasis as public health threat by 2025. The strategy for disease control includes mass drug treatment but also recognizes that targeting of the snail intermediate host is crucial to prevent transmission of new infections (World Health Organization, 2012).

The risk of schistosomiasis transmission in endemic countries correlates with poor socioeconomic conditions. Such circumstances hinder the provision of safe water supplies, education, and medication to populations at risk. Schistosome-infected people suffer from abdominal pains, diarrhea, and blood in the stools; advanced schistosomiasis causes inflammation and injury of the liver. Chronic infection causes fatigue, retarded development, and premature death. People at risk are commonly those that are exposed to water recreationally (children) or professionally for laundry, agriculture or fishing (Fletcher, LoVerde & Woodruff, 1981). Schistosomiasis infection imposes severe costs; treatment (frequently expensive) may not be available, and chronic disease comes with personal suffering and loss of productivity reducing the contribution of individuals to their community (King, Dickman & Tisch, 2005). Efforts to control schistosomiasis can lead to an overall socioeconomic improvement to endemic countries. In endemic Latin American countries (Venezuela, Brazil, Suriname), there are continuous efforts to control schistosomiasis. These include effective anti-parasite drug treatment (oxamniquine and praziquantel) of infected individuals, improvement of sanitation, health education, and control of intermediate host snails (Sarvel *et al*., 2011; Alarcón de Noya *et al*., 1999). However, effective snail control is elusive and the benefits of otherwise effective medications are diminished when cured individuals return to an environment where

they will be re-exposed and re-infected, demanding continuous treatment against human

schistosomiasis. Study of *B. glabrata* snails and their biology is important to reveal

information for how and where this snail persists in the environment such that we can

track and avoid or reduce parasite transmission by snails.

The extraordinary biology of the snail complicates schistosomiasis control efforts;

Biomphalaria snails easily (re)colonize water bodies, reproduce readily and persist under

conditions of pollution and drought (by remaining desiccated for months to re-emerge

after contact with water), all while having the ability to transmit schistosome parasites

(Richards, 1967; Barbosa & Barbosa, 1994). It is important to identify biological features

of the snail that explain distribution and survival in the environment to educate citizens

on types of water bodies to avoid, or environmental modifications that repel these snails.

Additional to this, focus on *B. glabrata* also permits the study of what makes an effective

snail host for the parasite and may provide alternative methods for control of

schistosomiasis. This dissertation concentrates on characterizing the unique biology of

the snail intermediate host, *B. glabrata*, which is distributed in several Latin American

countries and Caribbean islands, with consideration of potential targets for control

measures of snails in support of sustainable control of schistosomiasis.

INTRIGUING DISCOVERY OF HEMOCYANIN IN *BIOMPHALARIA*

GLABRATA

Biomphalaria glabrata is part of the Planorbidae, a family of gastropods that

employs hemoglobin (a soluble, iron atom-containing protein) as oxygen carrier. This is

unusual since most mollusks, including phylogenetic sister and ancestor groups use

hemocyanin (Fig. 2; Zapata *et al.*, 2014; Markl, 2013).

It was proposed that hemoglobin in the Planorbidae evolved from a pulmonate myoglobin to replace hemocyanin that has a lower affinity for oxygen (Lieb *et al*., 2006; Bugge & Weber, 1999). Still, electron microscope imaging of the hemolyph proteins in *B. glabrata* revealed the presence of a hemocyanin-like structure (Lieb *et al*. 2006) and hemocyanin-encoding sequences (indicated by several ESTs that contain partial sequences) have been retained in *B. glabrata*. Generally, molluscan hemocyanin is a multimeric protein composed of 10 hemocyanin protein subunits, known as a decamer.
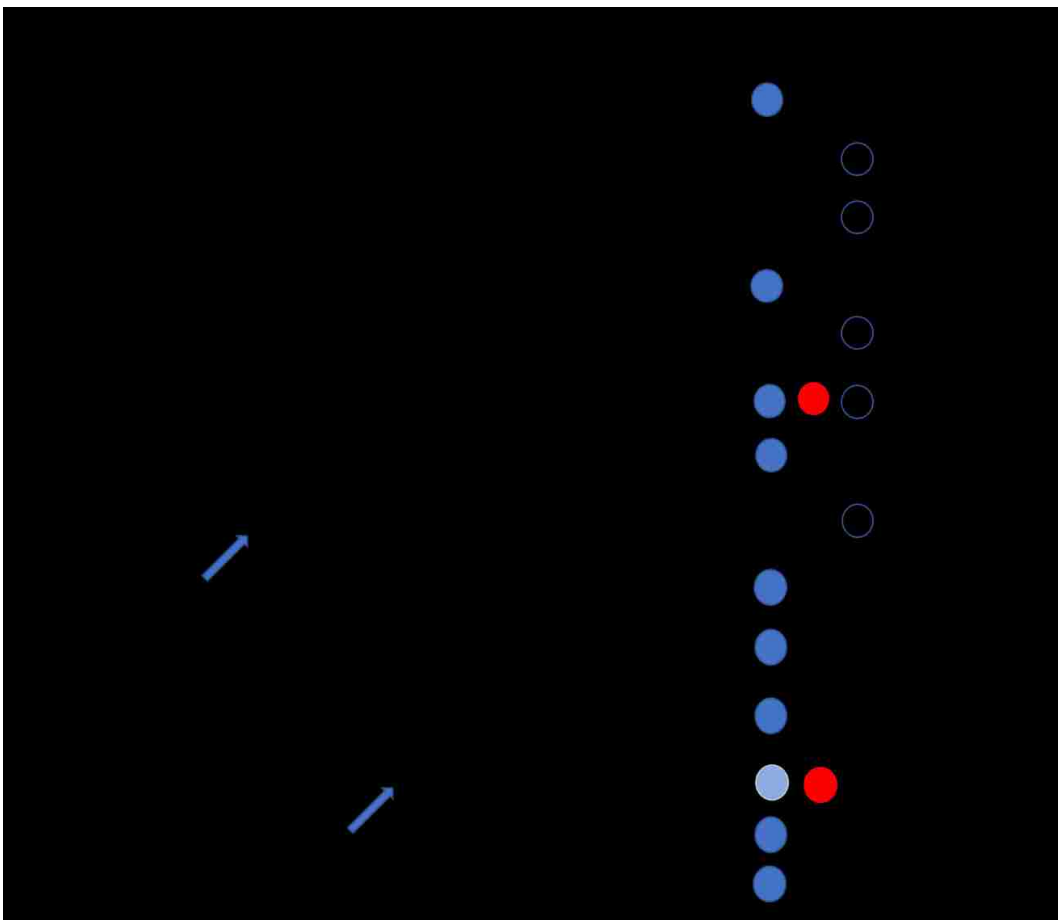
Figure 2. Phylogeny of Mollusca according to Zapata et al., 2014. Presence/absence of hemocyanin was derived from Markl (2013). The blue and red circles show the presence of hemocyanin and hemoglobin, respectively. The blank circles show no evidence or absence for any oxygen carrier.

Each subunit contains seven to eight functional units (FUs) denoted a-h; each FU contains six conserved histidines that bind two copper atoms (Markl, 2013; Fig. 3). Analysis of the *B. glabrata* hemocyanin-like EST sequences indicated that the encoded hemocyanin is atypical. The encoded protein is truncated at the C-terminus and has an amino acid mutation in a FU, switching a conserved histidine to an arginine. In chapter two of in this dissertation, we characterized two hemocyanin-like genes in *B. glabrata,* in particular, with further description of the EMF protein hemocyanin-like 1 (Hcl-1).
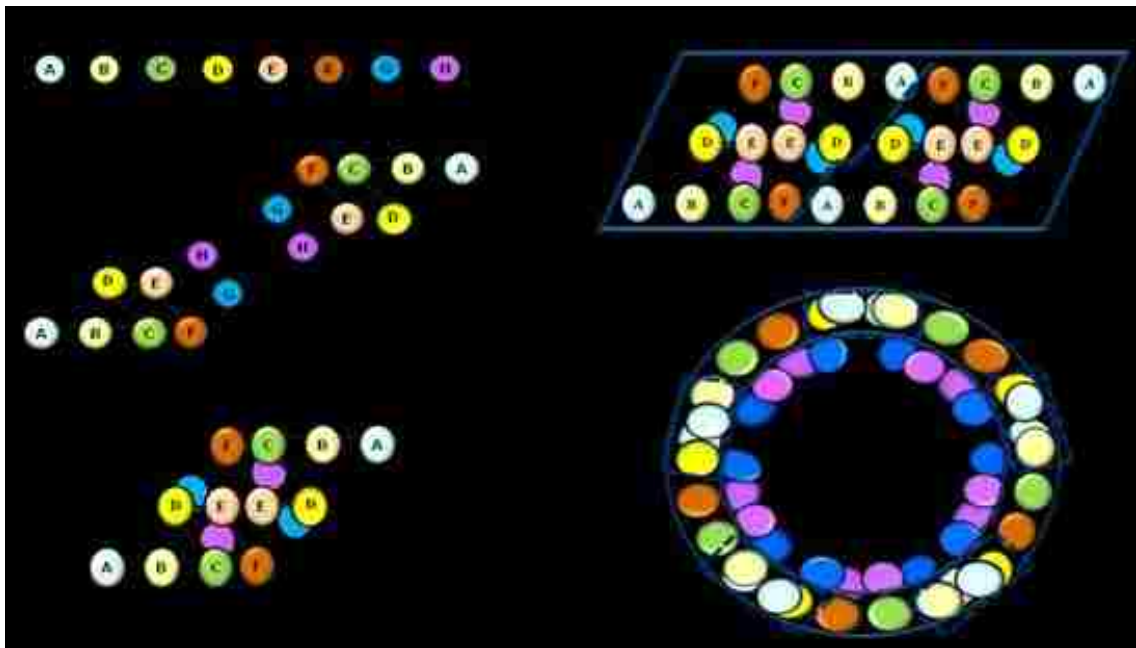
Figure 3. Gastropod hemocyanin protein structure. a) FUs present in a single subunit. b and c) Folding of two subunits that interact to form a dimer. d) Interaction of two dimers that form part of the "wall" complex to make the outside of the barrel shape protein. e) Top view of ten interacting subunits with inner collar complex, represented by FUs G (blue) and H (violet). Schematic adapted from Markl (2013).

PLANORBID SNAILS AND SCHISTOSOMES

A long, ongoing research tradition has investigated the role of *B. glabrata* snails in transmission of schistosome-caused disease (Toledo & Fried, 2011). The use of modern molecular techniques in biology provides many insights, ranging from *B.*

glabrata ecology to immunological and genetic suitability for schistosome parasites (Dejong *et al*., 2003; 2001). Remarkably, *S. mansoni* is not original to the New World; this species was introduced to Latin America from Africa by the slave trade (1400-1800; Fletcher, LoVerde & Woodruff, 1981). Genetic analysis showed that prehistorically, an ancestral South American *Biomphalaria* snail colonized Africa and became a highly suitable for *S. mansoni* transmission. Thus, upon the introduction of African slaves carrying *S. mansoni*, the parasites encountered suitable *Biomphalaria* snails and the schistosomiasis life cycle was established in the Neotropics (Dejong *et al*., 2003.).

  Biomphalaria glabrata, specifically, is employed for spearheading molecular host/pathogen interaction studies (Adema *et al*., 2010) and functional research with RNAi knockdowns (Qiu, Adema & Lane, 2005; Garcia *et al.*, 2010; Hanington *et al.*, 2010; Knight *et al.*, 2011; Adema, 2015) that have yielded comprehensive approaches and new, unique aspects of *B. glabrata* biology (Adema, 2015) that may help understand, predict and control distribution of these snails in the field to reduce transmission of schistosome parasites. Adding to this, the *B. glabrata* genome has recently become available, expanding the genomic information that is now available (Adema *et al.*, 2017). Such access to genomic data and research can provide insights, aiding the efforts of the WHO to eliminate schistosomiasis by integrating strategies that reduce parasite-transmitting snails. <u>Chapter three of this dissertation reports that Hcl-1 has an early origin in the planorbid lineage and is not present in close sister-taxa. Furthermore, Hcl-1 behaves different by functioning as a monomer when compared to respiratory molluscan hemocyanins that function as multimers.</u>

PUTATIVE FUNCTION OF HEMOCYANIN-LIKE 1

The hemocyanin sequences of arthropods and mollusks originated from ancestral sequences of the copper type 3 enzymes, phenoloxidase and tyrosinase. These enzymes are implicated in several functions such as oxygen transport and catalyzing the production of melanin from phenolic compounds and tyrosine (Decker *et al*., 2007; Jaenicke & Decker, 2004; Decker & Tuczek, 2000). The similarity in structure between these two enzymes and hemocyanin leads us to question whether hemocyanin may also have an immunological function that we are unaware of in the snail family Planorbidae. Bai et al. (1997) reported an involvement of phenoloxidase activity associated with 35kDa tyrosinase in formation of the eggshell in *B. glabrata* and the use of proteomics (Hathaway *et al.*, 2010) disclosed the presence of hemocyanin-like proteins in the albumen gland of *B. glabrata*, an immune-relevant organ (Jeong *et al.*, 1981; Vergote *et al.*, 2005). Additionally, arthropods have hemocyanin-derived proteins called hexamerins which are hypothesized to play a role in maintenance of arthropod physiology as a source of nutrition by providing amino acids to non-feeding larvae (Burmester, 2015; 1999). Hemocyanin-like peptides were also detected in the EMF that surrounds developing snail embryos inside the eggs, along with several immuno-protective proteins (antioxidants, proteases, protease inhibitors, antibacterial factors, and lectins; Hathaway *et al*., 2010) that may be important in successful development of *B. glabrata* embryos (Baron *et al*., 2013). The retention of hemocyanin sequences in *B. glabrata,* in the presence of hemoglobin, hints at a possible switch in function; <u>chapter four, through the use of RNA interference, establishes that Hcl-1 is important for reproductive output of *B. glabrata* snails, with knockdown stopping embryo development within 72 hours after egg laying.</u>

SIGNIFICANCE

This dissertation is significant from basic and applied perspectives. Planorbidae snails are an evolutionary enigma, unique in their employment of hemoglobin. All other sister-taxa only utilize hemocyanin. We provide insight into the dynamic history of the planorbid snail family by strengthening the notion that hemocyanin-like 1 derived from an ancestral respiratory hemocyanin, likely after the advent of hemoglobin. Relaxation of selective pressures to maintain respiratory function likely resulted in an example of gene repurposing, analogous to the fate of superfluous genes after a duplication event (Katju, 2012; Kondrashov, 2012). Understanding potential drivers or documenting occurrences that lead to gene repurposing can give insight into shared processes of genetic selective pressure that manage the fate of duplicated genes as part of genome evolution across phyla.

From an applied perspective, molluscicides that are employed in the field for schistosomiasis control have broad adverse effects upon many organisms in the aquatic ecosystems where they are utilized. Development of novel molluscicides is continuously needed, specifically in Brazil, where strengthening awareness of environmental preservation has led to a decreased use of the WHO-approved molluscicide for schistosomiasis control, Niclosamide (Li & Wang, 2017). This dissertation provides insight of a unique planorbid factor important for successful reproductive output of *B. glabrata*. This study opens the door to future development of an alternative molluscicide that can target Hcl-1 to potentially halt reproductive output of schistosome-transmitting planorbid snails.

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CHAPTER 2

THE PLANORBID SNAIL *BIOMPHALARIA GLABRATA* EXPRESSES A HEMOCYANIN-LIKE SEQUENCE IN THE ALBUMEN GLAND

ABSTRACT

The parasitic flatworm *Schistosoma mansoni*, causative agent of human intestinal schistosomiasis in South America, relies importantly on the freshwater snail *Biomphalaria glabrata* as intermediate host to achieve development of cercariae that infect humans. The recommendation from the World Health Organization (WHO) to integrate snail control in efforts to counter schistosomiasis transmission provides impetus for in depth study of *B. glabrata* biology. Our analysis indicates that two distinct hemocyanin-like genes (hcl-1 and hcl-2) are present in *B. glabrata*, a snail that uses hemoglobin for oxygen transport. Characterization of BAC clones yielded the full length hcl-1 gene, which is comprised of three functional unit (FU) domains at the amino acid level. Database searches and in silico analyses identified the second hcl gene (hcl-2), composed of six FU domains. Both genes are unusual for lacking canonical residues and having fewer FU domains than typical molluscan hemocyanins that contain 7–8 FUs. Reverse transcription PCR demonstrated that Hcl-1 is expressed in a manner that correlates with reproductive maturity in the albumen gland (AG), an immune- and reproduction-relevant organ. Immune cross-reactivity with anti-keyhole limpet hemocyanin (α-KLH) antiserum and tandem-mass spectrometry validated the presence of Hcl-1 protein in the AG and egg mass fluid (EMF). The evolutionary conservation of hemocyanin-like sequences in *B. glabrata* in the presence of the oxygen carrier hemoglobin, combined with our results, suggest that the Hcl-1protein has a functional role in general and/or reproductive biology. Further investigations are needed to explore Hcl-1 as a potential target for snail control.

INTRODUCTION

Human schistosomiasis is caused by parasitic blood flukes of the genus

Schistosoma that utilize freshwater snails as intermediate hosts for development and

asexual reproduction to progress to cercariae, the human infective stage. This parasitic

disease afflicts 208 million people worldwide and leaves 600 million at risk in endemic

areas [1]. *Schistosoma mansoni*, the causative agent of intestinal schistosomiasis in

endemic regions of South America, relies importantly on the planorbid snail

B*iomphalaria glabrata* as a major snail intermediate host for transmission of infection to

humans [2] and related *Biomphalaria* species transmit *S. mansoni* in Sub-Saharan Africa

[3]. The World Health Organization (WHO) recommends integrated control of both

parasite and snail intermediate host to reduce disease transmission [4]. In absence of

available vaccines [5], current approaches include mass-drug administration (MDA) [6],

management of snail habitats [7], use of molluscicides [8, 9], biological control of snails

[10, 11, 12] and public health education [13]. Unfortunately, MDA does not protect

against re-infection, hampering long-term control of schistosomiasis in parasite-endemic

regions [14]. Negative ecological impact may limit general application of non-specific

molluscicides [15].

Long-term, sustainable disease control requires further knowledge of biology of

the snail *B. glabrata* in order to develop and integrate alternative control methods to

counter the intermediate host and thereby transmission of schistosomiasis. *Biomphalaria*

glabrata belongs to the family Planorbidae; ramshorn snails that use iron-containing

hemoglobin to transport oxygen through their hemolymph, giving them red pigmented

blood [16]. This is unusual since other gastropods (basal taxa and sister families) do not

have hemoglobin but employ hemocyanin, a blue-pigmented protein that uses copper to bind oxygen for transport [17]. It was postulated that *B. glabrata* hemoglobin has evolved through gene duplications and fusion involving myoglobin, a protein crucial in oxygenation of muscle tissue [16]. Although hemocyanin is an efficient oxygen carrier [18]; hemoglobin is thought to possibly provide planorbid snails a greater diving capacity that may offer an evolutionary advantage over other snails that employ hemocyanin [19]. The advent of hemoglobin has not expunged all traces of hemocyanin; partial hemocyanin-like EST sequences have been recorded from *B. glabrata* and a hemocyanin-like protein ultrastructure was visualized from the hemolymph [16], and in mantle rhogocytes [20]. Hathaway et al. [21], using mass spectrometry, detected the presence of hemocyanin-like peptides in the albumen gland (AG) and egg mass fluid (EMF) of *B. glabrata*. A microarray study indicated increased expression of hemocyanin-like transcripts in immune-challenged *B. glabrata* [22]. The above raises a question regarding the origin and functional significance of hemocyanin-like sequences by *B. glabrata* in the presence of hemoglobin.

Molluscan hemocyanins belong to the copper type 3 protein superfamily. The functional unit (FU) domains of copper type 3 proteins have an active site that contains six canonical histidines that bind two copper atoms. Generally, molluscan hemocyanins are composed of monomers that have 7–8 FU domains that each have unique sequence features and are categorized by letters A-H [17]. Ten monomeric subunits multimerize to form a functional didecamer hemocyanin protein. The members of the copper type 3 protein superfamily, that also includes single-domain enzymes such as phenoloxidases and tyrosinases, may have one of a variety of biological functions ranging from pigment

formation, innate immunity, reproduction and, in the case of hemocyanin, oxygen transport [23–26]. Some molluscan and crustacean hemocyanins, which resulted independently from different subclasses of copper type 3 proteins through convergent evolution, are reported to have phenoloxidase activity in vitro[27–29], allowing them to produce melanin, an important compound involved in immunity of invertebrates[30] and in pigmentation [31].

This study explores the origins of hemocyanin-like sequences in *B. glabrata* and identifies two different hemocyanin-like genes (hcl-1 and hcl-2). *Biomphalaria glabrata* hcl-1 is characterized further because its presence in both the AG and EMF indicates a putative involvement in reproduction, and/or immunity and reveals insights into gastropod biology.

MATERIALS AND METHODS

Snails

Biomphalaria glabrata snails of the M line and BB02 strains and of two field isolates, referred to as VG2 and VG3, are maintained at the University of New Mexico [32]. Snails were housed in tanks containing artificial spring water [33] at 26°C and fed red leaf lettuce ad libitum and chicken pellets weekly. VG2 and VG3 were collected in 2009 from Virgem das Graças, Minas Gerais, Brazil, and identified as B. glabrata based on 16S, CO1 and ND1 sequences (JQ886405-10). The BB02 strain has been used to characterize the *B. glabrata* genome (VectorBase; www.vectorbase.org; assembly BglaB1).

Full length sequencing and computational analysis of hcl-1

Expressed sequence tags (CN779709, CN779680, CN655026, CN549169) obtained from M line *B. glabrata* [16] containing hemocyanin-like sequences were used to screen the BBO2 genomic BG_BBa BAC library [34]. Of several positive BAC clones, two contiguous inserts (BG_BBa50I14 and BG_BBa28B18) were sequenced to obtain a full length hemocyanin-like gene (hcl-1). Sequencing was performed in house at the Molecular Biology Facility at the University of New Mexico and commercially at The Genome Institute at Washington University, Saint Louis, MO. Sequence reads were assembled using Sequencher 5.1 (GC Codes). BAC insert sequences were submitted to GenBank (KU682269, KU682270). Open reading frame and BLAST analyses were used for in silico prediction of intron/exon splice junctions, also considering canonical CAG/GT motifs. The hcl-1 gene sequence was screened to identify the 5'UTR, Kozak sequence, start codon, stop codon and polyadenylation signal (AATAA). SignalP V 4.1 [35] was used for signal peptide prediction. The in silico assembled and annotated sequence was used to design primers for experimental confirmation with RT-PCR of cDNA sequence in BB02, M line, VG2 and VG3 strains of B. glabrata. Protein mass was predicted for Hcl-1 with the software Compute pI/Mw tool [36] for protein characterization. Public databases (GenBank and VectorBase) were also scanned with Hcl-1 for presence of additional hemocyanin-like proteins. Copper type 3 proteins from *B. glabrata* were aligned (ClustalW; http://www.genome.jp/tools/clustalw/) with additional gastropod hemocyanins and a tyrosinase from a bivalve (See legend Fig 3 for accession numbers). A gene-tree was generated using MEGA 6 [37], (Neighbor-joining) using default parameters and the software suggested model for evolution (Poisson correction method).

RNA extraction

For various experiments, RNA was extracted from adult *B. glabrata* snails (10–15

mm shell diameter), juveniles (4–9 mm), pooled newly hatched snails (within 2 days of

emergence), and pooled embryos from whole egg masses. The shell was removed from

individual snails to obtain whole body tissues, with exception of the newly hatched

snails. RNA from snail embryos was extracted from whole egg masses. Samples were

disrupted using a plastic pestle (Kontes RNase-free) in 1 mL of Trizol-Reagent

(Invitrogen) and processed according to manufacturer's instructions. The RNA was

treated with TURBO DNA-free (Ambion) to remove residual genomic DNA. The RNA

was quantified spectrophotometrically using the NanoDrop 2000c (Thermo Scientific).

