Developing novel ways of studying motility in *Schistosoma mansoni* and its potential contribution towards inhibiting Schistosomiasis

by

Sreemoyee Acharya

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biomedical Sciences (Cell Biology)

Program of Study Committee:
Timothy A. Day, Major Professor
Gunnar R. Mair
Michael J. Kimber
Steve Carlson
Douglas E. Jones

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2019

DEDICATION

For Baba, Ma and Riki-who mean the world to me.

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NOMENCLATURE

- 5-HT — 5-Hydroxytyrosine.
- ACC — Acetylcholine gated chloride channels.
- Ach — Acetylcholine.
- AKAP. — A-kinase-anchoring proteins.
- AMPA — α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.
- ART — Artemether.
- AsA. — Ascorbic acid.
- ATP — Adenosine triphosphate.
- ATPDase1 — Adenosine triphosphate Diphosphohydrolase 1.
- *B. glabrata* — *Bioamphalaria glabrata.*
- *B. sudanica* — *Bioamphalaria sudanica.*
- BBPS — Body bends per second.
- BgGRN — *Bioamphalaria glabrata* snail granulin.
- BgTLR — *Bioamphalaria glabrata* toll-like receptor.
- BLAST — Basic local alignment search tool.
- BRI — Biomedical Research Institute.
- *C.elegans.* — *Caenorhabditis elegans.*
- cAMP — Cyclic adenosine mono phosphate.
- CAT — Choline acetyltransferase.
- CDC — Centers for Disease Control and Prevention.
- CNS — Central nervous system.

- CQ — Chloroquine.
- *D. tigrina.* — *Dugesia tigrina.*
- DAG — Diacylglycerol.
- DALY — Disability-adjusted life years.
- DCAT. — Cathepsin D.
- DNA — De-oxy nucleic acid.
- DPAL. — Peptidyl-1-α-hydroxy glycine-α-amidating lyase.
- DPBS — Dulbecco's phosphate buffered solution.
- dsRNA — Double stranded ribose nucleic acid.
- *E.coli.* — *Escherichia coli*.
- ECM — Extracellular matrix.
- ELV — Extracellular like vesicle.
- FaRP. — FMRF amide related proteins.
- FC — Fold change.
- FITC — Fluorescein isothiocyanate.
- FLP — FMRFamide-like neuropeptides.
- FREP — Fibrinogen-related protein.
- *G. tigrina.* — *Giardia tigrina.*
- GABA — *gamma*-aminobutyric acid.
- GAR — G-protein coupled acetylcholine receptor.
- GDC — Glutamate decarboxylase.
- GIF — Genome Informatics Facility.
- GPCR — G protein coupled receptor.

- NIAID — National Institute of Allergy and Infectious Diseases.
- NMDA — N-methyl-D-aspartate.
- NTD — Neglected tropical diseases.
- OD — Optical density.
- OPZ — Oltipraz.
- OXAM — Oxamniquine.
- *P. falciparum* — *Plasmodium falciparum.*
- *P.vivax* — *Plasmodium vivax.*
- PCR — Polymerase chain reaction.
- PDK1. — Phosphoinositide-dependent protein kinase enzyme 1.
- PDMS — Polydimethylsiloxane.
- PKA — Protein Kinase A.
- PKC — Protein kinase C.
- PKCβ. — Protein kinase C β.
- PLC — Phospholipase C.
- PLC-β — Phospholipase C-β.
- PLS — Partial least squared.
- PNS — Peripheral nervous system.
- PS. — Phosphatidylserine.
- PZQ — Praziquantel.
- QN — Quinine.
- R-PZQ — Racemic structure of praziquantel.
- RNA — Ribose nucleic acid.

- RNAi — RNA interference.
- ROS — Reactive oxidative species.
- RPM — Revolutions per minute.
- RT-PCR — Reverse Transcription polymerase chain reaction.
- *S. haematobium* — *Schistosoma haematobium.*
- *S. japonicum* — *Schistosoma japonicum.*
- *S.mansoni* — *Schistosoma mansoni.*
- Ser — Serine.
- TCA — Tricarboxylic acid.
- TCBZ — Triclabendazole.
- TE Buffer. — Tris EDTA (Ethylene diamide triacetic acid) Buffer.
- TGFβ. — Transforming growth factor β.
- Tm. — Temperature of melting.
- TRPM8 — Transient receptor potential metastatin type 8 channel.
- VOCC — Voltage operated calcium channels.
- W — Watt.
- WHO — World Health Organization.
- WrMTrck — Wormtrack.

ACKNOWLEDGEMENTS

I have a long list of people to thank for my achievements. I would like to mention each person in detail, so please bear with me. Each and every person has contributed in their own way to make me the individual I am now, and they have molded me into a brave, strong, independent, honest, loyal and sensitive woman. I can never repay them, and I will forever be grateful to them.

My major professor, Dr. Tim Day for believing in me, even when I didn't believe in myself. He was my rock of Gibraltar, and he saved me at a point in my life, when I was under tremendous pressure and unhappiness. He gave me the freedom and independence to conduct my experiments, he never ever got angry or disappointed with me, even when I was mad at myself for not living up to his expectations. He helped me regain all the self-esteem and confidence that I had completely lost, he taught me the difference between right and wrong, the fact that science will fail from time to time, but being a good human being, is more important. I will never be able to repay him for all he has done for me— I will forever be grateful and so proud that I had Dr. Tim Day as my mentor. He is a celebrity in my family, and my whole extended family (especially my parents) admire him, even though they have never met him. Dr. Day made me laugh when I needed it badly and taught me that life is short, so one should live it with joy and happiness.

Dr. Michael Kimber for always supporting me, helping and guiding me, even though I was not his student. There is this one sentence which will forever be with me—it was during my search for a post-doctoral position and he said –" It is our responsibility to make sure you join a good lab, with a good PI, because we care for you, not just now, but also in the future". The fact that Dr. Kimber and I could talk about BBC sitcoms like Father Ted, IT

crowd, Countdown, Downtown abbey, Irish pop music—was a much-needed break from science and these were topics I loved discussing. I am very thankful that he was there, and I will always consider him one of the best people I have ever met.

Dr. Gunnar Mair who spent so much time and energy over my manuscripts, my abstracts and my thesis—all in such a short time. Again, I am not his student, he did not need to help me, but he did it out of kindness and generosity. Although there were times, he got upset with me, and rightfully so, but I never ever was bothered or affected—because he always stuck by the statement —"I want you to get your Ph.D." I always knew he was looking out for me. For many reasons, he reminded me of my Father, so I was able to understand him very well. Also, he introduced me to French and German pop music and although we have very different views on Enya's music (I love it, he absolutely hates it), Dr. Mair is still one of the best, nicest, kindest and most helpful people I have ever met, and I am extremely proud that he was one of my mentors. I cannot thank my lucky stars that he arrived at that point of my career when I needed him the most.

Dr. Steve Carlson, without whom I would not be at Iowa State University in the first place. I can proudly boast of 6 review papers, all thanks to him. There was this one semester, where I was teaching at DMACC at Des Moines, and I would drive back to Ames at break neck speed, just so that I could attend his class. I am still mystified by the fact that I was never ticketed during that entire semester. His love for Breaking Bad, makes him one of the relatable faculty members ever. Dr. Carlson's kindness, understanding, the fact that I could talk to him at any moment, about anything--- will always leave an everlasting impact on me. I would also thank his wife, Dr. Kristi Carlson, who always greeted me with a warm smile every time we met.

Dr. Douglas Jones, who has always been there from day 1, and was forced to make so many adjustments around my constantly changing schedules. I know I never really got to know him personally, but I always knew he was a wonderful person (also because I trust all of Dr. Day's decisions blindly, no questions asked).

Ms. Mary Madsen—my teaching supervisor when I taught human anatomy and human physiology as a graduate teaching assistant, who taught me to be a hardworking, sincere and effective teacher. Even after I left the position, she has always been my cheerleader and a very good friend. Mary is the perfect example of how a lab supervisor should be—and in my opinion, human anatomy and physiology will never be the same without her. The fact that she considers me as one of her best TAs, is a huge honor for me.

Glenn Clarke—I put him through so much work, constantly scheduling and rescheduling my plans, and he was always there. All my emails to Glenn would start with an apology and end with an apology. As someone who isn't very patient, I truly admire his calm, pacifying nature. I wish the very best for him and I am glad he is part of this department.

Cathy Martens—the guardian angel of the BMS department. It's pure magic how she saves all of us from trouble, places orders when we can't even locate the website, forget the product—Cathy is constantly under so much pressure, and has to respond to so many demands but not once have I seen her angry, or upset. Even when she was physically hurt, she would still come to Vet Med –her face adorned with her beautiful smile. And those cakes made by her---a piece of heaven. This department will never be able to function without Cathy.

Dr. Prince Agbedanu –my mentor, my spiritual advisor, my counsellor, my therapist and finally the older brother I never had. It was Prince who got me through the Day lab, it was Prince who taught me basic molecular biology , it was Prince who got me into DMACC, it was Prince who educated me on the life cycle of schistosomes, and finally thanks to him, me and Hiruni got addicted to Buffalo Wild Wings. As someone who has grown up being very pessimistic, Prince taught me how to look at a glass half full. In spite of the fact that he left the university some years ago, I still make it a point to update him on whatever is happening in my life and the university (based on our combined love for gossip)—bad or good. Prince is a very happy father now, with two beautiful children—and that is what I wish for him all his life.

Dr. Hiruni Hettiaracchi became more of a confidante and a close friend than a lab colleague. She introduced me to Sri Lankan cuisine, which I consider one of the finest cuisines in the world. Hiruni, along with Dr. Nicholas Wheeler and Dr. Mostafa Zamanian, were definitely the crème de la crème of the Day-Kimber labs. All three of them are brilliant scientists and they served as a source of inspiration for me. To say that I admire them immensely, would be an understatement.

I want to thank the people who have always been there, as lab colleagues, as office mates and close friends –Wang Yuan, Hannah Loghry, Carmen Reynolds, Sanjana Mahadev-Bhat, Shaunik Sharma, Niranjana Krishnan, Priyanka Bhandary, Luna KC and Kristina Feye. We have been through so much together—happiness, sorrow, frustration, pain and laughter. We talked about research (mostly the failures), funding (mostly the lack of it), relationships (again, lack of it) and miscellaneous topics which had nothing to do with research or work whatsoever. There were cakes, snacks, chocolates, stories of pets, gossip and in-depth

conversations about anything and everything. As someone who has depression, I always made it a point to come to Vet med, whether I had work or not. Some hours of laughter would cheer even the most morose of people and was probably the best anti-depressant on the planet. My graduate school experience was wonderful and memorable because of them.

Finally, this would not be complete without mentioning some of my closest friends and family—Chandni Saxena, Ishani Dasgupta, Sandesh Shankarappa, Sneha Bankar, my Aunt Jolly Roy, my favorite cousin Ruby Roy and my extended family, who have stood by my side, during some of the lowest phases of my life. I may not have everything in this world, I may not be the best scientist on the planet, but I consider myself one of the luckiest people in the universe. Thank you, with all my heart and soul, and I vow to be there for everyone, as everyone has been there for me.

ABSTRACT

Schistosomiasis, caused by *Schistosoma mansoni*, is responsible for infecting approximately 200 million people worldwide, mostly from low-income and middle-income populations; it is a key neglected tropical parasitic disease, second only to malaria as the most devastating parasitic disease in the world. An infection is initiated when the cercarial form of the parasite is released from its intermediary invertebrate host, a *Biomphalaria* snail, into the surrounding fresh water. Cercariae are non-feeding, free swimming, extremely infectious, highly motile schistosomal stage with bifurcated tails and they penetrate the mammalian skin tail-first, thus infecting the human host. Post attachment, the cercariae sheds its tail and the resulting schistosomule continues to develop within the host circulatory system. The parasites travel to the hepatic system, where they transform into adult worms, mate and lay eggs, most of which are excreted through the host's excretory system and the rest accumulating within the internal organs of the body. The spread of schistosomiasis relies heavily on the motility of the cercariae before human infection, as well as the movement of the schistosomules through the human body, post infection. For my doctoral dissertation, I have focused on the aspect of motility of the *S.mansoni* worms pre and post infection. The first part of my research deals with the design and development of a sensitive, simple, cheap biological assay i.e. a microfluidic platform to study the movement of the schistosomules as they travel through the host circulatory system. The complete navigation and the kinetics of the movement of the juvenile worms through the convoluted pulmonary, blood and hepatic vessels within the host remains largely unexplored. We believe that this novel approach will provide a highly efficient method for screening potential anti-schistosomal compounds and improving motility assays. The second part of my research concentrates on the qualitative

and quantitative proteomic analysis of the cercarial tails and cercarial bodies. Using mechanical separation of cercarial tails and bodies, and mass spectrometric analyses, we have identified a total of 945 proteins in the combined cercarial proteome from 4 independent samples: 791 proteins in the cercarial tails and 645 proteins from the somule bodies. Gene oncology analysis was conducted on the obtained proteomic data, and the peptide hits were classified based on molecular function, biological function and subcellular location. In conclusion, I believe that by preventing the motility of the parasitic worms at different stages of the life cycle is a novel, previously unexplored route for investigating potential drug targets which could interrupt the spread of the disease.

CHAPTER 1. INTRODUCTION

1.1.Schistosomiasis Is an Important NTD

Neglected tropical diseases (NTDs) are defined as a large, diverse group of infectious diseases, that are mostly present in tropical and subtropical countries [1-3]. According to the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC), more than one billion people, mostly from low-income and middle-income populations, are affected by NTDs in at least 149 countries [1-6]. Residents living in places without access to safe water, poor sanitation, contact with unhygienic contaminated water, limited education, food insecurity and lack of access to basic health services are prone to such infections [2, 4, 6]. NTDs are caused by a wide variety of microorganisms and metazoan pathogens which include viruses, bacteria, protozoa and helminth parasites [2, 3]. Helminth diseases are caused by two groups: nematodes and platyhelminths [7]. Schistosomiasis is an endemic NTD which is second-only to malaria as the most devastating parasitic disease in the world [8-11]. It is caused by a diverse group of helminth trematode parasites called schistosomes, which belong to the family *Schistosomatidae* within the phylum platyhelminthes [5, 12, 13].

This parasitic disease is responsible for the loss of 1.53 million disability-adjusted life years (DALYs) and up to 280,000 deaths annually in sub-Saharan Africa alone [14-16]. According to reports filed by the National Institute of Allergy and Infectious Diseases (NIAID) and CDC, these parasites are not found in the United States. Human schistosomiasis is distributed throughout Africa including sub-Saharan Africa, the Sudan, the Middle East and Egypt; in South America the disease is found in Brazil, Suriname and Venezuela; the Caribbean (for example, the Dominican Republic, Guadeloupe, Martinique and Saint Lucia); parts of China and South east Asia which includes Cambodia, Vietnam and Laos [3, 5, 9]. In

regions of high transmission, 60-80% of school-aged children and 20-100% of adults can be infected [4, 6, 7]. In 2014, the European Center of Diseases Prevention and Control reported an outbreak of urogenital schistosomiasis amongst a group of German tourists who had never visited a schistosome-endemic countries, in the French island of Corsica [17]. On further analysis, a large number of *Bulinus truncatus* snails were found along the shores of the river Cavu, which were infected with *S.haematobium* [17]. It was hypothesized that infected people had travelled from Senegal where this type of snail is abundantly present, to Corsica and in this way, the disease was spread [17]. The three primary species of the genus *Schistosoma* are *Schistosoma mansoni*, *S. japonicum* and *S. haematobium*. [4, 5, 8, 11, 12]. *S. mansoni* and *S. japonicum* are responsible for intestinal schistosomiasis, whereas *S. haematobium* causes urogenital schistosomiasis. Schistosomiasis is a prominent health concern in endemic regions and, unless detected early, can be responsible for high morbidity and mortality rates [9]. Of the three species, *S. mansoni* is most frequently maintained in laboratories in order to extensively study host-parasite interactions [18].

Life Cycle of Schistosomes

Schistosomes pass through two hosts in order to complete and maintain its life cycle: an intermediate snail host and a definitive mammalian host [3, 5, 13, 19]. Various freshwater snails can act as intermediate hosts for the different species of schistosomes [3, 6, 20]. These include *Bioamphalaria glabrata and Bioamphalaria sudanica* as hosts for *S. mansoni*, as well as *Bulinus* species as hosts for *S. haematobium* and *Oncomelania* species as hosts for *S. japonicum*. *B. glabrata* snails are common in South America such as Brazil and Venezuela, the Caribbean, Puerto Rico, Dominican Republic, Haiti, Guadeloupe and Suriname [18]. *B. sudanica* is more prevalent than *B.glabrata* in Africa, Madagascar and the Middle East [18].

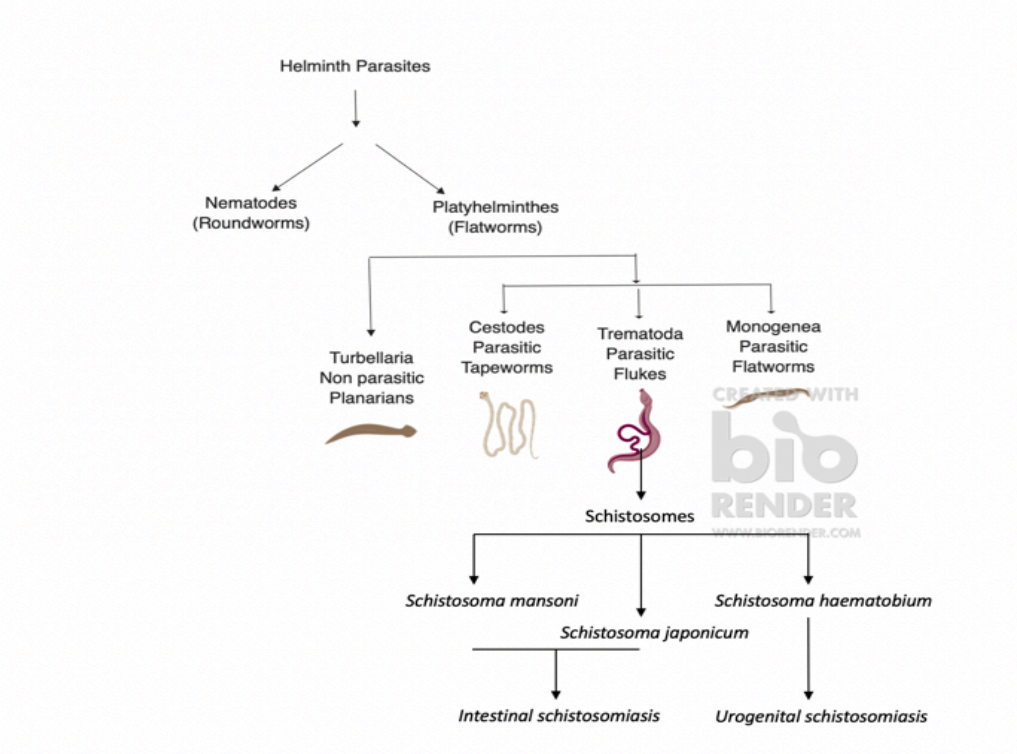


Figure number 1.1 :- The family of Helminth parasites. Helminth parasites are large macro parasites, visible to the naked eye. Many of them are intestinal worms that are soil-transmitted that infect the human gastrointestinal tract, whereas the schistosomes reside in the blood vessels. Helminth parasites are divided into two primary types :- nematodes and platyhelminthes. Nematodes such as roundworms have tubular digestive systems with openings at both ends. Platyhelminthes such as flatworms are simple, bilaterian, unsegmented, soft bodied invertebrates. They have no body cavities and are known as acoelomates. They also do not have any specialized circulatory and respiratory organs. Most have flattened shapes, which allow oxygen and nutrients to pass via diffusion. Platyhelminthes are further divided into two parts – Turbellaria which are non-parasitic; and parasitic cestodes, trematodes and monogenea. Schistosomes belong to the subclass trematodes, and there are broadly divided into *Schistosoma mansoni* and *Schistosoma japonicum*, which are responsible for intestinal schistosomiasis; and *Schistosoma haematobium* which is responsible for urogenital schistosomiasis.

This diagram was created using Biorender.com.

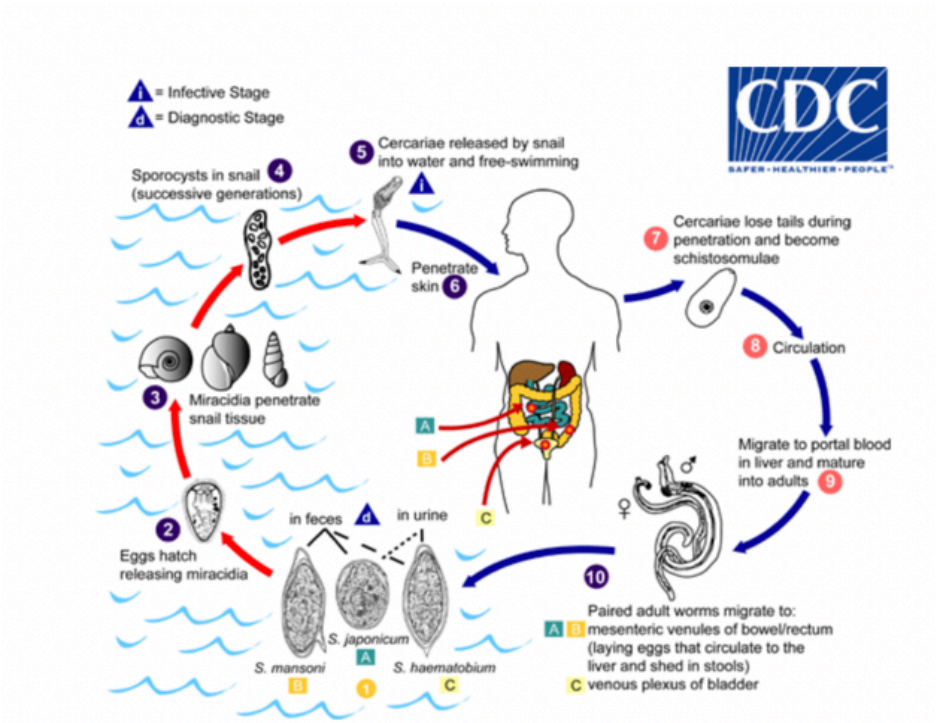


Figure number 1.2 :- The different stages of the life cycle of schistosomes. Eggs are eliminated from the patient, via feces or urine. 2. Under optimal conditions, the eggs hatch and release miracidia. 3. The ciliated, free- living miracidia swims and penetrates an intermediate invertebrate snail host. 4. Within the snail host, the miracidia develops into 2 generations of sporocysts –mother sporocysts and daughter sporocysts which ultimately develop into the cercariae. 5. The cercariae is released from the snail, marking the most infectious stage in the life cycle. 6. The cercariae penetrates the skin of the human host. 7. Once inside the human host, the cercariae sheds its tail and becomes schistosomules. 8. The schistosomules travel through the circulatory system. 9. They migrate and settle in their final residence- the liver. S.mansoni is found often in the superior mesenteric veins which drain the large intestine. 10 ABC. The schistosomules mature and develop into male and female adult worms. The paired worms mate and the female worms lay eggs in the small venules of the portal system and the perivesicle system. The eggs then gradually move towards the lumen of the intestine, and are eliminated via excretory products. However, a large number of eggs accumulate in the liver, leading to the formation of granulomas, which leads to the onset of schistosomiasis.

Source of the image :- https://www.cdc.gov/parasites/schistosomiasis/biology.html

After a person is infected with schistosomiasis, they release the parasitic eggs via feces or urine into water. The free-living, motile, non-feeding, non-reproducing and ciliated miracidia hatch from these eggs and they are attracted by a number of chemoattractants secreted by the intermediate snail host such as amino acids, magnesium ions, calcium, short-chain fatty acids, sialic acid, ammonia, glutathione, inorganic ions, D-glucose and

glycoprotein [21]. The apical papilla of the miracidia facilitates its attachment to the snail, and the proteases are secreted from the apical and lateral glands. These secretions along with mechanical movement allows the miracidia to penetrate the snail through the exposed head, foot, tentacle and mantle regions of the snail [3, 22-25]. Once inside the snail, the miracidia sheds its ciliated plates and a new syncytial tegument is formed. Asexual development of the larval stages of the parasite occurs within the intermediate snail host [22, 24, 26], producing two generations of multicellular sporocysts (the mother and the daughter), which finally differentiate into thousands of free swimming, infectious cercariae. Once mature, they are released from the birth pores of the daughter sporocysts into the fresh water triggered by sunlight [3, 20, 22-25].

However, certain specific populations of *B. glabrata* are resistant to transforming miracidia to sporocysts due to the presence of specific proteins like fibrinogen-related proteins (FREPs), *B.glabrata* snail granulin (BgGRN) and *B.glabrata* toll-like receptors (BgTLR), which activate the snail's immune system, thus killing the miracidia, reducing or preventing the formation of infectious cercariae [18, 25].

The approximate length of a cercaria is 300-500 μm with the width being approximately 70 μm [27, 28], The body of the cercaria is covered by a sugar-rich glycocalyx, upon a layer of body membranes known as the tegument [24]. These parasites are free-living, highly mobile, non-feeding parasites, present in the upper layers of the water body and they possess a sole aim of locating a suitable mammalian host to infect by using their forked tail which allow them to propel through the water [20, 25, 28, 29]. Apart from humans, the *S. mansoni* cercaria can also infect rodents and non-human primates [4]. Cercariae have two days (approximately 48 hours) to accomplish this feat and they rely

completely on glycogen stored in their bodies especially in the tail [3, 23, 24, 29-32] The energy derived from the glycogen in the tail fuels the movement, as well as use variety of ciliated sensory papillae located on the cercarial body [3, 23, 24, 29-32]. The stored glycogen undergoes a mechanism called glycogenolysis, where the enzyme glycogen phosphorylase converts glycogen to glucose-1-phosphate. Phosphoglucomutase acts on glucose -1-phosphate producing glucose -6- phosphate, which then proceeds to glycolysis, to provide energy for the cercariae. If the parasites are unable to locate a mammalian host, the glycogen store is completely used up and the cercariae die, as they cannot perform glycolysis to sustain themselves.

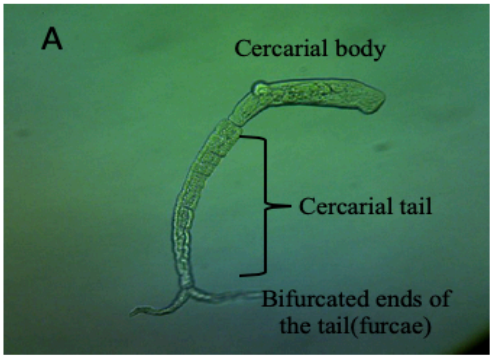
The human host is located through chemoattractants secreted by the human skin such as linoleic acid, ceramides and arginine. During infection, the cercaria penetrates the host skin by the mechanical activity of the tail as well as the secretion of proteolytic enzymes like proteinase, cercarial elastase and protease from the pre-acetabular cercarial glands, which allow the parasite to attach to and invade the mammalian skin [3, 13, 23, 24, 33-37]. The post acetabular glands secrete mucus for adhesion. During the invasive process, the cercariae shed their forked tails as well as the surface glycocalyx exposing the tegument underneath, thus transforming them into schistosomules or somules [3, 13, 23, 28, 29, 34, 36]. Along with the loss of the tail, the parasite also undergoes a change in physiology, which includes adapting to a different osmotic environment and a warm-blooded host [28, 29, 36].

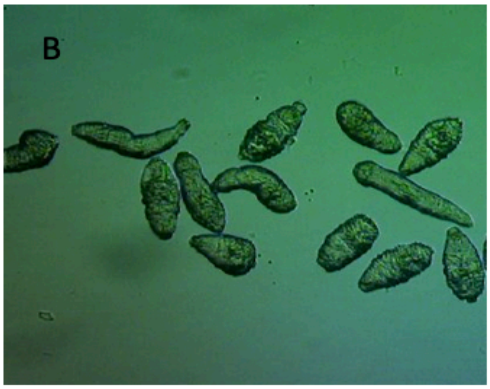
The schistosomules remain on the host skin for a short period of time, after which they tunnel through the host's dermis and travel through the circulatory system to the liver where they settle within the superior mesenteric venules that drain the large intestine gradually transforming into adult worms, capable of living up to 10 years in the body of the

host [8, 13, 14, 24, 35]. Schistosomes are dioecious by nature which means they develop either as male worm or as a female worm [6, 13, 24, 38]. Mature worms are approximately 7-20 mm in length, with a cylindrical shape and they possess suckers, digestive tracts and reproductive organs [3]. The male worms are slightly shorter than the female worms, and they mate in the ventral gynaecophoric canal of the male worm [3-5, 8, 23]. After mating, the adult females produce a large quantity of eggs (approximately 100-300 eggs per day) utilizing energy from the fatty acids from the human host by feeding on erythrocytes [4, 5, 8, 39]. This energy is produced exclusively via anaerobic glycolysis [3, 6]. Most of the eggs are excreted through the host's urine and stool [8, 39] but many eggs become permanently lodged in the liver and the small intestine of the body [4, 39].

Schistosomiasis goes through two phases: acute schistosomiasis followed by chronic schistosomiasis. In acute schistosomiasis, some of the initial symptoms of the disease include dermatitis as a result of cercarial penetration into the skin, followed by Katayama fever which is characterized by an immunological response to the antigens present during the maturation of the schistosome worms; abdominal symptoms such as enlarged liver and spleen (splenomegaly), followed by pulmonary hypertension, which are caused by the somules migrating through the human body [4, 13, 23, 40, 41]. The chronic version of schistosomiasis is identified by the deposition of collagen and extracellular matrix (ECM) components surrounding the eggs in the liver which result in granulomas, simultaneously activating the host immune system by bringing CD4+ T cells, CD8+ T cells, B cells, M2 macrophages, eosinophils and mast cells to the granulomatous inflammation [4, 13, 40, 42, 43]. The pathology of the disease is primarily caused by the host's immune response to the

accumulation of these schistosome eggs in the intestine and the liver, and not just by the juvenile or adult parasitic worms [4, 8, 13, 24, 40].