Quality was checked using 2100 Bioanalyzer with the RNA Nano Kit (Agilent).

Reverse transcription-PCR and sequencing

RNA from individual BB02, M-line, VG2 and VG3 snails was reverse transcribed

to cDNA (Omniscript RT Kit, Qiagen), using 2 μg of RNA per reaction, with an oligo

(dT) primer. AmpliTaq Gold (Applied Biosystems, Life Technologies) was used for

PCR, employing Hcl-1 primers that span introns, designed from the in silico transcript

prediction (S1 Table) and 1μL of RT-reaction. The *B. glabrata* eukaryotic translation

initiation factor eIF2α (GenBank KF648316) (Primers; F-

CCTGCAGGTTCTACAAAACA; R-TATCCTTACCTTTATCTTTGTCTACTC) was

used as positive control. Negative controls consisted of omitting the reverse transcription

step, and using water instead of template. The cycling conditions were as follows: 95°C

for 10 min for initial denaturation; 25 cycles at: 95°C for 60 s, 30 s at annealing

temperature (depending on primers used), 72°C for 60 s; then 72°C for 7 min for final

extension, ramping rate set at 1°/s. RT-PCR amplicons were purified (QIAquick PCR Purification Kit, Qiagen) and sequenced (BigDye® Direct Cycle Sequencing Kit v3.1, Applied Biosystems, Life Technologies) using amplicon-specific primers. Extension products were read using a ABI 3100 sequencer. Sequences were edited by eye and aligned using Sequencher 5.1(GC Codes).

Tissue specific expression of Hcl-1

The following tissues were dissected from an adult (14 mm shell diameter) M line snail; albumen gland, mantle, stomach, hepatopancreas, and ovotestis. RNA was extracted using Trizol-Reagent (Invitrogen, Carlsbad, CA). RNA quality checks and RT-PCR were performed as above, using Hcl-1 specific primers for a 440 bp amplicon with eIF2α as positive control. Amplicon presence was confirmed using 1% agarose gels stained with GelRed (Biotium). Two replicate experiments were performed with independent tissue samples.

Hcl-1 expression during ontogeny

RNA was extracted from M line snails at different developmental stages; newly laid egg masses (48hrs), embryos with shell (96 hrs); newly hatched snails (≤2 day), and snails of 4, 8, 9, 10, 11, 12, and 15 mm in shell diameter. To investigate expression, RT-PCR with Hcl-1 specific primers targeting a 757 bp sequence fragment were used, with eIF2α as positive control. Detection of amplicons was performed as mentioned above.

Immunodetection and mass spectrometry

The shell of an adult M line snail was crushed between glass slides and 10 μL of hemolymph were collected and centrifuged to remove cells (5 min, 15000g). The hemolymph supernatant was placed in Laemmli sample buffer (Bio-Rad) with increased

SDS content of 20% (w/v), boiled for 5 minutes and kept on ice until use. The AG was dissected from adult M line snails (14–15 mm diameter), and individually disrupted with a pestle in 100 μl of Laemmli sample buffer with an increased SDS content of 20% (w/v) [21], and boiled for 5 minutes. The perivitteline fluid surrounding the embryos, referred to as egg mass fluid (EMF), was collected from snail eggs and processed according to Hathaway et al. [21]. All samples were spun to remove debris prior to gel electrophoresis.

The protein components of AG, EMF, and hemolymph were separated by SDS-PAGE (Mini-Protean II system, Bio-Rad). The 1.0 mm thick gel consisted of a 4% acrylamide stacking gel (0.1% SDS in 0.125 M Tris-HCl pH 6.8) and a 5–20% gradient acrylamide separating gel (0.1% SDS in 0.375 M Tris-HCl pH 8.8) [32]. To increase resolution of large proteins, electrophoresis was continued until the 50kDa marker band (Precision Plus Protein Kaleidoscope, Bio-Rad) ran off the gels (~3 hours). Protein profiles were transferred to a nitrocellulose membrane using the Trans-Blot SD Semi-dry system (Biorad). The membrane was incubated in blocking solution (0.1% Tween 20 in 20mM Tris pH 7.5, 150mM Nacl, 5% w/v nonfat dry milk) for 1 hr, washed 3X in TBS and incubated with polyclonal α-KLH (anti-Keyhole Limpet hemocyanin; Sigma H0892) at a 1:1000 dilution overnight at 4°C. The membrane was washed and treated with AP-conjugated goat α-rabbit as secondary antibody to detect antiserum reactive protein bands with NBT/BCPI as substrate. Alternatively, SDS-PAGE gels were rinsed with DI water, stained in Coomassie blue G250 (BioRad) and photo-documented.

For mass spectrometry, Coomassie blue stained bands of 150 kDa in the AG and EMF were excised from gel, destained, and digested with trypsin, for details see [38]. Peptide mass fingerprints (PMFs) were obtained on a matrix assisted laser desorption

ionization tandem time-of-flight (MALDITOF-TOF) mass spectrometer (Ultraflex II; Bruker Daltonics Inc., Billerica, MA, USA). Porcine trypsin was used for internal mass calibration. Abundant peptides were subjected to tandem MS (MS/MS) to obtain information about the β- and γ-ions of the peptide sequence with a default setting of a precursor-ion mass tolerance of 0.6 Da recommended for MASCOT [38]. To identify proteins, we used Mascot (version 2.2; Matrix Science Inc., Boston, MA, USA) and combined PMFs and MS/MS in a search against the protein sequences of Hcl-1, Hcl-2 and proteins predicted from six frame translation of all *B. glabrata* ESTs in dbEST (NCBI). Our search allowed one missed cleavage during trypsin digestion. The molecular weight search (MOWSE) scores that indicated significant hits ($p < 0.05$) were 21 for EMF and 26 for AG. No other peptide sequences yielded significant hits against B. glabrata. After the above approach yielded positive identification of Hcl-1, the default precursor–ion tolerance was relaxed to 1 Da to investigate whether additional PMF -recovered peptide masses matched in silico predicted tryptic fragments of Hcl-1 (PeptideMass software, [39]).

RESULTS

hcl-1 *and* hcl-2 *gene structure*

A *B. glabrata* hemocyanin-like gene (hcl-1) encoding three FUs that were separated by two characteristic linker sequences was identified by sequencing of B. glabrata BB02 BAC clones (KU682269 and KU682270; Fig 1). Computational analysis
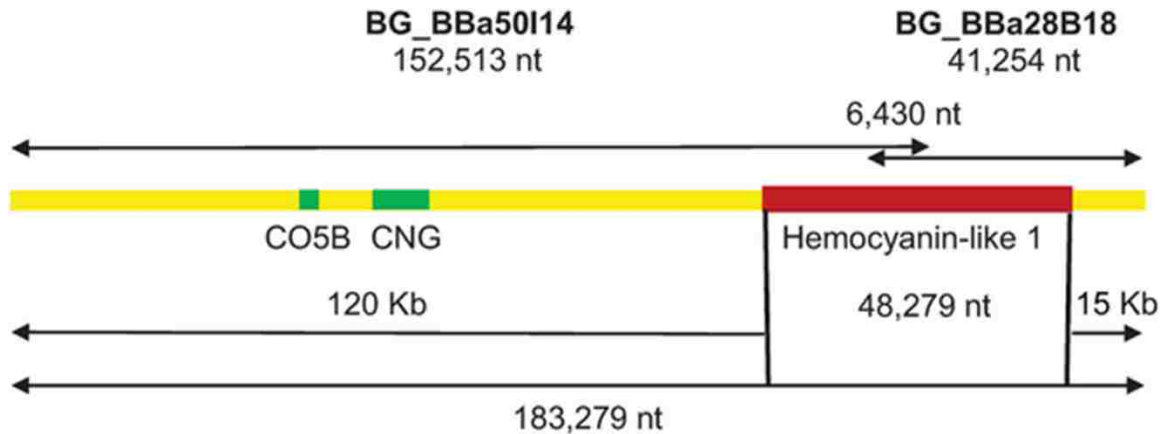
Fig 1. Genomic sequencing of hcl-1. Two contiguous BACs (BG_BBa 50I14 and BG_BBa 28B18) yielded a complete hemocyanin-like 1 gene (hcl-1). Additional genes were identified upstream of hcl-1 with BLAST similarity to CO5B (cytochrome oxidase subunit 5B) and CNG (cyclic nucleotide-gated olfactory channel-like isoform). https://doi.org/10.1371/journal.pone.0168665.g001

revealed a gene structure consisting of 23 exons. The predicted coding sequence was experimentally obtained, yielding a cDNA transcript of 3981 nt (KU682271). The hcl-1 gene model was annotated and is available in VectorBase (LGUN_ random _ scaffold 490; BGLB000218_RA). Inspection of predicted splice sites showed that intron phases were variable throughout the gene (Fig 2; S2 Table) and identified 11 non-consensus splice sequences (other than CAG), at the 3' termini of introns. As is typical for molluscan hemocyanin gene sequences [40], the linker sequences in the hcl-1 gene are preceded by phase 1 introns (interrupted after the 1st nucleotide of a codon; Fig 2; S2 Table).

BLAST searches with the predicted amino acid sequence of Hcl-1 identified a second hemocyanin-like gene (denoted hcl-2) in *B. glabrata*. The hcl-2 gene (~75000 nt) was reconstructed and annotated using genomic and RNA-seq data from the *B. glabrata* genome (NCBI Taxonomy ID 6526, and VectorBase assembly;
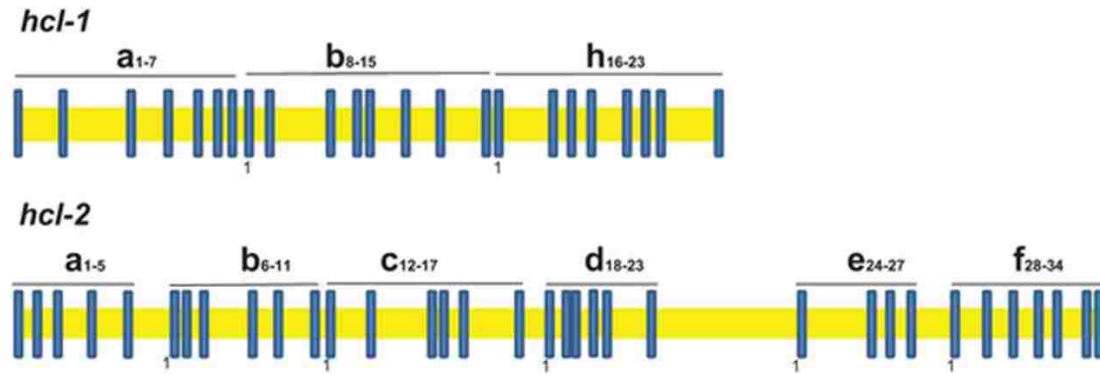
Fig 2. Intron-exon structure of hemocyanin-like genes of *B*. *glabrata*. The letters a-h designate the groups of exons that combine to encode a particular FU. The first exon of each FU is denoted with a one to indicate the conserved phase one intron upstream of the exons encoding the linker sequences that connect FUs. The FUs were annotated through comparisons with other gastropod hemocyanins. https://doi.org/10.1371/journal.pone.0168665.g002

LGUN_random_scaffold1005 28349–90182). Analysis of hcl-2 gene structure revealed 34 exons, yielding a predicted transcript of 6,027 nt, corresponding to six FUs, separated by linker sequences. The exons encoding linker sequences in the hcl-2 gene are also preceded by phase one introns (Fig 2; S3 Table), similar to hcl-1.

Hcl-1 and Hcl-2 are more similar to molluscan hemocyanins than to tyrosinases

Scanning of the *B. glabrata* genome assembly using Hcl-1 amino acid sequence revealed additional hits for putative copper type 3 proteins. Four complete gene sequences, each consisting of a single copper type 3 domain were described (by automated annotation) as putative *B. glabrata* tyrosinases (S1 Fig). Phylogenetic comparison of FU domain sequences of all these copper type 3 proteins indicated that Hcl proteins group with molluscan hemocyanins and identified FU domains A, B, H in Hcl-1 and A-F for Hcl-2. Both Hcl proteins grouped separately from the putative molluscan single-domain tyrosinases (Fig 3).

Amino acid sequences for Hcl-1 and Hcl-2

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The amino acid sequences for Hcl-1 and Hcl-2 are unusual compared to other molluscan hemocyanins. Generally, molluscan hemocyanins have seven or eight FU domains denoted A-H and each FU contains six canonical histidines that are required to form the copper-binding motif. Hcl-l has three FU domains classified as A, B, and H (Figs 2–4); each FU contains a conserved amino acid replacement of a canonical histidine. Hcl-2 consists of six FU domains (A-F; Figs 2 and 3), of which four FUs have one or more replacements of canonical histidines. The terminal domain F is incomplete, representing only half of a typical molluscan FU.

Experimentally obtained cDNA sequences confirmed that four different *B. glabrata* strains all have identical histidine replacements in Hcl-1. Using the BB02 Hcl-1 sequence as a reference, we identified additional differences in non-canonical amino acids among strains; six replacements occur in M line, six in VG2 and seven in VG3 (Fig 4).

Hcl-1 is expressed in the albumen gland of B. glabrata in an age-dependent manner

Among the tissues tested, Hcl-1 mRNA was only expressed in the albumen gland (Fig 5). This correlates with Hathaway et al [21], who detected hemocyanin-like peptides in the AG and EMF of *B. glabrata*. Hcl-1 is expressed in an age-dependent manner, commencing only after *B. glabrata* snails attain a size of 10 mm or greater (Fig 6), which is associated with reproductively mature snails.
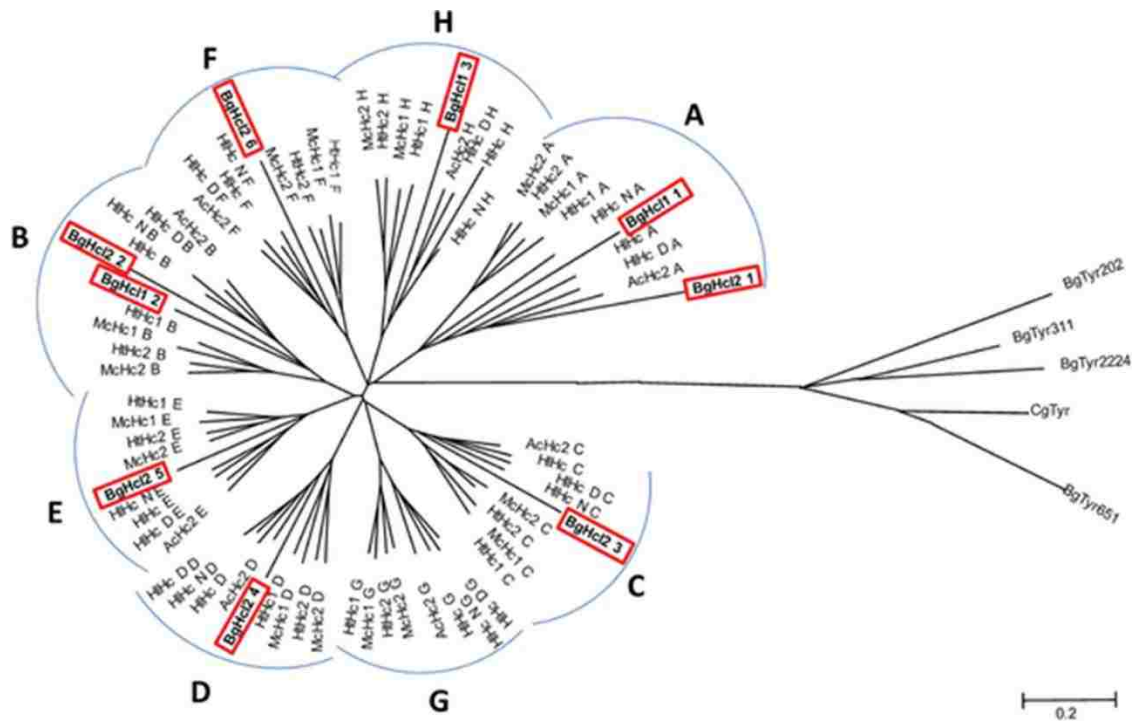
Fig 3. Identification of categories of functional unit domains by phylogenetic reconstruction.
Generally, molluscan hemocyanins are composed of monomers that have 7–8 FU domains that each have unique sequence features and are categorized by letters A-H [17]. To better understand the structures of the Hcl proteins of B. glabrata and their relationship to other copper type 3 proteins, a phylogenetic comparison of FU domains was performed to infer the categories of FU domains present in Hcl-1 and Hcl-2. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 73 amino acid sequences of FU domains from hemocyanins of 5 different gastropods; 2 vetigastropods (Megathura crenulata hemocyanin, McHc1 [CAG28309], McHc2 [CAG28308]; Haliotis tuberculata hemocyanin, HtHc1 [CAC20588], HtHc2 [CAC82192]), 3 heterobranchia (Helix lucorum hemocyanin, HlHc [AEO51766], HlHcD [AEO51767], HlHcN [AEO51768]; Aplysia californica hemocyanin, AcHc [CAD88977]; B. glabrata hemocyanin-like, BgHcl-1, BgHcl-2. Four B. glabrata predicted tyrosinase sequences (Tyr202 [XP_013065892], Tyr311 [XP_013078946], Tyr651 [XP_013065864], Tyr2224 [XP_013080599]) and a tyrosinase from the bivalve Crassostria gigas (CaTyr [XP_011420307]) were used for comparison against FU sequences from non-hemocyanin copper type 3 proteins. ClustalW (http://www.genome.jp/tools/clustalw/) was used for initial alignment; alignment screening and construction of the neighbor-joining tree were done in MEGA6 [37].
https://doi.org/10.1371/journal.pone.0168665.g003

Hcl-1 protein is present in the albumen gland (AG) and egg mass fluid (EMF)

The Coomassie stained gel profiles indicate differences in the protein composition between the samples. Yet, both AG and EMF contain an α-KLH antiserum reactive protein band at 150 kDa (Fig 7), the predicted molecular weight of Hcl-1. The protein profiles of Hemolymph and AG of *B. glabrata* showed reactivity with α-KLH at 250 kDa, the predicted molecular weight for Hcl-2 (Fig 7). The α-KLH polyclonal antibodies

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also cross-reacted with several protein bands of ~50kDa that were not analyzed further in this study.

Consideration of combined peptide mass fingerprinting (PMF) and tandem MS data from the trypsin-digested 150kDa protein bands yielded three peptide sequences (two shared between both samples, one only from EMF) that led to positive identification of Hcl-1 ($p < 0.05$). At a 1 Da precursor-ion tolerance, from 85 mass values recorded from AG and 73 from EMF, another eleven peptide masses from PMF matched *in silico* predicted tryptic peptide fragments of Hcl-1 (Fig 4; S5 Table). None of the peptides recovered matched the Hcl-2 sequence.

Discussion

The red-pigmented blood of *B. glabrata* underscores the fact that snails of the family Planorbidae are unique among gastropods in that they have replaced the ancestral (blue) hemocyanin as an oxygen-carrier with secondarily evolved hemoglobin. Nonetheless, hemocyanin-like (cDNA and peptide) sequences as well as a large blood protein that resembles the characteristic ultrastructural shape of multimeric molluscan hemocyanin have been described from *B. glabrata* [16, 20, 21]. We conclude that these observations do not stem from a respiratory molluscan hemocyanin, but rather, find a basis in two novel hemocyanin-like genes, termed hcl-1 and hcl-2.

The large hemolymph protein of *B. glabrata* with a hemocyanin-like shape [16, 20] originates from the hcl-2 sequence. An antiserum raised against the heterologous keyhole limpet hemocyanin (KLH) recognizes a *B. glabrata* hemolymph protein with same ~250 kDa molecular weight that is predicted from the amino acid sequence of Hcl-2. Additionally, the gene sequence of hcl-2 encodes a protein that comprises six FU

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Ht    --LVQFLLVALVVGAGADNVVRKDVSHLTVDEVQALHGALHDVTASTGP-LSFEDITSYHAAPASCDYKGRKIACCVHGMPSFPFWHRAYVVQAERALLSKRKTVGMPYWDW
BB02  MGTSRVLVLTTLVYLCRGLILRKDIDSLTSEDTINLRLSLQGVKYEYQLKKSYSYIASFYGYPTRCSVGNVAYSCSVHGMPTFEQWHRLYLAHLEQALTEKGGTVGIPYWDW
Mline MGTSRVLVLTTLVYLCRGLILRKDIDSLTSEDTINLRLSLQGVKYEYQLSYSYIASFTGYPTRCSVGNVAYSCSVHGMPTFEQWHRLYLAHLEQALTEKGGTVGIPYWDW
VG2   MGTSRVLVLTTLVYLCRGLILRKDIDSLTSEDTIVNLRLSLQGVKYEYQLKKSYSYIASFYGYPTRCSVGNVAYSCSVHGMPTFEQWHRLYLAHLEQALTEKGGTVGIPYWDW
VG3   MGTSRVLVLTTLVYLCRGLILRKDIDSLTSEDTINLRLSLQGVKYEYQLKKSYSYIASFYGYPTRCSVGNVAYSCSVHGMPTFEQWHRLYLAHLEQALTEKGGTVGIPYWDW
                                                      1                                                          2

Ht    TQTLTHLPSLVTEPIYIDSKGGKAQTNYWYRGEIAFINKKTARAVDDRLFEKVEPGHYTHLMETVLDALEQDEFCKFEIQFELAHNAIHYLVGGKFEYSMSNLEYTSYDPIF
BB02  SKPLQKMEAFLDDEKYNLEGEILDNPWHHTNISLSGVIHATNRTVDSRLWS-------LDLMEHIIHALEYPNYCQFVVQLEVLHSAIHFLVGGASKYSMSNIDFAAYDPLF
Mline SKPLQKMEAFLDDEKYNLEGEILDNPWHHTNISLSGVIHATNRTVDSRLWS-------LDLMEHIIHALEYPNYCQFVVQLEVLHSAIHFLVGGASKYSMSNIDFAAYDPLF
VG2   SKPLQKMEAFLDDEKYNLEGEILDNPWHHTNISLSGVIHATNRTVDSRLWS-------LDLMEHIIHALEYPNYCQFVVQLEVLHSAIHFLVGGASKYSMSNIDFAAYDPLF
VG3   SKPLQKMEAFLDDEKYNLEGEILDNPWHHTNISLSGVIHATNRTVDSRLWS-------LDLMEHIIHALEYPNYCQFVVQLEVLHSAIHFLVGGASKYSMSNIDFAAYDPLF
                                3   4                                                    5

Ht    FLHHSNVDRLFAIWQRLQELRGKNP------NAMDCAHELAHQQLQPFNRDSNPVQLTKDHSTPADLFDYKQLGYSYDSLNLNGMTPEQLKTELDERHSKERAFASFRLSGF
BB02  LVHHANLDRIYEVYEALYRERGSVPGTSCETDCEICDIKGFQMPLEPFNRDDNPFPNTRLLATGWNMTDKTVFDYNYDSLTFNGLGIADIKKRIEMKKKTDRAFAVFKLNGI
Mline LVHHANLDRIIYEVYEALYRERGSVPGTSCETDCEICDIKGFQMPLEPFNRDDNPFPNTRLLATGWNMTDKTVFDYNYDSLTFDGLGIADIKKRIEMKKKTDRAFAVFKLNGI
VG2   LVHHANLDRIYEVYEALYRERGSVPGTSCETDCEICDIKGFQMPLEPFNRDDNPFPNTRLLATGWNMTDKTVFDYNYDSLTLNGLGIADIKKRIEMKKKTDRAFAVFKLNGI
VG3   LVHHANLDRIYEVYEALYRERGSVPGTSCETDCEICDIKGFQMPLEPFNRDDNPFPNTRLLATGWNMTDKTVFDYNYDSLTLKGLGIADIKKRIEMKKKTDRAFAVFKLNGI
           6                                                                            6     7  8           9