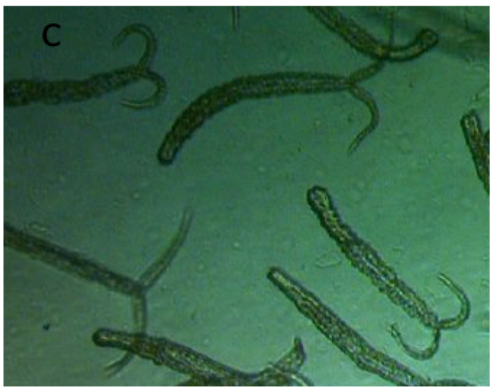


Figure number 1.3.A :- *Schistosoma mansoni* cercaria. Figure number 1.3.B :- *Schistosoma mansoni* schistosomules. Figure number 1.3.C :- *Schistosoma mansoni* cercarial tails A. *Schistosoma mansoni* cercaria was taken immediately after it was freshly shed from an infected host *Bioamphalaria glabrata* snail. The structure of the cercaria can be broadly divided into the upper head and the lower tail. The tail extends to form bifurcated ends, which help the highly infectious cercaria to swim and survive in the fresh water, until it can locate a mammalian host like a human being, to infect and penetrate. Once inside the host body, the tail is discarded and the cercarial body transforms into a schistosomula, which then continues on its journey through the host circulatory system. **B.** shows the *S.mansoni* bodies, or somules, after the cercarial tails were separated using a vortex and percoll gradient centrifugation. The somules will travel through the circulatory system of the human host towards the superior mesenteric veins which drain the large intestine, after which they will develop into adult male and female worms, and lay eggs. **C.** the discarded *S.mansoni* cercarial tails, which were separated from the main body using a vortex and percoll gradient centrifugation. All images were taken using a Nikon Eclipse TS 100 microscope with a 10X objective lens, attached to a 5.0 megapixel Nikon ED camera,

1.2 Old and New Methods For treating Schistosomiasis

1.2.1. Oxamniquine

Oxamniquine (OXAM), a tetrahydroquinolone compound, was developed by the pharmaceutical company Pfizer in the late 1960s and was sold under the brand name Vansil. It was introduced to the medical world in 1972 and was found to be effective only against *S. mansoni* schistosomules and adult worms (especially the adult male worms), and not against any other schistosome species.

Once administered, (OXAM) is converted to a sulfate ester by a sulfotransferase enzyme, which then dissociates and binds to the parasitic worm DNA covalently [44-46]. The binding leads to disruption of the cell cycle and inhibition of protein synthesis, as a result of nucleic acid alkylation, causing paralysis in the affected worms [44, 45]. The paralyzed worms are transported from the mesenteric circulation to the liver of the host, where the cellular host response kills the parasite [44]. OXAM also promotes the damage of the tegument, thus exposing the antigens on the surface of the worms, which are identified by the host immune system [4, 10, 44, 47, 48]. Another schistosomal drug called hycanthone was found to possess chemical and biological similarities with OXAM and its mechanism of action was caused by interfering with the synthesis of schistosomal nucleic acids. However, this drug also created the birth of hycanthone resistant schistosomes and was thus discontinued [49].

OXAM was used to treat approximately 10 million people in Brazil, however it led to resistance in the schistosomes, and thus had to be removed from the market in 2010 [45, 46]. It was found that a mutation in the enzyme of sulfotransferase made the drug ineffective [44,

45]. There were also reports of OXAM being responsible for seizures in epileptic patients and not being safe for pregnant women, hence the necessity to remove the drug quickly [4, 10, 47, 48]

1.2.2. Benzimidazole

Benzimidazoles are broad spectrum anthelmintics and are used in cestode, nematode, protozoan, and microsporidia infections [50, 51]. The first of these drugs was tiabendazole introduced in 1961 [51]. The role of these drugs is to interfere with the assembly of microtubules, specifically binding to β-tubulin, causing the unfolding of the protein leading to a conformational change, which inhibits the polymerization of tubulin, a protein subunit of the microtubules [50, 51]. This, in turn has a cascade effect by restricting the role of tubulin in the movement of the subcellular components and metabolites within the cytoplasm leading to interfering in spindle formation during the process of cell division [50, 51]. When administered at a high concentration, it converts fumarate to succinate, resulting in inhibition of microbial metabolism[51]. Triclabendazole (TCBZ) is a derivative of benzimidazoles and has been used to control o parasitic diseases such as *Fasciola* infections in veterinary medical sciences since the early 1980's [52]. TCBZ has been used to kill adult *S. mansoni* worms causing parasitic muscular contraction, destruction of tegument, ultimately ending in parasitic death within 24 hours [50, 52] . However, severe side effects have been observed post treatment like abdominal pain, nausea, vomiting, weakness, liver enlargement [52].

Another derivative of benzimidazoles is flubendazole when administered to *S. mansoni* infected mice leading to significant changes in adult worm recovery, reducing the load of eggs and decreasing the formation of hepatic granulomas [50, 51]. Albendazole was developed in the early 1970's and proved to be an effective broad spectrum antiparasitic

agent against nematodes, trematodes, cestodes, protozoan, ruminants and humans [51] However, this drug has been associated with teratogenicity and has been demonstrated to cause bone marrow toxicosis in dogs [51].

In order to survive in the human host, blood-feeding parasites such as *S. mansoni* and *P. falciparum* able to the bio crystallize of toxic heme molecules into inert and insoluble hemozoin (Hz), as evidenced by the presence of a significant quantity of Hz crystals obtained from the gut of the adult female *S.mansoni* worms [53]. Pyrido [1,2-a] benzimidazole derivatives (PBIs) possess both antimalarial as well as anti-schistosomal properties, examples of which are chloroquine (CQ), mefloquine (MQ) and quinine (QN) [53]. All these drugs have demonstrated antischistosomal properties such as the inhibition of the formation of hemozoin crystals, thus exposing the parasites to the toxic effects of hemoglobin, killing the worms and decreasing egg deposition [53]. Benzimidazoles tend to pose a threat at high doses, by causing unwanted side effects in the host [51]. These drugs can also have the potential of having teratogenic properties [51].

1.2.3. Praziquantel

Praziquantel (PZQ), which is sold under the brand name Biltricide and was introduced in 1978 [54], is one of the oldest and most popular drugs used to treat multiple parasitic worm infections, including schistosomiasis, related fluke infections such as liver and lung fluke infections caused by *Fasciolopsis buski*, various *Echinostoma* species, or *Heterophyses herephyses*, and also tape worm infections [3, 4, 55]. It can be used alone or in combination with corticosteroids and anticonvulsants [23]. PZQ is most effective against adult worms, but less effective against juvenile worms [4, 11, 54, 55]. The drug is used for mass administration (MDA) as it displays high efficiency and it is easy to administer, but

also due to the fact that it is well absorbed from the gastrointestinal tract [4, 6]. The exact molecular mechanism of PZQ is still unknown, however certain theories have been proposed to describe the drug's mode of action [6, 54].

It is hypothesized that PZQ increases permeability of the parasitic membrane by binding to voltage operated calcium channels (VOCC), thus allowing a rapid influx of calcium ions [56]. This in turn, could result in the prolonged contraction of the worm somatic muscles leading to paralysis, and is accompanied with dramatic tegumental damage [6, 54, 57]. The outer surface of adult *S. mansoni* worms is protected by a special double lipid bilayer tegument which plays an important role for parasitic survival and nutrition, signal transduction, osmoregulation, development, host-parasite interactions, by hiding surface proteins from the host immune system [14, 58, 59]. Damage to the tegument is believed to expose these very antigens, which are recognized by the host immune system and help to eliminate the injured worms from the body [6, 14, 57]. Another study states that PZQ is capable of disrupting the ion transport system, which could also lead to increased influx of calcium ions through the schistosomal cell membrane [3, 6, 57]. It has been also been suggested that PZQ causes the inhibition of nucleoside uptake and depletion of glutathione in *S. mansoni* worms [48, 60-62]. When glutathione is depleted from the parasite, it leads to an accumulation of glutaredoxin glutathione mixed disulfides in the cytoplasm, which in turn lead to the production of toxic reactive oxidative species (ROS), causing the death of the *S. mansoni* worms, thus making the thioredoxin glutathione reductase (which is replaced by SmTGR in schistosomes) an interesting potential drug target against schistosomiasis [48, 61-64]. In fact, two earlier drugs, potassium antimonyl tartrate (PAT) and oltipraz (OPZ) were initially used as antischistosomal drugs around 1917, and both have been discontinued since

then [61]. Although the exact mode of action of PAT and OPZ is not known, it was hypothesized that PAT stopped the action of the parasitic glycolytic enzyme, phosphofructokinase and OPZ inhibited the action of SmTGR, decreasing the glutathione levels and increasing ROS in adult worms [61].

Recently, it has been established that the racemic structure of PZQ (R-PZQ) can act as a ligand that can bind and interact with the worm's endogenous G protein coupled serotonin (5-HT)2B receptor in the human host [65]. In addition, R-PZQ may also be capable of selectively activating a human transient receptor potential metastatin type 8 channel (TRPM8) located on the plasma membrane, thus inducing extracellular calcium influx [60]. However, there are several problems associated with the usage of PZQ for schistosome control. PZQ never reaches 100% efficacy, and the drug does not prevent re-infection [66, 67]. PZQ is not effective against juvenile worms and acts only on adult worms, which makes it a big limitation of the drug. Drug-resistant *S. mansoni* worms have been reported from various African countries including Egypt and Kenya [6, 11, 66]. In addition, PZQ tablets are large in size and bitter, and are therefore often crushed, added to juice for children to consume [4]. Some of the common side effects of the drug include abdominal pain, headaches, dizziness and damage to the gastrointestinal (GI) tract with blood present in the stool [4, 60].

1.2.4. Artemether

Artemisinins such as artemether and artesunate have been popular for their anti-malarial properties since 1979 [52, 68]. Artemether (ART), when used in combination with PZQ, has been found highly effective for anti-schistosomal treatment purposes, as it can target juvenile parasites better than PZQ [4, 6, 10, 23, 52, 61, 68]. Once the parasites are

exposed to ART and haemin, highly toxic free radicals are released in the guts of the worms, eventually killing them [68]. However, a major disadvantage of using ART for the control of schistosomiasis is the gradual widespread risk for the development of malarial parasite drug resistance especially for *Plasmodium falciparum* and/or *P. vivax* [6, 10, 23, 61].

1.2.5. Molluscides

One of the earliest attempts to eradicate schistosomiasis was the use of broad spectrum molluscicides (e.g. niclosamide) for snail control, that would eliminate and kill the intermediate snail hosts; that procedure however was not successful in the long term since molluscides can be expensive, toxic and hard to maintain over a long term period [6, 11, 69].

1.2.6. Introducing Prawns as A Biological Mode of Control

Other resources such as environmental modification, education and improvement of sanitation are being explored to help curb schistosome infections [11]. A biological method of reducing schistosomiasis has been proposed by releasing predators of snails like prawns into endemic regions [15, 16, 69]. In fact, snails infected with miracidia, were found to demonstrate slow movement and detectable physiological changes, making them more susceptible to predators, as compared to uninfected snails [15, 16, 69]. An example of such a fresh water prawn found in the water bodies throughout West Africa, known as *Macrobrachium vollenhovenii*, can feed on these host snails, thus causing a drastic reduction in the number of schistosomiasis cases [69]. However, the idea of introducing prawns to schistosome contaminated regions has not been widely applied, due to lack of native species, to keep the prawns under biological control, as well as risking the chance of causing unwanted environmental effects [69].

Table number 1.1. Table depicting the different drugs used to treat schistosomiasis, along with their targets, mode of action and the reasons for being discontinued.

Name of drug/ Type of treatment	Works against	Mode of action	Reason for being discontinued.
Oxamniquine (Vansil) * Hycanthone	Schistosomules and adult male worms.	The enzyme sulfotransferase converts OXAM to sulfate esters. - Binds to the parasitic DNA. - Disruption of cell cycle. - Inhibition of protein synthesis. - Paralysis - Tegument damage.	- Mutation of enzyme. - Drug resistance in somules. - Seizures in epileptic patients. - Not safe for pregnant women.
Benzimidazole	Cestodes, nematodes, protozoans, trematodes.	- Interferes with the assembly of microtubules, binds to **β**-tubulin. - Unfolding of proteins, conformational change. - Interferes with spindle formation during cell division. - Muscular paralysis, tegument damage.	There are side effects. - Abdominal pain. - Nausea. - Vomit. - Weakness. - Liver enlargement.
*Triclabendazole	Adult worms.	- Muscular contraction, tegumental destruction.	
*Flubendazole	Adult worms	- Reduces load of eggs - Decreases the formation of hepatic granulomas.	- Teratogenic
*Albendazole	Nematodes, trematodes, cestodes, protozoans.		
*Pyrido (1,2-a) benzimidazole derivative (PBIs) Chloroquine, mefloquine, quinine.	Broad spectrum antiparasitic. Antimalarial + antischistosomal.	- Converts toxic hemozoin.	- Dangerous at high doses - Unwanted side effects - Teratogenic.
Artemisinins. *Artemether *Artesunate	Antimalarial + antischistosomal. Can target juvenile worms.	- Combined with praziquantel, it is antischistosomal. - Release of toxic free radicals released in the gut of the worm.	- Development of malarial parasite drug resistance.

1.2.7. New Approaches Towards Drug Therapy

With the epidemic of schistosomiasis spreading worldwide, it is of great importance that effective antischistosomal drugs are developed to combat the disease. In 2009, the genome of *S.mansoni* was sequenced, using whole genome shotgun sequencing and provided 11,809 putative genes, encoding 13,197 transcripts, making schistosomes the first of the phylum Platyhelminthes to be completed sequenced [66]. The identified genes included those which produced proteins such as cadherins (for cell adhesion), Notch/Delta (for tissue development), histone acetyltransferases (for histone modification), tetraspanins (for structural proteins), fucosyl and xylosyltransferases (glycans expressed at the host-parasite

interaction), invadolysins (for host invasion), cathepsins (blood feeding), thioredoxin glutathione reductase (for anti-oxidant activities), metabolic enzymes (glycolysis, TCA cycle, pentose phosphate pathway), kinases, proteolytic enzymes etc [66].

From the genome sequence, 92 putative G-protein coupled receptors or GPCR encoding genes were identified which included members from the rhodopsin family, amine receptors, neuropeptides and hormone receptors [66]. In 2011, the first comprehensive genome wide study of platyhelminth GPCRs was first published by Dr. Mostafa Zamanian and Dr. Tim Day's group at Iowa State University [70]. Other receptors identified were ligand gated ion channels, glutamate activated cation channels, ATP gated ion channels, nicotine acetylcholine receptors and voltage gated sodium, potassium and calcium channels [66]. Antischistosomal drugs which can bind to schistosomal receptors and channels which are capable of causing neuromuscular impairment are preferred targets for potential anthelminthic drugs [71]. Very recently, a paper published by the Marchant group at the University of Wisconsin-Madison, reported one such drug, nuciferine to be promising, the drug being an aporphine phytochemical found in the lotus plant [71]. It has been used in traditional medicine and is capable of blocking the serotonin or 5-hydroxytryptamine (5HT) receptor Sm5HTR, leading to a decrease in worm motility [71].

Considerable research is being carried out for the production of anti-schistosomal vaccines, by evaluating specific schistosome molecules which are exposed to the immune system of the host, and are important for the survival of the parasites as they travel through the host circulatory system, absorb nutrients and avoiding the host defense system [11, 34]. These targets include *S. mansoni* fatty acid binding protein, tetraspanin, and *S. haematobium* glutathione S-transferase which has presently reached phase II of clinical trials [3, 11, 40].

Other vaccine candidates include *S.mansoni* calpain, sodium/potassium ATPase, succinate dehydrogenase iron sulfur protein as well as *S. japonicum* associated with heat shock protein 70 (HSP70) ; however further research is necessary to allow them to be eligible for human trials [3, 11, 59]. Venom-allergen-like proteins (VALs) are abundant in many different helminth species, and have been used for trial runs to treat hookworm infections in humans, and could be considered as potential vaccine antigens to treat schistosomiasis [59].

The isolation and detection of extracellular vesicles or EVs or exosome like vesicles (ELVs) secreted by adult S. *mansoni* worms are found to contain a number of proteins whose functions include intercellular communication; activating the immune system; metabolic proteins involved in glycolysis; metalloproteases and elastase to allow host penetration [14, 37]. Some of the proteins which have been identified from parasitic ELVs such as ATP-diphosphohydrolase1 (ATPDase1), Sm 22.6, and cathepsin B can prevent blood coagulation, and thus allow the worms to migrate easily through the bloodstream [37]. Proteins and infection related biomarkers were identified from schistosome ELVs may be designed as targets for potential anti-schistosome vaccines [14, 72].

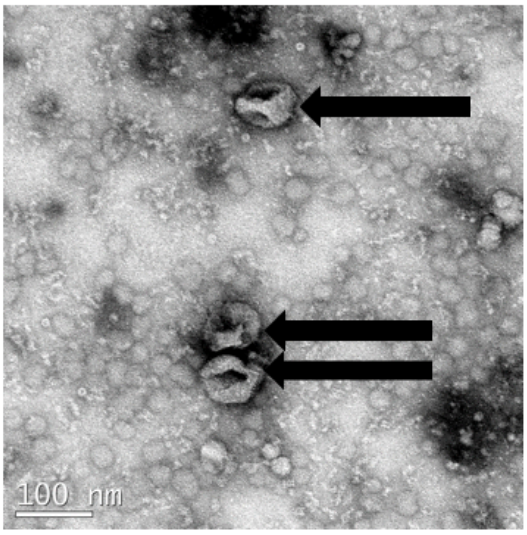


Figure number 1.4 :- Exosome like vesicles or ELVs isolated and purified from *Schistosoma mansoni* somules. *Schistosoma mansoni* schistosomules were incubated in Basch media, and the media was collected after 24-48 hours. The media was centrifuged at very high speed to remove the debris from the media, and then the supernatant was filtered through a 0.2 uM filter. This filtered supernatant was subject to 2 more rounds of centrifugation, and the images of the exosomes were taken using a transmission electron microscope using a JEOL 2100 scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA) at the Microscopy facility at Iowa State University, Ames, Iowa.

1.3. The Importance of Schistosomal Cercarial Motility in The Spread of Schistosomiasis

The non-feeding, short-lived and free-swimming *S. mansoni* cercaria is the most infectious stage of the life cycle of schistosomes, which bridges the gap between the intermediate snail and the mammalian definitive host [28, 73]. The motility of the cercariae is responsible for the spread of schistosomiasis to humans and thus the identification of the molecules responsible for the mechanism behind the motility of the cercarial tail, can be promising targets for the design of chemotherapeutic drugs.

1.3.1. The Role of The Muscular Organization of The Parasite on Cercarial Motility

Cercarial motility is largely dependent on the activity of the tail. The muscles in the furcae allow the cercariae to switch from backward to forward swimming and the swimming behavior has been broadly classified into three main categories (1) a tail-first mode where the cercarial tail fork is fully extended and the cercariae moves against gravity (2) a free-sinking method where the fork is partially extended and the cercariae is negatively buoyant and

lastly, (3) a head-first method where the cercarial tail fork is folded back, critical to host penetration [27, 30].

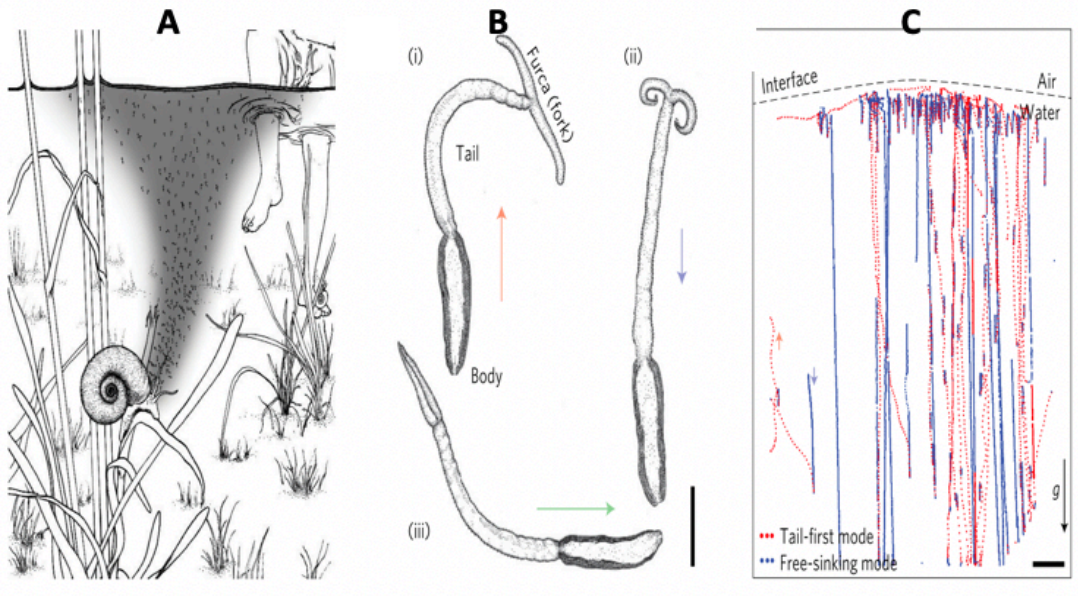


Figure number 1.5 :- Macroscale swimming behavior of *Schistosoma mansoni* cercariae. A. Artistic rendering (not to scale) of a typical transmission site where cercariae (immature, human infectious stage of the parasites) emerge into freshwater from snails and aggregate near the water surface. **B.** Artistic rendering of the three distinct modes displayed by cercariae in their intermittent swimming behavior. (i) Tail-first mode (ii) free-sinking mode and (iii) body-first mode. The arrows indicate the direction of motion. Scale bar, 100 µm. **C.** Computationally tracked snapshot of cercariae swimming in a vertical chamber showing tail-first swimming (red) and sinking (blue) trajectories for 10 minutes. The resulting tracks of organisms have a strikingly columnar nature with very little drift in the horizontal direction. This is due to the fact that cercariae are bottom heavy swimmers and undergo a gravitational realignment such that their longitudinal axis is aligned with the vertical with the body pointing down. Scale bar, 1 mm. Reproduced with permission from Krishnamurthy D., Katsikis G., Bhargava A. and Prakash M (2016). *Schistosoma mansoni* cercariae swim efficiently by exploiting an elastohydrodynamic coupling. Nature Physics Vol.13 pages : 266-271

The anatomy of the cercarial muscle tissues was studied using fluorescent microscopy, along with histological stains such as fluorescein isothiocyanate (FITC) labelled phalloidin which labels filamentous actin [24, 27]. The main body of the cercariae is made up of diagonal muscle fibers, longitudinal muscle fibers and circular muscle fibers [27]. The tail

is broadly divided into two main components: the upper portion, which is made up of striated tail muscles, and the lower bifurcated forked tail or furcae, which are composed of circular muscle fibers and longitudinal muscle fibers [24, 27, 28]. The motility of the cercarial tails depends on both thick and thin myofilaments, and they derive their energy from adenosine triphosphate (ATP) which is produced in abundance from mitochondrial cells [28]. The stored glycogen in the cercariae is converted to glucose-6-phosphate through glycogenolysis, which is then converted to glucose by the process of glycolysis, which is further transformed to energy in the form of adenosine triphosphate (ATP) using the Kreb's cycle [31, 32, 36].

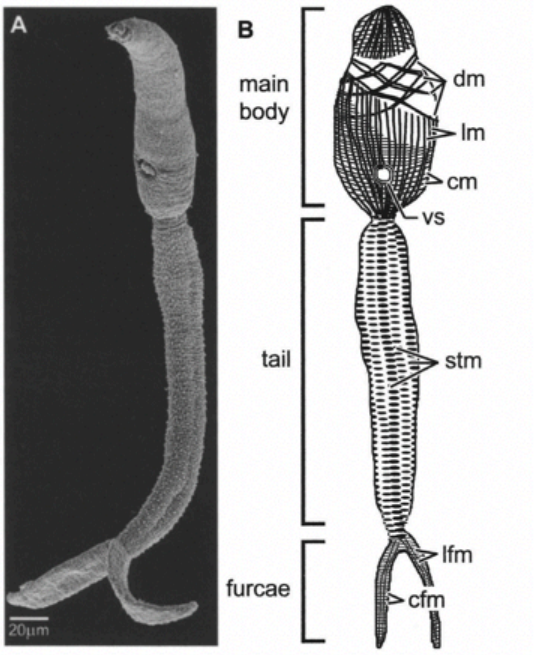


Figure number 1.6 :- Scanning electron micrograph of a *Schistosoma mansoni* cercaria. A Scanning electron micrograph of a *Schistosoma mansoni* cercaria (scale as indicated). **B** Schematic representation of its gross muscle fiber organization. Cfm, circular muscle fibers; dm, diagonal muscle fibers; lfm, longitudinal muscle fibers in the furcae; lm, longitudinal muscle fibers; stm, striated tail musculature; vs, ventral sucker. Reproduced with permission from G.R.Mair, A.G. Maule, B.Fried, T.A.Day and D.W. Halton (2003) Organization of the Musculature of Schistosome Cercariae. The Journal of Parasitology, vol 89, No 3, pp. 623-625.

1.3.2. The Role of Chemical Factors on Cercarial Motility

On exposure to light, the positively phototropic cercariae are shed from the infected *B. glabrata* snails and they accumulate along the surface of the water, where they can easily come in contact with humans or other mammalian hosts [74]. Free fatty acids (linoleic acids)

act as chemical signals which attract the cercariae to the skin of humans [74]. To assist in the invasion process, the cercariae uses its acetabular glands which are made up of four pre-acetabular glands and six post acetabular glands, located on the ventral part of the cercarial body [34]. The glands secrete mucous and proteolytic enzymes [24, 27, 28, 34, 74] which assist in host tissue invasion, breakdown of tissue barriers, and migration through the host body [24, 73, 74]. Once inside the mammalian skin, the tail is detached from the rest of the body and the schistosomule continues its journey through the host body [24, 28, 34, 74].

1.4 The Role of Neuropeptides and Neurotransmitters in Schistosomal Motility

Schistosomes are dependent on their neuromuscular system for a whole range of functions such as locomotion, penetration of cercariae through the human skin, migration of schistosomules through the circulatory systems, controlling the muscles of the suckers, reproductive, digestive, excretory systems and coupling of adult worms [75, 76]. Classical neurotransmitters and neuropeptides are key components in the regulation of the neuromuscular system of helminths [75]. The movement of the juvenile worms is dependent on the coordinated contraction of the longitudinal muscles and the elongation by the circular muscles [77]. Neurotransmitter receptors and neuronal proteins associated with the central nervous system (CNS) and the peripheral nervous system (PNS) have always been considered as potential targets for chemotherapeutic agents [21, 76].

1.4.1. Neuropeptides

With the availability of whole genome data, bioinformatic screens such as web-based basic local alignment search tool (BLAST), scientists have identified a plethora of putative amidated and non-amidated neuropeptide encoding genes in schistosomes and related platyhelminths [66, 78, 79]. The nuclear genome of *S. mansoni* was sequenced in 2009,

encoding 11,809 putative genes [66]. Schistosomes possess well-developed families of neuropeptides which consist of neuropeptide F (NPF) like peptides and myoexcitatory FMRFamide-like neuropeptides (FLPs), which have been identified and studied in whole worms, muscular strips and isolated individual muscular fibers of various flatworm species [76, 80, 81]. Schistosomal NPFs are polypeptides are prevalent platyhelminthes, however they are absent from the human genome, thus making NPF an attractive anthelmintic drug target [35, 66]. The genome of *S. mansoni* revealed the presence of NPP-21a and NPP-21b, concluding that NPFs and NPYs possess a common ancestry [66]. Eighteen distinct amidated peptides have been identified in the CNS and the PNS of *S. mansoni*, some of which have myoexcitatory functions, locomotion, reproduction and spread of infection [66, 78, 79]. This includes the *S. mansoni* neuropeptide precursor gene (Sm-npp-1) which is expressed in both larval and adult parasitic worms and encodes the myoexcitatory GFVRIamide. Classical neurotransmitters capable of affecting worm motility include serotonin (5HT), dopamine, histamine, glutamate and acetylcholine [35, 75, 82]. YIRF-amide and other similar peptides are potently myoexcitatory on *S. mansoni* dispersed muscle fibers [79]. Sm-NPP-1 (GFVRIamide) and Sm-NPP-5 (AAYMDLPWamide) were found in the schistosomal nervous system thus proving to be promising targets for future chemotherapeutic research [79].

1.4.2. Serotonin (5HT)

Like PZQ, 5HT has a myoexcitatory effect on whole worms, but the overall responses of both molecules are significantly different from each other [54, 65]. 5HT is an important myoexcitatory neurotransmitter for schistosomes, as it stimulates motility and controls the contraction of the muscles [77, 83]. 5HT also plays roles in larval growth, development and

maturation [75]. Muscle fibers isolated from adult *S. mansoni* worms proved the effect of 5HT on the contractility of the muscle fibers [82, 84]. The receptor for 5HT or Sm5HTR was the first to be identified as a functional serotonin receptor and is widely expressed in the nervous system of both juvenile and adult worms, [77]. The ligand 5HT when bound to the 5HT receptor, stimulates adenylate cyclase by activating the Gs (stimulatory) subunit of the trimeric G-proteins, thus increasing the concentration of cAMP, which releases calcium from intercellular storages such as the endoplasmic reticulum [77, 82]. Other functions of Sm5HTR include the uptake of glucose, glycogen utilization and carbohydrate metabolism, thus providing energy for muscular contraction [75, 82, 85].

The role of Sm5HTR in parasitic schistosomule and adult worm motility was confirmed by RNA interference (RNAi) [71, 77, 83]. When compared to untreated controls, the Sm5HTR dsRNA treated schistosomules were unable to elongate their bodies, and thus movement was affected resulting in hypomobility, thus identifying that GPCRs like Sm5HTR can be potential anthelmintic drug targets [71, 77, 83]. Nuciferine, D-glaucine and boldine are aporphines or tetracyclic alkaloids, are capable of blocking Sm5HTR, thus affecting schistosomal motility [71].

1.4.3. Dopamine and Histamine

Apart from 5HT, dopamine and histamine are the other two biogenic amine signaling neurotransmitters [75, 76, 81]. The rhodopsin-like GPCRs for the dopamine receptors are expressed in the parasitic musculature whereas the receptor for histamine is found in the tegument of the schistosomules [35, 75, 81]. Biogenic amines are also responsible for stimulating specific proteins such as activated protein kinase A (PKA), the functions of which include of activation of transcription factors, metabolic enzymes, signaling proteins,

neuromuscular communication, survival, muscular activity and can be stimulated by biogenic amines such as 5HT and dopamine, present in the nervous system of the worms [35].