Ht    GGSANVVVYACVPDDDPRSDDYCEKAGDFFILGGQSEMPWRFYRPFFYDVTEAVHHLGVPLSGHYYVKTELFSVNGTALSPDLLPQPTVAYRPGKGHL-—PPVHHRHDDDL-
BB02  QRSVNLRIQVCKTSSEDE-EDTCESAGDVFILGGSTEHPWMFRRPYYHDITKAVLKLGLKLDENFRVLTEMYGTDDKINSSEISPQPSVEFRPAVGKQD-APLSEKKKDVI-
Mline QRSVNLRIQVCKTSSEDE-EDTCESAGDVFILGGSTEHPWMFRPYYHDITKAVLKLGLKLDENFRVLTEMYGTDDKINSSEISPQPSVEFRPAVGKQD-APLSEKKKDVI-
VG2   QRSVNLRIQVCKTSSEDE-EDTCESAGDVFILGGSTEHPWMFRRPYYHDITKAVLKLGLKLDENFRVLTEMYGTDDKINSSEISPQPSVEFRPAVGKQD-APLSEKKKDVI-
VG3   QRSVNLRIQVCKTSSEDE-EDTCESAGDVFILGGSTEHPWMFRRPYYHDITKAVLKLGLKLDENFRVLTEMYGTDDKINSSEISPQPSVEFRPAVGKQD-APLSEKKKDVI-
                                      10                                    11

Ht    VRKNIDHLTREEEYELRMALERFQADTSVDGYQATVEYHGLPARCPRP--DAKVRFACCMHGMASFPHWHRLFVTQVEDALVRRGSPIGVPYWDWTKPMTHLPDLASNETYV
BB02  IRQDVDLLTEDEMNALRVAMENVQNNGTQNGYQAIAAHHGAPGQCPTPNPDVALTYSCSIRGLPSFPHWHRLFVMQLEDSLG---LSTGIPYWDWTKPGVQLPNLVKDATYQ
Mline IRQDVDLLTEDEMNALRVAMENVQNNGTQNGYQAIAAHHGAPGQCPTPNPDVALTYSCSIRGLPSFPHWHLFVMQLEDSLG---LSTGIPYWDWTKPGVQLPNLVKDATYQ
VG2   IRQDVDLLTEDEMNALRVAMENVQNNGTQNGYQAIAAHHGAPGQCPTPNPDVALTYSCSIRGLPSFPHWHRLFVMQLEDSLG---LSTGIPYWDWTKPGVQLPNLVKDATYQ
VG3   IRQDVDLLTEDEMNALRVAMENVQNNGTQNGYQAIAAHHGAPGQCPTPNPDVALTYSCSIRGLPSFPHWHRLFVMQLEDSLG---LSTGIPYWDWTKPGVQLPNLVKDATYQ

Ht    DPYG-HTHHNPFFNANISFEEGHHHTSRMIDSKLFAPVAFGEHSHLFDGILYAFEQEDFCDFEIQFELVHNSIHAWIGGSEDYSMATLHYTAFDPIFYLHHSNVDRLWAIWQ
BB02  IKDDGDSPKANPFYDAAIEFLRTGSRTSRSWPEQGVN------LDDLKDAVLLALEQDNFCDFEVQFEIAHNLIHALVGGNAPYGMSSLEYSAYDPIFYIHHSFLDKIWSIWM
Mline IKDDGDSPKANPFYDAAIEFLRTGSRTSRSWPEQGVN------LDDLKDAVLLALEQDNFCDFEVQFEIAHNLIHALVGGNAPYGMSSLEYSAYDPIFYIHHSFLDKIWSIWM
VG2   IKDDGDSPKANPFYDAAIEFLRTGSRTSRSWPEQGVN------LDDLKDAVLLALEQDNFCDFEVQFEIAHNLIHALVGGNAPYGMSSLEYSAYDPIFYIHHSFLDKIWSIWM
VG3   IKDDGDSPKANPFYDAAIEFLRTGSRTSRSWPEQGVN------LDDLKDAVLLALEQDNFCDFEVQFEIAHNLIHALVGGNAPYGMSSLEYSAYDPIFYIHHSFLDKIWSIWM
         12

Ht    ALQIRRHKPYQAHCAQSVEQLPMKPFAFPSPLNNNEKTHSHSVPTDIYDYEEVLHYSYDDLTFGGMNLEEIEEAIHLRQQHER-VFAGFLLAGIGTSALVDIFINKPGNQPL
BB02  SLQELRGKPYKAHCAQSYIFTLPSPNFSTTYNPNPKTYAHSTATNIYDHEKELGYTYDTLTFDGMNITELEHFIRFNVTSRPRMFVGVLLNGFNKSAKAEIHATLHTGERY
Mline SLQELRGKPYKAHCAQSYIFTLPSPNFSTTYNPNPKTYAHSTATNIYDHEKELGYTYDTLTFDGMNITELEHFIRFNVTSRPRMFVGVLLNGFNKSAKAEIHATLHTGERY
VG2   SLQELRGKPYKAHCAQSYIFTLPSPNFSTTYNPNPKTYTHSTATNIYDHEKELGYTYDTLTFDGMNITELEHFIRFNVTSRPRMFVGVLLNGFNKSAKAEIHATLHTGERY
VG3   SLQELRGKPYKAHCAQSYIFTLPSPNFSTTYNPNPKTYTHSTATNIYDHEKELGYTYDTLTFDGMNITELEHFIRFNVTSRPRMFVGVLLNGFNKSAKAEIHATLHTGERY
                         13 14                      15                        16

Ht    KAGDIAILGGAKEMPWAFDRLYKVEITDSLKTLSLDVDGDYEVTFKIHDMHGNALDTDLIPHAAVVSEP//VHRGGNHEDEHHDDRLADVL--------------------
BB02  IVGRFAVLGGPTELGWRLDRLYKVDITKAMFDAHLSWNDLFELSIEMFEFNGVSIETDLPLLQLIYQAP--EDSEIETQPAL--------------------------
Mline IVGRFAVLGGPTELGWRLDRLYKVDITKAMFDAHLSWNDLFELSIEMFEFNGVSIETDLPLLQLIYQAP--EDSEIETQPAL--------------------------
VG2   IVGRFAVLGGPTELGWRLDRLYKLDITKAMFDTHLTWNDLFELSIEMFEFNGVSIETDLPLLQLIYQAP--EDSEIETQPAL--------------------------
VG3   IVGRFAVLGGPTELGWRLDRLYKVDITKAMFDTHLTWNDLFELSIEMFEFNGVSIETDLPLLQLIYQAP--EDSEIETQPAL--------------------------
                                              17        18

Ht    IRKEVDFLSLQEANAIKDALYKLQNDDSKGGFEAIAGYHGYPNMCPERGTDKYPCCVHGMPVFPHWHRLHTIQMERALKNHGSPMGIPYWDWTKKMSLPSFFGDSSNNNPFY
BB02  LRKNIQELTDGESNNLRDALKKLQSETSADNFENIAGFHGAPNRCPPHGSDRFACSPHGLPIFPHWHRLLTVQFEQALSRLGASWGIPYWDWTDESTLPKLFSDPEDN-PFY
Mline LRKNIQELTDDESNNLRDALKKLQSETSADNFENIAGFHGAPNRQPHGSNRFACSPHGLPIFPHWHRLLTVQFEQALSRLGASWGIPYWDWTDESTLPKLFSDPEDN-PFY
VG2   LRKNIQELTDDESNNLRDALKKLQSETSADNFENIAGFHGAPNRCPHGSNRFACSPHGLPIFPHWHRLLTVQFEQALSRLGASWGIPYWDWTDESTLPKLFSDPEDN-PFY
VG3   LRKNIQELTDDESNNLRDALKKLQSETSADNFENIAGFHGAPNRCPHGSNRFACSPHGLPIFPHWHRLLTVQFEQALSRLGASWGIPYWDWTDESTLPKLFSDPEDN-PFY
                   19  20       21              23        24             25 26 27         28

Ht    KYYIRGVQHETTRDINQRLFNQTKFGEFDYLYYLTLQVLEENSYCDFEVQYEILHNAVHSWLGGTGKYSMSTLEHSAFDPVFMIHHSSLDRIWILWQKLQKIRMKPYYALDC
BB02  RYYIQAEKEWTDREVNLKQLNLLDPEGTKMLFHSALSILEEDQFCDFAVQFELLHYRLHALMGGTKKYSLATLDFSAFDPLFMILQSSFDRLWTMWQQLQYLRQKTVSGQCV
Mline NYYIQAEKEWTDREVNLKQLNLLDPEGTKMLFHSALSILEEDQFCDFAVQFELLHYRLHALMGGTKKYSLATLDFSAFDPLFMILQSSFDRLWTMWQQLQYLRQKPVSGQCV
VG2   RYYIQAEKEWTDREVNLKQLNLLDPEGTKMLFHSALSILEEDQFCDFAVQFELLHYRLHALMGGTKKYSLATLDFSAFDPLFMILQSSFDRLWTMWQQLQYLRQKPVSGQCV
VG3   RYYIQAEKEWTDREVNLKQLNLLDPEGTKMLFHSALSILEEDQFCDFAVQFELLHYRLHALMGGTKKYSLATLDFSAFDPLFMILQSSFDRLWTMWQQLQYLRQKPVSGQCV
                                          29

Ht    AGDRLMKDPLHPFNYETVNEDEFTRINSFPSILFDHYRFNYEYDNMRIRGQDIHELEEVIQELRNKDRIFAGFVLSGLRISATVKVFIHSKNDTSHEEYAGEFAVLGGEKEM
BB02  Y--KHVDSSMEPFRNPDINVNKMTRENSLPGLVSDHRRLGYKFDKLNLNVFSLKDLEDKIKLQKSKNRTYAGLMLRGVKNSVTLEVYLQDNQ-------VGTVNILGGPNEV
Mline Y--HHVDSSMEPFRNPDINVNKMTRENSLPGLVSDHRRLGYKFDKLNLNVFSLKDLEDKIKLQKSKNRTYAGLMLGVKNSVTLEVYLQDNQ-------VGTINILGGPNEV
VG2   Y--KHVDSSMEPFRNPDINVNKMTRENSLPGLVSDHRRLGYKFDKLNLNVFSLKDLEDKIKLQKSKNRTYAGLMLGGVKNSVTLEVYLQDNQ-------VGTVNILGGPNEV
VG3   Y--KHVDSSMEPFRNPDINVNKMTRENSLPGLVSDHRRLGYKFDKLNLNVFSLKDLEDKIKLQKSKNRTYAGLMLGGVKNSVTLEVYLQDNQ-------VGTVNILGGPNEV
                            30

Ht    PWAYERMLKLDISDAVHKLHVK-DEDIRFRVVVTAYNGDVVTTRLSQPFIVHRPAHVAHDILVIPVGAGHDLPPKVVVKSGTKVEFTPIDSSVNKAMVELGSYTAMAKCIVP
BB02  PWVFERPYKIDVTDAMKGAQLTTDKPVKLHLKTGTYDGSSSSEKDMEVFIIERPSGSHHDILVVPINK-KNPPPALKVVVKKDTQVKFVTDDVVVPMKDFNTFTAWKACNLP
Mline PWVFERPYKIDVTDAMKGAQLTTDKPVKLHLKTGTYDGSSSSEKDMEVFIIERPSGSDHDILVVPINK-KTPPPALKVVVKKDTQVKFVTDDVVVPMKDFNTFTAWKACNLP
VG2   PWVFERPYKIDVTDAMKGAQLTTDKPVKLHLKTGTYDGSSSSEKDMEVFIIERPSGSDHDILVVPINK-KNPPPALKVVVKKDTQVKFVTDDVVVPMKDFNTFTAWKACNLP
VG3   PWVFERPYKIDVTDAMKGAQLTTDKPVKLHLKTGTYDGSSSSEKDMEVFIIERPSGSDHDILVVPINK-KNPPPALKVVVKKDTQVKFVTDDVVVPMKDFNTFTAWKACNLP
                                           TGTYDGSSSSEK               31                                      32

Ht    PFSYHGFELDKVYSVDHGDYYIAAGTHALCEQNLRLHIHVEHE.
BB02  PSLQGSYDFGAVNPLIPGNYYMSPADVDLCNRGIKIHIFVEEE.
Mline PSLQGSYDFGAEHPLIPGNYYMSPADVDLCNRGIKIHIFVEEE.
VG2   PSLQGSYDFGAVHPLIPGNYYMSPADVDLCNRGIKIHIFVEEE.
VG3   PSLQGSYDFGAVHPLIPGNYYMSPADVDPCNRGIKIHIFVEEE.
                               33
```

A

B

H

31

Fig 4. Alignment of Hcl-1 from four B. glabrata strains to the functional hemocyanin of H. tuberculata. The alignment includes Hcl-1 sequences of two lab strains (BB02 and M line) and two wild type snails (VG2 and VG3). Canonical histidines are highlighted in yellow, conserved replacements in those sites are highlighted in black. The amino acid linkers separating the functional units are underlined in all sequences. The signal peptide of Hcl-1 is boxed (note: the H. tuberculata hemocyanin gene [CAC20588] does not include a methionine start); grey highlights additional amino acid differences among B. glabrata strains; conserved replacements (relative to BB02) are denoted by the black triangles. Synonymous mutations are highlighted in blue; the numbers below the aligned sequences refer to details of specific mutations indicated in S4 Table (S4 Table). The peptide sequences matching to peptide masses detected by PMF analysis are boxed with a dashed line. Bold peptide sequences highlight the three peptides recovered by tandem MS (MS/MS). S5 Table (S5 Table) shows the protein sample (AG or EMF) of origin of the recorded peptides mass data. Peptides identified by MS analysis of 150 kDa proteins from AG and EMF in a previous study [21] that match the Hcl-1 sequence from B. glabrata snails are highlighted in pink.
https://doi.org/10.1371/journal.pone.0168665.g004
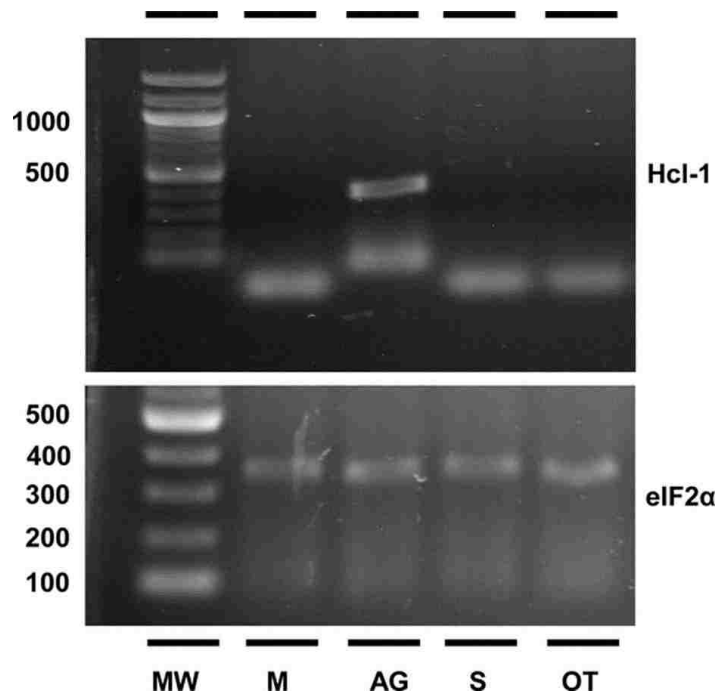
Fig 5. Hcl-1 is expressed in the albumen gland. Amplicons of 440 bp generated by RT-PCR using specific primers, (21F: TTGACGTTACTGACGCCATGA, 23R: AAGTCAAGTTTGTTACCGACGACG), demonstrate that Hcl-1 is only expressed in the AG. For positive control, eIF2α was used. MW: molecular weight, M: mantle, AG: albumen gland, S: stomach, and OT: ovotestis. https://doi.org/10.1371/journal.pone.0168665.g005

domains (A-F), two fewer than the eight FUs from bonafide molluscan hemocyanins.

This configuration is consistent with unusual aspects of the ultrastructural shape of the

hemocyanin-like blood protein of *B. glabrata*, resulting from the absence of the so-called

internal collar complex that is formed by the terminal FU domains G and H of regular

molluscan hemocyanins [17]. Kokkinopoulou et al. [20] provided structural evidence that

B. glabrata may express this protein (putatively encoded by hcl-2) in pore cells

(rhogocytes) of the mantle. The immune detection of a ~250kDa protein band also from

AG may indicate that Hcl-2 is also expressed in this organ, additional to rhogocytes [20].

Alternatively, this signal may be due to cross-reaction with an unrelated protein or the

blood circulation may cause a hemolymph protein as Hcl-2 to be present in the AG.

Relatively small amounts of the hemocyanin-like protein are present in the hemolymph,

compared to hemoglobin, such that it is less likely to function for oxygen-binding

alongside with hemoglobin [16, 20]. It may be expressed for a different purpose,

however, possibly related to the prophenoloxidase/tyrosinase activity of molluscan

hemocyanins [16].

The hcl-1 gene sequence characterized in this study was initially reported from

EST data and hemocyanin-like peptides recovered by mass spectrometry from *B.*

glabrata AG and EMF [16, 21]. Our immunoblot results with the anti-KLH antiserum

and novel, more extensive mass spectrometry data show that *B. glabrata* Hcl-1 is

expressed as a 150kDa protein, concordant with the predicted molecular weight of amino

acid sequence of the predicted mature protein. The hcl-1 sequence represents a novel type

of hemocyanin-like gene that has 3 FU domains, is expressed only in the AG and is

contributed to the EMF. Perhaps, after being replaced as respiratory pigment by the

evolutionary development of hemoglobin, the ancestral hemocyanin genes of planorbid

snails were repurposed to serve other functions, in analogy to functional diversification of
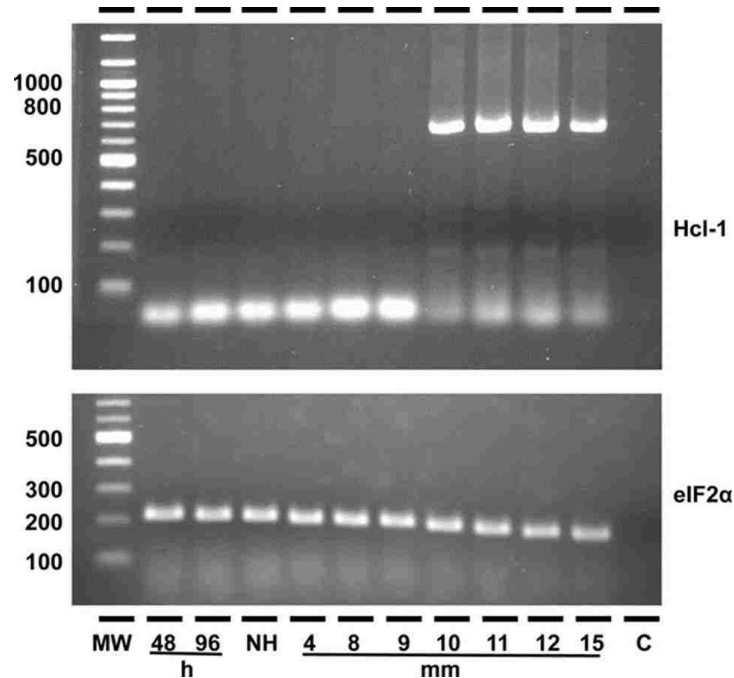
Fig 6. Hcl-1 is expressed in reproductively mature snails. Hcl-1 is only expressed in snails with a size of ≥10 mm. RT-PCR using Hcl-1 specific primers that span four introns (9F: TGATCCAATCTTTTACATTCATCAC, 13R: AAGTTGTCAGCGCTAGTCTCA) yielded an expected amplicon of 757 bp. eIF2α was used as positive control. MW: molecular weight, 48hrs, 96hrs, Nh: Newly hatched, 4, 8, 9, 10, 11, 12 and 15mm. C: water as negative control.
https://doi.org/10.1371/journal.pone.0168665.g006

duplicated genes [40–42]. In this analysis of these two hemocyanin-like genes of *B. glabrata*, we focus on an initial characterization of hcl-1 toward exploration of the hypothesis that hcl-1 may play a role in defense, reproduction and/or egg viability of *B. glabrata*.

Studies of other molluscs have revealed the general structure of hemocyanin genes to provide a frame of reference to track the evolution of hemocyanins [43–47]. Hemocyanin genes of cephalopods (*Octopus dofleini* & *Nautilus pompilius*; [43, 45], respectively), a bivalve (*Nucula nucleus*; [46]) and gastropods (e. g. *H. tuberculata*; [44]) share a characteristic feature; the exons that encode FUs and the downstream linker sequences are consistently connected by phase one introns. This shared feature was
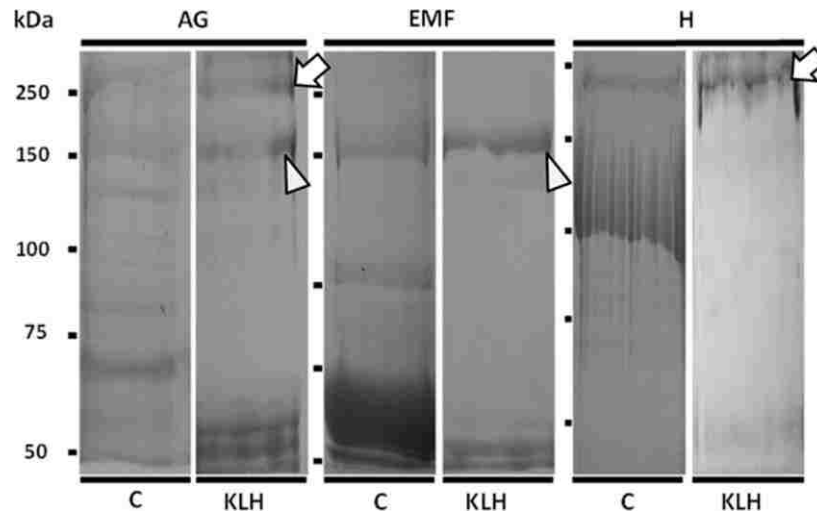
Fig 7. Hcl proteins in Biomphalaria glabrata. Protein samples of AG, EMF and hemolymph (H) were separated by 5–20% gradient SDS-PAGE and either stained with Coomassie (C) or probed with antiserum against Keyhole limpet hemocyanin (KLH). Electrophoresis was continued until the 50kDa marker reached the bottom of the gel to improve separation in the high molecular weight range. Coomassie staining shows the different protein compositions of the samples. Immunoblots show that the anti-KLH antiserum recognizes bands with the predicted molecular weight for Hcl proteins. Arrowheads indicate Hcl-1 (150kDa) in AG and EMF. Arrows indicate Hcl-2 (250 kDa) in the AG and H samples. The KLH antiserum also cross-reacted with smaller protein bands (~50 kDa) that were not analyzed in this study. Images shown are from separate gels, position of molecular weight markers in kDa is indicated with bars to the left of the images.
https://doi.org/10.1371/journal.pone.0168665.g007

proposed to serve a regulatory function and/or to facilitate duplication of single functional unit domains [43, 47]. Both hcl-1 and hcl-2 genes from *B. glabrata* also feature these phase one introns associated with linker-encoding sequences. This suggests that these genes are in fact evolutionarily related to oxygen-binding molluscan hemocyanins. The number of introns in the FU-encoding regions of the *B. glabrata* hcl genes is greater per FU than that of other traditional molluscan hemocyanins that have at most three introns within a FU [43–46, 48]. Intron gain and loss in orthologous genes is believed to occur at higher frequencies during major evolutionary transitions [49]. If hcl-1 and hcl-2 represent functionally diversified ancestral hemocyanins, the gain of introns associated with each FU may have aided in shaping a putative novel function for hcl

genes in B. glabrata upon the arrival of hemoglobin in planorbid snails, through provision of intron regulatory sequences.

Molluscan hemocyanins contain specific conserved features that distinguish them from other copper type 3 proteins. First, the organization of multiple FU domains provides a significant difference from single FU domain proteins of the same superfamily, such as tyrosinases and phenoloxidases [27]. Both *B. glabrata* Hcl proteins have linker sequences of 10–15 amino acids that connect multiple FU domains, similar to molluscan hemocyanins. Second, amino acid level comparison of single FUs showed that component FU domains of both Hcl proteins are most similar to those of other molluscan hemocyanins, more so than to tyrosinases (Fig 3). Despite these similarities with respiratory hemocyanins of other molluscs, differences in sequence and, in case of Hcl-1, site of synthesis and a developmentally-dependent expression in *B. glabrata* snails suggest that Hcl proteins may not function in the oxygen-carrying role of other molluscan hemocyanins. Both Hcl proteins contain a lesser number of FU domains, and these domains do not include the full complement of canonical histidines of the sequence motif that mediates copper binding to facilitate O2 binding [17]. In the case of Hcl-1, the presence of only 3 FUs may not permit Hcl-1 to assemble into a multimeric protein as afforded by the multidomain structure of seven or more FUs that characterize O2-transporting molluscan hemocyanins. Finally, one of the six conserved histidines from each of the copper-binding motifs of hemocyanin is absent in Hcl-1 sequences from all four *B. glabrata* strains tested (Fig 4). This may impair the ability of Hcl-1 protein to bind and transport oxygen. In summary, comparative analysis of these proteins suggests

that Hcl-1 and Hcl-2 are related to molluscan hemocyanins, but also support the notion that the purpose of B. glabrata Hcl-1 is not oxygen transport.

The synthesis of Hcl-1 uniquely in the AG is suggestive of the putative functional role that Hcl-1 may have in B. glabrata (Fig 5). The AG is an organ associated with the reproductive tract and it also has a role in immunity. Prophenoloxidase (PO) activity contributes to formation of egg shells in many invertebrates including *B. glabrata* [26]. A 35 kDa enzyme with PO activity from the AG of reproductively mature *B. glabrata* is critical for egg production, likely through crosslinking of egg shell proteins [50]. As a copper type 3 protein, Hcl-1 may also contribute PO activity needed for egg production in *B. glabrata*. However, the deposition of the 35 kDa tyrosinase and Hcl-1 in the EMF [21] suggests that these proteins may contribute to maintenance of shell structure and/or carry out other functions, perhaps immune-related.

In adult *B. glabrata* snails, the AG produces lectins also in response to parasitic infection [51]. Thus, Hcl-1 may play a role as an innate immune factor; some copper type 3 proteins possess PO activity towards melanization of pathogens [52, 53]. At the same time, protein components in the EMF of egg masses of *B. glabrata* have been implicated in immune defense of developing snail embryos against microbes. RNAi knockdown of the EMF component lipopolysaccharide-binding protein and bactericidal/permeability-increasing protein (LBP/BPI) from B. glabrata reduced fitness by lowering egg number per egg mass, and rendering egg masses highly susceptible to degradation by oomycetes [54]. Hcl-1 proteins are provided by adult *B. glabrata* to the perivitelline fluid surrounding developing embryos because the embryos themselves do not express Hcl-1 (Fig 6), this may be an example of vertical transfer of immunity in *B. glabrata*, providing

protection against microbes to increase fitness of embryos. Alternatively, Hcl-1 may provide a different type of protection by facilitating darker pigmentation through enzymatic production of melanin. Melanin pigment protects against UV radiation [55, 56] and may increase tolerance of *B. glabrata* embryos against exposure to sunlight, augmenting egg survival in the environment.

Another alternative possibility is that Hcl-1 functions similar to arthropod hexamerins, which are hemocyanin-derived proteins [57–59]. Hexamerins function in maintenance of arthropod physiology as a source of nutrition by providing amino acids to non-feeding larvae [60–62]. Hexamerins also lack at least one of the histidines of the copper-binding motifs that mediate O2 binding, rendering them non-functional for oxygen transport [59]. Hemocyanins in arthropods and molluscs resulted from convergent evolution and are only distantly related [57]; nevertheless, Hcl-1 may serve a similar function to hexamerins as a source of nutrition for developing *B. glabrata* inside the egg masses.

Finally, to our knowledge, Hcl-1 has been identified only in *B. glabrata*. Due to the occurrence of hemoglobin, it may have evolved from an ancestral hemocyanin after relaxed evolutionary pressure to function as the oxygen transporter for the family Planorbidae [16]. It remains to be determined if the development of Hcl-1 is unique to planorbid snails or if Hcl-1 diversified from hemocyanin in their ancestors such that it is also present in other gastropod families that do employ hemocyanin for oxygen transport, such as Physidae and Lymnaeidae, which are air-breathing freshwater snails in the clade Hygrophila together with the Planorbidae [63]. In that case, Hcl-1 may represent a novel category of copper type 3 related proteins not previously recognized in gastropods. In the

case of *B. glabrata*, we hypothesize that Hcl-1 supports successful development of embryos either by O2 transport, production of melanin for pigmentation/defense, serving as a source of nutrition for eggs, or all of these. Further studies are needed to investigate the distribution of this protein among gastropod snails, to better understand the function of Hcl-1 and to explore Hcl-1 as a potential target for snail control in aid of efforts to reduce transmission of schistosomiasis.

Acknowledgements

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SUPPLEMENTAL MATERIAL

Supplemental figure 1. *Biomphalaria glabrata* **Hcl-2, alignment of functional unit domains with copper type 3 enzymes.** The Hcl-2 FU domains (Hcl-2_1 through Hcl-2_6) were aligned with a tyrosinase sequence of the bivalve *Crassostrea gigas* (CrTyr) and four predicted tyrosinases of *B. glabrata* (Bgtyr). Amino acids relevant for the copper binding motif are highlighted in yellow; amino acid replacements are shown in red. Initial alignment was done with ClustalW and checked visually using MEGA 6.