1.4.4. Glutamate

Glutamate is a myoexcitatory amino acid classical neurotransmitter, responsible for the stimulation of isolated muscle contraction, interneuronal signaling within the central nervous system (CNS) and the control of egg production in female worms [75, 76]. The biochemical receptors for glutamate fall into one of four main categories: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors), N-methyl-D-aspartate receptors (NMDA receptor), metabotropic glutamate receptors and the less abundantly distributed kainite receptors.

1.4.5. Acetylcholine

Acetylcholine or ACh is a well-known excitatory neurotransmitter present in the neuromuscular junction of the nervous system of many types of animals and humans. In both vertebrates and invertebrates, ACh controls important functions of the body in both the central and the peripheral nervous systems, such as muscular contraction, secretion from the glands, memory formation and learning [86, 87]. The presence and role of this neurotransmitter in *S.mansoni* was first reported in the early 1950's and it was observed that the concentration of ACh in *S.mansoni* was similar to that present in the grey matter of human brain cortex [88]. However, the role of ACh in schistosomes is inhibitory, rather than excitatory, causing muscular relaxation leading to paralysis [86-88]. There are two main groups of ACh receptors: –ionotropic nicotinic receptors which are ligand-gated ion channels permeable to sodium, potassium and calcium ions; and metabotropic muscarinic receptors, which belong to the G-protein coupled receptors (GPCR) family [86, 87].

Nematode ligand gated nicotinic receptors have been previously used as targets for the manufacture of antiparasitic drugs [86]. A paper published in 2014 by Dr. Paula Ribeiro's group explored the nicotinic acetylcholine receptor (nAChR) to confirm the inhibitory role of these receptors in the *S.mansoni* motility [87, 89]. Using computational biology, it was discovered that 5 of the schistosome nAChR subunits were acetylcholine gated chloride channels (Sm ACC) located in the peripheral nervous system of the schistosomes [87]. Using RNAi, it was confirmed that SmACC activation was caused by an influx of chloride ions, which was responsible for the inhibitory response observed in schistosomes [87]. Based on this analysis, acetylcholine-gated chloride channels present a promising target for future antischistosomal drugs.

Muscarinic ACh receptors (mAChRs) are structurally within the Rhodopsin GPCR family. Invertebrates feature a closely-related family to as G-protein coupled acetylcholine receptors (GARs) [86]. GARs are related to, but distinct from mAChRs. The very first complete functional analysis of schistosomal GAR (SmGAR) was published in 2015 by Dr. Paula Ribeiro's group and it was demonstrated that by knocking down SmGAR, there was a significant reduction in schistosomal larval motility, suggesting the role of these receptors in the migratory process of *S. mansoni* [86].

An important question was raised regarding the role of these receptors—since ACh has an inhibitory effect on the schistosomes, knocking the SmGAR would hypothetically increase the movement of the somules, however the opposite effect was noticed [88]. It was suggested that a number of receptors stimulated all at once were responsible for the reduction in parasitic motility, and also there is a possibility of some of the receptors possessing a stimulatory effect on the schistosomes [86].

1.5 Microfluidics as A Novel Platform to Study Schistosomal Motility

Microfluidic systems are increasingly gaining a lot of attention due to the fact that they are designed to analyze the behavior and movement of organisms as they move through manipulated and controlled fluid flowing through the microchannels of the platform. The benefits of using such a device include the ease of manufacturing the device, small size of the chip, the use of less sample volume, thus not wasting excess sample, saving in chemicals, the ease of portability, conducting a number of experiments simultaneously on the same platform, improved sensitivity and increased resolution [90-92]. These chips have been used for screening anthelmintic drugs on free living and parasitic nematodes [91, 93]Using this approach, we studied and analyzed the movement of *S. mansoni* schistosomules in custom made microfluidic platforms with sinusoidal microchannels, produced using biologically inert, transparent and gas permeable polydimethylsiloxane (PDMS) on a glass slide, by standard lithography technique. This experiment was conducted in collaboration with the micro/nano laboratory of the Electrical and Computer Engineering Department of Iowa State University, who provided the microfluidic channels for our experiments. The goal of this research project was to establish a new screening platform where the microchannels mimicked human blood vessels, in order to test the effect of various agents on schistosomules, as they migrate through the body of the host.

1.6 The Proteomic Analysis and The Identification of *S. mansoni* Cercarial Proteins

The free-swimming, very motile and highly infectious cercarial forms of *S. mansoni* are responsible for bridging the gap between the invertebrate snail host and the human host, resulting in schistosomiasis. The proteins present in the cercarial tail are responsible for keeping the cercariae alive in the fresh water, to seek and penetrate a host. However, the proteins behind these functions are poorly understood. Functional proteomics, the large scale characterization of proteins in a biological sample, has gained importance in recent years, as it helps to identify a large number of interesting proteins as well as their functions, locations, modifications in a single sample simultaneously [22, 94]. Proteomic analysis of the material secreted by the cercariae post infection, have identified a carbohydrate rich glycocalyx, which would normally activate the host immune system, but is discarded once the cercariae invades the host [95]. Other identified proteins from the cercarial secretions include the proteolytic enzymes released by the acetabular glands, which helps the parasite to tunnel through the epidermis and enter the host circulatory system [95]. An analysis of the miracidium proteome revealed the presence of proteases, venom allergen like proteins (VALs), heat shock proteins, all of which function in the survival and development of the miracidia in the intermediate snail host [22].

Using mass spectrometry and proteomic analysis, we have attempted to present a complete analysis of the cercarial tail proteome, and a thorough comparison between the proteins located in the cercarial body and those present in the cercarial tail. By identifying novel proteins in the cercarial tail, we aim to identify possible drug targets, which can intervene and prevent host invasion.

1.7 Organization of The Dissertation

For my doctoral thesis, I have used two novel molecular techniques to study the motility of *S. mansoni* cercaria and *S. mansoni* schistosomules—microfluidics to improve motility platforms to study the movement of somules, and a thorough proteomic analysis of the cercarial proteins which may be responsible for the parasitic movement . To my knowledge, these techniques have not been tested before and we hope to use parasitic motility as a focus of future research studies, to combat the spread of schistosomiasis.

1.7.1. Objective Number 1

The first part of my thesis is based on the design and use of microfluidic devices to analyze parasitic movement [92]. Microfluidic assays with microchannels have been used previously to study the movement of *C.elegans*, in response to various drugs [91]. Specifically, we have implemented a novel lab-on-a-chip approach to study schistosomule motility in an environment more akin to that of the host's circulatory system. Our aim for this project was to improve motility platforms for the study of schistosomal movement, which would potentially assist scientists in tracking the parasitic movement, under the effect of drug.

1.7.2. Objective Number 2

The integration of novel techniques such as proteomics, functional genomics, and computational biology, have allowed scientists and researchers to make significant advances in the scientific world. For the second part of my doctoral thesis, we have extracted and analyzed the proteome of cercarial tails, which plays an essential role in the spread of the disease. Our goal for this project was to identify and compare the functions of the proteins in the cercarial bodies with those present in the cercarial tails, using mass-spectrometry to map

separate body and tail proteomes. Our analysis has confirmed the presence of 791 proteins in the cercarial tail as compared to 645 proteins in the cercarial body. Using this information, we have chosen specific cercarial tail proteins, not present in the body, which could be potentially used as potential targets for future drug research. By preventing the transport of the cercariae from the snail host to the mammalian host, we hope to reduce the spread of schistosomiasis.

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CHAPTER 2. USING MICROFLUIDIC ASSAYS TO STUDY THE MOTILITY OF *SCHISTOSOMA MANSONI* SOMULES

Modified from a paper to be submitted in PLoS Pathogens

Sreemoyee Acharya[1,3,4], Zach J. Njus[2,4], Prince N. Agbedanu[2], Santosh Pandey[2,4], Gunnar R. Mair[1], Tim A. Day[1,4]

2.1 Abstract

Schistosomiasis or Bilharzia is the second most important neglected tropical disease (NTD), after malaria, and is caused by parasitic helminth trematodes called Schistosomes, which belong to the phylum Platyhelminthes. According to a report filed by the World Health Organization (WHO) in 2016, schistosomiasis is responsible for approximately 200,000 deaths per year in sub-Saharan Africa alone. Praziquantel is the lone anthelmintic drug used to treat the disease, however in recent times, there is an urgent need for better and more efficient drugs to combat the disease. Although the life cycle of the causative agent of intestinal schistosomiasis, *Schistosoma mansoni* through the intermediate snail host and the definitive human host is well known, the navigation and kinetics of the movement of the post infection juvenile worms through the convoluted pulmonary and blood vessels in the human host remain largely unexplored. The study of schistosomal movement through the host has been hindered by the complex life cycle, high expense, poorly designed motility assays and the lack of high thorough put screening methods. In this paper, we have attempted to study the movement of *S. mansoni* somules through unique custom manufactured microfluidic chips with sinusoidal microchannels produced from polydimethylsiloxane (PDMS), so that the channels could provide a route similar to the human circulatory system. These microfluidic platforms have been used previously to analyze the movement of *C.elegans* successfully. The movement of the somules through the microchannels was recorded, and

these recordings were analyzed using a custom-made software. The goal of this research paper is to establish a novel and robust experimental device for screening potential anti-schistosomal compounds, and improving the definition of such motility assays, allowing researchers to distinguish between contractile activity and directional movement of organisms.

[1] Department of Biomedical Sciences, Iowa State University, Ames, IA, USA

[2] Department of Electrical and Computer Engineering, Iowa State University, Ames, IA, USA

[3] Primary authors

[4] Author for correspondence

2.2. Introduction

2.2.1. Life Cycle of Schistosomes

Neglected Tropical Diseases (NTDs) are a diverse group of highly infectious diseases that are mostly prevalent in tropical and subtropical countries [4]. The Centers for Disease Control and Prevention (CDC) reported that almost one billion people, mostly from low-income and middle-income populations, are affected by NTDs, in at least 149 countries. Schistosomiasis or Bilharzia is the second most important NTD, after malaria, and is caused by blood dwelling parasitic helminth trematodes called Schistosomes [4, 96]. These parasitic organisms belong to the phylum platyhelminthes and are responsible for infecting more than 200 million people worldwide [4, 12, 33, 96]. According to a report filed by the World Health Organization (WHO) in 2016, schistosomiasis is responsible for approximately 200,000 deaths per year in sub-Saharan Africa alone. The three important species of the genus Schistosomes are *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium*, of which the life cycle of *S.mansoni* is more frequently maintained in

laboratories for research purposes [12, 96]. *S. mansoni* and *S. japonicum* are responsible for intestinal schistosomiasis, whereas *S. haematobium* is responsible for urogenital schistosomiasis [4, 12, 96].

The life cycle of *S.mansoni* is complex as it needs to pass through two hosts—the first one being an intermediate invertebrate snail host and the second being a definitive vertebrate mammalian host [4, 33, 96]. When people are infected with schistosomiasis, they shed the parasitic eggs through their urine and/or feces into freshwater, where the eggs hatch to release free-living, non-feeding, highly motile ciliated miracidia [4, 33, 96, 97]. The miracidia then proceeds to infect an intermediate snail, *Bioamphalaria glabrata* in the case of *S.mansoni,* in which it undergoes asexual reproduction to produce a large number of sporocysts [4, 33, 96, 97]. The sporocysts mature in order to develop into tens of thousands of highly infectious and extremely motile cercariae, which are released into the water to locate a mammalian host [4, 33, 96]. The cercariae proceeds to invade the host skin by tunneling through the dermis, using both the mechanical force of its bifurcated tail as well as the proteolytic enzymes secreted from the cercarial acetabular glands [4, 33, 96, 98]. The extremely infectious cercariae is responsible for spreading schistosomiasis as it bridges the gap between the snail vector and the human host. Once inside the skin of the mammalian host, the cercariae discards its tail and transforms into schistosomules, which then travel first through the host circulatory system to the lungs, and then via the systemic circulation to the portal veins [33, 96, 97, 99]. Within the superior mesenteric veins, the dioecious parasites mature into either male adult worms or female adult worms, which mate, producing 100-300 eggs per day - some of which are excreted and most of which are accumulated within the host tissues and organs, leading to the pathogenesis of the disease [4, 8, 12, 33, 96, 99]. The

pathology of schistosomiasis is caused primarily by the host's immune response to the accumulation of schistosome eggs in the intestine and the liver, and not just by the presence of juvenile and adult parasitic worms [4, 8, 13, 97].

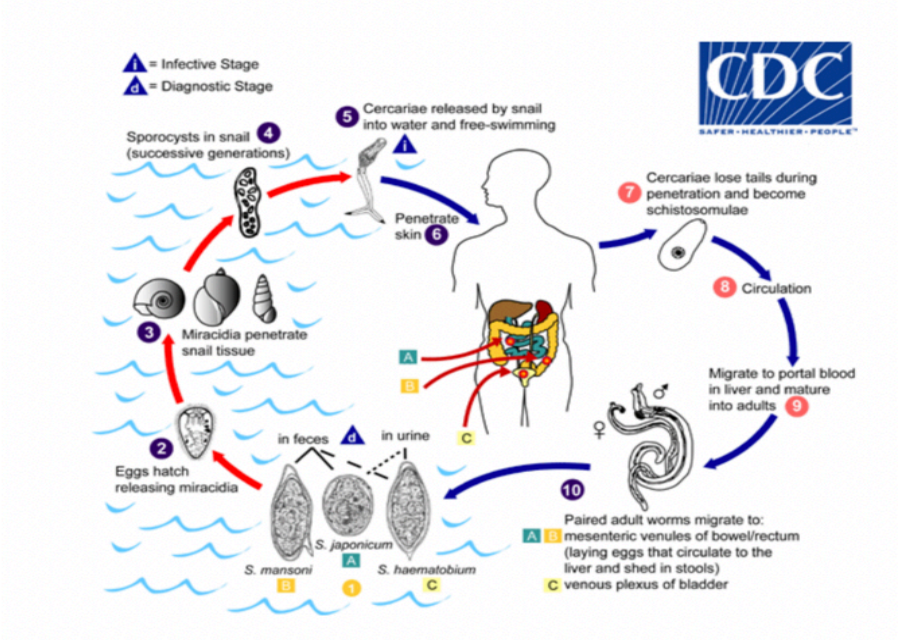

Figure number 2.2.A :- The different stages of the life cycle of schistosomes. 1. Eggs are eliminated from the. patient, via feces or urine. **2**. Under optimal conditions, the eggs hatch and release miracidia. **3.** The ciliated, free- living miracidia swims and penetrates an intermediate invertebrate snail host. **4**. Within the snail host, the miracidia develops into 2 generations of sporocysts –mother sporocysts and daughter sporocysts which ultimately develop into the cercariae. **5**. The cercariae is released from the snail, marking the most infectious stage in the life cycle. **6**. The cercariae penetrates the skin of the human host. **7**. Once inside the human host, the cercariae sheds its tail and becomes schistosomules**. 8.** The schistosomules travel through the circulatory system. **9.** They migrate and settle in their final residence- the liver. S.mansoni is found often in the superior mesenteric veins which drain the large intestine**. 10 ABC**. The schistosomules mature and develop into male and female adult worms. The paired worms mate and the female worms lay eggs in the small venules of the portal system and the perivesicle system. The eggs then gradually move towards the lumen of the intestine, and are eliminated via excretory products. However, a large number of eggs accumulate in the liver, leading to the formation of granulomas, which leads to the onset of schistosomiasis.

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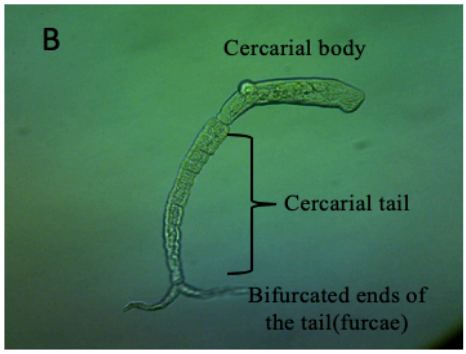
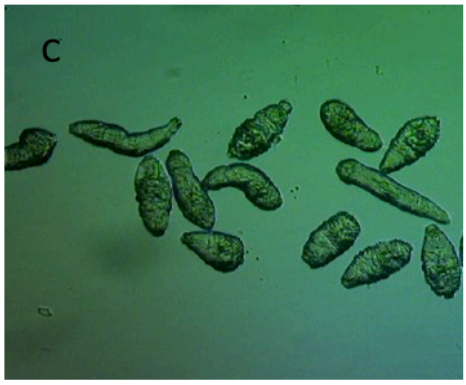




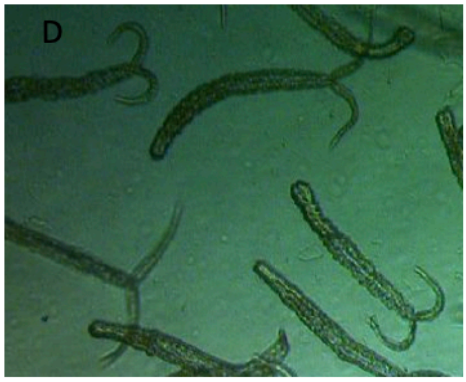


Figure number 2.2.B :- *Schistosoma mansoni* cercaria. Figure number 2.2.C :- *Schistosoma mansoni* schistosomules. Figure number 2.2.D :- *Schistosoma mansoni* cercarial tails

B. *Schistosoma mansoni* cercaria was taken immediately after it was freshly shed from an infected host *Bioamphalaria glabrata* snail. The structure of the cercaria can be broadly divided into the upper head and the lower tail. The tail extends to form bifurcated ends, which help the highly infectious cercaria to swim and survive in the fresh water, until it can locate a mammalian host like a human being, to infect and penetrate. Once inside the host body, the tail is discarded and the cercarial body transforms into a schistosomula, which then continues on its journey through the host circulatory system. **C.** shows the *S.mansoni* bodies, or somules, after the cercarial tails were separated using a vortex and percoll gradient centrifugation. The somules will travel through the circulatory system of the human host towards the superior mesenteric veins which drain the large intestine, after which they will develop into adult male and female worms, and lay eggs. **D.** the discarded *S.mansoni* cercarial tails, which were separated from the main body using a vortex and percoll gradient centrifugation. All images were taken using a Nikon Eclipse TS 100 microscope with a 10X objective lens, attached to a 5.0 megapixel Nikon ED camera.

2.2.2. Importance Of Schistosomal Motility In Spreading Schistosomiasis

Although the life cycle of the adult *S. mansoni* worms within the mammalian host is well understood, the navigation and the kinetics of the movement of the somules through the convoluted pulmonary and blood vessels remain largely unexplored. According to early

studies conducted using compressed tissue autoradiography, the migration of the somules through the pulmonary capillaries demonstrated a rather strenuous, protracted and slow process as the parasites squeeze through the tight walls of the of the blood vessels [33, 97, 99-101]. Schistosomes promote vasodilation of the blood vessels by generating specific metabolites such as kinins (bradykinins), biogenic amines (histamine and serotonin), ecoisanoids, and nitric oxide to allow the worms to travel freely [102].

The movement of somules through the body depends on a number of chemical and biological factors [99, 102]. Proteolytic enzymes like proteases, caspases and elastases secreted from the cercarial acetabular glands, assist in parasitic movement through the capillaries [33, 100]. Schistosomules are also guided by blood serum containing L-arginine and glucose as signaling molecules [98]. The tegument on the surface of the somules shields the worm antigens from being recognized and attacked by the host's immune system [33]. In order to survive, schistosomes can crystallize toxic heme molecules from human erythrocytes into insoluble hemozoin molecules (Hz), which allow the parasite to move through the blood vessels without any obstruction [53].

Once the somules reach the liver, they start to feed on blood cells, which provide energy in the form of adenosine triphosphate (ATP) for further migration and maturation [33, 101]. The maintenance of the *S. mansoni* life cycle and the spread of schistosomiasis depends on the migration of the somules through the body of the host as well as successful evasion of the immune system.

2.2.3. Neuromuscular Organization of Schistosomes.

Schistosomule motility depends upon a rhythmic pattern of alternating cycles of elongation and contraction of the body [103]. Central to the motility of the parasites is the

schistosome neuromuscular system, which is responsible for controlling the functions of its muscles [77, 103]. Confocal scanning laser microscopy of fluorescein isothiocyanate (FITC) labelled phalloidin staining of cercarial actin filaments, was used to demonstrate the detailed organization of the muscular system [27, 104]. The cercarial body is composed of diagonal muscles, longitudinal muscles and circular muscles which are responsible for locomotion [27, 104]. The tail is made up of two parts—the anterior portion is composed of striated tail muscles, and the posterior bifurcated forked tail or furcae, is composed of circular muscles and longitudinal muscles [27, 104]. The alimentary tract and the reproductive system of the parasite is made up of outer circular muscles and inner longitudinal muscles [105]. Once the cercariae invade the human host, they discard their tails and transform into schistosomules, which then continue on their journey. The mobility of the somules is dependent on the coordinated contraction of the longitudinal muscles and elongation of the circular muscles of the body [77]. Since the neuromuscular system plays a significant role in the maintenance and the survival of the parasites, this system is often the focus for potential anti-schistosomal drugs targets.

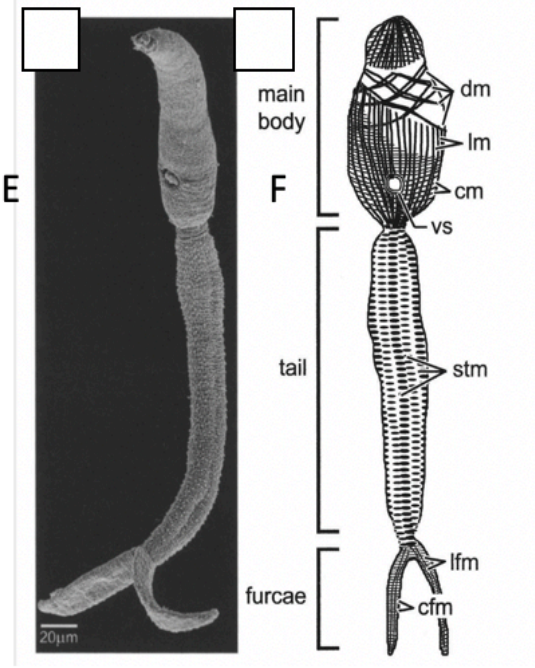


Figure number 2.2.E :- Scanning electron micrograph of a *Schistosoma mansoni* cercaria. E. Scanning electron micrograph of a *Schistosoma mansoni* cercaria (scale as indicated).
F. Schematic representation of its gross muscle fiber organization. Cfm, circular muscle fibers; dm, diagonal muscle fibers; lfm, longitudinal muscle fibers in the furcae; lm, longitudinal muscle fibers; stm, striated tail musculature; vs, ventral sucker. Reproduced with permission from G.R.Mair, A.G. Maule, B.Fried, T.A.Day and D.W. Halton (2003) Organization of the Musculature of Schistosome Cercariae. The Journal of Parasitology, vol 89, No 3, pp. 623-625.

2.2.4. Helminth Neuropeptides

Neuropeptides (NPs) are the primary neuromediators of the members of the platyhelminth family and the nematode family [106, 107]. NPs are small protein-like molecules, which are secreted and used by neuronal cells for interneuronal communication. Using a Basic Local Alignment Search Tool (BLAST) server on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST), more than 100 helminth neuropeptides have been identified in both platyhelminths and nematodes [79, 80, 108]. In platyhelminths, there are two families of NPs, FMRF-amide like peptides (FLPs) / FMRF-amide related peptides (FaRPs) and Neuropeptide F (NPFs) [106, 107, 109, 110].

FLPs or FaRPs are short peptides which are composed of 4-6 amino acids and are particularly abundant in nematodes with potentially myoexcitatory properties [106, 109-114]. In 1994, FLPs were demonstrated to inflict myoexcitatory effect on *S. mansoni* isolated

muscle fibers. Classical neurotransmitters and NPs are key components for regulating and coordinating the helminth neuromuscular system [75, 109, 114]. In 2007 the very first platyhelminth NP receptor was identified in *Giardia tigrina* and named GtNPR-1 (*G.tigrina* neuropeptide receptor-1) [106, 112]. It was demonstrated that GtNPR-1 was primarily responsible for neuromuscular excitation in platyhelminths, and the mode of action was via a G-protein coupled receptor (GPCR) pathway [106, 109, 112, 113]. NPs such as YIRF-amide, GYIRF-amide and RYIRF-amide were found to induce high levels of muscular contraction in parasitic flatworms, with GYIRF-amide being the most potent one amongst the three [105, 106, 108, 112, 114, 115]. However, McVeigh *et al.* in 2011, demonstrated that when YIRF-amide was applied to schistosomes, the parasites exhibited inhibition, which was contrary to the results derived by Day *et al*. in 1997 [80]. It was assumed that the reduction in motility was due to muscular paralysis caused by spastic (excitatory) or flaccid (muscular relaxation) [80]. When GtNPR-1 was screened against 220 neuropeptides it was found that three nematode FLPs, encoded by the *flp-1* gene, AAADPNFLRF-amide, SDPNFLRF-amide and SADPNFLRF-amide, were found to be more potent than GYIRF-amide, but since they were nematode FLPs, they could not be tested on helminths [106, 111, 112].

On the other hand, NPFs have 36-39 amino acids, making them longer in length than FLPs, and they possess GRPRF-amide (Gly-Arg-Pro-Arg-Phe)-amide at their carboxy ends [106, 107, 112]. A paper published in 2004 first identified the presence of NPF from *S.mansoni* and *S.japonicum* and it was demonstrated that that the NPF family caused a reduction in the cAMP concentrations, and had an inhibitory effect on the neuromuscular anatomy of the parasites [107]. In 2009 the genome of *S. mansoni* was first sequenced and was found to encode 11,809 genes with thirteen putative neuropeptides [66]. Since NPFs are

most abundantly distributed in parasites, they are considered potential targets for future anthelmintic drug therapy [107, 110, 112].

In 2011, a new family of helminth NPs was identified in both platyhelminths and nematodes, one of which was found to be expressed in both *S.mansoni* somules and adult worms-the NP precursor gene Sm-npp-1 which codes for GFVRI-amide and AFVRL-amide [80]. Using molecular techniques like immunocytochemistry (ICC) and confocal microscopy, GFVRI amide was found to be located in the neurons of the cerebral ganglion of *S. mansoni* and not detectable in somatic muscles or organs [80, 110]. These peptides demonstrated an inhibitory effect on both adult worms and somules, however when Sm-npp-1 was knocked out using RNAi, no phenotypic change in motility or morphology was observed [80]. Along with *S.mansoni* adult worms and somules, AFVRL-amide was also identified as one of the NPs in annelid worms, *Eisenia fetida,* using affinity chromatography combined with LC-MS/MS techniques [116]. However, the role of AFVRL-amide is not completely understood.

Parasitic helminth trematode members of the platyhelminth phylum such as schistosomes are completely dependent on their neuromuscular systems for the regulation of their activities such as locomotion, migration of somules through the host circulatory system, evading the host immune system, controlling the muscles of the suckers, reproduction, digestion, excretion and ultimately the maturation of somules to adult worms [75, 76, 106]. The examination of isolated muscle fibers from *S.mansoni* worms identified two types of major excitatory neurotransmitters: FaRPs/FLPs and serotonin (5-HT) [84, 105, 108, 115]. FaRPs/FLPs were denoted as the primary neurotransmitter on *S. mansoni* muscles responsible for neuromuscular activity [114, 115].

Myoexcitatory 5-HT is abundantly expressed throughout the central and peripheral nervous systems of the schistosomal body and it assists in increasing motility, sucker function and reproduction by acting on its muscle fibers, in a more modulatory role [77, 108, 117]. 5-HT signals through one of the few characterized G protein coupled receptors (GPCRs), which, when suppressed by RNA interference (RNAi), results in reduced motility as well as demonstrated resistance towards exogenous treatment of 5-HT [77]. RNAi suppressed worms were observed to appear round in shape and unable to elongate their bodies, thus proving that 5-HT has a very important role in maintaining the neuromuscular system of schistosomes [77].

2.2.5. Motility Assays

Screening techniques such as enzymatic, colorimetric and fluorescence assays have been tested to evaluate whole worm motility [118]. In a paper published in 2015, an assay called the xCELLigence worm real-time motility assay or xWORM (*ACEA Biosciences, San Diego, CA*) was used to monitor the movement of different life cycle stages of *S. mansoni* [118]. The study of parasitic movement has been hindered by expense, laborious nature and the lack of high thorough put screening methods, which is why it is necessary to develop a more affordable, easy and efficient way to analyze movement, in order to design anthelmintic drugs [118].

2.2.6. Microfluidic Assays

Microfluidic systems are designed to analyze the behavior, manipulation and control of fluids, which are transported via capillary forces, with practical applications in biotechnology. The system has gained a lot of attention as a novel and controllable technology for carrying out experiments at the small scale like high thorough put drug

screening [119]. Most microfluidic analysis devices are simply miniaturized versions of macroscale systems [92]. The development of a miniature biological or chemical analysis device, popularly known as “lab-on-a chip”, started from the field of MEMS (Micro-electro-mechanical systems) research [90]. Some of the advantages of microfluidic systems include the small size of the chip, the ability to work with small sample volumes, thereby saving on the cost of reagents and chemicals, but also allowing the development of highly sensitive devices [90]. Above all, microfluidic chips open up the possibility to conduct multiple experiments in parallel on the same chip with improved sensitivity, which facilitates the observation and the characterization of phenotypic characteristics in biological systems as well as correlate the change in motility associated with resistance [91]. These devices have been used for single cell handling, single cell culturing, microfluidic flow cytometry, cell sorters and macromolecular detection, as well as separation [33]. Multiparameter microfluidic bioassays with microchannels, created by lithography, have been utilized to research the movement of nematodes, *C. elegans* in particular, in response to various drugs [91]. Such experiments are typically recorded using video tracking, and with the help of specific tracking software, the changes in the kinetics of the worm’s locomotion is calculated [12].