```
Cu(A)binding.site

Hcl-2_1     ASIAAYHGYPAQCS...............AWGLKLGCSVHGVSIFPQWHRLYVVQMEQAFHEKGLTIGVPYWDWTRPLVRLPGLVSQLVFTARGG
Hcl-2_2     QAMAEFHGNPGMCP............HPTSQDRKYCSNHGQPSFPHWHRLMIVQLEDALCKRGSSIGVPYWDWTKHNTSIPLLAADPDYIDPYR
Hcl-2_3     QVIGRYHGGLEWCP............FPEAKVKKLCGHHGVATFPHWHRPVLLAMEQE....................................
Hcl-2_4     EDIATFHGKPGLCT...............HGGRTTACCSYGSATFPAWRRLYVKQVEDALLDRGSSVAVPYWDWTASFSKLPKLLDEATYYNWRK
Hcl-2_5     QALAAFHALPSLCP............NPRAEKRYVCSPYGTATFLPWHRLYTVQVEDALRRHGALVGLPYWDWTREGSELPEFLEDDNYHDPLT
Hcl-2_6     ..................................................WNIEQALRRHGSAIAVPYWDWTFPTTELPSIFTEEDYYDILK
CgTyr       QERANFHRAILLLKQDTTIRPNRYDALGLVHFRMVDNIHHGGA.FLAWHRLFITIFENALRQKVPDVTLPYWDSTMDEA....MIDPTQSVTWSP
BgTyr202    EERKRYHNAINTLKRDTSVRPNRYDAIAGIHTASISGTAHGGPGFLGWHRIFLFIYERALRQVDPRICLPYWDSTLESD....LNNPLESLIWSA
BgTyr311    KERARFHSAINTLKNDISVRPNKYDAIALIHSGGNLKVAHRGPGFLGWHRLYLMIYETALQEVDPSVCLTYWDTTIESR....LYDPSESSLWSH
BgTyr651    EEPTRYHNAVVALKRDTSVRPNKFDAIANIHTGQTNLIAHGGAGFLGWHRIFLLIYETALRQVDPRICLPYWDSSLEGQ....LARPLESSLWTP
BgTyr2224   DERDRFHNAINALKQDTTVEPNKYDAISLLHSGPSNFIAHGGPGFLGWHRIYLLIFETALREVDPSVCLPYWDSSLDNQ....LADPTSSLIWTP