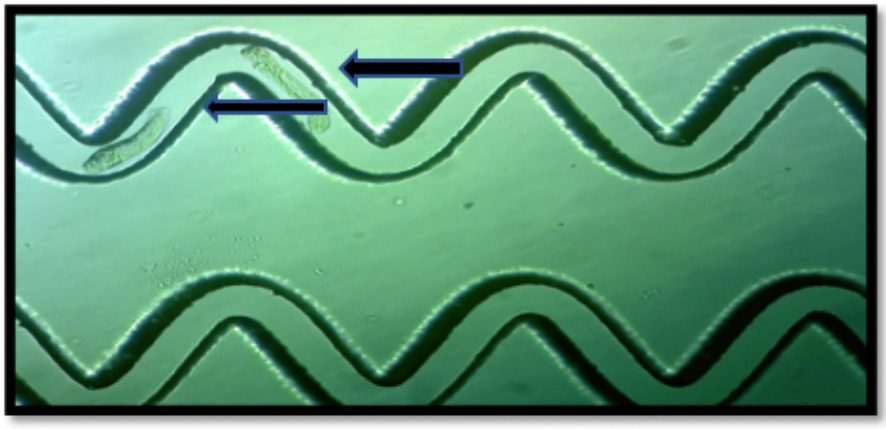


Figure number 2.2.G :- Microfluidic chip. The microfluidic platform with parallel, sinusoidal channels of width of each channel = 60 microns, amplitude = 220, channel wavelength = 360, as viewed through a 10X objective lens of a light microscope, with a pair of *Schistosoma mansoni* somules located in the upper channel, on the left-hand side.

In order to mimic the movement of *Schistosoma mansoni* schistosomules through convoluted blood vessels, we have used a unique microfluidic platform with sinusoidal microchannels produced from polydimethylsiloxane (PDMS) by a standard soft lithography technique [93, 120]. This platform has been previously used to study the movement of *C. elegans* successfully [35, 36]. The strong, irreversible bond between PDMS and standard glass slides prevents leakage, making PDMS an ideal chemical to use for manufacturing microchips [119]. PDMS provides large range of advantages over other chemicals including optical transparency, gas permeability, non-toxicity, inertness and compatibility with aqueous solutions, thus making it suitable for microscopic observations [119].

In this study, *S. mansoni* somule movement through such microchannels was evaluated and recorded, and these recordings were analyzed using a custom-made software. The aim of this paper is to establish a novel and robust experimental device for screening

potential anti-schistosomal compounds, and improving the definition of such motility assays, allowing researchers to distinguish between contractile activity and directional movement of organisms.

2.3 Materials and Methods

2.3.1. Experimental Animals

The NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (BRI, Rockville, MD) provided the *S. mansoni* infected *Biomphalaria glabrata* snails, through NIH-NIAID Contract HHSN272201000005I for distribution through BEI Resources. The infected snails were fed fresh lettuce leaves and maintained at 26°C-28°C in charcoal-filtered, aerated water.

2.3.2. Cercarial Shedding of Snails

In a 250 ml. glass beaker containing 40 ml. of charcoal-filtered, aerated water, approximately 30-40 *S. mansoni* infected *B. glabrata* snails were placed. The water was enough to cover the snails. The cercariae were shed from the snails, by exposing the snails to a 120V 60W light bulb for up to 2 hours. The water from each beaker was filtered through a glass funnel (*VWR catalog number #89428-960*) containing a 47-uM pore sized metal screen (*VWR catalog number # 89428-966*). Once the cercariae were transferred to a pre-chilled 50 ml. falcon tube placed on ice, the cercariae were allowed to settle at the bottom of the falcon tube. The supernatant was discarded, and 5-10 ml. solution with the pellet was left at the bottom of the tube.

2.3.3. Transformation Of Cercariae To Schistosomules, Separation And Collection Of The Somules

In order to produce schistosomules the cercarial solution was vortexed 6-7 times for 1 minute followed by 1 minute on ice thus manually separating the tails from the cercarial bodies. Percoll density gradient centrifugation was then used to separate the tails from the cercarial bodies. 40 ml. of a 60% Percoll solution were prepared from 24 ml. percoll (*Fisher Scientific catalog number # 45001754*), 4 ml. of 10X MEM Vitamin solution (*Life Technologies catalog number #11430030*), 1 ml of 85% (weight/volume) sodium chloride in 1M HEPES, 1.5 ml. of 100X antibacterial-antimycotic solution (*Thermo Fisher Scientific catalog number #15240062*) and 9.5 ml. of molecular grade water. The vortexed cercarial solution was pipetted on top of the percoll gradient solution. Centrifugation was carried out at 900g for 45 minutes at 4°C.

Post centrifugation, the tails accumulated at the top of the percoll were discarded, and the somules collected as a prominent pellet at the bottom of the tube. The somules were washed once with sterile Dulbecco's phosphate buffered solution (DPBS) (*Thermo Fisher Scientific catalog number #14190250*) and centrifuged at 20 minutes at 500g at 4°C. The recovered pellet containing the purified somules was dissolved in 500 µls. of sterile DPBS.

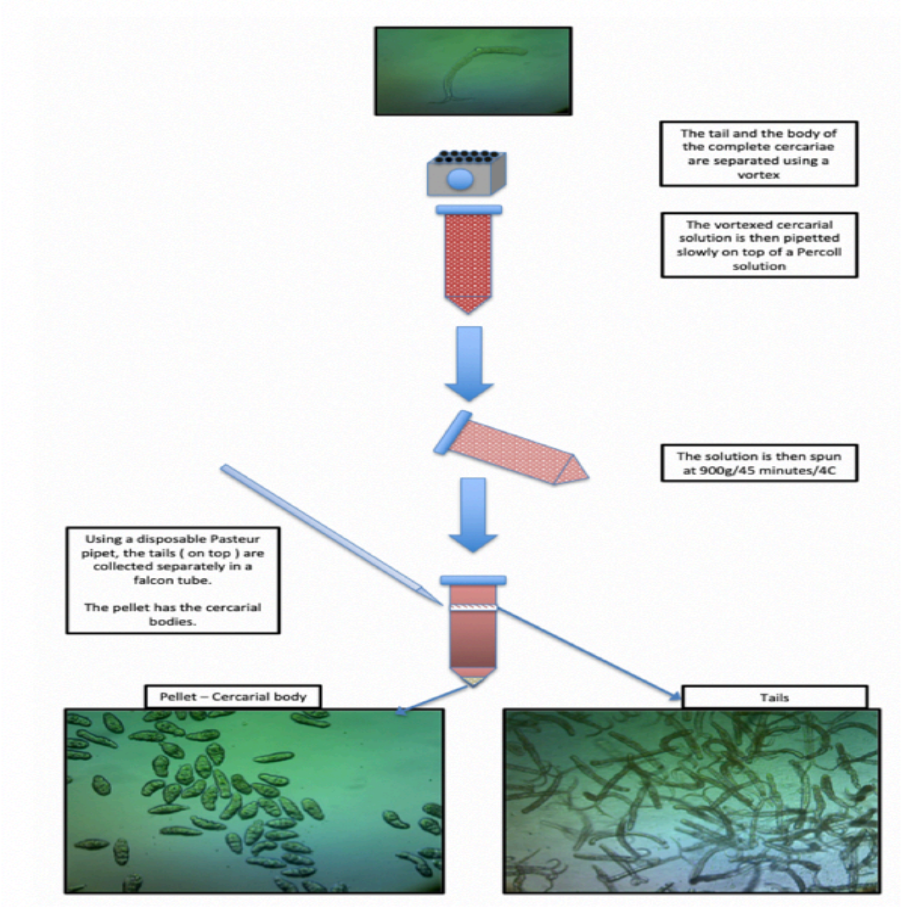


Figure number 2.3.A : - Diagrammatical representation of the process of cercarial shedding, separation and collection of *S. mansoni* somules and tails. Once exposed to bright light, the infected *B. glabrata* snails shed the larval parasites of *S. mansoni*, which are known as cercariae. The cercariae possesses an upper body and an active forked tail, which allows it to move through water to locate and infect a mammalian host. The bodies and the tails were separated using a vortex. The vortexed cercarial solution was then pipetted slowly on top of a percoll solution in a 50 ml falcon tube. The solution was then spun at 900g for 45 minutes at 4°C. The centrifugation resulted in the tails floating at the top of the percoll solution in the tube, and the bodies were saved as pellets at the bottom of the tube. The tails were collected using a disposable glass Pasteur pipette and the bodies were collected from the pellet. Both samples were washed with DPBS and prepared for further experiments.

2.3.4. Selection Of Neuropeptides

11 peptides were chosen and synthesized by the company Peptide 2.0 (Chantilly, VA 20153-3983, USA) : SAYPYV-NH2 (*Reference number #50647-649)*, SYFDPIIY (*Reference number #50647-923)*, VPPYITGGIRY (*Reference number # 50647-925)*,

AYHFFRL (*Reference number # 50647-926)*, FLFNLKDTRW-NH2 (*Reference number # 50647-653)*, GFVRI-NH2 (*Reference number # 50647-642*), AFVRL-NH2 (*Reference number #50647-641*), GMI-NH2 (*Reference number # 50647-643)*, YIRF-NH2 (*Reference number #50647-934)*, NYLWDTRL-NH2 (*Reference number # 50647-650)* and DDFRG-NH2 (*Reference number # 50647-651)* [80, 121]. All the peptides were delivered as lyophilized material and dissolved at a concentration of 1 mM in double distilled, molecular grade water. The diluted peptides were stored at -20°C.

2.3.5. Testing Neuropeptides on Somules In A 6 Welled Plate

Freshly isolated *S. mansoni* somules were added to 6 welled cell culture, non-pyrogenic, polystyrene plates (*Corning Incorporated catalog number #3506*) containing Basch Media (the protocol was provided by the Caffrey lab at the Skaggs School of Pharmacy and Pharmaceutical Science, University of California San Diego, USA as well as published) [122] . To the somules, a 1:1 dilution of neuropeptides (diluted in Basch media) of varying concentrations per well i.e. 0.1 μM, 1.0 μM, 5 μM, 10 μM, 50 μM and 100 μM, were added and were incubated for 30 minutes at 37°C. Motility of the somules were recorded for 5 minutes, at the rate of 10 somules per view through a 10X objective lens of a compound microscope (*Nikon Eclipse TS100*), equipped with a digital camera (*Zoom Nikkor ED Glass 5.0 Mega pixels 8X zoom*, *electric view finder*). The resulting videos were then imported and analyzed by the wrMTrck plugin of Image J. Serotonin hydrochloride (*Sigma Aldrich catalog number # H9523*) was used as a positive myo-excitatory control as it can stimulate movement [77] and carbamoyl choline chloride (*Sigma Aldrich catalog number # C4382*) was used as negative control, as it can inhibit movement [77] . Both products were used at the following concentrations: - 0.1μM, 1μM, 5μM, 10μM and 100μM. Animals are

classified as “motile” if they actively moved on the plates without stimulus, they are classified as “limited mobility” if they move with relatively slow movement or they are denoted “non-motile”, if the worms were immobile and did not respond to any external or internal stimuli [93, 120]. Unhealthy worms were ignored during recording.

2.3.6. Manufacture of The Microfluidic Platforms

S. mansoni somules were not capable of moving laterally in each well of the 6-welled plate, so microfluidic platforms with sinusoidal channels were designed. The protocol for the manufacture of the microfluidic platforms was followed as published before [101, 123, 124]. The microfluidic chips were fabricated in the lab of Dr. Santosh Pandey’s micro/nano systems laboratory of the Electrical and Computer Engineering department of Iowa State University, using a standard soft photolithographic process [123-127]. Polydimethylsiloxane polymer or PDMS (*Catalog number # Sylgard 184 Silicone Elastomer Kit, Dow Corning Corporation, Midland, MI*), which is known to be biologically inert, transparent and glass permeable, was mixed and poured over the SU-8 photoresist mold (*Catalog number # Microchem Corporation, Newton MA*), which was then allowed to solidify. Once solidified, the PDMS was peeled off the SU-8 mold, followed by input and output holes being punched into the device. Air plasma was used to bond the PDMS device to individual glass slides following a published protocol [123-127].

Table number 2.1. List of the neuropeptides, along with their associated npp genes, from which they originated.

Number	Name of the peptide	Reference number #	Origin from npp gene
1	SAYPYV-NH2	*50647-649*	*Sm-npp-15*
2	SYFDPIIY	*50647-923*	*Sm-npp-15*
3	VPPYITGGIRY	*50647-925*	*Sma-npp-27*
4	AYHFFRL	*50647-926*	*Sma-npp-28*
5	FLFNLKDTRW-NH2	*50647-653*	*Sm-npp-19*
6	GFVRI-NH2	*50647-642*	*Sm-npp-1*
7	AFVRL-NH2	*50647-641*	*Sm-npp-1*
8	GMI-NH2	*50647-643*	*Sm-npp-2*
9	YIRF-NH2	*50647-934*	*Sma-npp-23*
10	NYLWDTRL-NH2	*50647-650*	*Sm-npp-16*
11	DDFRG-NH2	*50647-651*	*Sm-npp-17*

Since *S. mansoni* somules in a well plate is unable to move laterally in the well, sinusoidal channels (width of each channel = 60 microns, amplitude = 220, channel wavelength = 360) were designed on the microfluidic chip which could imitate human blood vessels which aid in somule propulsion. The fixed amplitude and the wavelength allowed the

researcher to control the parasitic waveform and trajectory with limited latitudinal and longitudinal freedom [123-127].Adequately designed sinusoidal parallel channels were designed so as to allow multiple subjects to be viewed at the same time during a single microscopic recording. The *S. mansoni* somules were added to the reservoirs using a 1 ml. syringe, connected to a polyethylene tubing.

However, it was found that the somules would end up being flushed out of the channels whenever the chip was moved, or fluids were added to the ports. Microscale filters were attached to the reservoirs on either side of the wave like channels to prevent the rapid movement of fluids in the channel which could flush the somules out of the chip. The movement of somules was recorded for 5 minutes, using a 10X objective lens of a compound microscope (*Nikon Eclipse TS100*), equipped with a digital camera (*Zoom Nikkor ED Glass 5.0 Mega pixels 8X zoom*, *electric view finder*). The software used for recording the somule movement was Ken-a-Vision and the digitizer was connected to a USB 2760 camera. From previous research of studying the movement of *C. elegans* through similar microfluidic channels, the chips possessed certain advantages e.g. analyzing the unimpeded crawling motility of the schistosomules through the channels, the filters prevented the worms from being flushed out as well as high-thorough put pharmacological screening of the worms for potential anthelminthic agents as well as limiting the influence of the external environment

[125, 126].

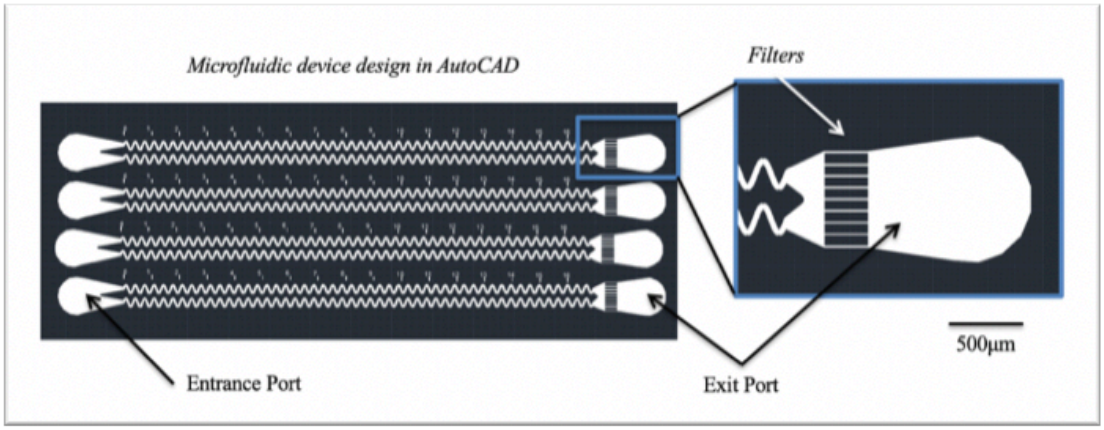


Figure number 2.3.B. Microfluidic chips with sinusoidal channels Design of the microfluidic platforms with the microscale filters attached to the reservoirs on either end of the channels. Image provided and designed by Dr. Zach Njus.

2.3.7. Media Used For The Microfluidic Platforms

Basch media was made in the following way: - 9.23 gms. of MEM with Earl's Salt, without L-glutamate, phenol red, sodium bicarbonate (*JRC catalog number # 56-119-000*) was dissolved in 1 liter of distilled water, followed by 1000 mgms. of glucose (*Fisher Scientific catalog number # D-16-3*) and 2400 mgms. of HEPES (*Fisher Scientific catalog number # 6003H*). 1000 milligrams of lactalbumin hydrolysate (*BRL Catalog number # 11800-025*) was added to the mixture and it was allowed to dissolve for 15-20 minutes on a magnetic shaker. Once dissolved, 2.2 gms of sodium bicarbonate (*Corning Cellgro catalog number # 90-009-PB*) was added to the media. 500 µls. of 1 mM hypoxanthine (*Sigma catalog number # H-9377*), 500 µls. of 1 mM hydrocortisone (*Sigma catalog number # H-0888*), 500 µls. of 1 mM triiodothyronine (*Sigma catalog number # T-5516*) and 900 µls. of bovine pancreas insulin (*Sigma catalog number # I-5500*) was added to the mixture, after

which the pH was adjusted to 7.3-7.4. 50 µls. of 1X Schneider's media (*Invitrogen catalog number # 11720-034*) and 5 ml of 100X MEM Vitamins (*Invitrogen catalog number # 11120-052*) were added and the pH was again calibrated to 7.3-7.4. The entire media was filter sterilized and to the sterile Basch media, 500 uls. of penicillin-streptomycin and 50 µls. of 5% re filtered, heat inactivated fetal bovine serum (*JRS catalog number # 43640-100*) was added. Aliquots of 50 ml were made and stored at 4°C.

One of the major problems of using a complete fluid media like Basch, was that somules were floating along with the flow of the media and not exhibiting any muscular function for locomotion, which is what we need to observe. In the human host, parasites cannot float that easily through blood vessels due to narrow size of the vessels and also because of the presence of red blood cells, white blood cells and platelets. To mimic this situation, different media were tested, to determine the ideal one in which the somules could move freely without any obstruction. We tested different concentrations of agar dissolved in water (*Fisher BioReagents OptiGrow Pre-Weighed LB Agar, Miller (Powder) catalog number #BP1425-10P1* – 0.1% 0.2% 0.3% 0.4%. The agar was maintained in a molten state, on a heated shaker. Somules were added to the agar, once it reached an ideal temperature so that the parasites could survive and freely move in it, without getting affected by the heat. Based on our trial runs, we believe maintaining the agar media at a temperature range of 50°C- 60°C was suitable for the somules to exist. When the temperature of the agar was higher than the observed range, it caused the heating up and destruction of the worms, and when the temperature of the agar was lower than the range, the media was solidified thus restricting the movement of the worms. In addition to agar, we also tested other media such

as collagen (*Advanced Biomatrix PureCol EZ Gel catalog number # 5074-G*), pluronic gel (*Sigma Aldrich number # 435422-250ML*).

2.3.8. Analysis of The Videos

The videos recorded from the 6 welled plates were imported into wrMTrck plugin of Image J software for analysis [77]. The recorded video images of worms are converted to binary objects through the software, which is then tracked from one frame to the next [77] . The phenotypic motility of the somules were measured in terms of body contractions, using the parameter Body Bends Per Second (BBPS). This parameter has been previously used to record the phenotypic motility of *C. elegans* [128]. Based on the definition of BBPS as provided by [128], the contraction of muscles which caused the *C. elegans* to bend on one side was considered as 1 body bend [128]. During analysis, when the somule made 3.5- 4.0 BBPS, this movement was considered as “normal swimming”. Movements recorded with fewer or more BBPS due to a different posture of the worm was considered “abnormal swimming” when compared to the control movement [128] . Based on the data obtained from the Image J wrM Trck software, graphs were created using Graph pad Prism version 8. Videos recorded from the microfluidic platforms were uploaded to the Iowa State University cloud Cybox storage, allowing collaborators access to the data. Dr. Zach Njus from Dr. Santosh Pandey’s lab wrote a custom computer program to interface with the scanner, in order to analyze the videos.

2.4. Results and Discussion

2.4.1 Testing The Neuropeptides On *S. mansoni* Somules Motility In A 6 Welled Plate

Based on initial experiments, 3 out of 11 neuropeptides were found to have a visible effect on the motility of the *S. mansoni* somules- GFVRI-NH2, AFVRL-NH2 and YIRF-NH2 (data not shown). These three peptides were chosen for further experiments. 50 *S. mansoni* somules were added to each well of a 6 welled-plate in 3 ml. of sterile Basch media, each well containing a different concentration of each neuropeptide ranging from 0 μM, 1 μM, 5 μM, 10 μM, 50 μM and 100 μM. For each peptide-treated plate, a positive control, 5-HT and a negative control, carbachol were maintained. All plates were incubated for 30 minutes at 37°C and the motility of the somules were recorded for 5 minutes, at the rate of 10 somules per view, using a compound microscope (*Nikon Eclipse TS100*), equipped with a digital camera (*Zoom Nikkor ED Glass 5.0 Mega pixels 8X zoom*, *electric view finder*).The resulting videos were imported into wrM Trck plug-in of Image J software for analysis.

Somules moved forward using a specific head-to-tail propagation of dorsal and ventral body bends [126]. In order to quantify the motility of worms, the software calculated the average number of subtracted pixels/frames to the total number of pixels in the binary object [77]. The phenotypic motility of the somules was measured in terms of body bends per second (BBPS).

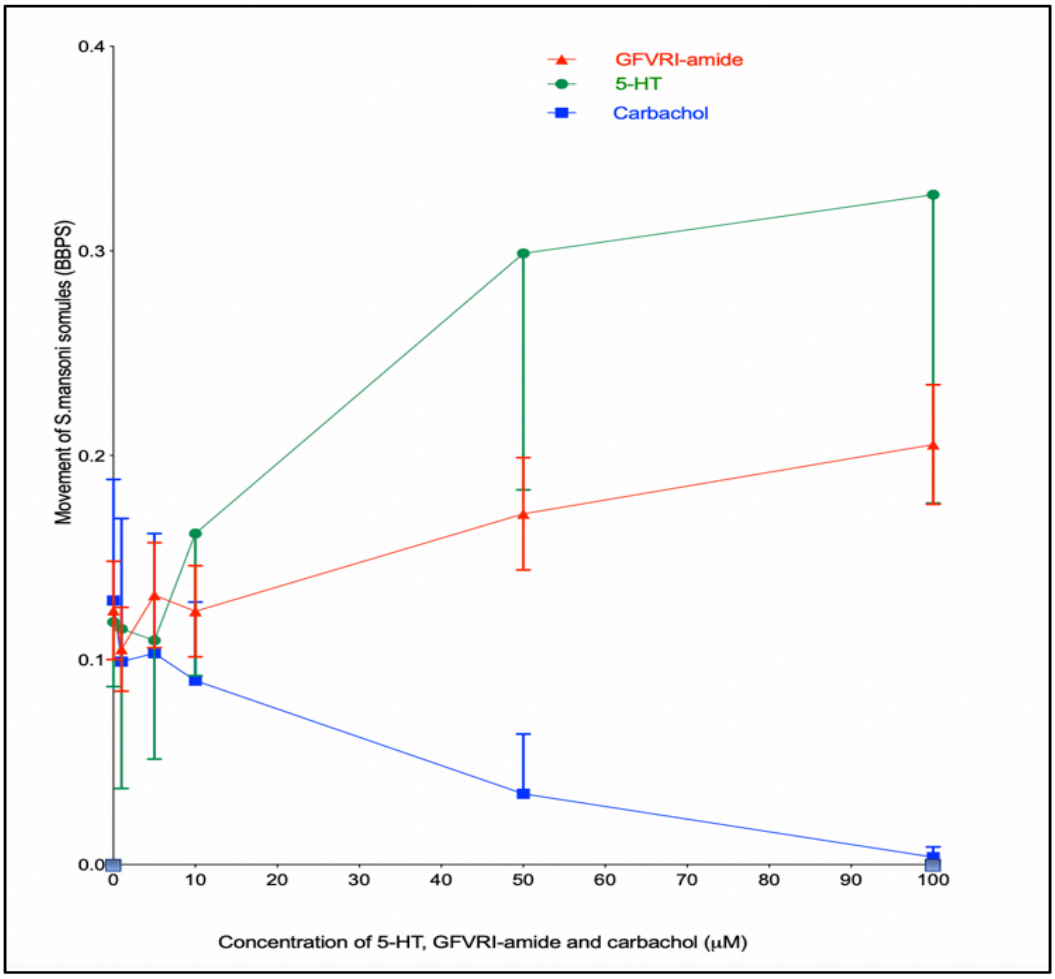


Figure number 2.4.A : - **The effect of neuropeptides on the activity of *S.mansoni* somules carried out in a 6 welled plate. A.** shows an initial increase in *S. mansoni* somule movement (measured in BBPS) at 1µM concentration of GFVRI-amide neuropeptide, followed by a slight fall in somule motility (measured in BBPS) at 5µM, followed by a significant increase in somule motility (measured at BBPS) between (10µM -100µM) concentration of GFVRI-amide. For the positive control, the somule motility increases with an increase in concentration of 5-HT (1µM-100µM) and for the negative control, the somule motility decreases with an increase (1µM-100µM) of carbachol.

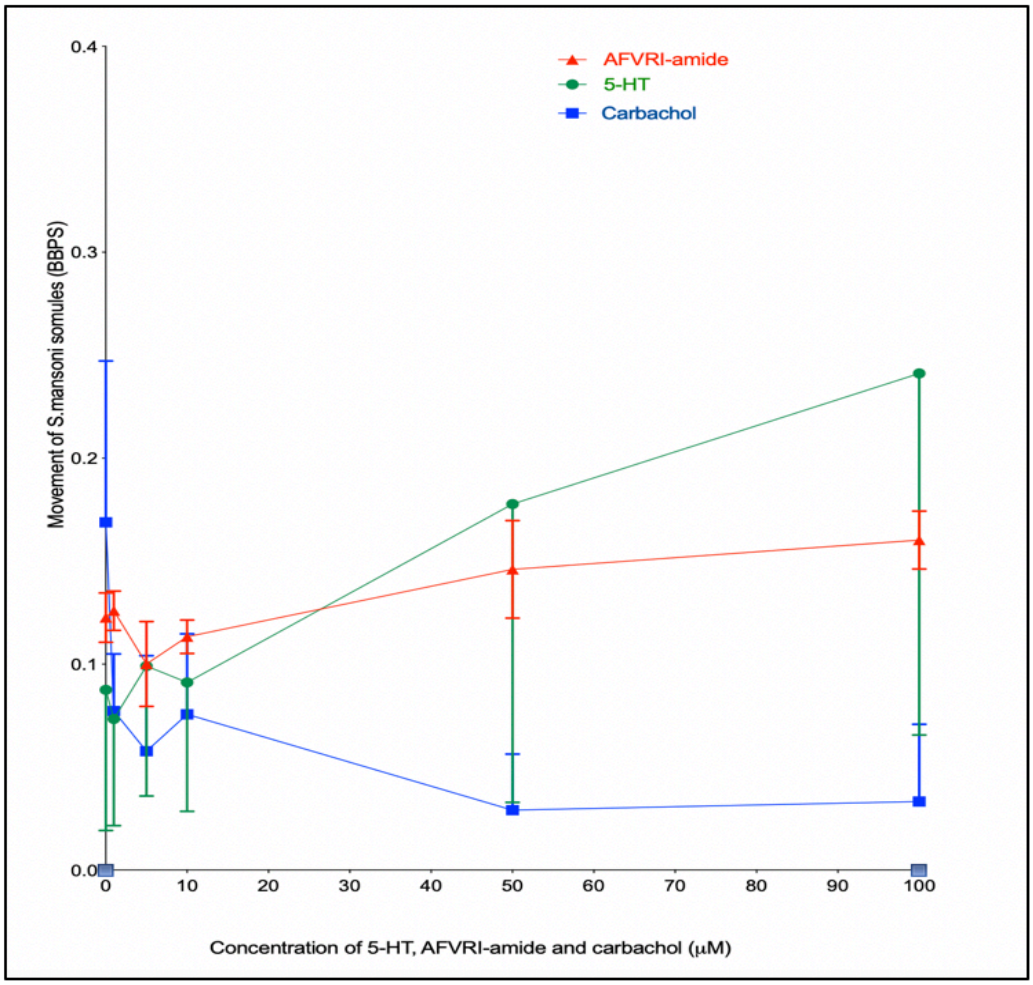


Figure number 2.4.B : - The effect of neuropeptides on the activity of *S.mansoni* somules carried out in a 6 welled plate. B. shows a slight decrease in somule motility (measured in BBPS) at 1µM concentration of AFVRL-amide neuropeptide, followed by a significant increase in somule motility (measured in BBPS) between (5µM-100µM) concentration of AFVRL-amide. For the positive control, the somule motility (measured at BBPS) increases with an increase in concentration of 5-HT and for the negative control, the somule motility decreases with an increase in concentration (5µM-100µM) of carbachol.

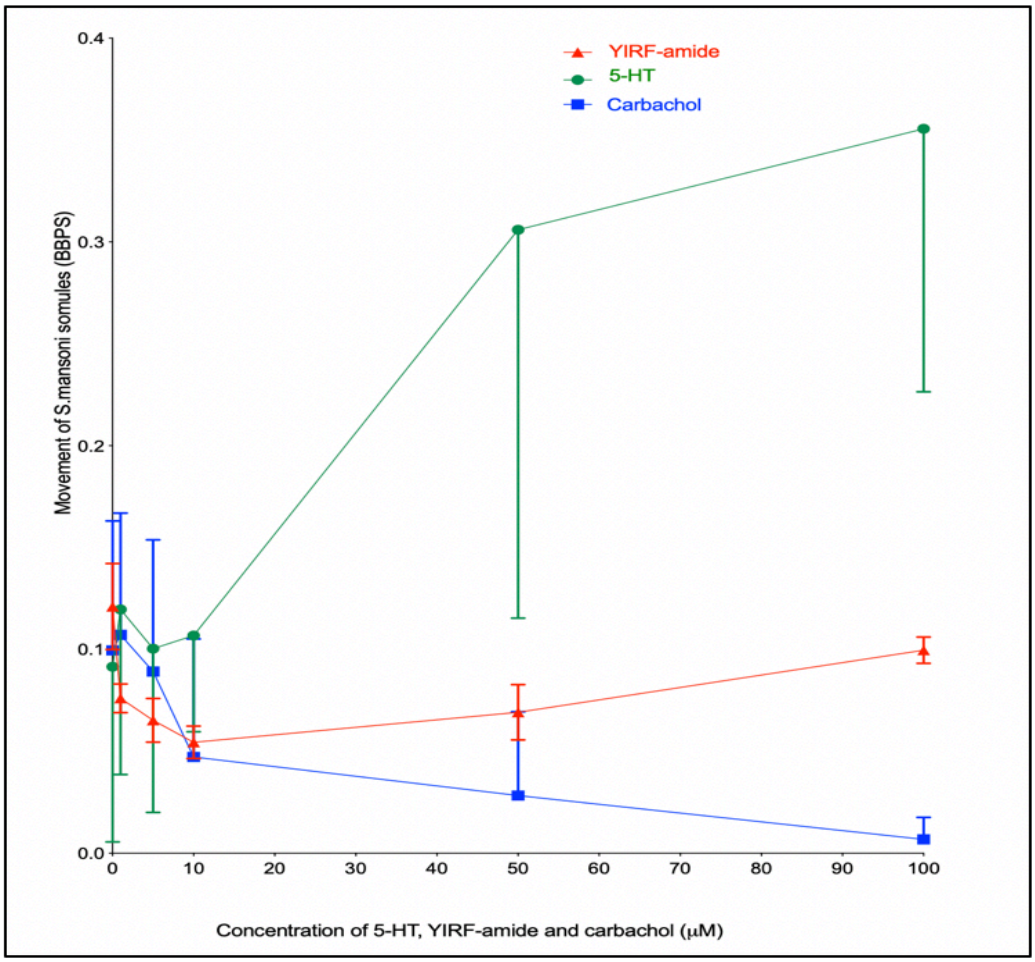


Figure number 2.4.C : - The effect of neuropeptides on the activity of *S.mansoni* somules carried out in a 6 welled plate. C. shows an initial decrease in somule motility (measured in BBPS) between (1µM-10µM) concentration of YIRF-amide neuropeptide, followed by a steady increase in somule motility (measured in BBPS) from (10µM-100µM) concentration of YIRF-amide. For the positive control, the somule motility increases with an increase in concentration (5µM-100µM) of 5-HT and for the negative control, the somule motility decreases with an increase in concentration (5µM-100µM) of carbachol.

In *S.mansoni* adult worms and cercariae, the neuropeptide precursor Sm-npp-1 encodes GFVRI-amide [80]. The neuropeptide GFVRI-amide is expressed in the cerebral ganglion neurons of the central nervous system (CNS) in *S.mansoni* adult worms and schistosomules and is believed to be responsible for interneuronal communication [80]. The

application of GFVRI-amide to isolated muscle fibers from somules, caused a visible inhibitory effect on the somule motility, which was assumed to be a result of spastic (myoexcitatory) or flaccid (muscular relaxation) paralysis [80].

In our experiments, when GFVRI-amide was applied to *S. mansoni* somules in a concentration dependent manner, there was an initial reduction in somule motility at 1μM concentration, followed by a significant increase in somule motility starting from 5μM concentration to 100μM concentration of GFVRI-amide. When compared to the untreated control, GFVRI-amide had a significant increase in somule motility. Unfortunately this result seems confusing and does not correspond with previous published data, which displayed an inhibition of *S.mansoni* adult and somule muscular movement [80].

In *S.mansoni* adult worms and cercariae, Sm-npp-1 precursor also encodes the AFVRL-amide [80]. AFVRL-amide was also identified in *Eisenia fetida,* however not much information is available regarding the function of this peptide in helminths [116]. When we treated schistosome somules with AFVRL-amide, there was a slight reduction in somule motility at 10μM concentration, after which there was an increase in motility starting from 50μM-100μM concentration of AFVRL-amide. When compared to the untreated control, AFVRL-amide seems to increase somule motility.

YIRF-amide is a FMRF-amide like protein or (FLP), which was first isolated from a horseshoe crab commensal *Bdelloura candida* and the NP caused a dose-dependent contraction in turbellarian muscle fibers [129]. YIRF-amide is abundantly distributed through the central nervous system of platyhelminths as well as in the nerves innervating the reproductive and somatic muscular systems [106, 107, 112]. In 2007, the very first platyhelminth NP receptor, GtNPR-1was identified from *G.tigrina* and is believed to be the

main receptor responsible for neuromuscular excitation in platyhelminths [106, 112]. The mode of action of GtNPR-1 is via the GPCR signal transduction pathway, in which the receptor is coupled to the $G\alpha_{i/o}$ subunit, and once the FLP binds to the receptor, the phospholipase C-β (PLC-β) mediated downward pathway is activated, leading to the activation of protein kinase C (PKC) [106, 107, 112, 130]. Upon activation, PKC translocates to the cellular plasma membrane, resulting in muscle contraction in platyhelminths [106, 130]. In 1997, YIRF-amide was found to increase muscular contraction in somule and adult schistosomes, thus being myoexcitatory in nature [112, 115]. However, this result was contradicted in 2011, since YIRF-amide was found to inhibit schistosome worm movement [80].

Our experiments demonstrate that when YIRF-amide was applied to *S. mansoni* somules in a concentration dependent manner, there was an initial reduction in somule motility starting from 5μM concentration, followed by an increase in somule motility starting from 10μM concentration of YIRF-amide. Overall, there seemed to be an inhibition in motility of the treated somules, when compared to the untreated controls. This seemingly corresponds with the results published in 2011 by McVeigh's group, which displayed an inhibition of somule muscular movement [80]

In this assay, the motility of the parasites was analyzed and expressed using the parameter body bends per second (BBPS). However, this parameter is not sufficient to understand and analyze the effect of drugs on the directional motility of the worms. In order to ensure the effectiveness of a newly synthesized anti-schistosomal drug, it is necessary to study if the drug has any effect on the forward movement of the somule, and not just on its motion at a singular point. This is an important deficiency of this assay, since the 6-welled

plates are unable to mimic the host environment in which somules can survive and travel through the body.

2.4.2 Analysis of The Movement of *Schistosoma mansoni* Somules Through the Microfluidic Channels Using A Custom Computer Program Designed by Dr. Zach Njus

In recent years, microfluidic technology has gained a lot of attention as a more ideal form of motility assays. The effect of chemotherapeutic agents on organisms within custom-designed chips can help researchers study and analyze the resultant chemotactic behavior of the animals [92]. For our experiments, it was necessary to study the migration of *S. mansoni* somules through narrow channels that could mimic the mammalian circulatory system and these platforms were manufactured by Dr. Zach Njus and Dr. Santosh Pandey's micro/nano systems laboratory of the Electrical and Computer Engineering department of Iowa State University. We hypothesized that these microfluidic channels could help researchers develop better and more reliable drug screening assays while assessing worm viability.

The microfluidic channels are organized as sinusoidal channels and the width of each channel is 60 microns with an amplitude of 220 and a channel wavelength of 360. After transforming freshly shed *S. mansoni* cercariae into somules, the somules were added to the opening pores of the microfluidic channels using a 1 ml. syringe connected to a polyethylene tubing. The movement and migration of the worms through the channels were recorded for a period of 5 minutes. Since the somules are in a constant state of occlusion with the side walls of the sinusoidal channel of the microfluidic chip, Dr. Pandey's group designed a custom-made software program which characterized the structure of the somules based on 3 parameters, texture, shape and size. Initially, the bright field video of the somule moving through the microfluidic channel was observed, which was then segmented based on

intensity and texture from the video, and finally a visual representation of the data in video format was used for verification and analysis. This program tells the scanner when to take a picture, how often to take pictures, how many pictures to take and then analyze the pictures when done. A climate control system was created because these experiments took so long, the climate in the lab could be subject to fluctuating temperatures. The protocol of software analysis was divided into a number of steps. As per previous publications, a body movement is defined by a displacement of pixels in between two consecutive frames [77]. The unit "pixel" is used to measure the width or height of a worm image, and hence in this study, the term has been utilized to measure length and distance [131].

The first step of the software analysis involved channel segmentation, a background image was made by looping through the video frames and recording the brightest pixel value taken during the video, thus effectively erasing the worm from the frame. This allowed the channel of the microfluidic device to be segmented based off of color and size. The second step of the software analysis involved segmenting somules from the video. A standard deviation filter was taken across the original image to highlight areas with a high texture value.

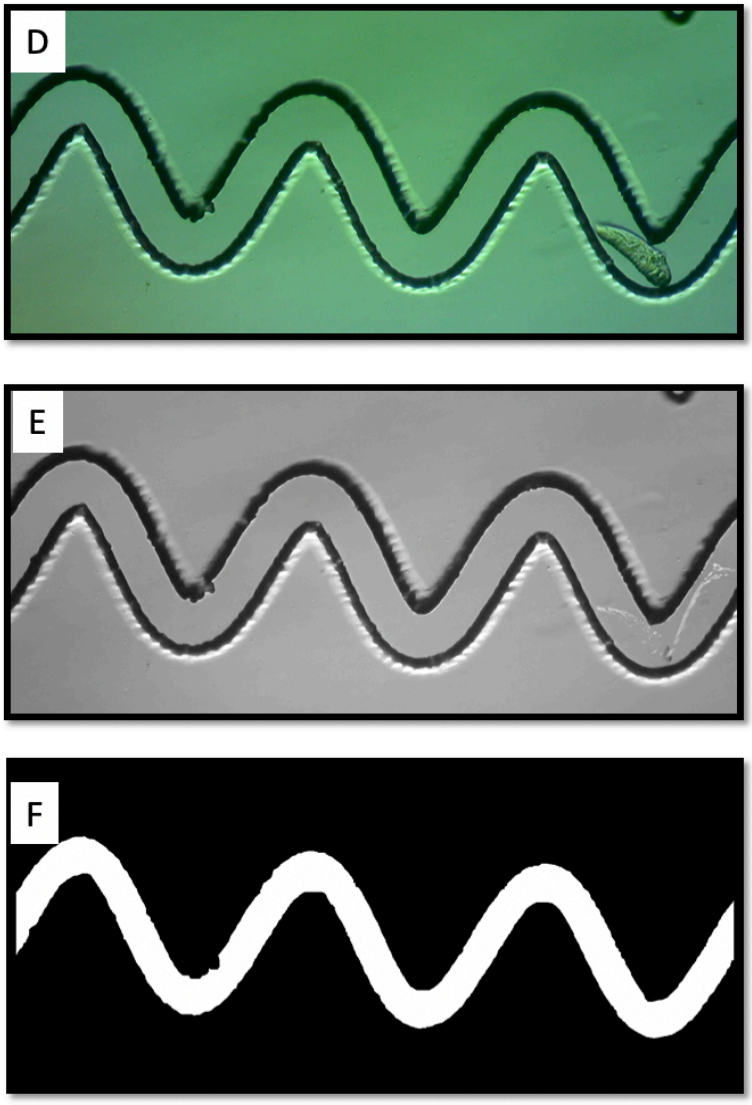


Figure number 2.4.DEF : -Analysis of the movement of *S.mansoni* somules through the microfluidic channels Step 1. D. depicts the raw video image and channel segmentation **E.** recording the brightest pixel value taken during the video **F.** erases the worm from the frame. The software was designed by Dr. Zach Njus and Dr. Santosh Pandey.

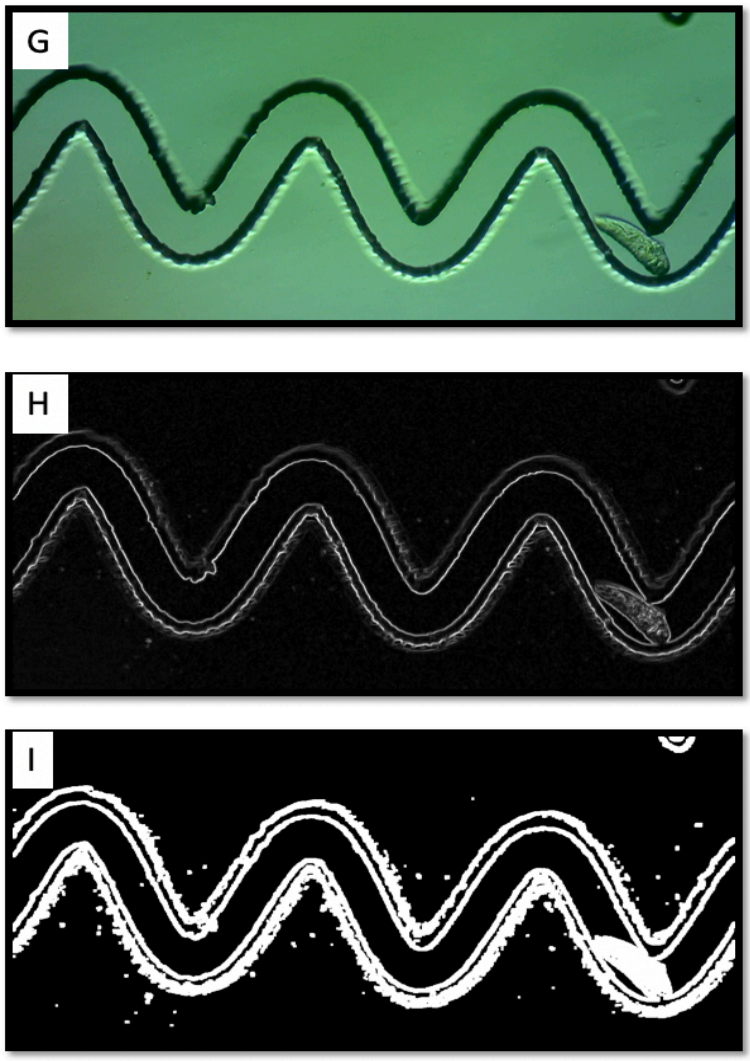


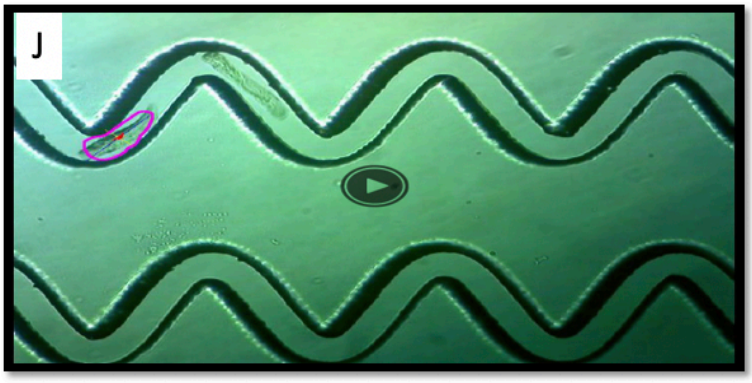
Figure number 2.4 GHI : -Analysis of the movement of *S. mansoni* somules through the microfluidic channels Step 2. G. Segmenting the somules from the video **H.**. Standard deviation filter applied. **I.** Highlights the areas with a high texture value so the somule in the channel is highlighted. The software was designed by Dr. Zach Njus and Dr. Santosh Pandey.

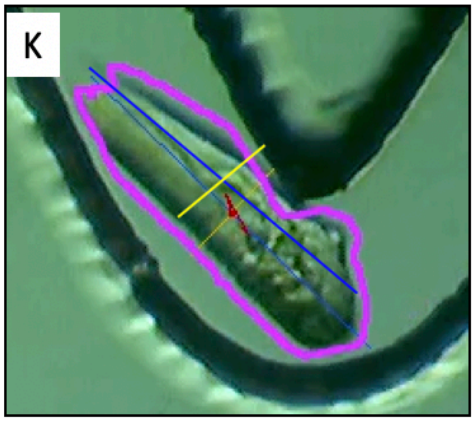
The two key parameters used for expressing the movement of somules within the microfluidic channels were (1) Centroid Distance and (2) Track Distance. These terms were introduced by Dr Zach Njus.

(1) Centroid Distance is defined as the point whose coordinates demonstrate the mean value of the coordinates of all the points (expressed in pixels) which in turn, represents the subject under study i.e. somule in this case (as opposed to 'background') in a binary worm image [131]. The centroid distance represents the body movement of the somule, as it expands and contracts at one point .Once the schistosomules were placed into the microfluidic channels by a wide-bore pipette, the movement of the worms was tracked over a specific time period (typically 10-15 minutes); this provided the velocity of the body centroid [103].

(2) Track distance represents the distance moved by a somule from one point to the second point. The ratio of the distance traversed by the worms through the sinusoidal channels from one point to the second point, to the net change in time, was calculated to provide the track distance [120].

For our experiments, the movement of somules through the channels was recorded for 5 minutes, using a 10X objective lens of a compound microscope (Nikon Eclipse TS100), equipped with a digital camera (Zoom Nikkor ED Glass 5.0 Mega pixels 8X zoom, electric view finder).





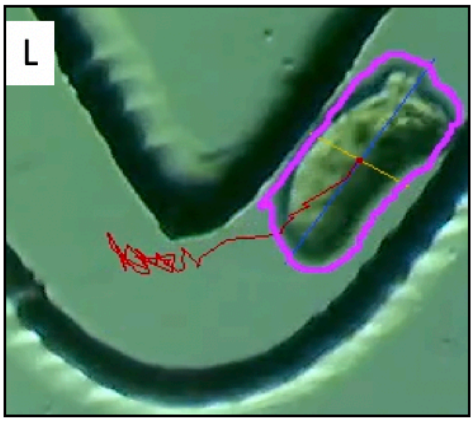


Figure number 2.4 JKL. : Centroid distance and track distance are the two parameters used to express the movement and migration of the somules as they travel through the sinusoidal channels of the microfluidic chip. J. Tracing *S. mansoni* somule as it travels through the sinusoidal channel of the microfluidic chip. **K.** The parameter used for the expressing the movement of the somule at a single point is centroid distance, denoted by red . In the somule. the major axis is denoted by blue and minor axis is denoted by yellow. The shape of the somule is denoted by pink. **L.** The parameter used for the expressing the migration of the somule from one point to another track distance, denoted by red. In the somule. the major axis is denoted by blue and minor axis is denoted by yellow. The shape of the somule is denoted by pink. The software was designed by Dr. Zach Njus and Dr. Santosh Pandey.

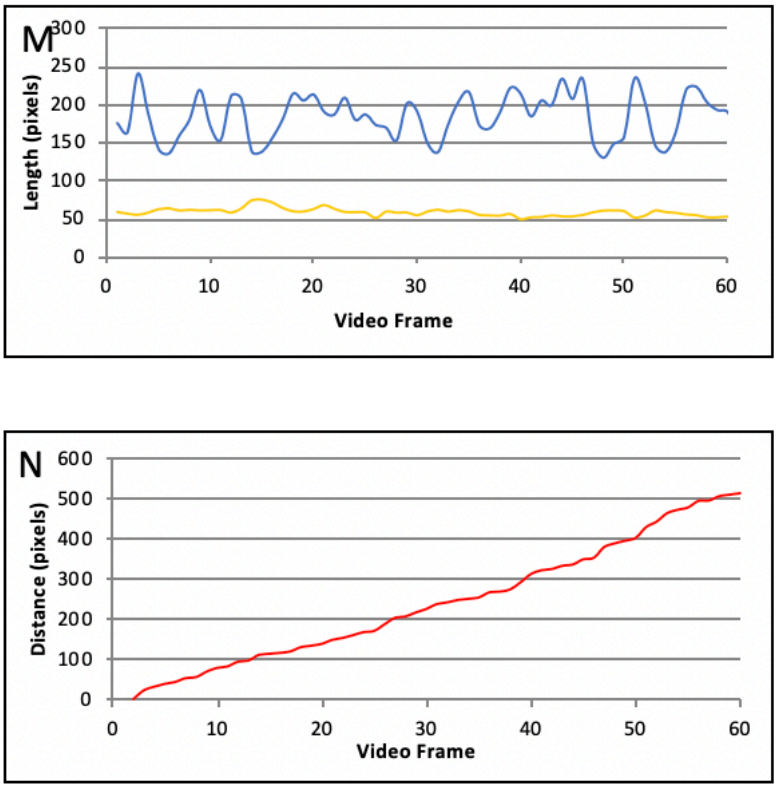


Figure number 2.4. MN. M. Graphical representation of the axis length (expressed in pixels) of the *S.mansoni* somules versus time (expressed in seconds). The major axis of the somule is shown in blue and the minor axis of the somule is shown in yellow **N.** Graphical representation of the total centroid distance (expressed in pixels) travelled by the *S. mansoni* somule versus time (expressed in seconds). The red line represents the centroid track distance.

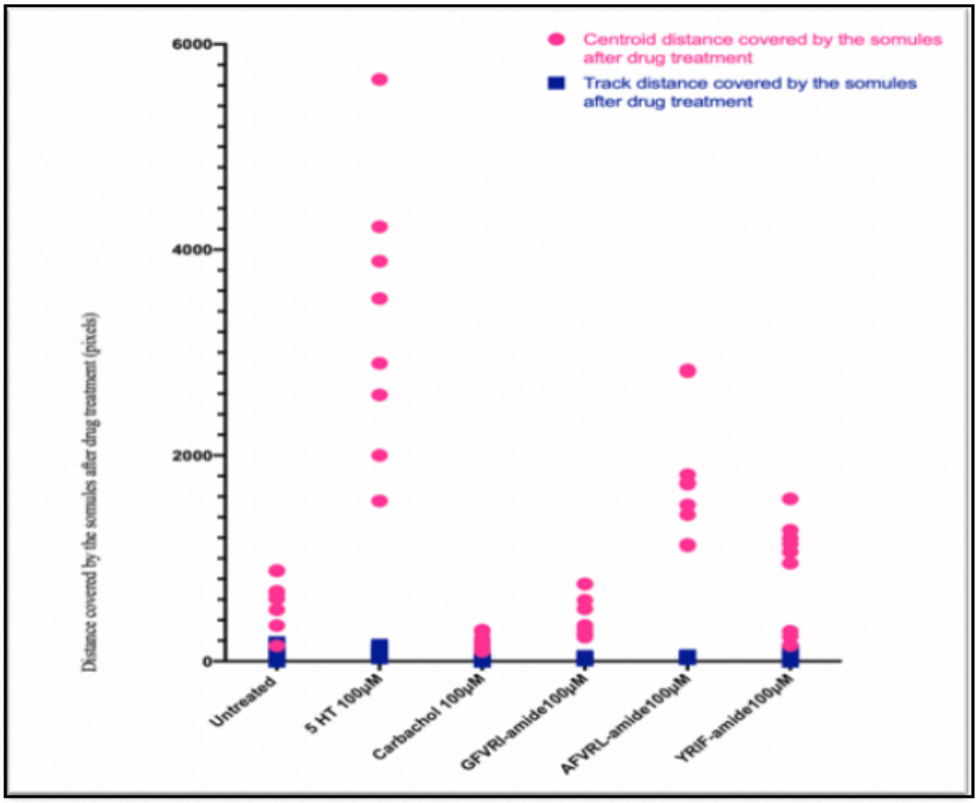


Figure number 2.4.O. Graphical representation of the comparison of the centroid distance (expressed in pixels) versus the track distance covered (expressed in pixels) by *S.mansoni* somules (n=9), post drug treatment (the concentration of each drug is expressed in µM). The somules exhibit maximum motility at a single point when exposed to AFVRL-amide, followed by YIRF-amide and then GFVRL-amide. The somules migrated the greatest distance after being exposed to YIRF-amide, followed by exposure to AFVRL-amide and the least distance was covered by those somules treated with GFVRL-amide. When the parasites were treated with 5-HT, they demonstrated very high muscular expansion and contraction, however, they did not seem to migrate much ahead. On the other hand, the parasites treated with carbachol displayed minimum motility at one position, and they were equally unable to move ahead.

The videos were analyzed using the software custom made by Dr. Njus and Dr. Pandey. The centroid distance (expressed in pixels) and the track distance (expressed in pixels) of the *S. mansoni* somules treated with 100µM of each neuropeptide were compared against the untreated control, a positive control of 100µM 5-HT and a negative control

of100μM carbachol .5-HT was chosen as the positive control in this assay because it causes hypermotility in *S. mansoni* via rapid contraction of the longitudinal muscles and cross-sectional constriction of circular muscle, leading to rapid elongation, by modulating neuronal output to the parasitic muscles [77] . Carbachol is a cholinomimetic agent and an inhibitor of acetylcholinesterase which binds directly to mammalian post synaptic nicotinic cholinergic receptors and can produce flaccid paralysis in schistosomes, making carbachol an ideal negative control [132]. Based on the results we obtained, *S. mansoni* somules treated with a high concentration of AFVRL-amide, showed the maximum movement at a single point when compared to the untreated control, however the distance moved by these treated somules was less, when compared to the untreated control. Since the effect of the neuropeptide AFVRL-amide on helminths has not been much explored, we can assume that the peptide has a myoexcitatory effect on the somules.

A literature review conducted on previously published research on the effect of YIRF-amides on *S. mansoni* somules, gave us contradictory results. In 1997, it was demonstrated that YIRF-amide had a myoexcitatory effect on somules, causing rapid muscular excitation and contraction in the parasite [115]. But in 2011, the same peptide was found to inflict inhibitory effect on the muscular movement of the somules [80]. Our results correspond with the data published in 1997 by the Day group, since there seemed to be a visible increase in centroid distance when the somules were exposed to YIRF-amide, when compared to the untreated control. Hopefully this data will help to support the hypothesis that YIRF-amide is myoexcitatory for parasites.

Previously it was established that the neuropeptide GFVRI-amide has an inhibitory effect on the muscular activity of *S. mansoni* adult worms and somules [80]. The data we

present here, corresponds with the previously published data. When the somules were treated with GFVRI-amide, there was a reduction in the motility of the worms, when compared to the untreated controls.

From the data we obtained above, we can hypothesize that motility of a parasite at a singular point in not indicative of its directional movement. Just because the parasite displays rapid myoexcitatory activity, does not essentially mean that the parasite is capable of moving ahead. We demonstrated that AFVRL-amide treated *S. mansoni* somules displayed a high contractile activity, however it is unable to move ahead, when compared to the untreated controls. Similarly, the parasites treated with YIRF-amide showed high muscular contraction, but again the distance travelled is insignificant, when compared to the untreated controls. The pattern was repeated in the somules treated with GFVRI-amide. It can be assumed that the somule uses up all its energy during rapid muscular expansion and contraction, and it is possible that this high activity exhausts the parasite, as a result of which it is unable to move ahead. We can thus hypothesize that contractile activity is not directly proportional to directional motility. To prove the hypothesize, we conducted the next set of experiments, the results of which confirmed our proposed theory.

2.4.3 Analyzing the Relationship Between Contractile Activity and Directional Motility of *S. mansoni* Somules

Contractile activity is defined as the contraction of the cell, depending on the activity of the major axis along the length of the cell and minor axis along the width of the cell [44]. Directional cell motility is initiated as a result of a functional asymmetry between the front and the rear of the cell, due to the presence of a steering mechanism, which produces persistent self-propagated polarization [121]. In order to differentiate between the two

parameters, *S. mansoni* somules were treated with different concentrations of 5-HT (1µM, 5µM, 10µM, 50µM and 100µM). As mentioned before, the motility and migration of the somules through the channels were recorded for 5 minutes, using the same equipment as used before, and the videos were analyzed using the Njus-Pandey software.

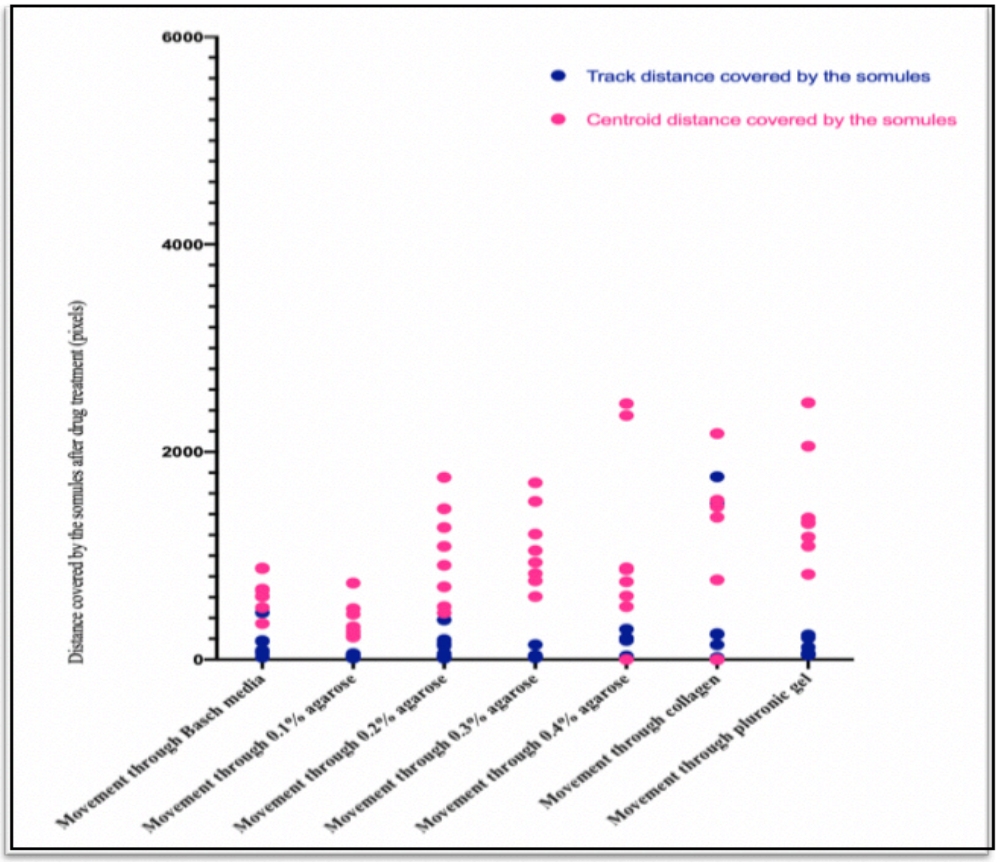


Figure number 2.4.P. Graphical representation of *S.mansoni* somule contractile activity is inversely proportional to *S.mansoni* somule (n=5) directional motility. A rapid increase in the muscular activity (expansion and contraction) of the somules were observed with an increase in the concentration of
5-HT (1µM-100µM) However, there was a significant reduction in the track distance covered by the somules with the similar increase in the concentration of 5-HT (1µM-100µM). It can safely be assumed that the somule contractile activity is inversely proportional to the somule directional motility. More the movement of the parasite at a singular point, less is the distance travelled by it.

From figure number 2.4.O, it is obvious that the activity of the somule increases proportionally when exposed to increasing concentration of 5-HT, however the distance moved by the somules decrease at the same concentrations of 5-HT. Figure 2.4.P suggests that the contractile motility of the parasite is inversely proportional to the directional motility. This supports our proposed hypothesis that when the activity of the somule increases, it uses up its energy, which leaves it exhausted and unable to follow directions to move ahead through the host circulatory system. We can assume that while 5-HT is essential for the overall survival of the schistosome, too much of it can actually lead to its death. Although this looks like a possible route for designing chemotherapeutic drugs to prevent schistosomiasis, the consequence of having excessive serotonin in the mammalian body, is dangerous. At high doses of serotonin (70-200 mg/kg), a person can experience symptoms ranging from tremor, nausea, dizziness, delirium to severe side effects like muscle rigidity, seizures and in extreme cases, it could lead to death [133].