Cu(B)binding.site

Hcl-2_1     DYNQFEVQLEIAQNTIHHLVGGRNKYSMSNLDYASYDPIFFLHHANVDRIYTIYERYQDLTLNGLDSMALQKLIKERKKKPRAFAVFRLNSFRTS
Hcl-2_2     ....IFIQFEMAHNLIHSLVGGRHPYSMATLEYTAFDPIFYIHHSGLDRIWAIWTALQEL.RGK.PYKAHCAQSYTNTPMKPFAFSSPYNNNNKT
Hcl-2_3     DFCDFEVQFEIAHNCINVHIGGSELYSMSSLKYAAFEPLFVLHHANADRIWAIWQALQKLRNKPYLTANCAQGLMQTPLSPYNLTDGINRYSNTQ
Hcl-2_4     .FCDFEIQFEVFCSAIHFLVGGRSLFSLSSLDYSAFDPVFILHRTNTDRIWAIWQELQRYRRLPYSHADCAINKMKKHLEPFDRPELNKNKDTAK
Hcl-2_5     NYCDFQVQLEVIKQVLSVWIGGSETHSMSHLHFASYDPLFFVHHANVDRLWAVWQALQKHRGHDPDKSNCALEMMKKPLKPFS...FGSPYNLNE
Hcl-2_6     ......FQ..VVHSTILNSLGGDQLYSLSTLEYSAYD.IFIIHSFMDKIWLIWQELQKERHLPSLGTECALNYMNQPLQPFN....SELNMNTF
CgTyr       PGAPDQYNIENYHGDAHTWIGGQMEP....METSAFDPVFYLHHAFVDYVWEIFRQQQRAMGIDPTQDYPQNFGPQSHAPFTPTGFGNLPNVFGI
BgTyr202    EESGRQYSILSNKGSTHMYVGGMMAS....LRMSTYDPIFFMHHAFIDYIFERFRARLRSLGVDPTVYPKWGNSN..LHEPNAPINIFNYTVKDG
BgTyr311    NTTKPKFDLESFHEGVHAFCGGAMKL....FATAAFDPLFYLLHTFIDLIWSLFRDNLKKHGVDPETFPVIKNSSHLHSSDSPTY.FGNLTHADG
BgTyr651    SA.SPQYDIELQHGTAHMYVGGVMTR....LDTAAFDPIFFMHHAFIDYIFEMFRNRLRSIGVNPAQYPRSNSNFRHEPTATTG..FGSYTQEYG
BgTyr2224   DATEPRYDLEFHHASVHVYVGGAMTR....LDTAAFDPVFFMHHAFIDYVWDLFRTNLRNNNANPETYPEIVNVDPRHIATAPTG.FGDLTQADG
```

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Supplemental table 1: Hcl-1 primers used for sequencing. Asterisks identify primers used for RT-PCR of tissue-specific (*) and age-dependent (**) experiments (v = a/c/g; n = a/t/c/g).

Hcl-1 primers	5'-3'
5'UTR	TTCAGTACAGCCCATGGACTGGTTTAAAG
2R	GCAAGATACAGCCTGTGCC
2F	TGCCGAGGCCTGATTCTTAGAAAAG
5R	AGTCAAAGACGGTCTTGTCAGTCATG
5F	ATCCCAACTATTGCCAGTTTGTGGTG
9F**	TGATCCAATCTTTTACATTCATCAC
12R	ATATGGACCAGATCTTATC
12F	TGATCCAATCTTTTACATTCATCAC
13R**	AAGTTGTCAGCGCTAGTCTCA
15R	AAGTTGTCAGCGCTAGTCTCA
15F	AGAAAGAACATCCAAGAGCTGACAG
20R	ACTCAAAGACCTGGAGAC
21R*	TTGACGTTACTGACGCCATGA
23R*	AAGTCAAGTTTGTTACCGACGACG
PolyTanchor	TTTTTTTTTTTTTTTTTTTVN

Supplemental Table 2: Structure of *hcl-1* intron phases. The hcl-1 gene contains 23 exons. The FUs have a greater number of introns when compared to other molluscan hemocyanins. An asterisks (*) identifies introns between FUs that contain the conserved phase one codon interruption characteristic of in molluscan hemocyanins.

	Hcl-1		
Intron #	Intron '5-3'	Intron phase	FU
1	GT…CAG	-	sp
2	GT…CAG	1*	A
3	GT…GAG	0	A
4	GT…CCG	1	A
5	GT…TAG	2	A
6	GT…GAG	0	A
7	GT…AGC	1*	B
8	GT…CAG	0	B
9	GT…TAG	0	B
10	GT…GAG	2	B
11	GT…CAG	0	B
12	GT…CAG	2	B
13	GT…TCA	1	B
14	GT…CAG	2	B
15	GT…CAG	1*	H
16	GT…CAG	1	H
17	GT…TAG	0	H
18	GT…TAG	0	H
19	GT…CAG	0	H
20	GT…TAG	0	H
21	GT…CAG	0	H
22	GT…CAG	0	H

Supplemental table 3. Features of *hcl-2* introns. The *hcl-2* gene contains 34 exons. The FUs have greater number of introns when compared to other molluscan hemocyanins. Introns between FUs contained the conserved phase one codon interruption previously recognized in molluscan hemocyanins (highlighted by asterisks).

	hcl-2		
Intron #	Intron 5'-3'	Intron phase	FU
1	GT...TAG	2	A
2	GT...CAG	0	A
3	GT...TAG	2	A
4	GT...CAG	0	A
5	GT...CAG	1	*
6	GT...CAG	0	B
7	GC...TAG	0	B
8	GT...CAG	0	B
9	GT...CAG	2	B
10	GT...CAG	2	B
11	GT...CAG	1	*
12	GT...CAG	0	C
13	GT...TAG	0	C
14	GT...CAG	0	C
15	GT...CAG	0	C
16	GT...TAG	2	C
17	GT...TAG	1	*
18	GT...TAG	0	D
19	GT...TAG	2	D
20	GT...CAG	2	D
21	GT...CAG	2	D
22	GT...CAG	0	D
23	GT...TAG	1	*
24	GT...TAG	2	E
25	GT...CAG	0	E
26	GT...CAG	0	E
27	GT...CAG	1	*
28	GT...TAG	0	F
29	GT...TAG	0	F
30	GT...TAG	0	F
31	GT...CAG	2	F
32	GT...CAG	0	F
33	GT...AAG	1	F

Supplementary table 4. Synonymous mutations among Hcl-1 transcripts of four *B. glabrata* strains.
The numbers refer to locations indicated in the alignment of Hcl-1 sequences among four *B. glabrata* stains (Figures 4).

#	Codon sequences	Amino acid
1	TTG/TTA	L
2	GTC/GTT	V
3	CTG/CTT	L
4	CCC/CCA	P
5	AGC/AGT	S
6	TCG/TCT	S
7	GGT/GGA	G
8	CTA/CTT	L
9	CGA/AGA	R
10	CCC/CCT	P
11	GGT/GGC	G
12	GAT/GAC	D
13	TTT/TTC	F
14	ACT/ACC	T
15	ACA/ACT	T
16	TTT/TTC	F
17	GAA/GAG	E
18	CTG/TTG	L
19	AGA/AGG	R
20	TCG/TCC	S
21	CTA/CTG/CTT	L
22	AAC/AAT	N
23	TTC/TTT	F
24	CCC/CCA	P
25	CTT/CTG	L
26	ATC/ATT	I
27	TAC/TAT	Y
28	CCG/CCA	P
29	CTA/CTC	L
30	CTG/CTT	L
31	GCA/GCG	A
32	AAC/AAT	N
33	GAC/GAT	D

Supplemental table 5. Hcl-1 peptides recovered from Peptide Mass Fingerprinting (PMF) and MS/MS analysis of 150 kDa protein band from AG and EMF samples of B. glabrata. Consideration of combined peptide mass fingerprinting and tandem MS data (PMF/MS) yielded three peptide sequences that led to positive identification of Hcl-1 (p <0.05). At a 1 Da precursor-ion tolerance, another additional eleven peptide masses (PMF) matched in silico predicted tryptic peptide fragments of Hcl-1. FU= Functional unit of Hcl-1 (A,B and H) where peptide sequences are located in Fig.4, AG= Albumen gland, EMF= Egg mass fluid. (lines 752-759).

Sample	Peptide Sequence	Method	FU
EMF	SYSYIASFYGYPTR	PMF	A
AG, EMF	IYEVYEALYR	PMF	A
EMF	DDNPFPNTR	PMF	A
AG, EMF	RPYYHDITK	PMF	A
EMF	QDVDLLTEDEMNALR	PMF	B
AG, EMF	GLPSFPHWHR	PMF	B
EMF	ANPFYDAAIEFLR	PMF	B
EMF	AEIHATLHTGER	PMF/MS	B
EMF	LQSETSADNFENIAGFHGAPNR	PMF	H
AG, EMF	LLTVQFEQALSR	PMF/MS	H
AG, EMF	LFSDPEDNPFYR	PMF/MS	H
EMF	HVDSSMEPFR	PMF	H
EMF	ENSLPGLVSDHR	PMF	H
EMF	TGTYDGSSSSEK	PMF	H

CHAPTER 3

COMPARATIVE ANALYSIS OF THREE FAMILIES OF HYGROPHILID SNAILS SHOWS THAT THE EGG MASS FLUID PROTEIN HEMOCYANIN-LIKE 1 (HCL-1) IS UNIQUE TO PLANORBIDS.

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ABSTRACT

Previous description of hemocyanin like-1 (Hcl-1) from the planorbid snail *Biomphalaria glabrata* raises questions regarding evolutionary origin of this unusual type-3 copper protein component of the egg mass fluid (EMF; also known as perivitelline fluid) that surrounds developing embryos. SDS-PAGE protein profiles of EMF differ among *Physella acuta*, *Lymnaea elodes* and *Bulinus globosus*, snails of the hygrophilid families Physidae, Lymnaeidae, and Planorbidae, respectively. Immunoblotting with α-Keyhole Limpet Hemocyanin (α-KLH) antiserum showed a cross-reactive band of 148kDa (predicted molecular weight of Hcl-1), only for EMF of the planorbid snail. Similarly, analyses of next generation sequence data yielded Hcl-1-encoding sequences from *Bu. globosus* and *Planorbella duryi* (representing the two subfamilies Bulininnae and Planorbinae that span the range of planorbid phylogeny, with *Planorbella* considered sister genus to *Biomphalaria*) but not from *P. acuta* and *L. stagnalis* (physid and lymnaeid). Computational prediction of 3-D protein structure indicated that the C-terminus of planorbid Hcl-1 differs structurally from the multimerization domain that causes molluscan respiratory hemocyanins to assemble as native didecamer molecules. A monomeric configuration of native Hcl-1 was confirmed by immunoblotting. Molecular clock analysis estimated that Hcl-1 proteins diverged from gastropod respiratory hemocyanins at ~267 $\pm$ 143 mya. These results are concordant with the hypothesis that Hcl proteins originated in the ancestor of the planorbid lineage, associated with secondary evolution of respiratory hemoglobin. Altered selective pressures for maintaining original function may have led to mutation and repurposing of the ancestral

planorbid hemocyanin. Unique features of EMF composition may provide specific targets for potential development of control options aimed at a specific family of snails.

INTRODUCTION

Freshwater pulmonate snails are hygrophilid gastropods (Jörger *et al.*, 2010) that abundantly inhabit lakes, ponds, and streams. Hygrophila are capable of effective (re)colonization of habitats due to resilience to persist in polluted environments and periods of drought, ability for selfing (hermaphrodism) and high rates of reproduction (Richards, 1967; Barbosa & Barbosa, 1994; Rozendaal, 1997). Snails of the families Planorbidae, Lymnaeidae and Physidae are of further interest because they may serve as intermediate host for digenean trematodes, parasites of medical and veterinary importance (Rozendaal, 1997; Mera y Sierra *et al.*, 2009; Horák *et al.*, 2015). Several species of planorbid snails are intermediate host for human schistosomes (Rozendaal, 1997). The planorbid snail *Biomphalaria glabrata* (Say 1818)*,* with an annotated genome available, serves as a model to study host-parasite interactions with *Schistosoma mansoni,* the causative agent of intestinal schistosomiasis in humans (Adema *et al.*, 2017), with 206.5 million people in need of treatment in 2016 (WHO, 2017). Along with planorbids, both lymnaeids and physids also transmit avian schistosomes, blood flukes that target birds but can accidentally penetrate humans and cause cercarial dermatitis (Horák *et al.*, 2015). Study of reproductive biology can yield insights to help interpret biogeography or to develop targeted approaches for control of particular snails in the environment.

The family Planorbidae is unique among hygrophilid snails because of the usage

of iron-containing hemoglobin (Hb), an oxygen transporter that gives them red-pigmented hemolymph. The planorbid lineage acquired Hb through convergent evolution following duplication of a myoglobin gene (Lieb *et al*.; 2006). Most other molluscs (and arthropods) utilize hemocyanin (Hc), a member of the type-3 copper protein superfamily to transport oxygen through their blood (Senozan, 1976; Markl, 2013; Burmester, 2015). Still, two hemocyanin-like genes have been previously identified from *Bi. glabrata*, but these are different from the respiratory Hc pigments used by other gastropods (Peña & Adema, 2016). The *Bi. glabrata* hemocyanin-like 1 protein (BgHcl-1) is synthesized only in the albumen gland (AG) of reproductively mature snails only and packaged into the eggs as part of the egg mass fluid (EMF). Like other EMF components, Hcl-1 may have a nutritive function and/or serve in a protective role as a type of parental transfer of immunity (Hathaway *et al.*, 2010; Baron *et al.*, 2013).

Possibly, the evolutionary appearance of Hb in the ancestor of the planorbid family altered the selective pressures upon Hc to maintain function as an oxygen carrier, similar as for duplicated genes, allowing mutation and subsequent functional repurposing of Hc genes to yield Hcl-1. Arthropods provide examples where duplication events have led to the appearance, in different clades, of pseudo-Hc genes such as cryptocyanins and hexamerins that are thought to serve as transport/storage proteins, play a role in immunity and may be important for cuticle formation (Burmester, 2015). Alternatively, Hcl-1 may have evolved independently, preceding the emergence of Hb in planorbid snails. In that case, Hcl-1 may represent a novel member of the type-3 copper binding protein superfamily, that has eluded characterization to date, and that serves as a general

component of gastropod EMF, perhaps encoded in the genome of multiple families of Hygrophila.

To investigate the phylogenetic distribution of Hcl-1, previously described only from *Bi. glabrata*, this study applied protein level analyses to compare protein profiles of EMF from eggs produced by *Bulinus globosus* (Morelet, 1866). This species was selected to sample across the phylogenetic range of the Planorbidae (see Morgan *et al*., 2002), consisting of the subfamilies Bulininae (represented by *Bu. globosus*) and the subfamily Planorbininae (contains *Bi. glabrata*), and blue-blooded snails from the closely related sister-families Physidae (*Physella acuta* [Draparnaud, 1805]) and Lymnaeidae (*Lymnaea elodes* [Say, 1821]), (Kokkinopoulou *et al*., 2015; Thorp & Covich, 2010). Presence of Hcl-1 protein among EMF proteins was assessed with immunoblotting employing cross-reactive antiserum raised against Keyhole Limpet Hemocyanin (α-KLH; see Peña & Adema, 2016). Bioinformatic searches for Hcl-1 were performed using available genomic and transcriptomic Illumina and 454 (NGS) data of planorbid snails from two different genera (*Bu. globosus*, *Planorbella duryi* [Wetherby, 1879]*)*, and one physid and lymnaeid snail (*Ph. acuta* and *Lymnaea stagnalis* [Linnaeus, 1758]*, respectively)*, as representative of major families from the clade Hygrophila. Amino acid level sequences were used computationally predict 3D structure of Hcl-1 protein. Our results indicate that EMF protein profiles for the three snail families are different and that Hcl-1 uniquely occurs in the planorbid snails screened in this study. The timing of origin of Hcl-1 in planorbid snails was investigated using molecular clock analysis. It is hypothesized that evolution of planorbid Hcl-1 associated closely with advent of the family Planorbidae through repurposing of hemocyanin, possibly triggered by the advent of hemoglobin.

MATERIALS AND METHODS

Snails

     Planorbella duryi and *Lymnaea elodes* were obtained from Shady Lakes, Albuquerque NM, USA, September 2016. Taxonomic identification was based on morphological features and sequence information (*Pl. duryi:* complete mitochondrial genome KY514384; *L. elodes*: 16S sequence genbank accession MH426843). Previously collected snail species, available at the University of New Mexico (UNM), included *Bulinus globosu*, *Physella acuta* isolate A (New Mexico, USA, 2010; Nolan, Bergthorsson & Adema, 2014) and *Biomphalaria glabrata* (M line). All snail species were kept at UNM in tanks containing artificial spring water at 26°C and fed red leaf lettuce *ad libitum* and chicken pellets weekly.

Extraction of Egg Mass Fluid (EMF) proteins

     Egg masses from *Bi. glabrata, Bu. globosus, Ph. acuta* and *L. elodes* were removed from tanks between 24-48 hours after deposition. The distinct shape and consistency of egg masses (eggs are surrounded by a raft or capsule of gelatinous material) of snails from these families of Hygrophila called for different methods to extract the EMF. Similar to *Bi. glabrata* snail eggs that were processed according to Hathaway et al. (2010), eggs of *Bu. globosus* that were held within the raft of an egg mass, were punctured with an G27 hypodermic needle. Egg masses treated in this manner were placed in a 0.6ml Eppendorf tube with a small hole in the bottom and spun in an intact 1.7ml Eppendorf tube (5 minutes, 800 x g) to collect the EMF. For *Ph. acuta* egg masses, cotton swabs were used to absorb away the viscous, gelatinous material that surrounded the eggs. Using tweezers, twenty individual *P. acuta* eggs were placed in the

cap of an Eppendorf tube and then punctured with a G27 hypodermic needle. Drained

egg membranes containing embryos were removed from the cap and an equal volume of

0.01% w/v SDS was added to the EMF, liquid in cap was spun down (14,000 x g, 1

minute) and protein content was quantified using a Qubit fluorometer (Life Science).

Eggs from *L. elodes* were isolated by cutting and macerating egg masses with dissection

scissors to remove the viscous material surrounding the eggs. Intact eggs were then

processed in the same manner as for *Ph. acuta* to isolate and quantify the EMF.

SDS-PAGE, native PAGE and immunoblotting

EMF samples were placed in sample buffer (62.5 mM Tris-HCl, pH 6.8, 40%

glycerol, 0.01% w/v bromophenol blue) containing 20% sodium dodecyl sulfate (SDS)

and 5% β-mercapto-ethanol (BME), boiled for 10 minutes, spun to remove debris, and

subjected to SDS-PAGE gel electrophoresis using a 1.0 mm thick gel consisting of a 4%

acrylamide stacking gel (0.125 M Tris-HCl pH 6.8) and a 5–20% gradient acrylamide

separating gel (0.375 M Tris-HCl pH 8.8) (Loker & Hertel, 1987). Gels were stained

using Coomassie Brilliant Blue G250 (Biorad).

Native form EMF from *Bi. glabrata* was prepared using the same sample buffer

but without SDS and BME (reducing agent) and separated on a native PAGE gel (1.0 mm

thickness) consisting of a 4% stacking gel (pH 6.8) and a 5–20% gradient acrylamide

separating gel (pH 8.8), according to Adema *et al*. (1997). For immunoblotting, proteins

were transferred to nitrocellulose (Trans-Blot SD Semi-dry system, Biorad). The

membrane was incubated in blocking solution (0.1% Tween 20, 20mM Tris pH 7.5,

150mM Nacl, 5% w/v nonfat dry milk overnight, 4C), then transferred to room

temperature, washed 3X in TBS (20mM Tris pH 7.5, 150mM Nacl) and incubated with

polyclonal α-KLH (anti-Keyhole Limpet hemocyanin; Sigma-Aldrich H0892) for 2

hours. The membrane was washed (3X in TBS) and incubated 1 hour with HRP-

conjugated goat α-rabbit IgG (Sigma-Aldrich 1:30,000 TBS) as secondary antibody.

Protein bands that were reactive with the KLH antiserum were visualized with DAB

substrate (Vector Labs).

DNA extraction and Illumina genome sequencing

DNA from *Pl. duryi* was extracted using a CTAB-based protocol

(Winnepenninckx B, Backeljau T, 1993) and treated with RNase A (Thermo Scientific).

Quality and quantity were checked by agarose gel electrophoresis and

spectrophotometrically (NanoDrop 2000c; Thermo Scientific). A genomic sequencing

library was generated at the Molecular Biology Facility at the University of New Mexico

using the KAPA Biosystems Library Prep kit. Paired-end (PE) reads of 150bp were

obtained on an Illumina Nextseq500 sequencer.

Bioinformatics, sequence datasets and computational searches for Hcl-1

The raw Illumina PE reads were subjected to quality control (FastQC, Andrews,

2010; Trimmomatic,(Bolger, Lohse & Usadel, 2014) and assembled using SOAPdenovo2

with default parameters (Luo *et al*., 2012) as representation for the genome of *Pl. duryi*.

In total ~ 22.24 Gbases were collected for an estimated *15.5-fold coverage, considering* a

1.42 Gb genome size for the related *Planorbella tenuis* (Gregory, 2018; Animal

Genome Size Database. *http://www.genomesize.com*). Also available for analysis were

transcriptome (454) and genome (Illumina) data from *Ph. acuta* strain A (Schultz &

Adema, 2018; Genbank accessions SRX1041853 and SRX1041811). Advance access

was provided to transcriptome data from *Bu. globosus* (Dr. Zhang, UNM), and *L.*

stagnalis (Dr Seppälä, Swiss Federal Institute of Technology ETH Zurich), as well as to

relevant genome scaffolds from *L. stagnalis* (Dr. Davidson, University of Notthingham;

Davison *et al*., 2016). *L*ocal blast databases were built for each snail species and searched

using tblastn with the amino acid sequence of the previously characterized BgHcl-1

(GenBank accession *KU682272*). NGS sequences (raw reads and assembled sequences)

with significant similarity (*E-values ≤ 1e-10*) to Hcl-1 were assembled relative to BgHcl-

1 and other gastropod hemocyanin sequences using MUSCLE *(*Edgar*, 2004; the multiple

sequence alignment tool incorporated in the phylogenetics package MEGA 6; Tamura *et

al*., 2013*)* and Sequencher 5.1 (GC codes) to check for full length open reading frames

and (as possible) to determine *in silico* exon/intron splice sites (R/GT-intron-YAG/G).

Intron sequences were not characterized full-length. GENscan

(http://genes.mit.edu/GENSCAN.html; Burge & Karlin, 1998) was used for protein

sequence prediction to check manual analysis only for scaffolds of *L. stagnalis*. For

identification of types of function unit (FU) domains in experimentally obtained

hemocyanin-related sequences, these sequences were aligned with select regions of

gastropod hemocyanins (AA level) using MUSCLE (Edgar, 2004) and analyzed with

MEGA 6 (Tamura *et al.*, 2013).

Prediction of 3-Dimensional structure of Hcl-1

The 3-D structures of full-length Hcl-1 proteins and separately of the terminal FU

H were predicted from primary AA sequences employing I-Tasser (Zhang, 2008; Roy,

Kucukural & Zhang, 2010; Yang *et al.*, 2014) with default parameters. PyMol

(http://www.pymol.org/, Version 1.7.4.5; (DeLano, 2002) was used to visualize and

superimpose the predicted structures onto the structures of respiratory hemocyanin

proteins of *Megathura crenulata* (Hc1 [CAG28309], Hc2 [CAG28308]) . The root-mean-square deviation (RMSD), a measure for structural similarity, was calculated from the output program database (PDB) files.

Molecular clock

The multiple sequence alignment tool MUSCLE (Edgar, 2004) was used for alignment of amino acid sequences of two FU domains (A and B), the only domains planorbid Hcl-1 and Hcl-2 share with all other gastropod hemocyanins. Specifically, FU domains A and B of other panpulmonates (*Helix lucorum* [Hcβ, AEO51766; HcαD, AEO51767; HcαN, AEO51768], *Theba pisana* [Hc1CL21795, Contig4_All and overlapping Unigene20531_All and Unigene86883_All; http://thebadb.bioinfo-minzhao.org/index.html], *Aplysia californica* [Hc1, XP_012938879; Hc2, XP_012938878], *Ph. acuta*, *L. stagnalis*; *M. crenulata* [*Hc1, CAG28309; Hc2, CAG28308]*, *Haliotis tuberculata* [Hc1,CAC20588; Hc2, CAC82192] and *Haliotis rubra* [Hc1, ANE23704; Hc2, ANE23705]), and as outgroup, hemocyanins from the cephalopods *Octopus dolfleini* (AAK28276), *Nautilus pompilius* (CAF03590), *Todarodes pacificus* (BAS69907), *Sepia officinalis* (ABD47515), and *Euprymna scolopes* (AIL00901) were used. Output was used for Maximum Likelihood best-fit model and inference of tree topology with MEGA 6 and Prottest3 (Darriba *et al.*, 2011). MEGA 6 was used for Tajima's rate test (Tajima, 1993). Maximum Likelihood method was used for phylogenetic analysis by running 1000 bootstraps (Whelan & Goldman model). The timetree was generated using the RelTime method (Tamura *et al*. 2012). MEGA 6 was used for Tajima's rate test (Tajima, 1993) and for evolutionary analyses. Three well established clade splits defined by the fossil record (Benton *et al*., 1993) were used for

calibration (Cephalopoda/Gastropoda split at 520 ± 10 mya; the

Vetigastropod/Heterobranchia split at 380 ± 10 mya and the split between the

vetigastropod genera *Megathura* and *Haliotis* at 280 mya).

RESULTS

Hcl-1 is uniquely present in snails of the family Planorbidae

The EMF that surrounds developing embryos in eggs of hygrophilid snails

differed considerably in protein profiles among from the closely related families

Planorbidae, Physidae and Lymnaeidae (Fig. 1). The pattern observed from *Bu. globusus*

EMF was highly similar to that of another planorbid *Bi. glabrata* (Hathaway *et al*., 2010).

Immunoblotting revealed an anti-KLH antiserum-reactive protein band of 148 kDa, the

predicted molecular weight of *Bi. glabrata* Hcl-1 (Peña & Adema 2016), only in the EMF
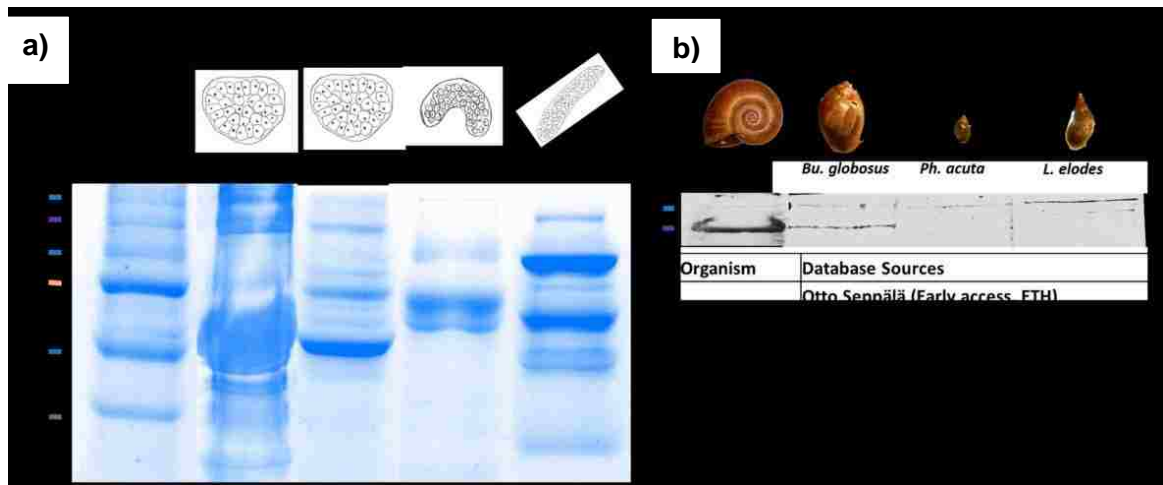
Figure 1. Hcl-1 is uniquely present in planorbid snails. a) EMF protein samples from *Bulinus globosus*, *Physella acuta* and *Lymnaea elodes* show profile differences in snails from the clade Hygrophila. Proteins were separated by 5–20% gradient SDS-PAGE and stained with Coomassie. Egg mass images were acquired from http://northamericanlandsnails.org/WIFreshwaterSnailskey/snaileggmasses.html. To illustrate differences in egg mass arquitecture. b) Immunoblot probed with antiserum against Keyhole limpet hemocyanin (KLH) cross-reacts with a 150kDa band only in the EMF samples of *Pl. duryi* and *Bu. globosus*, predicted molecular weight of Hcl-1 protein. Phylogenetic relationship is shown with scale bar representative of 2 mm to illustrate difference in shell size of adult hygrophillid snails from sister-families. Table informs on genomic and transcriptomic database sources that were used to screen for presence of Hcl-1.

from the planorbid *Bu globusus*. The EMF profiles of *Ph. acuta* and *L. elodes* did not

show a protein at this molecular weight in either SDS-PAGE or immunoblots.

Similarly, inspection of available genomic and transcriptomic sequence data for

hemocyanin-related sequences yielded Hcl protein-encoding sequences from planorbid

snails but not from physid or lymnaeid snails. An Hcl-1 transcript was recovered from

Bu. globosus and the genomic reads from *Pl. duryi* included sequences coding for Hcl-1.

Planorbella duryi also yielded genomic sequence for Hcl-2 (containing 6 FU but this

sequence was not analyzed here further. *Physella acuta and L. stagnalis (*representing

non-planorbid families of Hygrophila) did have respiratory hemocyanins with eight FU

domains, but such sequences were not evident from the planorbid snails. Different from

the seven-eight FU domains contained in molluscan respiratory hemocyanins, the

planorbid Hcl-1 sequences comprise a typical three FUs domain structure, consisting of

Fu-a, b and h (C-terminal) categories (Fig. 2, Fig. 3) with predicted molecular weights

(based on amino acid backbones) of 154kDa for *Bu. globosus* and 150 kDa for *Pl. duryi*,

comparable to the 148 kDa for Hcl-1 of *Bi. glabrata*. The FU domains of *Bu. globosus*

Hcl-1 each contain a six histidine motif that is similar to the copper-binding sites that

facilitate oxygen binding in respiratory hemocyanins. These motifs are not present in

Hcl-1 from *Pl. duryi* (like *Bi. glabrata*) due to amino acid replacements of one or more

histidines in some of the FU domains. (Fig. 2).

Native Hcl-1 is monomeric, unlike multimeric respiratory hemocyanins.

The primary amino acid sequences of planorbid Hcl-1 proteins from *Bu.

globosus* and *Pl. duryi,* were not identical relative to *Bi. glabrata* (59-73% amino acid

sequence identity, 74-84% similarity) yet computational modeling provided highly

comparable 3-D protein structure predictions for FU-a FU-b, and FU-h, generally
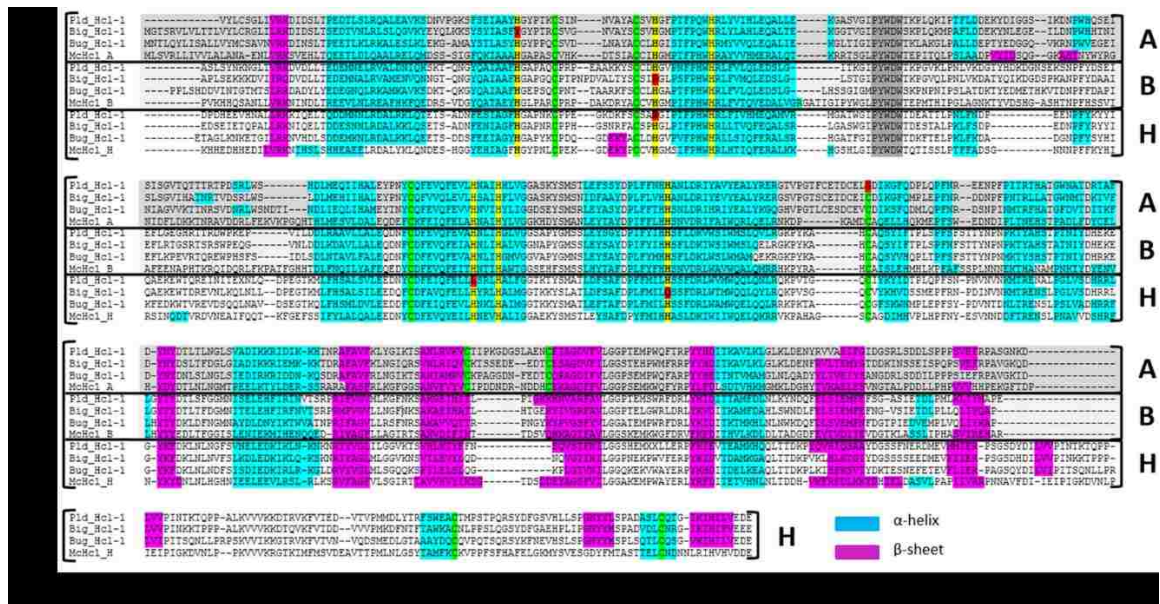
Figure 2. Functional unit domain alignment of planorbid Hcl-1 against the respiratory hemocyanin of *Megathura crenulata*. Alignment represents full Hcl-1 sequences and aligns FU a (dark gray), b (gray) and h (white) against each other to highlight shared and unique features between the planorbids *P. duryi* (Pld_Hcl-1), *Bi. glabrata* (Big_Hcl1) and *Bu. globosus* (Bug_Hcl-1) and the vetigastropod *M. crenulata* (McHcl1). Letters to the right of alignment represent type of FU in domain alignment. Weight matrix used was BLOSSUM for identification of conserved amino acids; *****=conserved residues, **:**=conserved amino acid properties,**,**=weekly conserved. Canonical histidines are highlighted in yellow and conserved cystines that may be involved in disulfide bridges or thioester binding are shown in green. Any replacements of conserved amino acids are shown in red. Structural predictions done with I-Tasser (Zhang Y. 2008) were used to highlight α-helices (blue) and β-sheets (pink).

similar with regards to secondary structures of FU domains from respiratory Hc (Fig. 4).

Detailed analyses showed that the 3D structures of C-terminal FU-H domains of the

planorbid Hcl-1s were also highly similar to those of respiratory Hc (Fig. 4A), as

indicated by low RMSD values ranging from 0.117 to 0.324 for FU-h comparisons of *Bi.*

glabrata Hcl-1 and *M. crenulata* Hc1 and Hc2 (Fig. 3b). The C-terminal FU-h domains

of planorbid Hcl-1 proteins, however, contain two predicted β-sheet regions (indicated in

Fig. 2,4B-C) such that they are structurally distinct from the so-called cupredoxin-like

sequence in FU-H domains of respiratory hemocyanins (using *M. crenulata* as example)

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that facilitates multimerization of gastropod hemocyanin subunits into functional didecamer protein molecules (Fig 4). Moreover, the primary sequence of the unique β-sheet regions of FU-H of *Bi. glabrata* FU-H (AA regions 482-487 and 500-506), contained only four of the six amino acids and two of five amino acids, respectively, that are conserved in the multimerization site of gastropod respiratory hemocyanins (Jaenicke *et al.*, 2010).

Immunoblotting, performed to investigate the apparent lack of a functional multimerization site showed that BgHcl-1 protein occurs in native form as a monomer, not as a multimeric protein. Cross-reactive α-KLH antiserum detected no protein bands greater than 148 kDa, the predicted molecular weight of the amino acid sequence of Hcl-1, from the protein profile of *Bi. glabrata* EMF that was generated using native PAGE (Fig 3d).

Molecular clock: evoltionary emergence of planorbid Hcl proteins.

Phylogenetic analysis of respiratory gastropod hemocyanins and Hcl proteins, using amino acid sequences of the FU-a and FU-b domains that are common to all sequences, yielded a tree with taxon groupings in line with accepted gastropod taxonomy (Jörger *et al.*, 2010). An exception is that the hemocyanins of the euopistobranch *A. californica* were placed in a clade together with those from the stylommatophoran snails rather than as an independent taxon. This pattern, however, was previously observed also in a study of land snail hemocyanin from *Helix lucorum* (De Smet *et. al*. 2011). Both Hcl-1 and Hcl-2 branch off from the respiratory hemocyanins in a manner that is consistent with the placement of the family planorbidae within gastropod phylogeny.
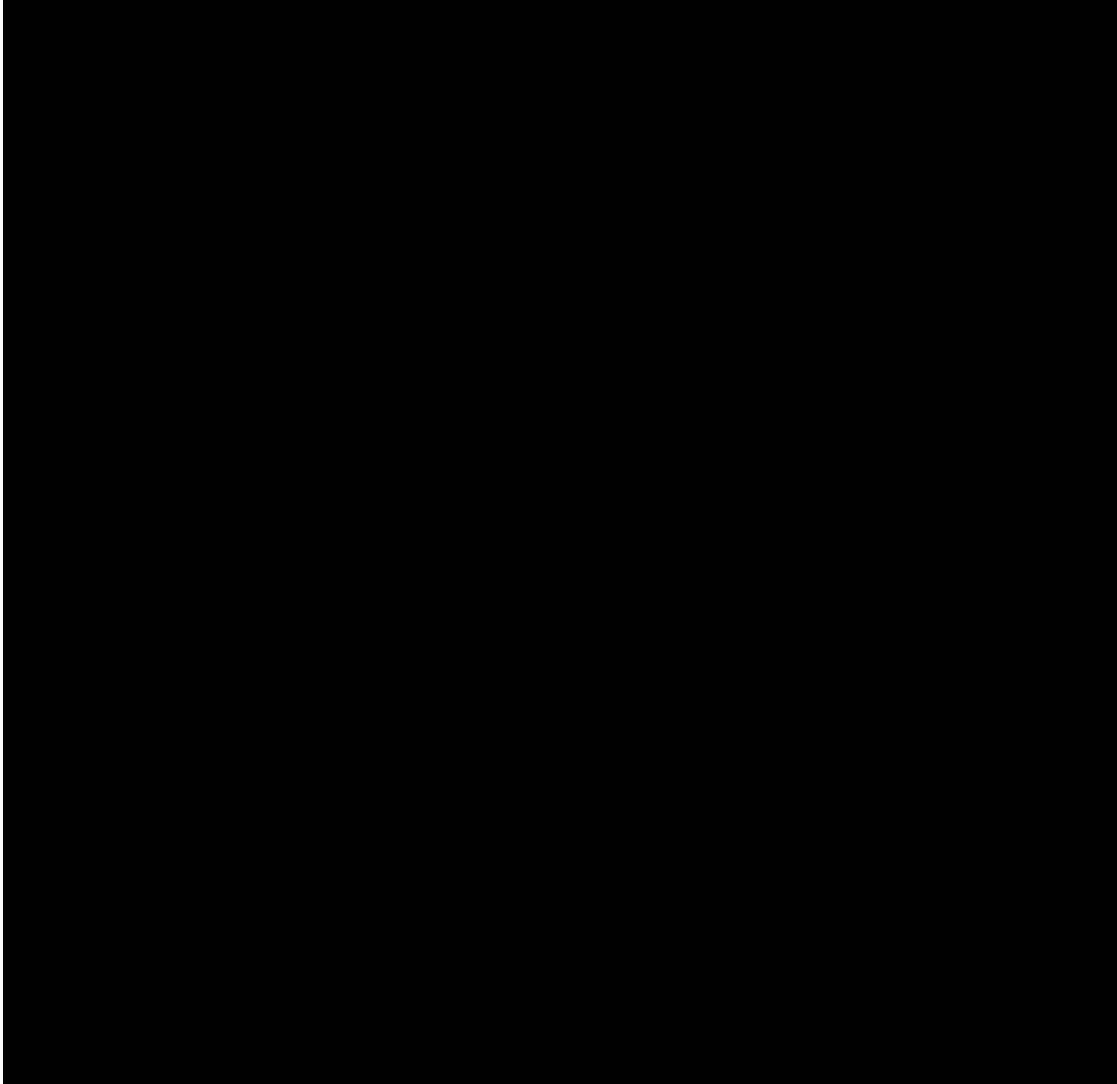
Figure 3. Identification of FU domains of hemocyanin from hygrophilid snails. Phylogenetic comparison of FU domains was performed to infer the categories of FU domains using public molluscan hemocyanin sequences as well as sequences identified in this study (including hemocyanin -like sequences from planorbids). Analysis involved sequences of FU domains from hemocyanins (*Helix lucorum* [HlHc; Hcβ, AEO51766; HcαD, AEO51767; HcαN, AEO51768], *Theba pisana* [TpHc; Hc1CL21795, Contig4_All and overlapping Unigene20531_All and Unigene86883_All], *Aplysia californica* [AcHc; Hc1, XP_012938879; Hc2, XP_012938878] *Ph. acuta* (PaHc), *L. stagnalis* (LsHc), *Bu. globosus* (BugHcl), *Pl.duryi* (PdHcl) and *Bi. glabrata* (BigHcl; Hcl-1, KU682272; Hcl-2, XM_013215044); *M. crenulata* [Hc1, CAG28309; Hc2, CAG28308], *Haliotis tuberculata* [Hc1,CAC20588; Hc2, CAC82192] and *Haliotis rubra* [Hc1, ANE23704; Hc2, ANE23705]). MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) was used for initial alignment; alignment screening and construction of the neighbor-joining tree were done in MEGA6.

Molecular clock analysis indicated that Hcl-1 protein emerged 267 ± 143 mya followed by Hcl-2 thereafter at 260 ± 148 mya; diverging away from panpulmonate respiratory hemocyanins, early at the base of the planorbid lineage.

DISCUSSION

The role of the *Bi. glabrata* as major intermediate host snail for transmission of schistosomiasis has motivated numerous studies of this planorbid snail. Additional to immunobiological investigations to gain understanding of parasite/host compatibility (Coustau *et al.*, 2015), the biology of *Bi. glabrata* is explored more broadly (e.g. by genome characterization, Adema *et al*., 2017), also for features that determine snail distribution and persistence in the field. A means to interpret or even modify the abundance of schistosome-transmitting *Biomphalaria* in endemic areas may integrate with existing control strategies aimed at human schistosomiasis. Current approaches rely largely on mass drug administration (MDA) to treat existing infections but that cannot protect humans from re-infection (Mutapi *et al.*, 2017; WHO, 2017).

Previous studies uncovered that, similar to other Gastropoda, reproductive biology of *Bi. glabrata* includes parental investment effort towards (immune)protection of offspring. Proteins are contributed to the EMF inside eggs to support reproductive success; they not only provide nutrition to developing embryos but also offer protection against predators. Eggs of *Pomacaea caniculata* contain the neurotoxin PcPV2 (Sun *et al*., 2013) or common microbiological pathogens (for instance through antimicrobial factors like LBP/BPI; Hathaway *et al*., 2010, Baron *et al*., 2013). Considering the importance of EMF components for successful development of snail embryos,

characterization of species-specific EMF proteins can identify factors that determine reproductive output and success of a particular snail.

Hemocyanin-like 1 (Hcl-1) from *Bi. glabrata* is such a candidate EMF protein based on the following considerations. The presence of Hcl-1 in *Bi. glabrata* was unexpected because the family of planorbid snails lacks respiratory hemocyanin, using hemoglobin for respiration instead. Furthermore, Hcl-1 is expressed by *Bi. glabrata* only after sexual maturation. As member of the copper type 3 protein superfamily, the EMF protein Hcl-1 may have immune or nutritive function (yet to be characterized). Finally, Hcl-1 was a novel sequence, not previously recorded from any other species (Lieb *et al*., 2006; Peña & Adema, 2016).

The selection of snail species other than *Bi. glabrata*, for investigation of the occurrence of Hcl-1 among families of hygrophilid gastropods, was largely defined by availability of comprehensive next generation sequence datasets and of live animals as source of snail eggs. The Physidae were represented by *Ph. acuta*, Lymnaeidae by (live) *L. elodes* and *L. stagnalis* (sequence data). The phylogenetic range the planorbids, encompassing both subfamilies, is represented by *Pl. duryi* (*Planorbella* is sister genus to *Biomphalaria* [subfamily Planorbinine]), and by *Bu. obstructa* (live snails and sequence data) a member of the subfamily Bulininae (Morgan *et al.*, 2002; Albrecht, Kuhn & Streit, 2007).

Inspection of protein compositions of EMF for presence of Hcl-1 uncovered considerable differences in molecular weights of prominent proteins among EMF of the three families of hygrophilid snails (Fig. 1). Essentially similar protein profiles were obtained from EMF of the planorbids *Bu. obstructa* and *Bi. glabrata* (Hathaway *et al.*,
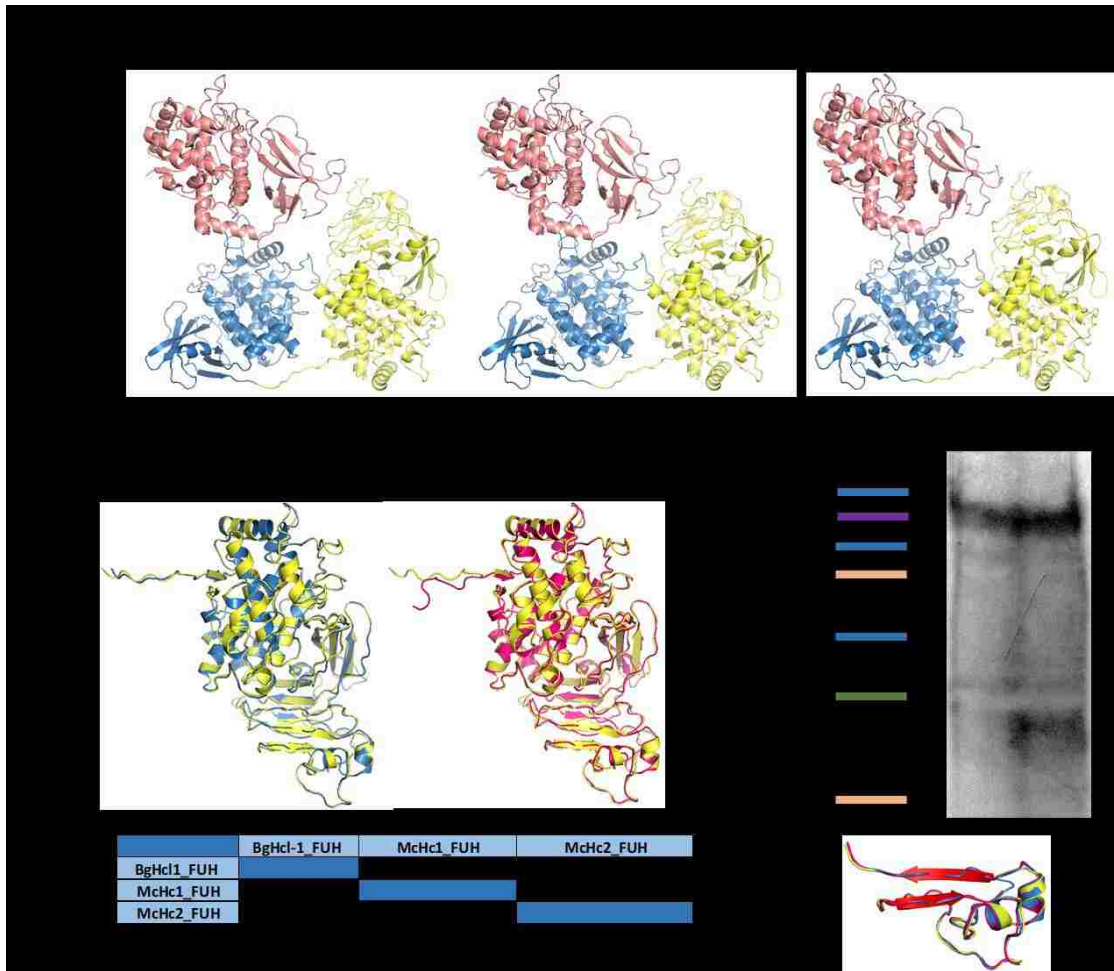
Figure 3. Hcl-1 copper binding sites of planorbid snails have different histidine replacements. a) Structural predictions of planorbid Hcl-1 from *Bu. globosus* (BugHcl-1), *Pl. duryi* (PdHcl-1) and *Bi. glabrata* (BigHcl1). Functional units (FU) as colored according to the type of FU in the structure; a=pink, b=blue and h=yellow. b) Superimposition of FU-h of *Bi. glabrata* (yellow) against two FU-h isoforms from *M. crenulata*. Table shows measure of "best-fit" with root-mean-square deviation (RMSD) from aligned carbon atoms for superimposed FU-h structure predictions. **c)** Immunoblot of native-PAGE shows BigHcl-1 from EMF doesn't shift from the expected 150kDa MW, highlighting lack of multimerization. Modelling of cupredoxin-like domain from FUH predicts two β-sheets (red) in BigHcl1(yellow), different from the superimposed isoforms of *M. crenulata* isoforms.

2010). This indicates the likelihood that the protein composition of EMF differs for each

hygrophilid family. The remarkable level of distinction suggests that different types of

EMF proteins can support embryological development of snail eggs, or that eggs of each

of the snail families experience uniquely different stressors and pathogens. These

questions warrant more detailed characterization of potential taxon-specific features of EMF among hygrophilid snails. This study, however, already provides an initial answer by indicating that Hcl-1 is an EMF protein of planorbids, not of physid or lymnaeid snails. Protein-level studies showed a ~150 kDa protein (predicted molecular weight of Hcl-1) that was immune-reactive with anti-keyhole limpet hemocyanin antisera (as reported for *Bi. glabrata*; Peña & Adema 2016) only from *Bu. globosus,* and not from *Ph. acuta* or *L. elodes.* Comprehensive, detailed sequence similarity searches of extensive genomic- and transcriptomic-level datasets uncovered hemocyanin-related sequences from all Hygrophilids tested. Nevertheless, assembly of these sequence fragments did not yield Hcl-1 but only respiratory hemocyanins for *Ph. acuta* and *L. stagnalis* snails, (Physidae and Lymnaeidea, respectively). By contrast, *Bu. globusus* or *Pl. duryi* both did not have respiratory hemocyanins, but each provided clear evidence for Hcl-1 in planorbid snails. *Planorbella duryi* also yielded Hcl-2, another hemocyanin-related sequence, different from respiratory hemocyanins and not involved in reproduction, previously described from *Bi. glabrata* (Kokkinopoulou *et al*., 2014; Peña & Adema, 2016). In summary, two independent lines of investigation within this study (analysis of protein composition of EMF and computational bioinformatics) indicated that Hcl-1 is unique to planorbids among hygrophilid snails.

The availability of Hcl-1-encoding sequences from three different species of planorbid snails (with representation of the phylogenetic diversity of the Planorbidae) enabled comparative study of common features and evolutionary origin of Hcl-1. The three Hcl-1 proteins are highly similar in primary (AA level) sequence among *Bu. globusus*, *Pl. duryi* and *Bi. glabrata* with each comprising three FU domains. Alignment

shows consistent (modestly sized) indels in Hcl-1 sequences versus respiratory

hemocyanin (exemplified by *M. crenulata*), but the three FU domains are readily

categorized as the N-terminal FU-a, FU–b and the C-terminal FU-h domains that have

been defined for gastropod respiratory hemocyanins, Likewise, the FU domain are

connected by linker sequences similar to those present in respiratory hemocyanins. (Fig.

2). Whereas the *Bu. globusus* Hcl-1 sequence contains all canonical histidine amino acids

(six in each FU) of the copper-binding domain that facilitates oxygen binding in

respiratory hemocyanins (Markl, 2013), amino acid replacements of some of these

histidines are evident from *Pl. duryi* Hcl-1, similar to *Bi. glabrata* Hcl-1 (Peña & Adema,

2016). Accordingly, it remains unclear whether oxygen-binding is required for the

function of planorbid Hcl-1. Secondary structure predictions for the FU domains are

highly similar regarding location of α-helices and β-sheets, also compared to equivalent

FU domains of *M. crenulata* respiratory hemocyanin. Similarities among the three Hcl-1s

and *M. crenulata* respiratory hemocyanin extend further to the predicted 3D-structures of,

the three FU domains. Differences in the C-terminal FU-h domains, however, are a likely

cause for distinct quaternary structures of Hcl-1 and respiratory hemocyanin of

gastropods. The structural features of the so-called cupredoxin-like domain in the

downstream region of the FU-h domain of respiratory hemocyanins facilitate stable

multimerization of hemocyanin proteins into large didecamer molecules to result in

functional respiratory hemocyanins (Jaenicke *et al.*, 2010). The predicted structures for

comparable regions of planorbid Hcl-1s are largely similar but they diverge from

functional cupredoxin due to altered formation of two α-helices and to two novel

β–sheets the predicted structures (Fig. 2 and Fig. 4). The impact of these *in silico*

predictions may prevent the FU-h domain of Hcl-1 from harboring a functional multimerization site. Indeed, respiratory hemocyanins assume complex multimeric quartenary structure, whereas this study showed the native configuration of Hcl-1 as a monomeric molecule in *Bi. glabrata* EMF (Fig 4).

The newly characterized sequences (including respiratory hemocyanins of *Ph. acuta* and *L. stagnalis*) were employed in a molecular clock analysis to investigate when the hemocyanin-like proteins (Hcl-1, and also Hcl-2) of planorbids may have evolved from gastropod respiratory hemocyanin as is suggested by extensive similarities in sequence and structural features (also see Peña & Adema, 2016). A gene tree based on FU-a and FU-b domains (align-able for all hemocyanin-related sequences) showed that the evolution of these gastropod hemocyanin-related sequences generally tracks the generally accepted taxonomy of gastropods, grouping vetigastropoda separate from a clade that includes separate branches for Hygrophila, Eupulmonata, and Euopisthobranchs (e.g. Jörger *et al*., 2010). In the clock tree, branches that encompass the planorbid Hcl-1 and Hcl-2 sequences diverge from the respiratory hemocyanins 267 and 260 mya, respectively. Considering estimates based on fossil records that the Planorbidae originated within the panpulmonate lineage 200-300 mya (Baker, 1945) and that Hcl-1 occurs in both planorbid subfamilies (Bulininae and Planorbininae), this indicates that Hcl-1 first emerged in an early, common ancestor of the family Planorbidae. In turn this suggests the following speculative yet attractive scenario for the evolutionary development of Hcl-1. Likely, all gastropods employed hemocyanin as respiratory pigment before convergent evolution, through duplication of myoglobin, yielded a gastropod hemoglobin (Lieb *et al*., 2006) in the ancestor of planorbid snails. Hemoglobin
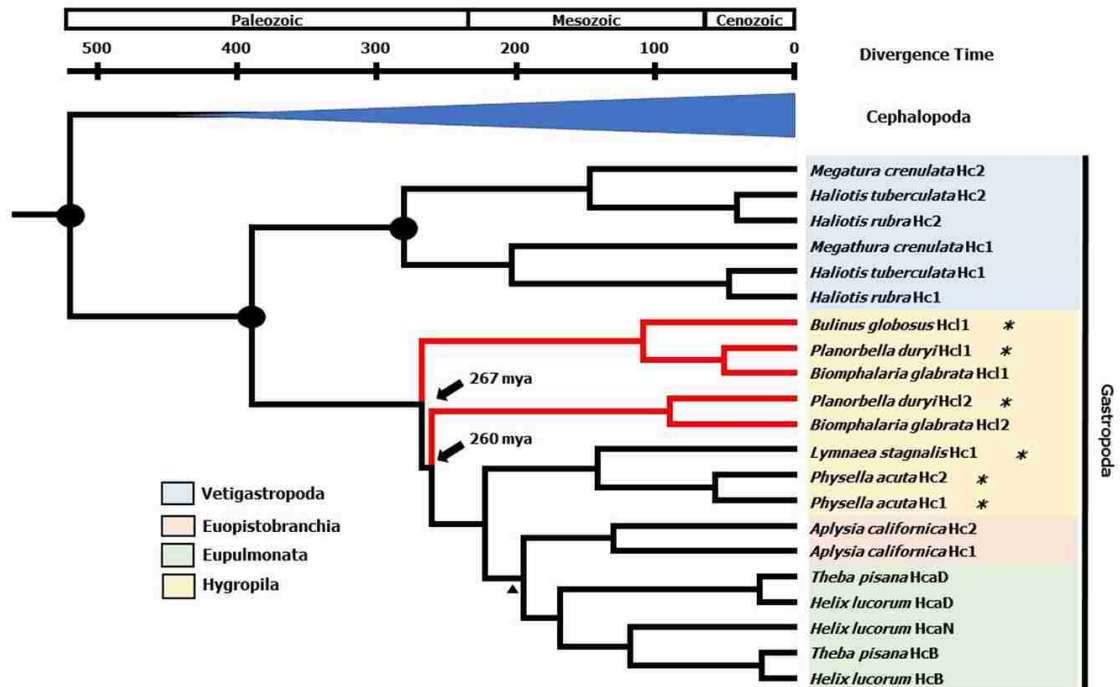