2.4.4 Choosing the Ideal Media for Microfluidic Assays

To design a suitable and efficient microfluidic assay, it is important to choose an ideal media which can mimic the environment in mammalian blood vessels [101]. Human blood is made up of fluid (plasma) and cells (erythrocytes, leukocytes and platelets), so after schistosomules invade humans, they have to navigate through a complex network to spread the disease. Controlling of the flow of the media or fluid is important in biological assays [92]. One drawback of using liquid media like Basch media in microfluidic assays, is the tendency for the media to produce an internal fluid pressure in the channels that could directly affect the somules' natural movement; as a result, the somules may float in the medium, rather than move normally, which can be observed under a light microscope [134] .

An additional problem with Basch media is that it tends to contaminate quickly as a result of its rich composition. To improve our microfluidic setup, it was necessary to imitate the conditions undertaken by the somules as they travel through the human system to their final destination. In a previous publication, the Pandey group studied the movement of *C. elegans* in 0.7% agarose dissolved in M9 buffer within the microfluidic channels, their reasoning being agarose provided a suitable resistance for the worms unlike fluid media [134]. In our study, 0.7% agarose dissolved in distilled water was found to be too dense for the worms to move freely (data not shown). We therefore tested the motility of somules in lower concentrations of agarose dissolved in distilled water, collagen and pluronic gel, using fluid Basch media as the control for comparison. The motility of the somules traveling through the microfluidic platforms was recorded as per our previous protocol and the videos were analyzed using the Pandey-Zach software program.

In Basch media the schistosomules tend to float, rather than move on their own through the channels. The drawback of using different concentrations of agarose is maintaining each at reasonable temperatures, to prevent the agar from solidifying.
The third media which was tested was biocompatible pluronic F-127 gel solution which had been previously used to study the movement of *C.elegans* through microfluidic channels [135]. Unfortunately, pluronic gel was not very helpful because of its constant fluctuation between fluid media and gel media at the slightest change in temperature.

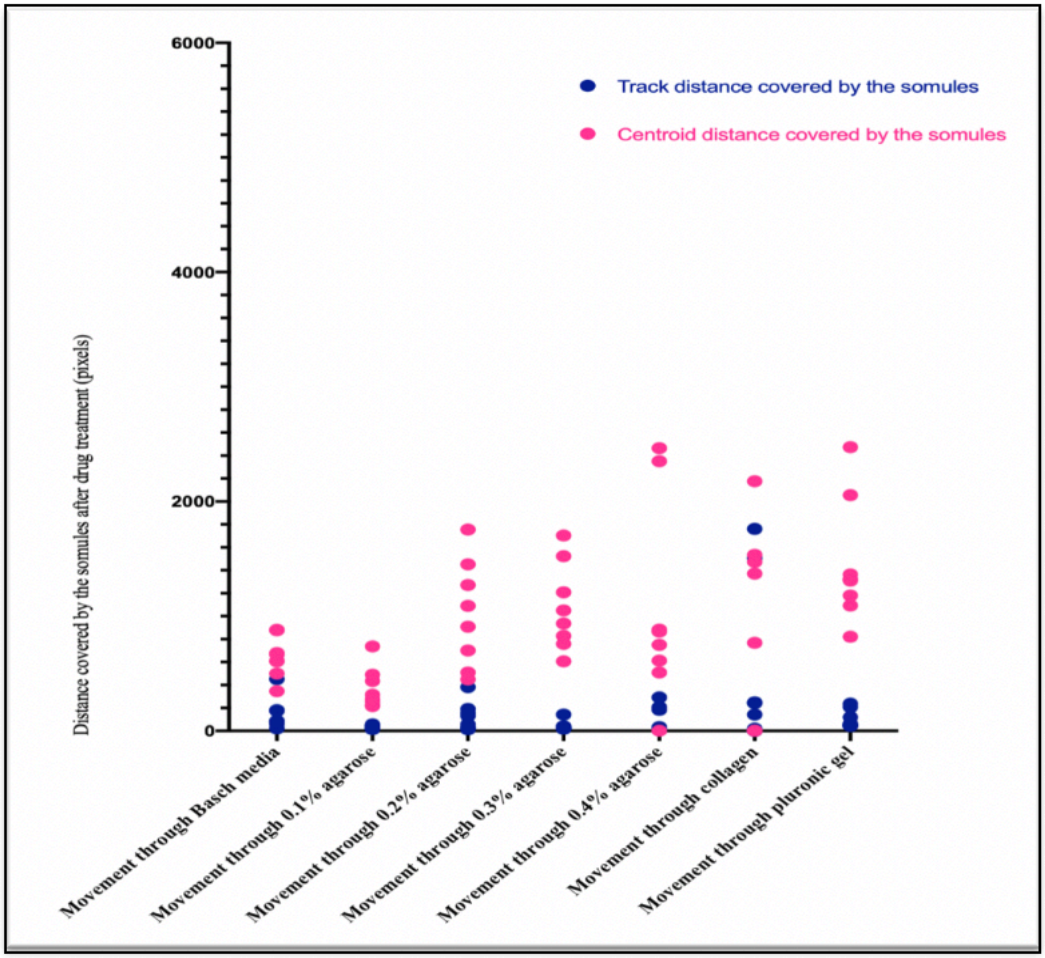


Figure number 2.4.Q. Identification of an ideal media to use in microfluidic platforms to study the movement and migration of *S.mansoni* somules (n=10). Untreated somules were allowed to move through the sinusoidal channels of a microfluidic chip, using Basch media, 0.1% agarose media, 0.2% agarose media, 0.4% agarose media, collagen and pluronic gel. Each sample had n=10, and the average of the values of each sample was calculated. The average centroid distance (expressed in pixels) of the somules in different media was plotted against the track distance covered by the somules (expressed in pixels) in different media. Based on the graph, we believe that collagen is the ideal media for microfluidic assays.

Maximum contractile activity of the somules was observed in pluronic gel and minimum contractile activity was seen in 0.1% and 0.3% agarose media. The distance travelled by the somules was highest in collagen and the minimum distance was covered by the somules in 0.1%-0.3% concentration of agarose. But it was observed that when somules were in pluronic gel, the track distance covered by the somules was very low. This indicates that the somules had restricted movement in pluronic gel. However, the centroid distance covered by the somules in collagen and the track distance travelled by the somules were high. This would indicate that although collagen provided some amount of restriction to the somule mobility, it also allowed the somule to move ahead easily. One drawback of using collagen was its tendency of solidifying at a slight increase in temperature. Fortunately, this solidified media could be reversed immediately to fluid media by placing the collagen on ice. Therefore, we can conclude that collagen is a good media to test the movement and activity of worms in microfluidic assays.

2.5. Conclusion

Schistosomiasis is a freshwater borne parasitic neglected tropical disease (NTD) caused by parasitic worms belonging to the genus *Schistosoma*. According to reports filed by World Health Organization (WHO), NTDs have been reported in at least 149 countries, where it affects more than one billion people worldwide. Intestinal schistosomiasis is caused by *Schistosoma mansoni*, which is widely prevalent in Africa, the Middle East, parts of South America as well as Asia. The recommended drug to combat this parasitic disease is the affordable and relatively safe anti-parasitic drug praziquantel (PZQ) [67].

Table number 2.2. Table representing the advantages and disadvantages of different media to use in microfluidic devices.

Number	Media	Affordability	Ease of utilization	Advantages	Disadvantages	Preference
1	Basch media	Affordable	Easy to add to the chips	Less resistance to movement	Worms tend to float away due to the fluid. Worms also get flushed out of the chips.	
2	0.1% agarose	Affordable	Not easy to add to the chips. Tends to solidify	Nothing noticeable	Constant heating and stirring to prevent complete solidification. Tends to solidify within the channels. Somules get stuck.	
3	0.2% agarose	Affordable	Not easy to add to the chips. Tends to solidify	Nothing noticeable	Constant heating and stirring to prevent complete solidification. Tends to solidify within the channels. Somules get stuck.	
4	0.4% agarose	Affordable	Not easy to add to the chips. Tends to solidify	Nothing noticeable	Constant heating and stirring to prevent complete solidification. Tends to solidify within the channels. Somules get stuck.	
5	Collagen	Affordable	Easy to add to the chips	Becomes opaque when it starts to solidify, so visually easy to work with.	Chances of contamination. Needs to be kept at 4 C. Tends to solidify if kept at RT for long.	Preferred media
6.	Pluronic gel	Expensive	Not easy to add to the chips	Has been used previously to test motility of *C elegans* by the Pandey lab	Tends to solidify easily at RT. Keep it on ice to make it fluid again.	

PZQ acts by increasing the permeability of the parasitic membrane, thus allowing a rapid reflux of calcium ions through voltage operated calcium channels (VOCC) [54]. Recently there have been reports of drug resistant *S. mansoni* worms, from various African countries like Egypt and Kenya [8, 11]. Another major disadvantage of PZQ is that the drug can act against adult worms but is not very effective against juvenile worms. There is an urgent need for new, specific and highly effective anti-schistosomal drugs, and also the pressure to develop efficient platforms for easy detection of the disease [70]. Early detection of schistosomiasis can help researchers to design better assays in order to test the effect of novel drugs on parasites.

Both nematodes and flatworms express a large number of helminth neuropeptides however, the functions of all the peptides is not completely known .Many of these neuropeptides have been shown to be widely distributed throughout the nervous system of flatworms and are involved in the contractile and regulatory activities of the organisms [79, 80, 114]. These worm-specific neuropeptides and their as yet unidentified receptors have the potential of being possible anthelminthic drug targets, capable of overcoming the emergence of PZQ drug resistance and introducing better anthelmintic therapeutic agents.

One of the major challenges when it comes to monitoring and treating of parasitic diseases, is the lack of a sensitive, simple and cheap biological assay [136]. In recent times, microfluidic devices or “Lab-on-a-chip” technology, which are miniaturized versions of macroscale systems, have become popular in biological applications such as electrophysiology, pharmaceutical analysis, screening of drugs, analysis of cells and cellular

materials, separation and screening of biomolecules, diagnostics, and tissue engineering [92]. Microfluidics have been used previously to study the movement of *C. elegans* [93, 120]. This assay has many significant advantages such as the integration of an entire lab on a single small chip; working with reduced chemical and fluid volumes, thus eliminating wastage and lowering costs, reduced reaction times, faster screening of novel compounds, simpler, quicker, portable, affordable manufacture as well as the ability to study several experiments simultaneously [96, 119, 137]. In addition, the process of fabrication of the microfluidic chips is standardized with consistent reproducibility with increased sensitivity and increased resolution, allowing easy handling, which would otherwise be a time consuming and laborious process [33, 119, 137] . Hence its popularity has sky rocketed in the world of modern biology and biotechnology.

In this paper, we have presented and demonstrated specially manufactured microfluidic chips to study the movement and behavior of *S. mansoni* larval parasites as they move through the sinusoidal channels designed on the chips, thus mimicking the host environmental conditions. The microfluidic platforms were made of gas permeable and visible PDMS irreversibly bound to glass slides using soft lithography. Each chip had a number of sinusoidal channels running parallel to each other, so that multiple experiments could be carried out simultaneously. To analyze somule motility, the Njus-Pandey research group created a custom software program using two parameters (1) centroid distance to measure the activity of the somule at one point, and (2) track distance to measure the distance covered by the worm from one position to the other. Of the 11 chosen neuropeptides, 3 peptides were selected for further studies to test the effect of each NP on somule motility. The 3 peptides were AFVRL-amide, GFVRI-amide and YIRF-amide.

When the first set of experiments were conducted in 6 welled plates, it was observed that the motility of the somules increased with an increase in concentration of GFVRI-amide as well as AFVRL-amide and there was a substantial decrease in somule motility with an increase in the concentration of YIRF-amide. The results we obtained from the GFVRI-amide treated somules did not correspond with previously published data, which had stated that GFVRI-amide has an inhibitory effect on *S. mansoni* adults and somules [80]. In addition, the data we obtained from the YIRF-treated somules did not match with those published in 2011, which stated that the peptide had an inhibitory effect on the worms [80]. However our results did agree with those published earlier in 1997 by *Day et al*, which suggested that YIRF-amide had a myoexcitatory effect in somules [115].

In our second state of experiments, we introduced the drug treated somules to microfluidic channels, and the movement was recorded for 5 minutes. Using Dr. Njus' custom software program, the video recordings were analyzed, and it was confirmed that the schistosomules treated with 100μM of AFVRL-amide displayed maximum centroid distance and those treated with 100μM of GFVRI- amide displayed minimum centroid distance, when compared to the controls. In other words, AFVRL-amide caused the worms to undergo high levels of contraction and expansion at one point, whereas GFVRI- amide caused the somules to demonstrate minimum expansion and contraction. This result corresponds with the data published by McVeigh *et al*. in 2011 [80]

On the other hand, somules treated with 100μM of YIRF-amide demonstrated the maximum track distance i.e. these schistosomules covered the maximum distance from one point to the second point. The somules treated with 100μM of GFVRI-amide demonstrated the least track distance i.e. the schistosomules covered minimum distance between two

points. These results introduced another novel hypothesis i.e. contractile activity of worms was inversely proportional to directional motility of the worms. In other words, the more a parasite exhibits activity at one point, less is the distance it can travel. To prove this hypothesis, we tested somules with increasing concentrations of 5-HT and it was observed that centroid distance of the somules increased whereas the track distance decreased. This conclusively proves that contractile activity was inversely proportional to directional motility. 5-HT is widely distributed throughout the central and peripheral nervous system, and plays an important role in worm motility, glucose uptake, carbohydrate metabolism to produce energy and directly affect the body wall muscles of the worm [77]. It was proposed that the hyperactivity of the 5HT treated somules caused a depletion in the energy stores, and this lack of energy prevented the somule from performing other activities, like migration. So, although the viability of the somule is dependent upon 5-HT, exposure to higher concentrations of 5-HT would destroy the somule.

In order to improve the microfluidic chip, it was of great importance to choose a suitable media, through which the schistosomules could move ahead easily, with minimum resistance. Of all the different types of media tested, we believe that collagen was the ideal media to use in microfluidic assays.

Although this microfluidic assay seems promising for future research, it is not a perfect device, and requires improvement. The addition of a chemoattractant at the end of the channel might be a good idea to test for future experiments, so that the parasites have incentive to move forward and not get stuck at one place. Here, we present a novel assay to analysis and quantify the movement of *S. mansoni* somules through microfluidic chips. We hope that our method will potentially help researchers to study the parasitic motility and its

role in the spread of the disease, assisting scientists in developing better and effective drugs therapy.

2.6. Bibliography

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CHAPTER 3. A QUANTITATIVE AND QUALITATIVE PROTEOMIC ANALYSIS OF *SCHISTOSOMA MANSONI* CERCARIAL TAIL PROTEINS

Modified from a paper to be submitted to PLoS Pathogens

Sreemoyee Acharya[1,3,4], Gunnar R. Mair[1], Tim A. Day[1,4]

3.1 Abstract

Schistosomiasis is caused by the blood fluke *Schistosoma mansoni* and is responsible for infecting 200 million patients worldwide; it is a key neglected tropical parasitic disease (NTD) [4, 12, 71, 95, 96] . An infection is initiated when the larvae, known as the cercariae, are released from its intermediary host, a *Biomphalaria* freshwater snail, into the surrounding water. Cercariae are free swimming, highly motile forms with specialized bifurcated tails that approach the host tail-first and penetrate the mammalian skin thus infecting the human host [27, 95, 138]. Following attachment and during invasion, the cercaria sheds its tail and the resulting somule continues to develop inside the circulatory system of the host body [32]. Cercarial tail motility plays an essential role in host localization and survival of the parasite outside the mammalian body, yet the proteins supporting this prolonged and vigorous movement are poorly understood. In this study, we have extracted and identified the proteome of isolated cercarial tails and bodies using mechanical transformation of cercariae into somules. Using mass spectrometric analyses, we identified a total of 945 proteins in the combined cercarial proteome from four independent samples: 791 proteins accumulated in the cercarial tails and 645 proteins were identified from the somule bodies. By determining the function and localization of specific proteins in the cercarial body, we hope to propose possible therapeutic mechanisms via which host skin invasion by these parasites can be avoided.

[1] Department of Biomedical Sciences, Iowa State University, Ames, IA, USA

[3] Primary authors

[4] Author for correspondence

3.2 General Introduction

3.2.1. Life Cycle of Schistosomes

Schistosomiasis is a major neglected tropical diseases (NTDs), only second to malaria in the list of devastating parasitic diseases. According to the World Health Organization (WHO) and the Centers of Disease Control (CDC), more than one billion people are affected by NTDs in at least 149 countries [1-4]. The disease can be either acute or chronic [4] .The three species belonging to the genus *Schistosoma* capable of infecting humans are *Schistosoma mansoni* and *Schistosoma japonicum* that can cause intestinal schistosomiasis and *Schistosoma. haematobium* that causes urogenital schistosomiasis [4, 12, 96]. As per reports filed by WHO schistosomiasis is very prevalent in tropical and subtropical countries, in poor communities with no access to safe drinking water, limited scope for education, food insecurity and poor sanitation [3, 6, 139] . People who are involved in routine agricultural or domestic occupations and school-aged children are especially prone to infection, since they are constantly in contact with contaminated water.

The eggs of the parasites hatch in freshwater, releasing free-living, non-feeding miracidia which then proceed to infect an intermediate snail host [4, 22] . Each species completes the asexual phase of the life cycle in the first host e.g. *S. mansoni* and *S. haematobium* develop inside the aquatic freshwater *Bioamphalaria* and *Bulinus* snails, whereas *S. japonicum* needs a freshwater *Oncomelania* species of snails [4]. The cercariae matures and develops into multicellular sporocysts, which then transform to produce

thousands of free swimming cercariae [6]. The approximate length of a cercaria is between 300-500 μm and the width are about 70 μm [27, 28]. Upon release from the snail, the highly infectious cercariae seeks to penetrate a mammalian host within a time span of 2 days or 48 hours because they rely on the glycogen stored in their bodies to for swimming [3, 4, 23, 30-32]. During the process, they shed their bifurcated tails and develop into schistosomules [4, 8, 13, 22]. The somules course through the host circulatory system to the liver, where they settle within the superior mesenteric venules, and transform into adult male and female worms [4, 8, 13, 22]. The adult male and female mate and the female worm produces around 100-300 eggs per day [4]. Some of the eggs are secreted via urine or stool into freshwater, whereas others accumulate in the host tissues and organs where they can lead to inflammation, resulting in the spread of the disease [4].

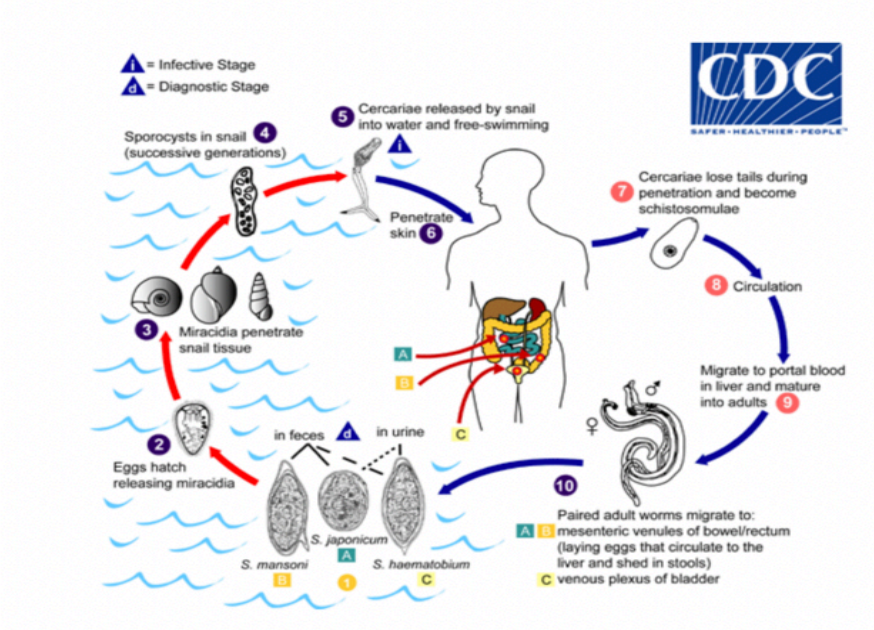

Figure number 3.2.A :- The different stages of the life cycle of schistosomes. 1. Eggs are eliminated from the. patient, via feces or urine. **2**. Under optimal conditions, the eggs hatch and release miracidia. **3.** The ciliated, free- living miracidia swims and penetrates an intermediate invertebrate snail host. **4**. Within the snail host, the miracidia develops into 2 generations of sporocysts –mother sporocysts and daughter sporocysts which ultimately develop into the cercariae. **5**. The cercariae is released from the snail, marking the most infectious stage in the life cycle. **6**. The cercariae penetrates the skin of the human host. **7**. Once inside the human host, the cercariae sheds its tail and becomes schistosomules. **8.** The schistosomules travel through the circulatory system. **9.** They migrate and settle in their final residence- the liver. S.mansoni is found often in the superior mesenteric veins which drain the large intestine. **10 ABC**. The schistosomules mature and develop into male and female adult worms. The paired worms mate and the female worms lay eggs in the small venules of the portal system and the perivesicle system. The eggs then gradually move towards the lumen of the intestine, and are eliminated via excretory products. However, a large number of eggs accumulate in the liver, leading to the formation of granulomas, which leads to the onset of schistosomiasis.

Source of the image :- https://www.cdc.gov/parasites/schistosomiasis/biology.html

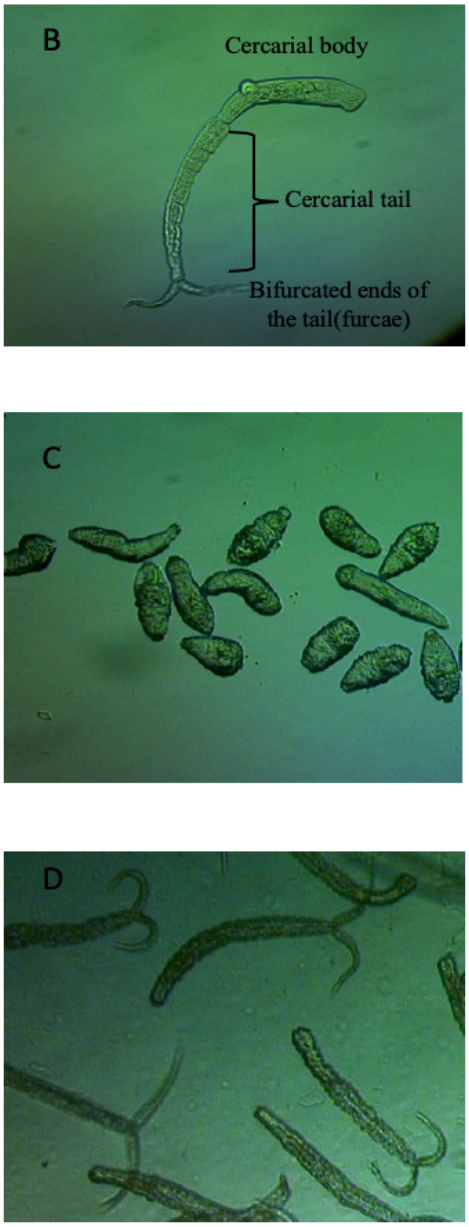

Figure number 3.2.B :- *Schistosoma mansoni* cercaria. Figure number 3.2.C :- *Schistosoma mansoni* schistosomules. Figure number 3.2.D :- *Schistosoma mansoni* cercarial tails B. *S. mansoni* cercaria was taken immediately after it was freshly shed from an infected host *Bioamphalaria glabrata* snail. The structure of the cercaria can be broadly divided into the upper head and the lower tail. The tail extends to form bifurcated ends, which help the highly infectious cercaria to swim and survive in the fresh water, until it can locate a mammalian host like a human being, to infect and penetrate. Once inside the host body, the tail is discarded and the cercarial body transforms into a schistosomula, which then continues on its journey through the host circulatory system. **C.** shows the *S.mansoni* bodies, or somules, after the cercarial tails were separated using a vortex and percoll gradient centrifugation. The somules will travel through the circulatory system of the human host towards the superior mesenteric veins which drain the large intestine, after which they will develop into adult male and female worms, and lay eggs. **D.** the discarded *S.mansoni* cercarial tails, which were separated from the main body using a vortex and percoll gradient centrifugation. All images were taken using a Nikon Eclipse TS 100 microscope with a 10X objective lens, attached to a 5.0 megapixel Nikon ED camera.

3.2.2. The Application of Proteomics in Parasitology

Proteomics is the large-scale experimental analysis of proteins of a cell line, tissue or organism, and it allows an in-depth study of cellular systems e.g. the phenotype of cells, biological mechanisms, protein modifications, protein localization and compartmentalization, protein-protein interactions and the novel drug targets [94]. With improvements in mass

spectrometry, the development of databases and the creation of new computer algorithms, there have been significant advances in proteomic interpretation and protein expression profiling [94]. The genomes of *S. japonicum* and *S. mansoni* as well as the genome of the *Bioamphalaria glabrata* have been sequences and analyzed, thus assisting in better understanding of parasite biology [22, 66, 95, 140]. A large transcriptome database containing resources for peptide sequences for *S. mansoni* was published in 2003 [141]. The proteomic analysis of different subset protein groups belonging to the interaction between the snail and the parasite has also been established, providing exclusive protein information of immature eggs [142, 143], secretions from the egg [144, 145] as well as its contents [142, 146], the proteins involved in the excretory and secretory activities of the miracidia [147] and the proteins from the sporocysts [148, 149]. From the egg secretome, chaperone proteins such as heat shock proteins (HSP) as well as proteins involved in redox balance, protein folding [145] were identified [146] and from the hatch fluid defense proteins were isolated [142]. Cytoskeletal proteins and proteases were observed from the immature eggs and the mature egg contents [142, 144]. From the miracidium to sporocyst stage, 127 proteins were identified such as those involved in larval transformation, protease inhibitors, chaperone proteins, antioxidant enzymes, ion binding proteins and venom allergen like proteins [142]. Novel biomarkers such as transferrin, α-1-antitrypsin and glutathione-S-transferase were identified in the serum of mice infected with *Schistosoma mansoni* parasites [150].

3.2.3. The Significance of *S. mansoni* Cercarial Motility in Spreading Schistosomiasis

The *S.mansoni* cercariae is highly mobile and infectious in fresh water for approximately 2 days or 48 hours [151]. Fluorescein isothiocyanate (FITC) labeled phalloidin staining of whole cercariae combined with confocal scanning laser microscopy

revealed the presence of diagonal muscles, longitudinal muscles and circular muscles in the cercarial body; striated muscles in the tail and longitudinal as well as circular muscles in the furcae [27] .

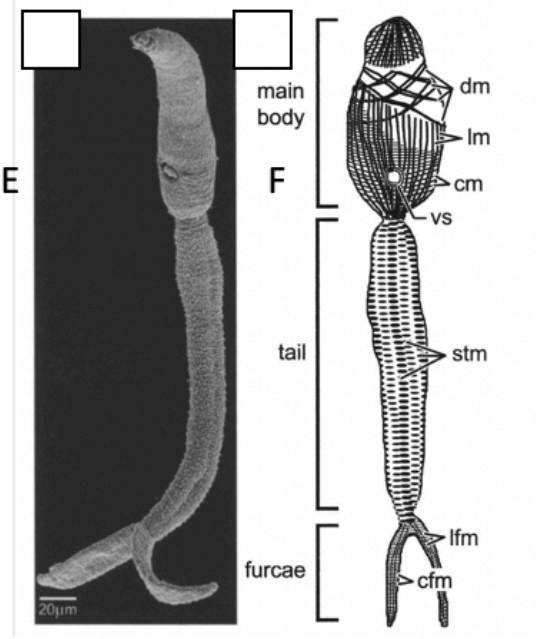

Figure number 3.2.E.F.:- Scanning electron micrograph of a *Schistosoma mansoni* cercaria. E. Scanning electron micrograph of a *S.mansoni* cercaria (scale as indicated). **F.** Schematic representation of its gross muscle fiber organization. Cfm, circular muscle fibers; dm, diagonal muscle fibers; lfm, longitudinal muscle fibers in the furcae; lm, longitudinal muscle fibers; stm, striated tail musculature; vs, ventral sucker. Reproduced with permission from G.R.Mair, A.G. Maule, B.Fried, T.A.Day and D.W. Halton (2003) Organization of the Musculature of Schistosome Cercariae. The Journal of Parasitology, vol 89, No 3, pp. 623-625.

Cercarial motility drives the rate of cercarial infectivity towards human skin thus dividing the movement into two phases (1) an active phase where the cercariae swim upwards and (2) a passive phase where the cercariae uses its forked tail as a drag anchor to sink down to the bottom of the fresh water body [152-154] . The swimming motion of the cercariae was first analyzed in 1967 by high speed photography [155]. Very recently, using a novel theoretical model and robotic structure designed by Dr. Manu Prakash's group at Stanford University, the swimming behavior of cercariae was divided into three modes (1) a tail-first mode where the cercarial tail fork is fully extended and the cercariae moves against gravity (2) a free-sinking method where the cercarial tail fork is partially extended, making the parasite negatively buoyant and (3) a head-first method where the cercarial tail fork is

folded back meant for host penetration [152]. The cercarial tails are found to contain more glycogen as compared to the cercarial bodies, and the rate of glycogen use is significantly higher in the tail than in the body, which explains the aggressive motility of the tails [151]. In addition, the very active motility of cercariae depends on a number of environmental factors such as gravity, light, temperature and water turbulence as well as chemical gradients of skin and blood compounds of the human host [154, 156, 157]. These factors compel the cercariae to accumulate in the upper water layer which where they can come in contact with human hosts [152, 158, 159] . Once the cercariae penetrates into the human skin, the secretion of the acetabular glands occur in response to the host cues and chemical host signals, thus allowing the parasite to penetrate into the [158, 159].

With the rise in the parasite resistance against commonly used drugs, there is an urgent need to identify novel chemotherapeutic targets. Since the cercarial tail is essential in identifying and penetrating the human host, we decided to concentrate on the cercarial proteins. Here, we present a completed proteomic analysis of the *S. mansoni* cercariae along with a comparison of the proteomic data between the parasitic bodies and the tails. We have also identified several proteins significantly abundant in the *S. mansoni* cercarial tail which have never been explored before and might possibly be potential drug targets.