Figure 5. Hemocyanin-like proteins in planorbids diverged early in the Heterobranchia. Alignment of 752 amino acids from FUs A and B was done using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). Specifically, FU domains A and B of gastropods listed in Fig. 3 and as outgroup, cephalopod hemocyain from *Octopus dolfleini* (AAK28276), *Nautilus pompilius* (CAF03590), *Todarodes pacificus* (BAS69907), *Sepia officinalis* (ABD47515), and *Euprymna scolopes* (AIL00901). Maximum Likelihood best- fit model and inference of tree topology with MEGA 6 and Prottest3. Maximum Likelihood method was used for phylogenetic analysis by running 1000 bootstraps (Whelan and Goldman model). The timetree was generated using the RelTime method (Tamura K et al. 2012). MEGA 6 was used for Tajima's rate test (Tajima, 1993). Three well established clade splits were used for calibration (Cephalopoda/Gastropoda split at 520 ± 10 mya; the Vetigastropod/Heterobranchia split at 380 ± 10mya and the split between the vetigastropods *Megathura* and *Haliotis* at 280 mya; Benton, 1993) and are represented by the black circles in each split. Evolutionary analyses were conducted in MEGA6. Red branches highlight red blooded planorbids and black arrows point to timed Hcl protein divergence. Numbers in clade splits show branch support from ML analysis, if support values were 100, values were not shown. Asterisks highlight new data added by this study. Black triangle represents support values <60%.

is generally a more efficient respiratory pigment (Bugge & Weber, 1999) and provided

evolutionary benefits that supported the rise of a new taxonomic clade of red blooded

snails, the family Planorbidae. The establishment of respiratory hemoglobin rendered the

ancestral gastropod hemocyanins redundant as respiratory proteins (Fig.6). The release

from selective pressures to maintain function led the ancestral hemocyanins to incur
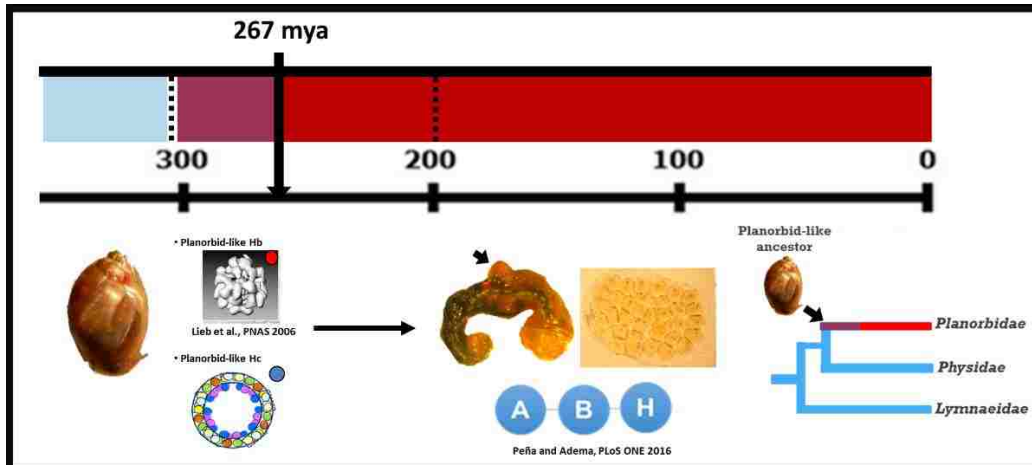
Figure 6. **Scenario for the evolutionary development of Hcl-1**. Prior to our current-day planorbids, an ancestor snail that utilized hemocyanin as its oxygen carrier underwent pressures that allowed the development of gastropod hemoglobin through duplication of myoglobin (Lieb et al., 2006). For some period, there may have been two oxygen carriers in this ancestral planorbid, represented by the purple color in timeline. Fossil records suggest planorbids originated within the panpulmonate lineage 200-300 mya (Baker, 1945). Considering that Hcl-1 occurs in both planorbid subfamilies (Bulininae and Planorbininae) and its predicted emergence is 267 mya; Hcl-1 first emerged in an early, common ancestor of the family Planorbidae. However, being that hemoglobin provides biological benefits because of its oxygen carrying efficiency (Bugge & Weber, 1999), biological pressures to conserve hemocyanin as an oxygen transporter were relaxed, allowing for the takeover of hemoglobin as oxygen carrier, making ancestral gastropod hemocyanins redundant as respiratory proteins and allowing for its integration into reproductive biology of planorbid snails.

mutations, akin to the fate of duplicated genes that may be lost or repurposed (Katju, 2012; Kondrashov, 2012). Mutational processes acting upon a respiratory hemocyanin to reduce the number of FU domains and inactivate the C –terminal cupredoxin site led to evolution of Hcl-1 as a monomeric protein component of EMF. Similarly, modifications of another ancestral hemocyamin may have resulted in planorbid Hcl-2. The evolutionary retention and repurposing, instead of loss of a redundant hemocyanin, suggests that Hcl-1 integrated functionally in the biology of planorbid snails. Further characterization will clarify if Hcl-1 contributes to snail reproduction in a manner that can be manipulated to study or modify reproductive biology, specifically of planorbid snails.

In summary, comparative analysis showed differences in composition of EMF among three families of hygrophilid snails, indicating family-level differences in parental

investments in success of their offspring. Moreover, Hcl-1 protein is unique to EMF of planorbid snails. Hcl-1 originated early during the evolutionary origin of the planorbid lineage, likely deriving from ancestral gastropod respiratory hemocyanin. The evolutionary retention of this planorbid lineage-specific EMF protein suggests that Hcl-1 is important for reproductive biology of planorbid snails and warrants functional studies to better characterize Hcl-1.

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CHAPTER 4

HCL-1 IS A DETERMINANT OF REPRODUCTIVE SUCCESS OF THE SNAIL

BIOMPHALARIA GLABRATA

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ABSTRACT

Previous research indicated that hemocyanin-like 1 (Hcl-1) protein, a constituent of egg mass fluid (EMF) is present only in the Planorbidae. This study undertook characterization of the role of Hcl-1 in the reproductive biology of *Biomphalaria glabrata*, vector snail of the human parasite *Schistosoma mansoni*. qRT-PCR showed no transcriptional response following exposure to Gram (+) *Micrococcus luteus*, but Hcl-1 expression increased up to 5-fold in response to *Escherichia coli* Gram (-). RNA interference effected knockdown of Hcl-1 expression for about 7 days and inhibited reproductive output of *B. glabrata* by causing either reduced number and hatching success of eggs produced or complete cessation of egg production. Likely, Hcl-1 contributes to the egg deposition process of fertilized embryos and/or provides essential support (nutrients or oxygen) for the successful development of embryo's inside eggs. As a planorbid-specific protein, Hcl-1 may be targeted to develop molluscicides that target only planorbid snails of the genera *Biomphalaria* and *Bulinus* that transmit *Schistosoma* parasites in support of control efforts aimed schistosomiasis in humans.

INTRODUCTION

Global human health is severely impacted by the infectious disease schistosomiasis. Flatworm parasites of the genus *Schistosoma* cause chronic infections that over time may result in death, but associate with high morbidity leading to significant loss of disability-adjusted life years (DALYs; Hotez *et al*., 2014). Over 200 million people are infected with 600 million more at risk of infection in endemic tropical regions (Bruun, Aagaard-Hansen & Watts, 2008). Treatment of schistosomiasis largely relies on mass drug application (MDA) of praziquantel (PZQ), an antihelminthic that

effectively cures existing schistosome infections but cannot protect against (rapid)

reinfection (Sarvel *et al.*, 2011; Ezeamama *et al.*, 2016). More sustainable disease control

will require strategies to reduce transmission of schistosomes by freshwater snails that are

intermediate hosts for schistosomes. *Schistosoma mansoni,* causative pathogen of

intestinal schistosomiasis is only transmitted in regions that are endemic for particular

planorbid snails of the genus *Biomphalaria* because the parasite cannot develop in other

snails species (Caldeira *et al.*, 2016; Buddenborg *et al.*, 2017). Thus, characterization of

(lineage-specific) aspects of snail biology that determine snail distribution and

persistence in the field may assist in interpretation and perhaps modification of

abundance of intermediate host snails in support of control efforts aimed at

schistosomiasis.

Previous investigations have identified hemocyanin-like 1 (Hcl-1) protein as

constituent of the egg mass fluid (EMF) surrounding embryos as they develop inside

snail eggs. (Peña & Adema, 2016), unique to planorbid snails (chapter 3). Based on

sequence and structural properties It was proposed that Hcl-1 evolved from an ancestral

respiratory hemocyanin that was rendered functionally redundant by convergent

evolution of a gastropod hemoglobin in the founder of the planorbidae as taxonomic

family of the hygrophylid snails (chapter 3). The modification and repurposing of the

modified hemocyanin-related sequence over evolutionary time as an EMF protein, as

opposed to loss of a redundant sequence, strongly suggest that Hcl-1 serves an important

function for *B. glabrata.* The functional role of Hcl-1 remains unclear to date but Hcl-1

belongs to the copper type 3 superfamily and may have enzymatic activity similar to that

of other members of this superfamily that include tyrosinases, phenol oxidases and

hemocyanins (Decker & Tuczek, 2000; Decker *et al.*, 2007). EMF of snail eggs contains a diverse set of nutritive and (immuno-) protective proteins that represent snail parental investment toward protection and successful development of offspring. EMF of *Pomacea caniculata* includes a neurotoxin to discourage consumption of its brightly red colored eggs (Sun *et al.*, 2012; Mu *et al.*, 2017). *Biomphalaria glabrata* eggs contain several categories of common antimicrobial proteins (Hathaway *et al.*, 2010), including LBP/BPI that was functionally characterized using RNA interference to protect against infestation by oomycetes (Baron *et al.*, 2013). The unique association with planorbid snails makes the EMF protein Hcl-1 an interesting candidate for investigation of a potential role in reproductive biology of *B. glabrata.*

To test broadly for potential function of Hcl-1 as EMF protein, this study employed qRT-PCR to determine expression levels of Hcl-1 in the albumen gland (that produces EMF components) of egg-laying *B. glabrata* after exposure to Gram (-) or Gram (+) bacteria. RNA interference, first shown feasible in *B. glabrata* by Jiang et al. (2006) and capable to alter gene expression in the AG (Baron *et al*., 2013), was applied to study the effect of Hcl-1 knockdown in *B. glabrata* upon the reproductive output and hatching success of eggs.

MATERIALS AND METHODS

Snails and pathogens

Biomphalaria glabrata snails of the M line strain are maintained at the University of New Mexico. Snails are routinely housed in tanks containing artificial spring water (ASW) at 26° and fed red leaf lettuce *ad libitum* and chicken pellets weekly. *Escherichia coli* and *Micrococus luteus* (ATCC 14948 and 9341, respectively) were available from

the microbiology teaching facility at UNM. Bacteria were cultured in LB broth overnight prior to experimental use.

Bacterial exposure and RNA extraction.

For exposure to bacteria according to the methods of Deleury *et al*. (2011) mature *B. glabrata* (14-16 mm shell diameter) were individually placed in wells of 12 well tissue culture plates (Greiner) filled with 3 ml ASW containing *E. coli* or *M. luteus* suspended at an OD600 = 0.6. Snails were removed after 1 h, rinsed and placed in tank with clean ASW. RNA was extracted at 1, 4, 6, 12, 24 and 48 hours post- exposure. Nine snails were used for each time point, another nine snails were used as unexposed controls. For each time point, the albumen glands (AG) were dissected from 9 snails and pooled as three triplicate samples of 3 AGs for RNA extraction (Fig. 1).

Figure 1. Bacterial exposure assay. *B. glabrata* snails were bathed in *E. coli* for one hour. Albumen glands (AG) were extracted at 0, 4, 6, 12, 24, 48 hours post-exposure. Untreated (Unt) snails were placed in ASW for 1 hour. Nine snails were used, three AGs were pooled/sample, resulting in 3 replicates/timepoint. Three replicate experiments were done.

The tissue structure of each AG sample was disrupted using a plastic pestle

(Kontes Rnase-free) in 1 mL of Trizol-Reagent (Invitrogen) and RNA was extracted

according to manufacturer's instructions, using TURBO DNA-free (Ambion, Grand

Island, NY) to remove residual genomic DNA. The RNA was quantified

spectrophotometrically (NanoDrop 2000c; Thermo Scientific) and quality was checked

using 2100 Bioanalyzer with the RNA Nano Kit (Agilent).

qRT-PCR

Primers were designed from the *B. glabrata* Hcl-1 sequence (GenBank accession

KU682271) using recommendations from the manual from the Biorad CFX96 Touch

system. Primers for *B. glabrata* L23, previously validated as housekeeping gene, were as

designed by Larson *et al*. (2014). See Table 1 for primer sequences. Primers were

validated as detailed in the Biorad manual with CFX96 Touch system.

Table 1. Primer sequences used for RT-PCR and qRT-PCR.

Target	Primers (5'-3')		Assay
eIF2α	F011: CCTGCAGGTTCTACAAAACA		PCR
	R247: TATCCTTACCTTTATCTTTGTCTACTC		
Hcl-1	E9F: TGATCCAATCTTTTACATTCATCAC		qPCR
	E10R: ATGGAGTGAAGATGTAGGAC		
L23	L23F: CAGATATGGGAAAGTTAAGCCAAA		
	L23R: CCATAACCAATGTTTGGCATC		

Two µg of RNA from each AG sample was reverse transcribed to cDNA using an

oligo (dT) primer (Omniscript,Qiagen). Successful cDNA synthesis was confirmed prior

to qRT-PCR by PCR generating sequence fragments *B. glabrata* eukaryotic translation

initiation factor (eIF2α, GenBank accession AAH81784; see Table 1 for primers used);

with AmpliTaq Gold (Applied Biosystems, Life Technologies). The cycling conditions

were as follows: 95 °C for 10 min for initial denaturation; 25 cycles at: 95 °C for 60 s,

30 s at annealing temperature (depending on primers used), 72 °C for 60 s; then 72 °C for 7 min for final extension, ramping rate set at 1°/s. Presence of amplicons was confirmed on Gelred (Biotium)-stained 1.1% agarose gels.

qRT-PCR reactions (10 uL volume containing 10ng cDNA template, 100nM of each primer) were performed with SsoAdvanced SYBR Green Supermix with precision Blue PCR dye (Bio-Rad) on a Biorad CFX96. Three technical replicates were tested for each of the three biological replicates per time point, and the unexposed control. Melting curve analysis was used to confirm amplicon specificity. Results were analyzed using the Biorad CFX Manager software that employs the 2^(-delta (delta Ct)) method to determine expression levels relative to the controls (Livak & Schmittgen, 2001).

RNAi-knockdown of B. glabrata *Hcl-1*

Short interfering RNA (siRNA) oligonucleotide sequences (purchased from Integrated DNA technologies) were designed according to manufacturer's instructions to target each of the three functional unit (FU) domains present in Hcl-1 protein and GFP-conytol (AAB51348.1) were employed for control of RNAi (Fig. 2). To reduce likelihood of off-target reactivity (Qiu, Adema & Lane, 2005), BLAST was used to check target specificity of siRNA sequences against publicly available genomic and transcriptomic data from *B. glabrata* (Adema *et al.*, 2017).

The protein of interest, Hcl-1 is only expressed in the AG of sexually mature *B. glabrata.* Adult *B. glabrata* snails (12mm shell diameter) were maintained in individual containers to confirm that they were reproductively active as indicated by egg laying. Snail- conditioned water was used to encourage separately kept *B. glabrata,* a simultaneous hermaphrodite that is preferentially out crosser (Escobar *et al.*, 2011) to
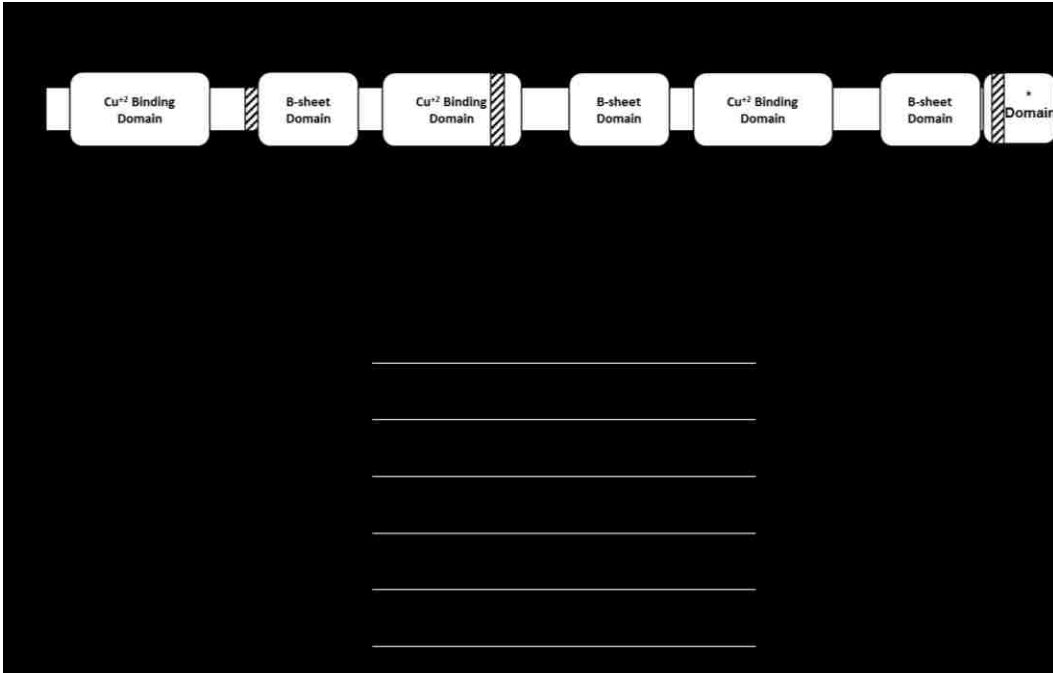
Figure 2. Hcl-1 siRNA targets for knockdown in *B. glabrata*. Schematic of BgHcl-1protein showing all three functional units (FU; a,b,h) with their respective protein domains; Cu +2 Binding domain, B-sheet Domain, and uniquely to FU-h, the cupredoxin Domain (*). Striped blocks within domains show target sites for siRNA knockdown. B) Table containing siRNA sequences for Hcl-1 knockdown and control siRNA sequences from GFP.

continue egg laying.

Day 0, twenty snails were injected into the cardiac sinus with 5μL containing 10μg of an equimolar cocktail of the three 27-mer siRNA specific for Hcl-1 using a G27 hypodermic needle. Snails were kept in separate 300ml tanks and egg masses produced by each individual snail were collected for inspection, about every other day (see timeline, Fig. 3). Five snails were sacrificed at both day 2 and day 7 to monitor Hcl-1 protein expression in the AG of each individual snail (see below). On day 12, the remaining 9 snails each received an additional injection with the anti-Hcl-1 siRNA. Groups of four snails were sacrificed to obtain individual AGs, each on day 14 and 21 after the first injection. For controls, 10 snails were injected with siRNA oligonucleotides targeting GFP (Fig. 2) using the same methods on day 0 and day 12. For the controls,

egg masses were collected and evaluated as indicated in the timeline (see Fig. 3). Five snails on day 2 and four remaining snails on day 21were sacrificed for recovery of the AGs.

Hcl-1 protein expression in AG after RNAi

To investigate knockdown of Hcl-1, AG tissues dissected from individual snails were disrupted with a plastic pestle (Kontes) in 50 μL 0.01% w/v SDS, boiled for 10 minutes and spun to remove debris. Extracted protein was quantified using a Qubit fluorometer (Qubit Protein Assay kit, Life Science). Then an equal volume of Biorad sample buffer (62.5 mM Tris-HCl, pH 6.8, 40% glycerol, 0.01% w/v bromophenol blue) enriched to yield a final 20% w/v SDS concentration (see Hathaway et al., 2010) was added and the sample was stored at -80C. Protein samples were subjected to SDS-PAGE gel electrophoresis using a 1.0 mm thick precast 4-15% gradient gel (Biorad). Gels were stained using Coomassie Brilliant Blue G250 (Biorad). Protein profiles were scanned using densitometric analysis (Imagelab 5.2.1, Biorad) to determine the expression levels of Hcl-1 protein (a distinct band at 150kDa, confirmed as Hcl-1 by bioinformatics, immunoblotting and mass-spec analysis; Peña & Adema, 2016) relative to the abundant LBP/BPI (~60kDa, previously characterized by Hathaway *et al*., 2010; Baron *et al*., 2013) in AG of snails following injection with siRNA targeting Hcl-1 and GFP, respectively.

Impact of RNAi treatment on reproductive output and hatching success.

For each individual snail, the number of egg masses and the number of eggs per egg mass were determined from the collected egg masses. Egg masses were kept in ASW and inspected 8 days after deposition for proportion of unhatched eggs. Embryo's in eggs

that had not hatched were evaluated for level of development and damage using criteria

from (Nakano *et al.*, 2003; Shartau *et al.*, 2010). The aspect of egg masses from routinely

maintained M line *B. glabrata* was used as a general reference.

Statistics

Statistical analysis was done using the data analysis toolpak in Excel. For both,

qPCR and protein densitometry, ANOVA was done followed by two-sample t-Test

assuming unequal variance for mean comparison between samples, alpha was set at .05.

Figure 3. Hcl-1 knockdown experiment timeline. Twenty **s**nails, confirmed to be reproductively mature, were injected (I) with a cocktail of Hcl-1 siRNAs. Protein from albumen gland of five snails/timepoint was extracted at day 2, 7, 14 and 21 days post-injection. At day 12, a "booster" injections was given to all remaining snails.

RESULTS

Hcl-1 *expression in the AG following bacterial exposure.*

Experimental exposure to Gram (+) *M. luteus* did not result in significant changes

of *Hcl-1* expression (p-value range= 3.3 to .1). On average, Gram (-) *E. coli* exposure

associated with upregulated expression in the AG of *B. glabrata* at 12 (4-fold median

upregulation, p-value= 0.036) and 24h post treatment (p-value= 0.0248). Individual snails

showed considerable differences in the amplitude of this response but the time kinetics of

the response was similar (Fig. 4).

RNAi knockdown of Hcl-1 *impact on reproductive output.*

The injection of siRNA had minimal effect on survival of *B. glabrata* snails, only three of

twenty-four experimentally-treated snails died. These were Hcl-1 siRNA injected snails
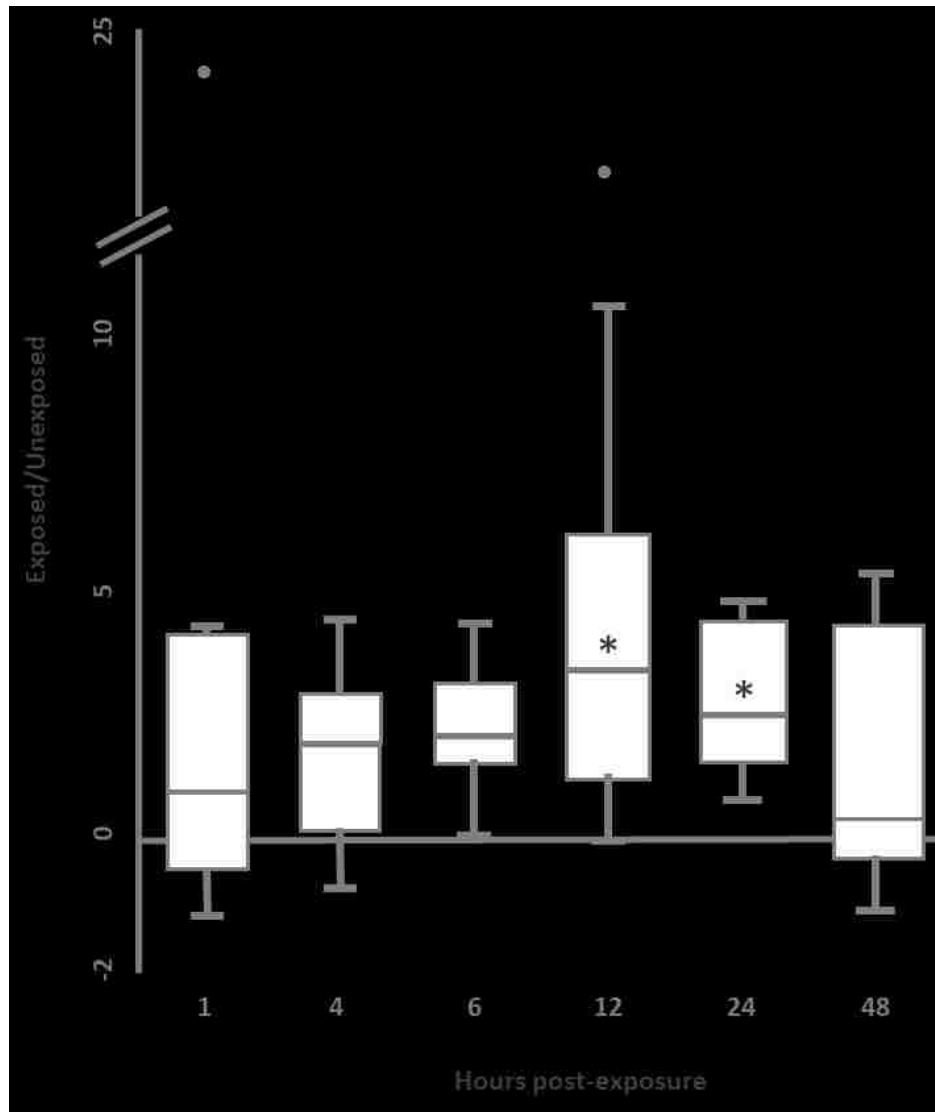
Figure 4. Hcl-1 is upregulated after bacterial exposure with *E. coli*. Exposure to Gram (-) *E. coli* was associated with upregulated expression in the AG of *B. glabrata* at 12 (4-fold median upregulation, p-value= 0.036) and 24h post treatment (p-value= 0.0248) relative to untreated snails, depicted by *. A two-sample t-Test assuming unequal variance was used for mean comparison of unexposed and exposed samples, alpha was set at .05.

on day 2 after the first injection and on day 10 (after the second injection), and one GFP

siRNA injected snails on day 2 (Fig. 5).

Densitometric analyses of AG protein profiles indicated that RNAi treatment

effected knockdown of Hcl-1 protein, although not to the same extent in individual snails,

with Hcl-1 levels in individual snails ranging from 25 to 70% to those in the GFP

controls (Fig. 6). Protein profiles of AG samples were otherwise similar such that siRNA reagents used did not appear to alter expression of other proteins. After the first siRNA injection, Hcl-1 knockdown was evident at day 2 and more pronounced at day 7. Increased levels of Hcl-1 on day 14 (two days after the second "booster" siRNA injection), still low compared to controls, suggest that the snails begin to recover from RNAi knockdown after a week, however, low levels at day 22 indicate that RNAi knockdown can be effected repeatedly in the same snail. (Fig. 6).

Six out of 14 (43%) remaining snails with RNAi-mediated (none complete) knockdown of Hcl-1 ceased production of egg masses by day 6 after the first injection. Production of egg masses was then reinitiated and yet again reduced following the second injection with siRNA targeting Hcl-1. (Fig. 5).

Knockdown of Hcl-1 also reduced the number of eggs per egg mass; egg masses containing 5-25 eggs were observed throughout the duration of the experiment with exception of the last few time points that resulted in the normal range of 15-55 eggs/egg mass (Table 2).

Inspection of eggs from snails after Hcl-1 knockdown revealed unusual morphologies of embryos. Embryo's did not progress in size, different from what was observed from the GFP control snails and as would be appropriate for the gastrula stage that is attained normally within 72 h (the time interval between collection; Nakano et al., 2003; Shartau et al., 2010).

Alternative morphologies suggested damaged or destroyed embryos. At eight days after production of egg masses, the percent hatching was also reduced in eggs from snails with Hcl-1 knockdown (Fig. 7, Table 3).
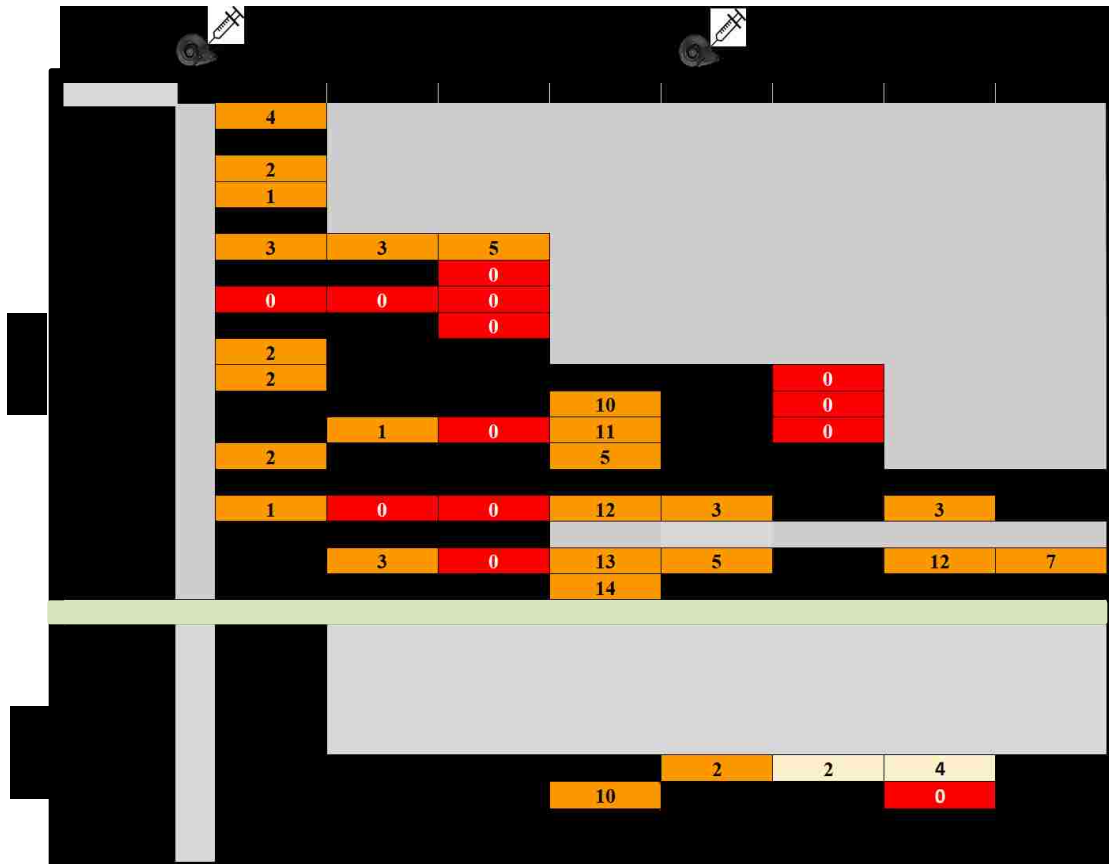
Figure 5. Hcl-1 knockdown affects egg mass output. Egg masses were collected at the days shown in timeline. Checkmarks correlate with timepoints for albumen gland protein extraction in Fig. 3. Cells colored in red highlight lack of egg mass production, orange cells show aberrant egg masses and yellow cells are snails that had a low proportion of aberrant egg masses (>30%). Snails that died after first injection are not included in the image (14b and 21e-GFP).

Considering all reproductive output from the different cohorts of RNAi-treated *B. glabrata* (grouped by day of sacrifice, averaged for living snails in a group up until sacrifice), knock down of Hcl-1 versus controls produce similar number of egg masses, but these contained fewer eggs and the hatching success of these eggs was reduced by 20-30 %. Overall, reproductive output, expressed as the average number of hatched juvenile snails per adult snail was reduced by 68% (day 7) and 57% (day 22) for the HCl-1 knockdown *B. glabrata* versus snails treated with GFP siRNA (Table 3).
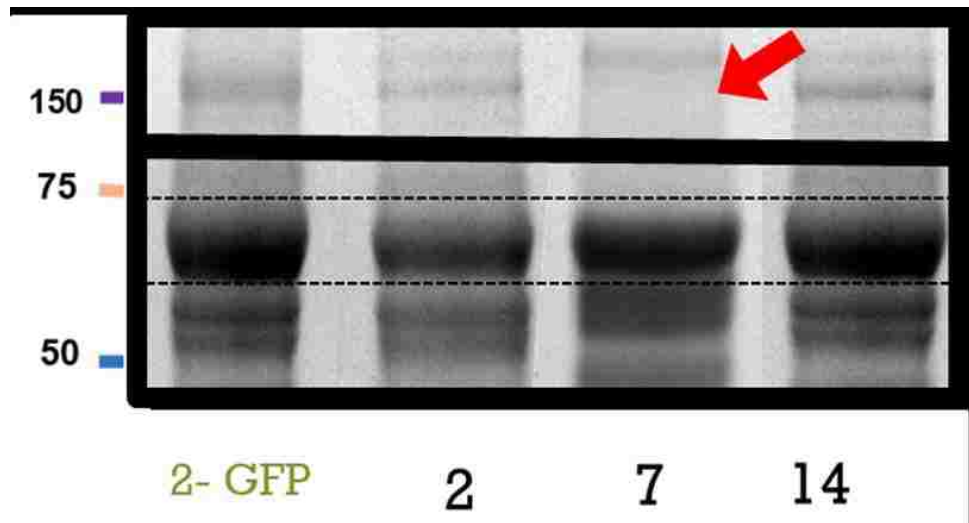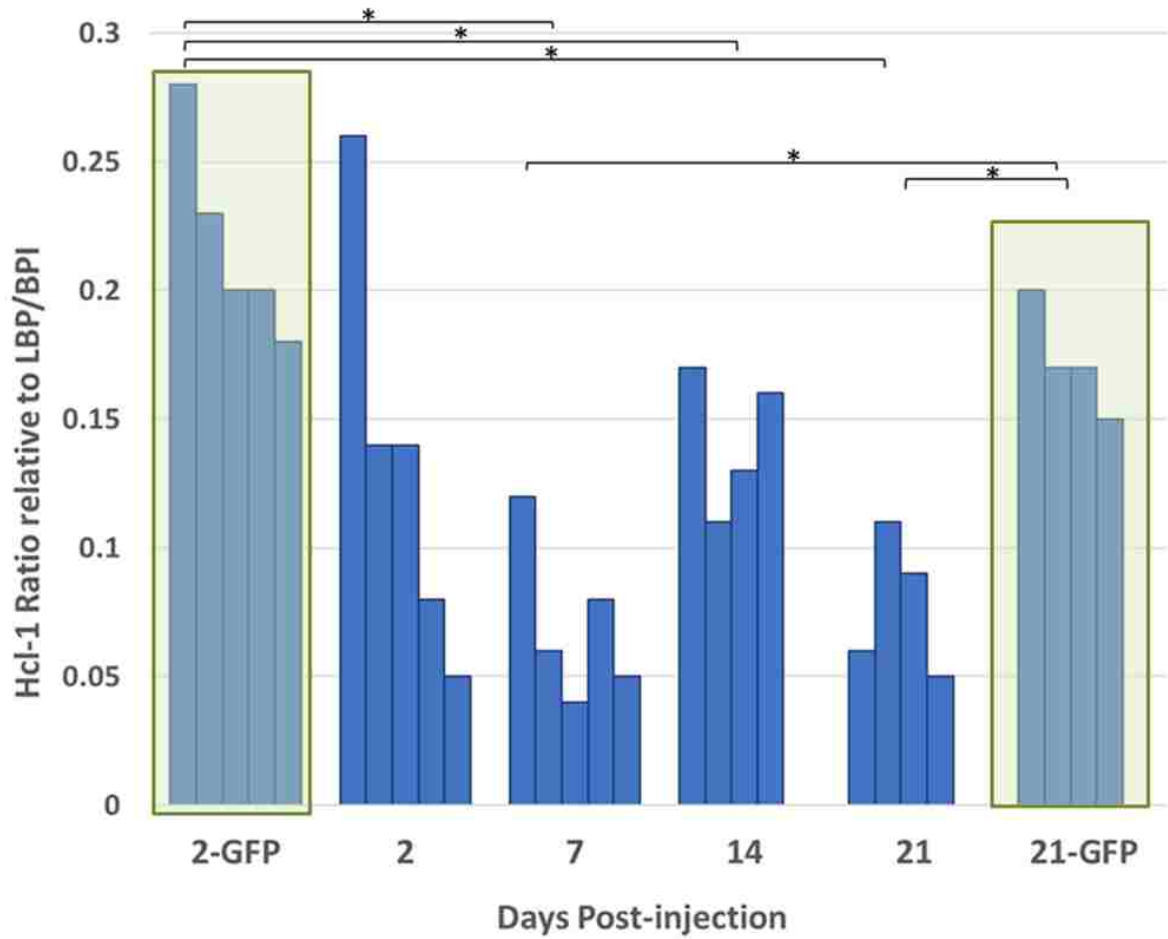
Figure 6. Hcl-1 protein is reduced after siRNA knockdown. Densitometric analyses (Top) of AG protein profiles indicated that Hcl-1 protein was reduced most at seven days after injection; relative to LBP/BPI. Hcl-1 levels/individual snails ranged from 25 to 70% compared to those in the GFP controls. Asterisks show significant differences (p-value=<.05). Coomassie stained gradient gel (5-20%) shows reduction of Hcl-1 presence (150kDa; red arrow). LBP/BPI is highlighted by the strong band between two dashed lines.

Table 3. Egg output is reduced in Hcl-1 knockdown snails. Last column to the right shows percentage of juveniles produced by the Hcl-1 knockdown snails relative to GFP controls.

	Hcl-1 knockdown					GFPsiRNA					
Day	#EM	#eggs/EM total	Total eggs	%hatch	juveniles	#EM	#eggs/EM total	Total eggs	%hatch	juveniles	Hcl-1/ Control
2	2	5	10	(-)	(-)	3	13	39	(-)	(-)	(-)
7	7	16	112	69	77	7	36	252	95.8	241	31.90%
14	19	25	475	82	390	18	55	996	96.2	958	40.70%
21	36	19	684	80.9	554	25	54	1350	96.8	1306	42.40%

DISCUSSION

The properties of Hcl-1 protein justified further characterization: over evolutionary time the protein derived from gastropod respiratory hemocyanin, yet it is expressed in the AG and became involved in reproductive biology as a constituent of EMF of eggs from snails only in the family Planorbidae of the hygrophilid taxon of freshwater panpulmonate snails (Peña & Adema, 2016; Chapter 3).

Biomphalaria glabrata Hcl-1 was investigated as a potential antibacterial response factor because Hcl-1 belongs to the copper 3 type protein superfamily that includes sequence- related hemocyanins and phenoloxidases with antibacterial immune function (Terwilliger, 2007). Additionally, several other components of *B. glabrata* EMF were previously identified as likely immune factors (Hathaway *et al.*, 2010). When exposed to bacteria, however, qRT-PCR did not show upregulated expression of *Hcl-1* transcripts in the AG of *B. glabrata* in response to the Gram (+) *M. luteus* whereas Gram (-) *E. coli* evoked a transient response, elevated to a maximum of 5-fold expression (at 12h and 24h, returning to baseline at 48h). This is in line with detection of hemocyanin related sequences in response to bacterial infection using an oligo-based microarray (Adema *et al*. 2010). Another EMF protein, *B. glabrata* LBP/BPI was also found to protect eggs against a single, specific category of environmental pathogens, oomycetes but not bacteria (Baron *et al.*, 2013). Perhaps bacterial stress causes a modest, temporary

increase of parental investment by *B. glabrata* into immune defense of offspring by elevated contribution of Hcl-1 (and possibly other EMF components) to eggs, or, *B. glabrata* expresses more AG proteins to supply an increased number of eggs produced under stress from Gram (-) but not Gram (+) bacteria similar to the response of some ecdyzosoans to bacteria (Adamo, 1999; Mosser, Matic & Leroy, 2011).

The application of experimental RNA interference-mediated knockdown, previously shown effective for several *B. glabrata* genes including AG expressed LBP/BPI (Baron *et al.*, 2013; Garcia *et al.*, 2010; Hanington *et al.*, 2010; Knight *et al.*, 2011; Jiang *et al.*, 2006) provided significant advances in functional characterization of Hcl-1 by indicating a prominent, critical role for Hcl-1 in the reproductive biology of *B. glabrata*.

Considering that RNA transcript levels rarely correlate directly with expression of the encoded proteins (Vogel & Marcotte, 2012; Hotez *et al.*, 2014), we confirmed effectiveness of RNAi knockdown directly by showing reduced levels of Hcl-1 protein in the AG of snails injected with siRNA targeting Hcl-1, versus control. The RNAi knockdown of Hcl-1 was evident after two days and lasted for about 7 days, similar to observations reported by Jiang *et al* (2006). The efficiency of RNAi knockdown was variable, yielding between 25-70% reduction of the Hcl-1 amount in AGs of experimental *B. glabrata*. Nevertheless, knockdown of Hcl-1 yielded a dramatically altered phenotype consisting of inhibited reproduction in *B. glabrata*. Within 2-7 days after RNAi treatment, individual *B. glabrata* snails either produced egg masses with reduced numbers of eggs or ceased egg laying all together compared to controls. Snails of the latter category resumed egg laying during the experiment, but this must be considered

in light of the temporary effect of RNAi knockdown (about 7 days). In fact, egg laying by the snails was again halted after a second injection with SiRNA targeting Hcl-1.

Potentially, the loss of reproductive activity of *B. glabrata* would be irreversible with permanent absence of Hcl-1. This study, aimed at Hcl-1, represents the second example of functional characterization of a component of *B. glabrata* EMF. Baron *et al* (2013), in using a similar experimental approach involving RNAi knockdown of AG-expressed LBP/BPI, showed that this EMF protein provides immune protection to snail eggs. Loss of LBP/BPI had an indirect impact, however, affected egg masses and embryos developed normally in presence of several opportunistic pathogens, except for having increased susceptibility only to oomycete infestation. Rather than providing optional immunoprotection for a specific pathogen that may not be encountered, Hcl-1 is involved at a more fundamental level in the reproductive biology of *B. glabrata.* Significantly, RNAi knockdown (even reducing, not fully abrogating AG expression) of Hcl-1 severely inhibited or even halted reproductive output of *B. glabrata.* The phenotypic manifestations ranged from halted production of egg masses (no reproductive output) to egg masses that held fewer eggs with lower hatching success compared to control snails. Inspection of non-hatched eggs indicated embryological morphologies consistent with death early during development (not progressing beyond the gastrula stage), with damage to embryo's similar as resulting from exposure to toxic chemicals and anoxic conditions (Nakano *et al.*, 2003; Shartau *et al.*, 2010). These effects suggest that Hcl-1 may have a role in regulating the successful packaging of fertilized eggs with EMF and subsequent addition of the egg shell before fully formed eggs are embedded in the matrix of egg masses. Additionally, Hcl-1 may provide immediate vital support for
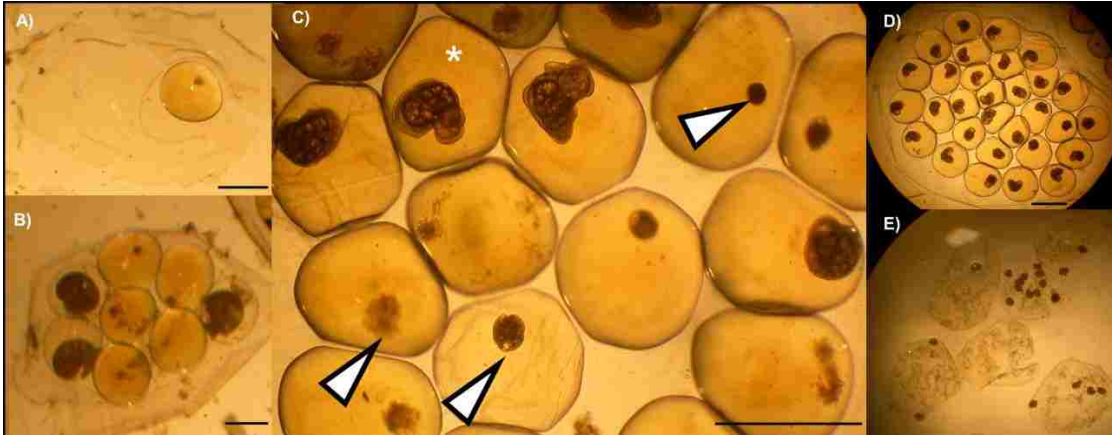
Figure 7. Hcl-1 knockdown affects reproductive output in *B. glabrata*. A) Unusual egg mass deposited with a single egg. B) Egg mass with eight egg shows only 37% hatching success. C) Egg mass from Hcl-1 knockdown snails six days after deposition. Both, developing and halted embryos are present in a single egg mass. White arrows point to disintegrated or paused embryos. Asterisks (*) denotes the expected wild-type development of *B. glabrata* embryos. D) GFP-control snails produce egg masses similar to wild-type snails, embryos show appropriate development 7-8 days post-oviposition. E) Eggs produced by controls hatched successfully, shown by multiple disrupted egg masses with no remaining embryos. Scale bar represent measurement of 1mm.

embryo development inside eggs, such as regulation of transcription, nutrition or provision of oxygen for respiration. These considerations warrant further functional characterization of the biological activities of Hcl-1.

The reproductive output of each group of *B. glabrata* with experimental Hcl-1 knockdown, expressed as average number of hatched eggs per individual snails, including snails that did not cease egg laying, was lower than that of control snails at all time points (Table 2). The phylogenetic distribution of Hcl-1 EMF proteins is restricted to the representative samples of planorbid snails, excepting other families of the Hygrophila (freshwater panpulmonate snails of the families Lymnaeidae and Physidae). The Hcl-1 proteins differ in sequence among genera (even at species and strain levels) of planorbid snails (Peña and Adema, 2016; Chapter 3). Already, several types of chemical inhibitors exist for tyrosinases, essentially the copper binding FU domains (Deri *et al.*, 2016) that characterize all members of the copper 3 type protein superfamily, including

Hcl-1. This may provide direction to development of specific inhibitors of the FU

domains of Hcl-1 of different species of the genera *Biomphalaria* and *Bulinus* that

transmit schistosome parasites, *Schistosoma mansoni* and *Schistosoma haematobium,*

Specific molluscicides that inhibit reproduction and consequently distribution in the field

of snail that transmit schistosome parasites may supplement MDA treatment of human

schistosome infections towards more sustainable, integrated control of schistosomiasis.

ACKNOWLEDGEMENTS

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SUMMARY

This dissertation investigates the egg mass fluid (EMF) factor, hemocyanin-like 1 (Hcl-1) of *Biomphalaria glabrata*., vector snail of schistosomiasis. Potentially, unique features of *B. glabrata* may be employed to interpret or reduce the distribution of this snail to aid in control efforts aimed at reducing impact of schistosome parasite infections on human health. Taken all together, the major findings are as follows:

1)   *Hemocyanin-like genes in planorbid snails differ from molluscan respiratory hemocyanins.*

In chapter two, two hemocyanin-related genes of the snail *B. glabrata* were characterized. These genes were of interest because *B. glabrata*, like other planorbid snails, employs hemoglobin as oxyen carrier and hemocyanin was not expected to be present. One gene, encoding Hemocyanin-like 1 (Hcl-1) protein was characterized in detail because it occurs in the albumen gland (AG) and egg mass fluid (EMF), indicating a role in snail reproductive biology. Based on having copper-binding functional unit (FU) domains, Hcl-1 belongs to the copper type 3 protein superfamily that includes hemocyanins (Peña & Adema, 2016). Different from respiratory hemocyanins, *B. glabrata* Hcl-1 only has three FU domains and it is expressed in a developmentally-dependent manner only in reproductively mature snails. The FU domains of Hcl-1 may not bind oxygen because canonical histidine amino acids in the copper-binding motif are lacking in *B. glabrata*. However, they are conserved in the planorbid species *Bulinus globosus* (Chapter 3). Despite considerable similarities in sequence and predicted structural features, other than subunits of respiratory hemocyanins, Hcl-1 molecules do not multimerize, likely due to structural differences in the C-terminal FU domain.

2)   Hemocyanin-like 1 (Hcl-1) genes are found only in representative samples of planorbid freshwater snails. Likely, evolution of a gastropod hemoglobin early in the planorbid lineage allowed mutation and repurposing of hemocyanins that were no longer needed for respiration.

Chapter three describes that comparative analyses of EMF and next generation sequence data indicate that Hcl-1 is broadly present in planorbid snails (additional to *B. glabrata*), but not in the closely related families of lymnaeid and physid freshwater snails. Also, considering the absence of hemocyanins in planorbids along with similarities between Hcl-1 and respiratory hemocyanins (sequence and predicted 3D structures; also see Chapter two), these findings strengthen the hypothesis that Hcl-1 derived from an ancestral hemocyanin. Molecular clock analysis of hemocyanin-related sequences from hygrophilid snails (Planorbidae, Physidae and Lymnaeidae) and other gastropod clades indicates that Hcl-1 diverged away from gastropod respiratory hemocyanins ~267 mya, consistent with the origin of Planorbidae within the panpulmonate lineage 200-300 mya (Baker, 1945). Hypothetically, convergent evolution yielded a respiratory hemoglobin in the ancestral planorbid, evident by the red blood of extant Planorbidae. Rendered functionally superfluous, rather than being lost, respiratory hemocyanins mutated and found new purpose as EMF proteins in the reproductive biology only of planorbid snails.

3)   Hcl-1 is important for successful reproductive output of B. glabrata embryos.

In Chapter four, we report that Hcl-1 protein, a unique feature of planorbid snails, is important for reproductive success of *B. glabrata.* The expression levels of *Hcl-1* transcripts in the AG do not change in response to Gram (+) bacteria but increase up to 5-

fold in a transient manner when encountering Gram (-) *E. coli*, indicating that Hcl-1 may be part of general stress response.

Reduction of Hcl-1 expression in the AG of *B. glabrata*, effected experimentally by RNA interference, strongly inhibits reproductive output of *B. glabrata*. Additional to decreased numbers of eggs per egg mass, eggs also had lowered hatching success rates with embryological development halted within 72 hours following oviposition. Moreover, almost half of the experimental snails ceased production of egg masses completely. The RNAi knockdown of Hcl-1 was temporary (~ 7days) and reduced rather than obliterated expression of Hcl-1 protein. Likely complete absence of Hcl-1 would completely inhibit reproductive output of *B. glabrata.*

CONCLUSIONS AND IMPLICATIONS

The complex biology of planorbid snails that vector schistosomiasis challenges and often defeats control efforts of schistosomiasis-transmitting snails in order to reduce (re)infection risk for human populations in disease endemic regions (WHO, 2017). Molluscicide application with potential broad negative environmental impact (Li & Wang, 2017) may have only short term effects; *Biomphalaria* snails easily (re)colonize water bodies, as simultaneous hermaphrodites they have the ability to reproduce readily, persist in polluted environments and can endure drought by remaining desiccated for months (estivation) to re-emerge after contact with water, all while transmitting schistosome parasites (Richards, 1967; Barbosa & Barbosa, 1994).

Study of all aspects of snail biology may identify unique targets that can be developed towards specific understanding or even control of schistosome vector snails like *B. glabrata.* This is exemplified by characterization of Hcl-1 from *B. glabrata*. Gene

discovery efforts revealed, unexpectedly, hemocyanin-related sequences that initially

seemed out of place in the perception of biology of *B. glabrata* and planorbid snails in

general. The research described in this dissertation provides an interpretation of the

emergence of Hcl-1 as a unique feature of planorbid snails that resulted over millennia

through complex evolutionary processes. Gastropod hemoglobin, that emerged through

myoglobin gene duplication, left the universal gastropod respiratory hemocyanins

redundant in ancestral planorbid snails. The hemocyanins underwent alterations to result

in Hcl-1 some 267 million years ago, this protein attained new function through

integration as EMF protein in reproductive biology of planorbids. Retention of Hcl-1

over this interval of evolutionary time suggests an important contribution to the success

of planorbid snails like *B. glabrata*. This is confirmed by dramatic reduction of

reproductive output following RNAi knockdown of Hcl-1 in *B. glabrata*. The resulting

phenotype in *B. glabrata* is starkly evident from egg masses with few eggs that contain

embryos with low hatching rates, or from complete cessation of egg laying.

As an aside, this phenotype associated with Hcl-1 function may provide a useful

marker or indicator to track the success of efforts to adopt the CRISPR/Cas technology

for genome editing as a research tool to the study of planorbids (Wanniger & Wollesen,

2018). Once functional, this technique will revolutionize functional studies of many as

yet uncharacterized genes of gastropods that have been revealed by full genome

characterization of *B. glabrata* (Adema et al., 2017) as well as many other gastropod

species (e.g. Schultz and Adema, 2017).

At this time however, presence in planorbids only, with species-specific

sequences, and importance for reproductive biology, make Hcl-1 a prime candidate target

for development of selective molluscicides aimed at reducing field abundance of planorbid snails that transmit schistosome parasites. Resolution of exact molecular functions is warranted to understand how *B. glabrata* Hcl-1 is involved in egg production and support of embryological development. The FU domains contained within Hcl-1 are typical for the copper type three protein superfamily. Already, extensive pharmaceutical information exists regarding inhibitors of tyrosinases, enzymes that essentially consist of a single FU domain. This may provide an effective jumping off point for developing chemical molluscicides that can withstand the rigors of environmental application while maintaining function to interact with planorbid snails in a manner that uniquely inhibits reproduction of locally occurring planorbid vector snails of schistosomiasis.

Indeed, study of basic snail biology may well provide novel avenues that can combine into strategies for long term, sustainable integrated control of schistosomiasis as threat to global human health.

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