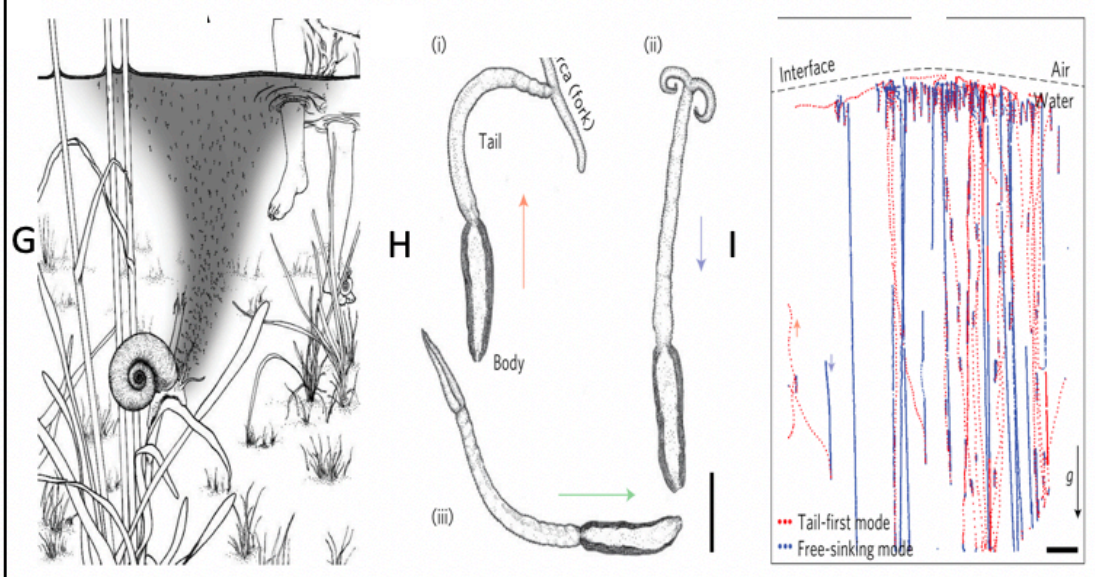

Figure number 3.2.G.H.I. :- Macroscale swimming behavior of *Schistosoma mansoni* cercariae. **G.** Artistic rendering (not to scale) of a typical transmission site where cercariae (immature, human infectious stage of the parasites) emerge into freshwater from snails and aggregate near the water surface. **H.** Artistic rendering of the three distinct modes displayed by cercariae in their intermittent swimming behavior. (i) Tail-first mode (ii) free-sinking mode and (iii) body-first mode. The arrows indicate the direction of motion. Scale bar, 100 µm. **I.** Computationally tracked snapshot of cercariae swimming in a vertical chamber showing tail-first swimming (red) and sinking (blue) trajectories for 10 minutes. The resulting tracks of organisms have a strikingly columnar nature with very little drift in the horizontal direction. This is due to the fact that cercariae are bottom heavy swimmers and undergo a gravitational realignment such that their longitudinal axis is aligned with the vertical with the body pointing down. Scale bar, 1 mm. Reproduced with permission from Krishnamurthy D., Katsikis G., Bhargava A. and Prakash M (2016). *Schistosoma mansoni* cercariae swim efficiently by exploiting an elastohydrodynamic coupling. Nature Physics Vol.13 pages : 266-271

3.3. Material and Methods

3.3.1. Experimental Animals

S. mansoni infected *B. glabrata* snails were provided by the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract HHSN272201000005I for distribution through BEI Resources. The snails were maintained at 26°C-28°C in charcoal-filtered, aerated water and fed fresh lettuce on a regular basis.

3.3.2. Cercarial Shedding Of Snails

Approximately 30-40 *S. mansoni* infected *B. glabrata* snails were placed in a 250 ml glass beaker containing 40-50 ml of charcoal-filtered, aerated water (enough to cover the snails). Cercarial shedding was induced by exposing the snails to strong light for up to 2 hours. The water from each beaker was filtered through a glass funnel (*VWR catalog number # 89428-960*) containing a 47-μM pore metal screen (*VWR catalog number # 89428-966*). The collected cercariae were transferred into a 50 ml. falcon tube and kept on ice causing the cercariae to settle at the bottom of the tube. Most of the supernatant was discarded, leaving 5-10 ml. at the bottom of the tube.

3.3.3. Transformation Of Cercariae To Schistosomules

In order to remove the tails from the cercarial bodies, the 5-10 ml. of cercarial solution was vortexed vigorously for 1 minute and then kept on ice for 1 minute. This cycle was repeated for 6-7 times. To check whether the tails had separated successfully from the cercarial bodies, 50 μl. of the sample was observed under a light microscope.
Tails were separated from bodies by Percoll density gradient centrifugation. 40 ml. of a 60% Percoll solution were prepared from 24. ml Percoll (*Fisher Scientific catalog number #45001754*), 4 ml. of 10X MEM Vitamin solution (*Life Technologies catalog number #11430030*), 1 ml. of 85% sodium chloride in 1M HEPES, 1.5 ml. of 100X antibacterial-antimycotic solution (*Thermo Fisher Scientific catalog no #15240062*) and 9.5 ml. of molecular grade water. The vortexed cercarial solution containing separated bodies and tails was layered on top of the Percoll solution, taking care that the solution was not disturbed in any way. It was then centrifuged at 900g for 45 minutes at 4°C separating the tails from the cercarial bodies.

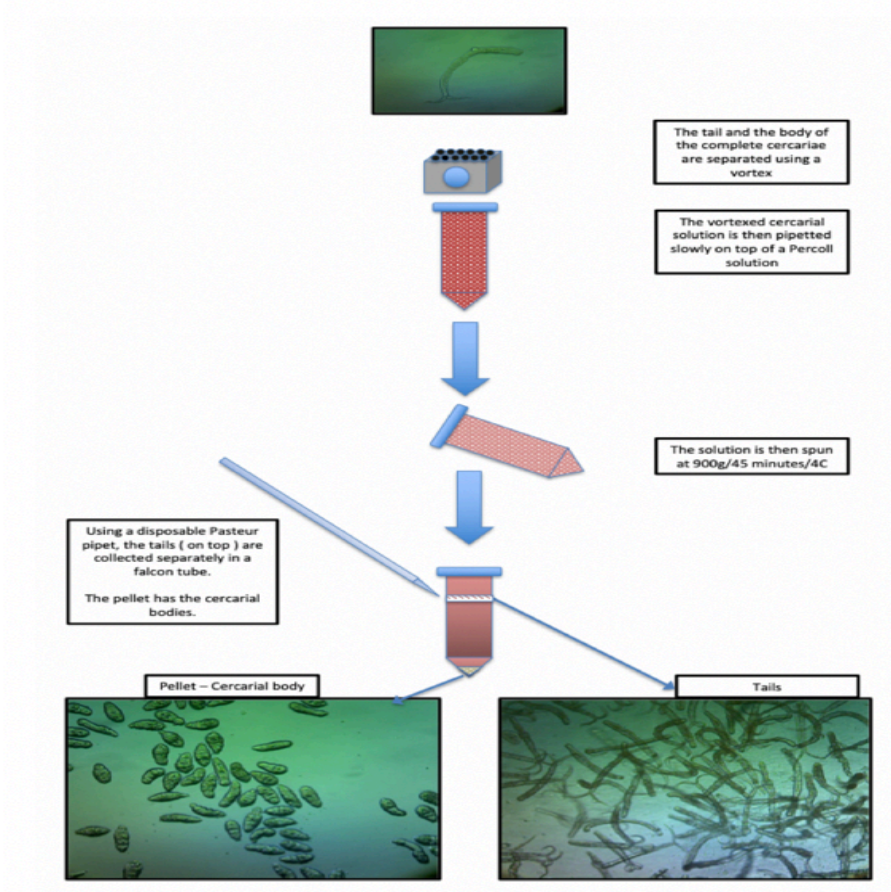

Figure number 3.3.A : - Diagrammatical representation of the process of cercarial shedding, separation and collection of *S. mansoni* somules and tails. Once exposed to bright light, the infected *B. glabrata* snails shed the larval parasites of *S. mansoni*, which are known as cercariae. The cercariae possesses an upper body and an active forked tail, which allows it to move through water to locate and infect a mammalian host. The bodies and the tails were separated using a vortex. The vortexed cercarial solution was then pipetted slowly on top of a percoll solution in a 50 ml falcon tube. The solution was then spun at 900g for 45 minutes at 4°C. The centrifugation resulted in the tails floating at the top of the percoll solution in the tube, and the bodies were saved as pellets at the bottom of the tube. The tails were collected using a disposable glass Pasteur pipette and the bodies were collected from the pellet. Both samples were washed with DPBS and prepared for further experiments.

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3.3.4. Collection Of Cercarial Tails And Cercarial Bodies

Post centrifugation, the tails were collected from the top of the percoll solution, whereas the cercarial bodies (somules) were observed as a thick pellet at the bottom of a tube. Using a disposable Pasteur pipet (*VWR catalog #14672-380*), the tails were transferred

carefully from the top of the solution into a new 50 ml. falcon tube. The tails and the cercarial bodies were washed with sterile Dulbecco's phosphate buffered solution (DPBS) (*Thermo Fisher Scientific catalog #14190250*) and centrifuged at 500g. for 20 minutes at 4°C. The pellets from each sample—tails and cercarial bodies—were dissolved in 0.5 ml of DPBS.

3.3.5. Protein Extraction

Three independent cercarial sheddings were conducted on alternate days and the three tail samples were pooled into one tube. Similarly, three cercarial body samples were pooled into another tube. The tails and bodies (approximately 15,000-20,000 as counted using a light microscope) were lysed using a *Fisher Scientific 60 Sonic Dismembrator* with an output power at 100-Watt, frequency set at 22.5 KHz (*Fisher Scientific 60 Sonic Dismembrator catalog # F60*]. Each cycle was lysed for 1 minute, then kept on ice for 1 minute. In total there 4-5 cycles of lysing were conducted overall. The samples were centrifuged at 13,200 RPM for 30 minutes at 4°C and the supernatant was collected as the crude extract. The pellet containing the remnants and debris was discarded. The concentration of protein in each tube was analyzed using the Bradford test, and the samples were submitted for mass spectrometry. 3 repeats were performed for each individual body part of the cercariae (body n=3, tail n=3).

3.3.6. Mass-spectrometric Proteomic Data Of Cercarial Tails And Cercarial Bodies. Crude protein extracts were digested in solution with trypsin/Lys-C. After digestion, PRTC standard (*Pierce part #88320*) was spiked into the sample to serve as an internal control. The peptides were then separated by liquid chromatography (*HPLC: Thermo Scientific EASY nLC-1200 coupled to a Thermo Scientific Nano spray FlexIon source; Column: Pulled glass emitter 75um X 20 cm (Agilent capillary, part #160-2644-5),* packed with *Agilent SB-C18*

Zorbax 5um packing material (part #820966-922). Buffer A is composed of *0.1*% formic acid/water and buffer B is composed of 0.1% formic acid/acetonitrile. The samples were analyzed by MS/MS (*Thermo Scientific Q Exactive Hybrid Quadrupole-Obrbitrap Mass Spectrometer with an HCD fragmentation cell*) by fragmenting each peptide. The resulting intact and fragmentation pattern was compared to a theoretical fragmentation pattern (from either MASCOT or Sequest HT) (Proteome Discoverer 2.1; Mascot against database of interest; Sequest HT against the PRTC database.) to find peptides that could be used to identify the proteins. The peak-area based quantification used precursor ions to assess the relative abundance of the identified peptides. The relative abundance of the identified proteins was based on the areas of the top three unique peptides for the identified protein. The PRTC areas were used to normalize the data between samples. Each sample had the same amount of PRTC spiked in.

3.3.7. Analysis of The Proteomic Data

The analysis of the proteomic data obtained from the mass spectrometry facility was conducted in the following way. We identified each peptide hit using a genome database for eukaryotic and prokaryotic pathogens www.genedb.org. This data base provides information on the type of RNA of the peptide hit, the potential position, the length, the polypeptide domain, the product of the gene, the translation sequence as well as the FASTA files. The other database which was used for analysis was www.uniprot.org, which is a protein database. The accession number of each peptide hit was entered into each database, and based on the information, we were able to conduct gene ontology studies for each peptide hit, based on 3 parameters -biological function, molecular function and subcellular location. The gene ontology analysis (molecular function, biological function and subcellular location) of

each peptide hit was graphically represented based on abundance in the sample, using MS Excel.

3.4. Results and Discussion

3.4.1. Mass-spectrometric Analyses Of Schistosome Tail and Body samples

The data, specifically the gene accession numbers, obtained from the mass spectrometric analysis of the *S. mansoni* cercarial body and tail samples were analyzed using the parasitic genome database (www.gene.db) and it was found that there were 791 peptide hits in the cercarial tail and 645 peptide hits in the cercarial body. The cercarial tails and the cercarial bodies shared 491 proteins.

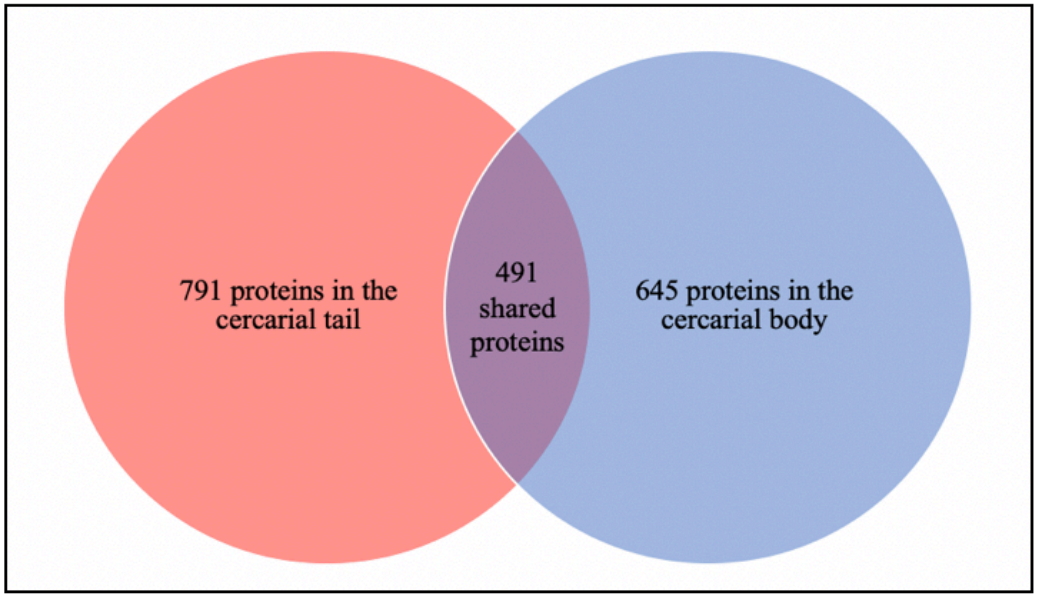

Figure no 3.4.A: -Venn Diagram showing the overlap of the peptide hits in S.mansoni cercarial tails and *S. mansoni* cercarial bodies. The red circle indicates the 791 peptide hits in the cercarial tail samples (n=3) and the blue section indicates the 645 peptide hits in the body sample (n=3). 491 proteins are shared between the cercarial tails and cercarial bodies.

3.4.2. Results Of The Analysis Of The Proteomic Data Using Gene Ontology

Using GO analysis, the peptide hits were divided into 3 main categories – molecular function i.e. the activities of the gene product at the molecular level; biological function i.e. a set of molecular events pertaining to the functioning of the specific gene product and finally cellular location i.e. the approximate location of the gene in the parts of the cell, both intercellular and extracellular spaces. Based on the analysis, *S. mansoni* cercarial tail peptide hits had 368 molecular functions, 324 biological functions and 134 subcellular locations. The cercarial body had 315 molecular functions, 270 biological functions and 128 subcellular locations.

In case of the *S. mansoni* cercarial tails (Figure number 3.4.B.), the top ten molecular functions were ATP binding, metal ion binding, calcium ion binding, GTPase binding, RNA binding, GTP binding, unfolded protein binding, actin binding, DNA binding, and magnesium ion binding.

For the same sample (Figure number 3.4.C), the top ten biological functions were ubiquitin dependent protein catabolic process, signal transduction, tricarboxylic acid cycle, carbohydrate metabolic process, protein folding, cell redox homeostasis, cilium assembly, positive regulation of ATP dependent microtubule motor activity, plus end directed, proteasome mediated ubiquitin dependent protein catabolic process and transport along microtubule.

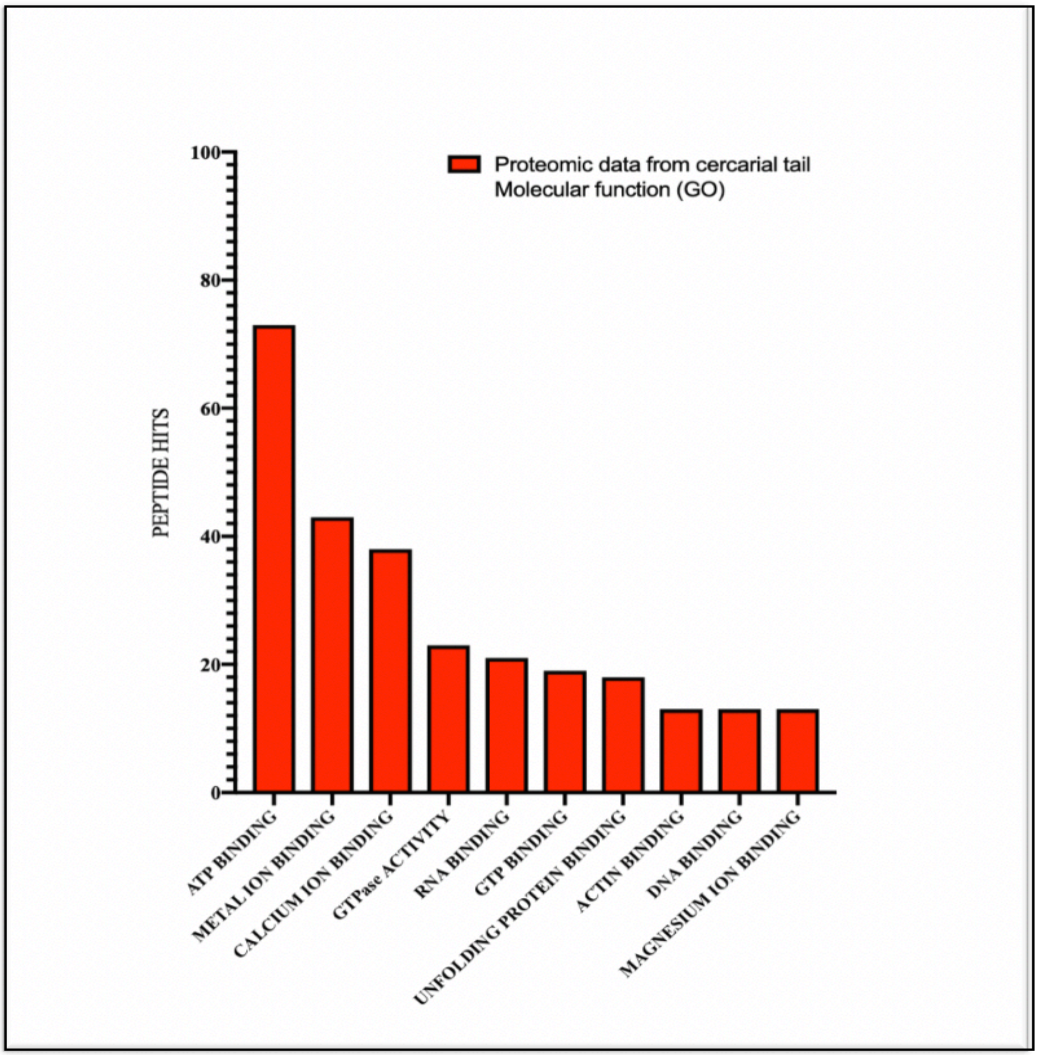

Figure no 3.4.B.: Graphical representation of the gene ontology *of* *S.mansoni* cercarial tails – molecular function. The highest expressed molecular functions are ATP binding, metal ion binding, calcium ion binding, GTPase activity, RNA binding, GTP binding, unfolded protein binding, actin binding, DNA binding and magnesium ion binding.

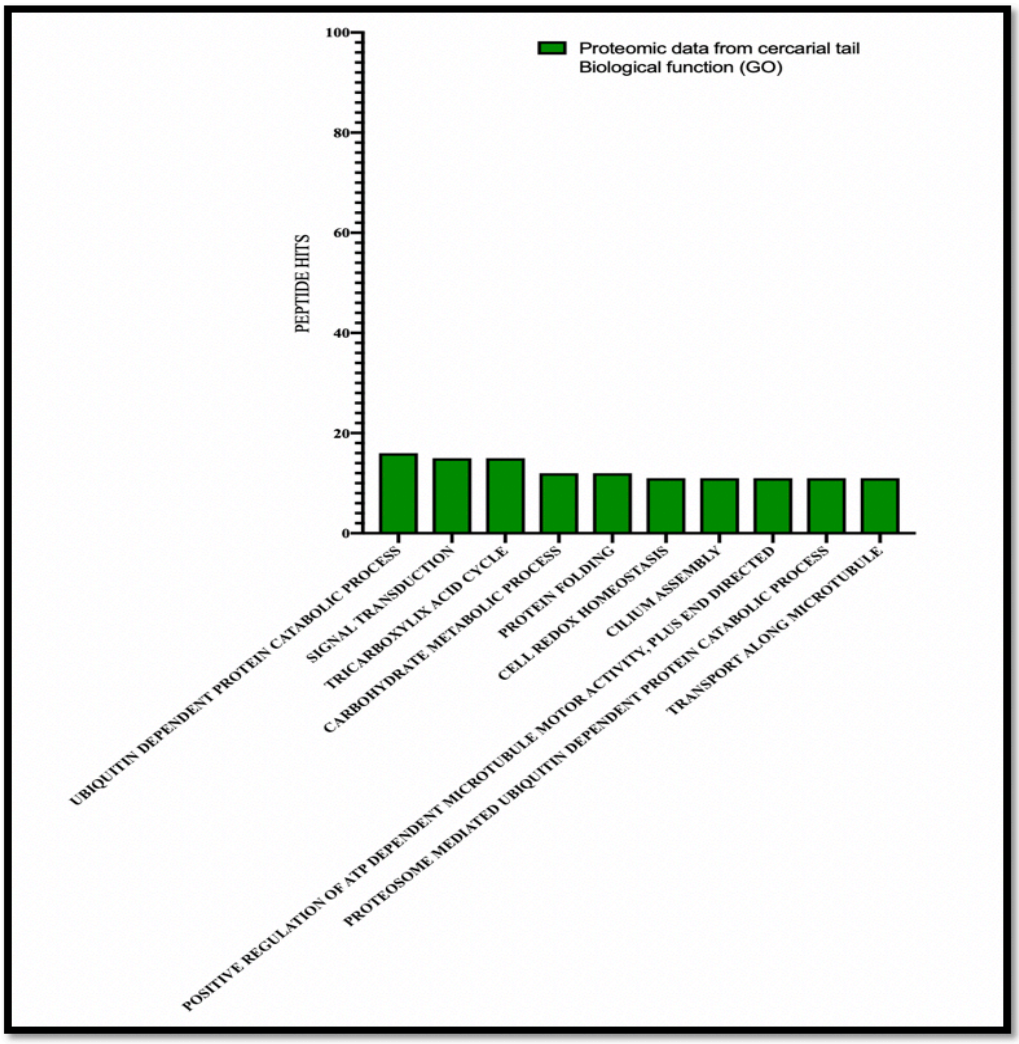

Figure no 3.4.C.: Graphical representation of the gene ontology *of* *S.mansoni* cercarial tails – based on biological function. The highest expressed biological functions are ubiquitin dependent protein catabolic process, signal transduction, tricarboxylic acid cycle, carbohydrate metabolic process, protein folding, cell redox homeostasis, cilium assembly, positive regulation of ATP dependent microtubule motor activity, plus end directed, proteasome mediated ubiquitin dependent protein catabolic process and transport along microtubule.

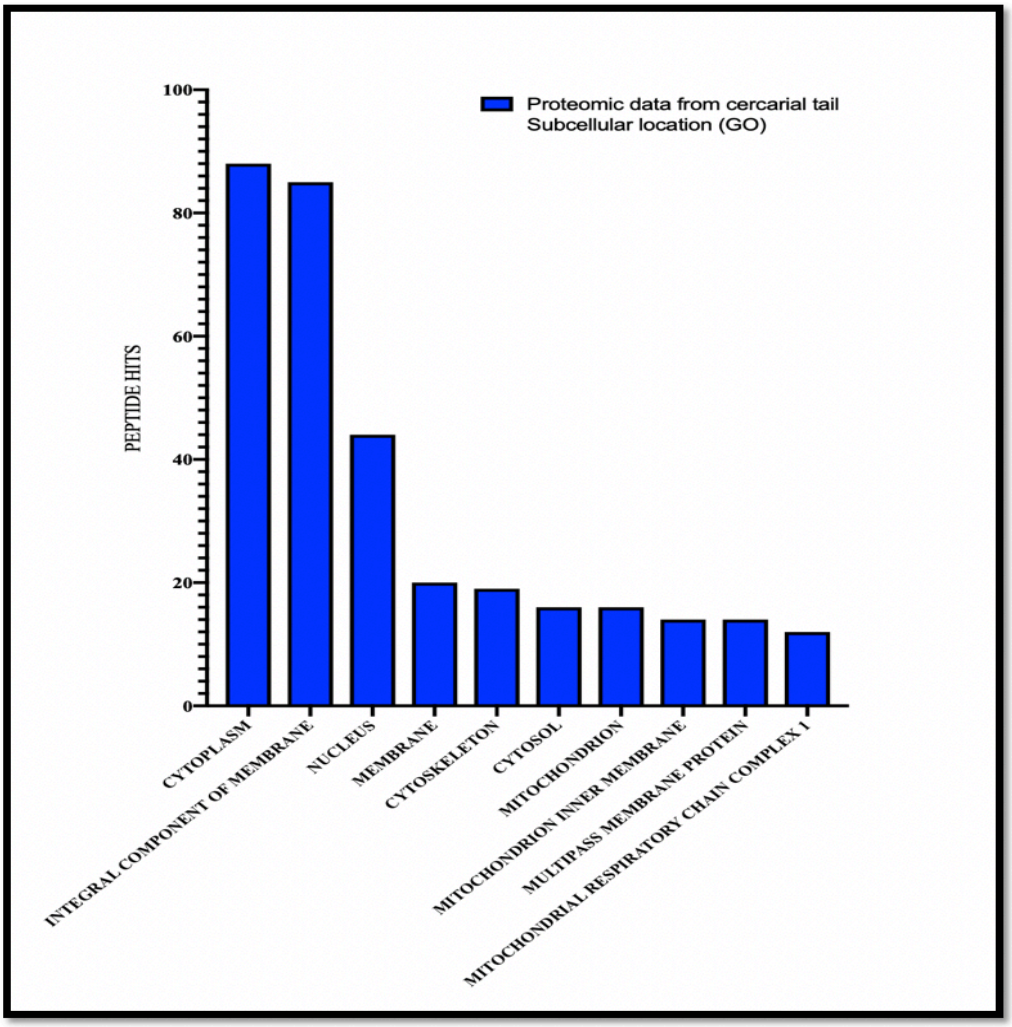

Figure no 3.4.D: Graphical representation of the gene ontology of *S.mansoni* cercarial tails based on subcellular function. The proteins are maximally located in the cytoplasm, integral component of membrane, nucleus, membrane, cytoskeleton, cytosol, mitochondrion, mitochondrion inner membrane, multi pass membrane protein and mitochondrial respiratory chain complex 1.

The top ten subcellular locations (Figure number 3.4.D), for the peptide hits to be abundantly present are cytoplasm, integral component of membrane, nucleus, membrane, cytoskeleton, cytosol, mitochondrion, mitochondrial inner membrane, multipass membrane protein and mitochondrial respiratory chain complex 1.

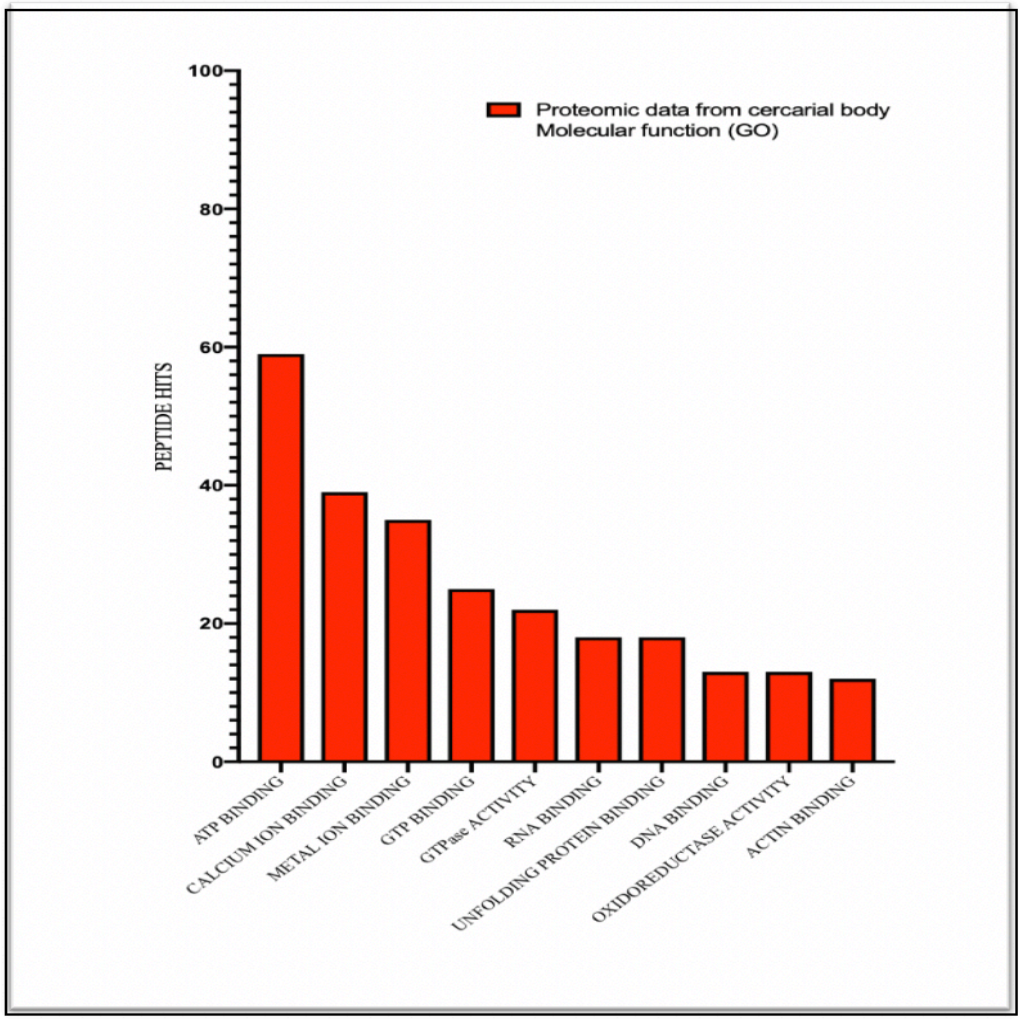

Figure no 3.4.E: Graphical representation of the gene ontology of *S.mansoni* cercarial bodies – based on molecular functions. The highest expressed molecular functions are ATP binding, calcium ion binding, metal ion binding, GTP binding, GTPase binding, RNA binding, unfolded protein binding, actin binding, DNA binding, oxidoreductase activity and actin binding.

In case of the *S. mansoni* cercarial bodies (Figure number 3.4.E) , the top ten molecular functions were ATP binding calcium ion binding, metal ion binding GTP binding GTPase binding activity RNA binding unfolded protein binding DNA binding oxidoreductase activity and actin binding

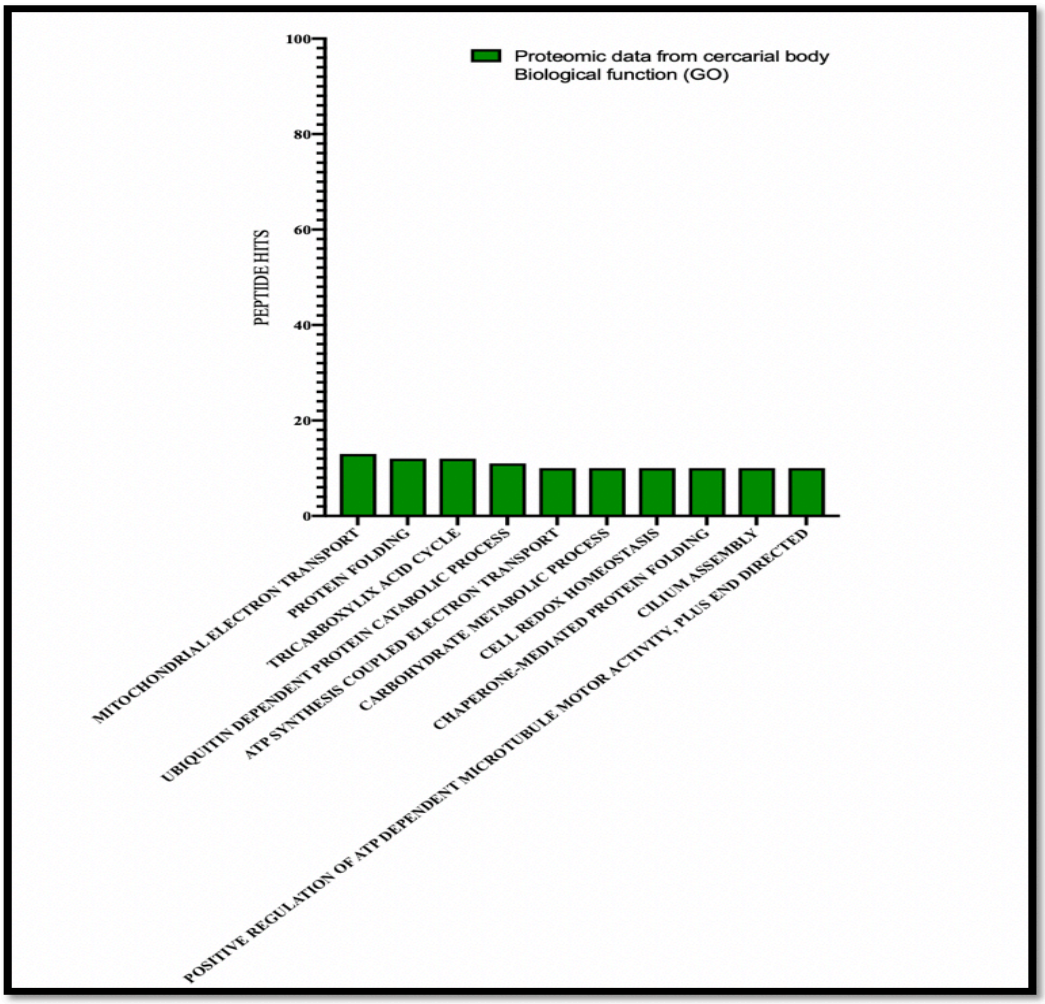

Figure no 3.4.F: Graphical representation of the gene ontology of *S.mansoni* cercarial bodies – based on biological function. The highest expressed biological functions are mitochondrial electron binding, protein folding, tricarboxylate acid cycle, ubiquitin dependent protein catabolic process, ATP synthesis coupled electron transport, carbohydrate metabolic process, cell redox homeostasis, chaperone mediated protein folding, cilium assembly and positive regulation of ATP dependent microtubule motor activity, plus end directed.

For the same sample (Figure number 3.4.F), the top ten biological functions were mitochondrial electron transport protein finding tricarboxylic acid cycle, ubiquitin dependent protein catabolic process, ATP synthesis coupled electron transport, carbohydrate metabolic process, cell redox homeostasis, chaperone mediated protein

folding, cilium assembly and positive regulation of ATP dependent microtubule motor activity, plus end directed.

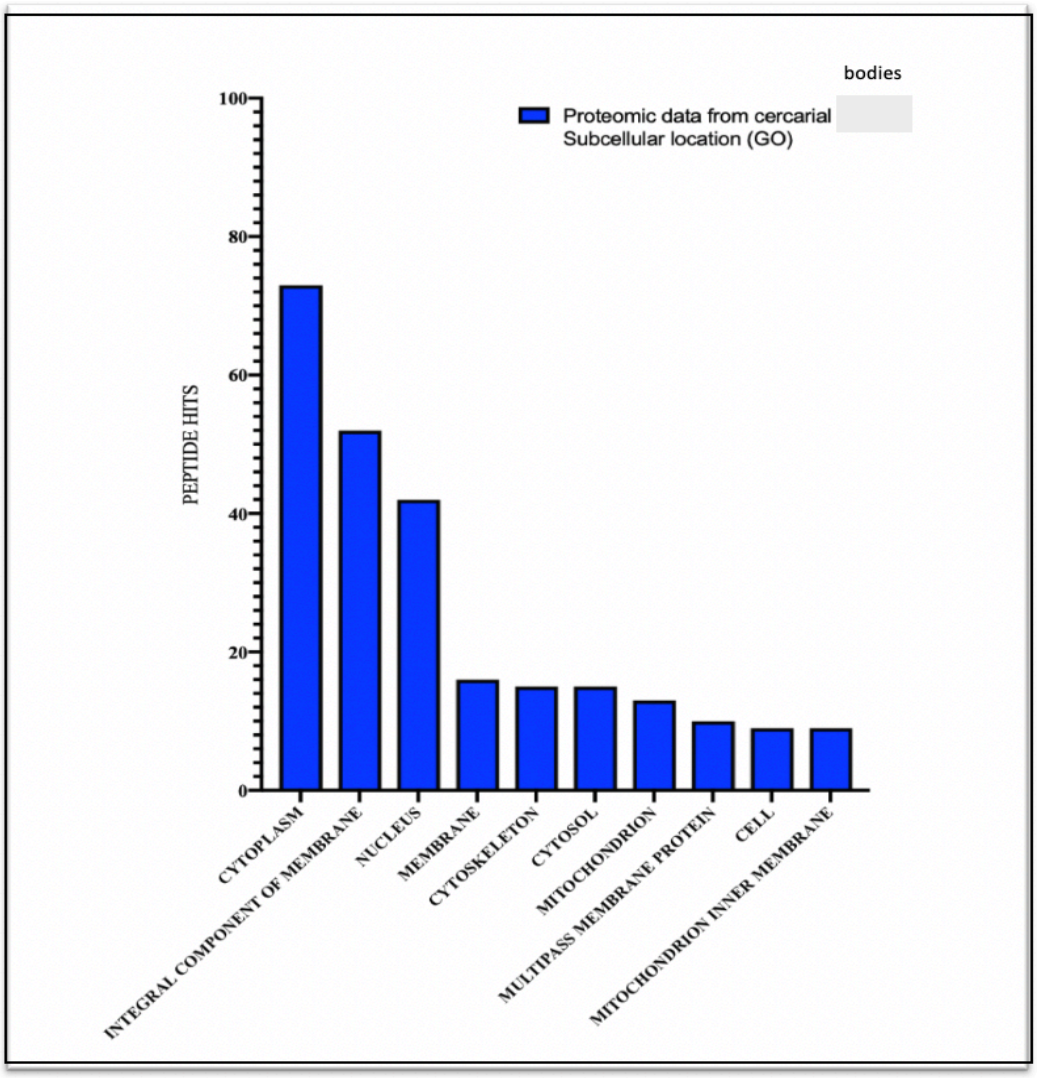

Figure no 3.4.G: Graphical representation of the gene ontology of *S.mansoni* cercarial bodies – based on subcellular locations. The proteins are maximally located in the cytoplasm, integral component of membrane, nucleus, membrane, cytoskeleton, cytosol, mitochondrion, mitochondrion inner membrane, cell and mitochondrial inner membrane.

From the figure number 3.4.G, the top ten subcellular locations for the peptide hits to be abundantly present are the cytoplasm , integral component of the membrane , nucleus ,

membrane , cytoskeleton , cytosol , mitochondria , multi pass membrane protein cell and mitochondrion inner membrane .

ATP binding cassettes or ABCs are transport proteins which belong to a large family of membrane proteins, that interact selectively and non-covalently with ATP, and many of the functions include the transportation of diverse compounds such as peptides, metabolites, hormones, signaling molecules, immunomodulators, cholesterol, iron, drugs, toxins etc, [160-162]. Some of the proteins characterized in schistosomes include SmDR2 and Sm MRP1. [160-162]. SmDR2 has been previously established as a schistosomal homolog of P-glycoprotein, the expression of which is upregulated in response to praziquantel concentration [161]. Similarly, SmMRP1, a schistosomal homolog of mammalian MRP1, was found to be upregulated in adult worms, when exposed to low concentrations of praziquantel [161] One of the theories behind the increasing praziquantel resistance in schistosomes may be due to the fact that the drug can bind to P-glycoprotein (a subset of ABC proteins) and these multidrug resistance (MDR) associated proteins can be flushed out of the parasitic body [160-162]. In fact, schistosomal worm isolates from Egypt which demonstrated praziquantel resistance, displayed a very high expression of SmDR2 protein [161]. According to our data analysis, the number of peptide hits for ATP binding proteins in the cercarial tails is higher than that present in the cercarial bodies. Both SmDR2 and SmMRP1 could be potential targets for future drug design and therapy.

Table number 3.1. Table representing the comparison of the proteomic data from the peptide hits from the *S. mansoni* cercarial bodies versus the peptide hits from the *S.mansoni* cercarial tails, using Gene Ontology.

CERCARIAL BODIES

Molecular Function	ATP Binding (59 Hits)	Calcium Binding (39 Hits)	Metal Ion Binding (35 Hits)	GTP Binding (25 Hits)	GTPase Binding Activity (22 Hits)
Biological Function	Mitochondrial Electron Transport (13 Hits)	Protein Folding (12 Hits)	TCA Cycle (12 Hits)	UBQ Dependent Protein Catabolic Function (11 Hits)	ATP Synthesis Coupled Electron Transport (10 Hits)
Subcellular Location	Cytoplasm (73 Hits)	Integral Component Of The Membrane (52 Hits)	Nucleus (42 Hits)	Membrane (16 Hits)	Cytoskeleton (15 Hits)

CERCARIAL TAILS

Molecular Function	ATP Binding (73 Hits)	Metal Ion Binding (43 Hits)	Calcium Binding (38 Hits)	GTPase Activity (23 Hits)	RNA Binding (21 Hits)
Biological Function	UBQ Dependent Protein Catabolic Function (16 Hits)	Signal Transduction (15 Hits)	TCA Cycle (15 Hits)	Carbohydrate Metabolism (12 Hits)	Protein Finding (12 Hits)
Subcellular Location	Cytoplasm (88 Hits)	Integral Component Of The Membrane (85 Hits)	Nucleus (44 Hits)	Membrane (20 Hits)	Cytoskeleton (19 Hits)

Calcium binding proteins or CaBPs are vital in the growth, development and maintenance of organisms. From our proteomic analysis, CaBPs are abundantly distributed in both the cercarial tails and the cercarial bodies. Based on literature review, a number of these proteins have been characterized. In 1990, Sm20 was identified as a 20-kilodalton functional calcium binding protein, expressed in *S.mansoni* schistosomules tegumental membranes and adult worms [163]. In 1992, SmE16 was characterized as a 16-kilodalton calcium binding protein expressed in the *S.mansoni* eggs [164]. In 2002, SmTCTP (translationally controlled tumor protein) was isolated as a 23-kilodalton protein from all stages of the *S.mansoni* life cycle, the homologs of which had been previously found in *Brugia malayi* and *Wuchereira bancrofti* [165]. SmTCTP can bind to the heme portion of the host hemoglobin, as well as tubulin molecules, causing a release of histamine and secretion of interleukin-4 from basophil/mast cells, resulting in the development of allergic inflammation associated with schistosomiasis [165]. A proteomic analysis of *S.mansoni* cercarial secretions was conducted in 2005 and a number of proteins were identified, many of which were found to be responsible for the cercarial infection [95]. Some of the proteins were characterized as those involved in calcium binding, immune evasion (paramyosin), degradation of host skin barriers (proteases) and cytosolic proteins (heat shock proteins) [95]. In 2015, an 8-kilodalton intracellular calcium binding protein named SjCa8 was identified in *S.japonicum* cercariae and early stage schistosomules [166].This protein was found to have a significant inhibitory effect on the migration of macrophages, and potentially prevented the release of nitric oxide (NO) from the cells, thus allowing the somules from evading the host immune system, however the mode of action of SjCa8 is not completely known [166]. It is highly

probable that a homolog of this protein would be present in *S.mansoni* however, it has not been identified as of yet. In 2016, a family of calcium binding proteins were discovered in *S.mansoni* and these SmTAL proteins (tegumental allergen like proteins) were found to interact with praziquantel, however the exact mechanism of action is unknown [167]. Due to their abundant distribution, and their role in parasitic activity, calcium binding proteins can be considered as potential targets for the design of promising chemotherapeutic drugs.

Metal binding proteins are distributed throughout the *S.mansoni* cercarial bodies, with more peptide hits identified in the cercarial tails (43%) compared to the cercarial bodies (35%). One example of a schistosomal metal binding protein which has been successfully characterized, is SmPCS or *S.mansoni* phytochelatins synthase (PCS), belonging to the papain family of cysteine proteases [67].This enzyme cleaves glutathione to release oligopeptides known as phytochelatins, which can scavenge and sequester toxic heavy metals leading to metallic detoxification [67]. SmPCS is found in plants, cyanobacteria, algae, ferns, fungi, nematodes but are absent in human[67]. The ability of SmPCS in regulating the availability of metal ions, thus contributing towards pathogen survival, makes it a potential target for designing antiparasitic drugs [67]. Another example of a metal ion binding protein is SmCREB-binding protein (cAMP response element binding protein), which can activate gene transcription by interacting selectively and noncovalently with zinc ions. Based on their function, metal binding proteins are vital for the development and maintenance of the schistosomal life cycle.

The fourth most abundant peptide in the *S.mansoni* cercarial body was identified as a low molecular weight Rab related GTP binding protein (SmRab), using proteomic

data analysis [168]. Once activated, these proteins are essential for membrane trafficking, vesicle formation, membrane fusion and vesicle movement along actin, tubulin networks [168]. RNA binding proteins such as Y-box binding proteins or YBPs have also been isolated and characterized in schistosomes [169]. Located within the cytoplasm of parasitic cells, SmYB1 is responsible for gene expression, translational regulation, transcriptional regulation, DNA repair, cellular proliferation, drug resistance ,stress response, mRNA processing and post translational gene regulation. [169]. All these above-mentioned proteins can be considered as potential targets for designing and manufacturing antischistosomal chemotherapeutic drugs.

In the *S.mansoni* cercarial bodies, the top biological functions identified from the proteomic data analysis were mitochondrial electron transport, protein folding, tricarboxylic acid (TCA cycle), ubiquitin dependent protein catabolic process and ATP synthesis coupled electron transport. In the cercarial tails the top five biological functions were ubiquitin dependent protein catabolic process, signal transduction, TCA cycle, carbohydrate metabolism and finally protein folding. One of the top biological functions of the peptide hits in both cercarial bodies and cercarial tails is the tricarboxylic acid (TCA cycle). As mentioned before, the cercarial stage of *S.mansoni* relies on its glycogen store for its supply of energy. Once the glycogen store is exhausted, the parasite is unable to survive due to lack of glycolytic activity. The process in which glycogen is converted to glucose is defined as glycogenolysis, in which glycogen is cleaved by glycogen phosphorylase to provide glucose -1- phosphate, which in turn is converted to glucose-6-phosphate by phosphoglucomutase. The glucose-6-phosphate undergoes glycolysis to provide energy in the form of ATP, and this fuels the movement of the muscular cells of

the cercariae. The pyruvate is produced as a by-product of glycolysis, which is oxidized during the tricarboxylic acid cycle into carbon dioxide and water. The total number of ATP molecules produced during glycolysis is 8, and in the TCA cycle, is 24. These ATP molecules provide energy which in turn can make the cercariae hyperactive. The number of peptide hits involved in the TCA cycle are higher in the cercarial tails (15 hits) as compared to the cercarial bodies (12 hits), which explains the very high level of movement in the tails. The number of peptide hits involved in the ubiquitin dependent protein catabolic process were found to be higher in *S.mansoni* cercarial tails (16 hits) as compared to cercarial bodies (11 hits). This biological process is responsible for the breakdown of a protein or a peptide by hydrolysis of the peptide bonds, caused by the covalent attachment of a ubiquitin group to the protein. The other top biological functions present in the cercarial tails were signal transduction conducted by protein kinases, and carbohydrate metabolism. The biological functions can explain the hyperactivity of the cercariae, which in turn assist in identifying, infecting and penetrating the vertebrate host. In addition, since glycogenolysis and glycolysis occur in the cytoplasm of the cell, it makes sense that the top subcellular location for the peptide hits in both cercarial bodies and cercarial tails is the cytoplasm.

3.5. Conclusion

Schistosomiasis is a life threatening neglected tropical disease caused by schistosomes , second only to malaria responsible for the loss of 1.53 million disability-adjusted life years (DALYs) as well as 280,000 deaths annually in sub-Saharan Africa alone [14-16]. According to reports published by the World Health Organization (WHO), 220.8 million people required preventive treatment in 2017, out of which more than 102.3 million

people were reported to be treated. Presently, praziquantel is the only drug which is used to treat patients, however in recent years, there has been a gradual increase in parasitic drug resistance [67]. Although a lot of scientific research is being carried out all over the world, with the sole aim of eliminating the disease, there is still a long way to go.

The *S.mansoni* parasitic eggs are released in the excretory system by an infected patient, which then hatch in fresh water to form miracidia. The miracidia infect the first invertebrate vector—*B. glabrata* snails, within which they develop and are shed as highly motile cercariae—the most active and infectious stage of the parasitic life cycle. The expansion and the contraction of the parasitic muscular system contributes towards its movement e.g. the diagonal muscles, longitudinal muscles and circular muscles in the cercarial body; striated muscles in the tail and longitudinal as well as circular muscles in the furcae [27] . Other factors include gravity, light, temperature, water turbulence and chemoattractant gradients of skin and blood of the host [154, 156, 157]. Once the cercariae penetrates into the human skin, the secretion of the acetabular glands occur in response to the host cues and chemical host signals, thus allowing the parasite to penetrate into the host skin [158, 159, 166]. Post infection host, the cercariae loses its tails and transforms into schistosomules which then proceed to migrate through the body's lungs and portal circulatory system [166]. The somules develop into adult worms in the hepatic portal system, mate and lay eggs, thus leading to the symptoms of the disease in the patient. Since the cercarial tail plays a significant role in identifying and penetrating a potential mammalian host, this paper attempts to present a qualitative and quantitative proteomic analysis of cercarial tail proteins in detail, with the hope of identifying specific proteins as potential targets for designing chemotherapeutic drugs.

The world of proteomics allows large-scale experimental investigation of biological material, specifically focusing on the proteins as well as an conducting an in-depth study of cellular systems [94]. The popularity and advances in this field is largely due to improvements in mass spectrometry, the development of databases and the creation of new computer algorithms [94]. In fact, mass spectrometry is an indispensable tool for this field of study. In case of schistosomiasis, the genomes of *S. japonicum*, *S. mansoni* and that of the intermediate snail host, *Bioamphalaria glabrata*, have been extensively documented [22, 66, 95, 140]. A vast transcriptome database containing resources for peptide sequences for *S. mansoni* was published in 2003, thus providing clues for research in parasite cell biology [141]. In 2005, a proteomic analysis of cercarial secretions was carried out and a number of proteins were identified, which were found to be responsible for the spread of the disease [95]. Some of the proteins were calcium associated proteins (e.g. calcium binding, calcium regulation, calcium activation), immune evasion (e.g. paramyosin), degradation of the host skin barrier (e.g. proteases) and heat shock proteins (e.g. cytosolic proteins, heat shock proteins) [95].For our paper, we carried out a mass spectrometric analysis of the proteins isolated and purified from the cercarial tails as well as the cercarial bodies. Using the parasitic genome database (www.gene.db) it revealed the presence of 791 peptide hits in the cercarial tail and 645 peptide hits in the cercarial body, with an overlap of 491 proteins. With the help of gene ontology (GO) analysis, the peptide hits were divided into three groups – those based on molecular function , those based on biological function and finally those based on cellular location. Based on the analysis, *S. mansoni* cercarial tail peptide hits had 368 molecular functions, 324 biological functions and 134 subcellular

locations. In comparison, the peptide hits in the he cercarial body had 315 molecular functions, 270 biological functions and 128 subcellular locations.

For *S. mansoni* cercarial tails, the top molecular functions were ATP binding, metal ion binding, calcium ion binding, GTPase binding, RNA binding, GTP binding, unfolded protein binding, actin binding, DNA binding, and magnesium ion binding. In case of the *S. mansoni* cercarial bodies, the top ten molecular functions were ATP binding calcium ion binding, metal ion binding GTP binding GTPase binding activity RNA binding unfolded protein binding DNA binding oxidoreductase activity and actin binding . In cercarial tails, the top ten biological functions were ubiquitin dependent protein catabolic process, signal transduction, tricarboxylic acid cycle, carbohydrate metabolic process, protein folding, cell redox homeostasis, cilium assembly, positive regulation of ATP dependent microtubule motor activity, plus end directed, proteasome mediated ubiquitin dependent protein catabolic process and transport along microtubule. On the other hand, for cercarial bodies, the top ten biological functions were mitochondrial electron transport protein finding tricarboxylic acid cycle, ubiquitin dependent protein catabolic process, ATP synthesis coupled electron transport, carbohydrate metabolic process, cell redox homeostasis, chaperone mediated protein folding, cilium assembly and positive regulation of ATP dependent microtubule motor activity, plus end directed. The fact that more ATP binding peptide hits were identified in the cercarial tails than in the bodies is significant , because this result can explain the energy involved in the hyperactivity of the tails. The top biological functions of the peptide hits in both cercarial bodies and cercarial tails is the tricarboxylic acid (TCA cycle), followed by ubiquitin dependent protein catabolic process, and protein folding.

The entire responsibility of localizing and penetrating a mammalian host is undertaken by the cercarial tails. The free-living, non-feeding cercariae swim tail-first, depending on its glycogen source for bursts of energy. The parasites continue to seek a suitable vertebrate mammalian host, until the cercarial glycogen source is completely exhausted. The process in which glycogen is broken down to produce glucose is known as glycogenolysis. Since cercariae are incapable of carrying out glycolysis on their own, the depleting of glycogen ends up killing the parasite. These particular proteins can be considered as potential chemotherapeutic drug targets, for designing future antischistosomal agents. Other ATP binding proteins could include specific transport proteins such as SmDR2 and SmMRP1 that interact with ATP, and some of the functions include the transportation of peptides, metabolites, hormones, signaling molecules, immunomodulators, cholesterol, iron, drugs, toxins etc, [160-162].

In the cercarial tails, the peptide hits are abundantly distributed in cytoplasm, integral component of membrane, nucleus, membrane, cytoskeleton, cytosol, mitochondrion, mitochondrial inner membrane, multipass membrane protein and mitochondrial respiratory chain complex 1 whereas in cercarial bodies, the peptide hits are in the cytoplasm , integral component of the membrane, nucleus , membrane , cytoskeleton , cytosol , mitochondria , multi pass membrane protein , cell and mitochondrion inner membrane . Since glycogenolysis and glycolysis occur in the cytoplasm of the cell, it makes sense that the top subcellular location for the peptide hits in both cercarial bodies and cercarial tails is the cytoplasm.

Based on gene ontology analysis, calcium binding proteins or CaBPs, metal binding proteins and GTP binding proteins are always distributed throughout the cercarial

system, and they play essential roles in the overall development and growth of the parasite. These peptide hits could be studied further as possible targets for manufacturing antiparasitic drugs. If the movement of cercariae can be prevented or slowed down, this could stop them from infecting mammalian hosts, thus decreasing the spread of schistosomiasis.

Additional analysis of the proteomic data was conducted by our colleagues at the Genome Informatics Facility (GIF) of Iowa State University, using a software called MetaboAnalyst 4.0 to process metabolomic data, provide visualization of data, statistical analysis and functional interpretation [170-172]. NCBI-Gene IDs for all the proteins that were significant (P value <0.1) were mapped to the Kyoto Encyclopedia of Genes and Genomes or KEGG pathway, which is an integrated comprehensive database resource developed for the biological interpretation of large-scale datasets as well as the molecular functions stored as a functional ortholog of genes and proteins. (Data not shown).

In conclusion, we have attempted to present a completed qualitative and quantitative proteomic analysis as well as a thorough comparison of the peptide hits in the *S. mansoni* cercarial tail versus those in the cercarial bodies.

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CHAPTER 4 : A MINIATURIZED SCREEN OF A *SCHISTOSOMA MANSONI* SEROTONERGIC G PROTEIN-COUPLED RECEPTOR IDENTIFIES NOVEL CLASSES OF PARASITE-SELECTIVE INHIBITORS

Modified from a manuscript published in PLoS Pathogens (2016).

John D. Chan [1], John D. McCorvy [2], Sreemoyee Acharya [3], Malcolm E, Johns [1], Timothy A. Day [3], Bryan L. Roth [2,4,5], Jonathan S. Marchant [1,6.]

[1] Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota, USA.

[2] Department of Pharmacology, University of North Carolina at Chapel Hill, North Carolina, USA.

[3] Department of Biomedical Sciences, Iowa State University, Ames, Iowa, USA.

[4] Division of Chemical Biology and Medicinal Chemistry, Eshelmann School of Pharmacy, University of North Carolina at Chapel Hill, North Carolina, USA.

[5] National Health of Mental Health Psychoactive Drug Screening Program (NIMH PDSP), School of Medicine, University of North Carolina at Chapel Hill, North Carolina, USA.

[6] Stem Cell Institute, University of Minnesota, Minneapolis, Minnesota, USA.

4.1. Summary

Schistosomiasis is the second most dangerous neglected tropical disease, after malaria and is caused by water borne parasitic worms called schistosomes. *S.mansoni* is responsible for causing intestinal schistosomiasis, which is presently prevalent in more than 149 countries. According to a report filed by the World Health Organization (WHO) in 2016, schistosomiasis is responsible for approximately 200,000 deaths per year in sub-Saharan Africa alone. The lone drug responsible for treating patients is praziquantel, however with the recent reports of increasing drug resistance, it is highly important that more effective

drugs be designed and manufactured. New generation anti schistosomal drugs are difficult to create due to the lack of library screening of flatworm G-protein coupled receptors or GPCRs. This paper presents a novel, non-destructive and highly efficient method of pharmacological profiling of *S.mansoni* GPCRs. Serotonin or 5-hydroxy tyrosine (5-HT) is a ligand which binds to Sm5HTR , leading to receptor activation, causing an increase in the concentration of adenylyl cyclase enzyme, which in turn causes an increase in the concentration of cAMP. This increased cAMP can bind to a luciferase construct resulting in enhanced luminescence. A number of drugs were tested using this experiment and it was determined that selective inhibition of Sm5HTR was caused by alfuzosin, orphenadrine, atomoxetine and rotundine, of which the latter was found to be most sensitive. My contribution towards the paper was in the Materials and Methods section, where I isolated *Schistosoma mansoni* cercariae from infected *Bioamphalaria glabrata* snails. I transformed the cercariae into schistosomules, purified them and tested a set of chemical compounds of varying concentrations on them, to study their motility under the effect of the drugs. I also carried out the euthanization of the *Schistosoma mansoni* infected Swiss Webster mice, and assisted Dr. John Chan in isolating and purifying the adult worms from the mesenteric veins of the mice.

4.2. Materials and Methods

4.2.1. Experimental Animals

The NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (BRI, Rockville, MD) provided the *S. mansoni* infected *Biomphalaria glabrata* snails, through NIH-NIAID Contract HHSN272201000005I for distribution through BEI Resources (*Catalogue number #NR-21961)*. The infected snails were fed fresh lettuce leaves and

maintained at 26°C-28°C in charcoal-filtered, aerated water. Swiss Webster mice exposed to *Schistosoma mansoni* cercaria at 5-7 weeks old were obtained from BEI Resources (*Catalogue number # NR-34792*) and maintained at the laboratories animal resources (LAR) facilities at Iowa State University College of Veterinary Medicine. The protocol of animal work was approved by the LAR facility.

4.2.2. Cercarial Shedding of Snails

In a 250 ml. glass beaker containing 40 ml. of charcoal-filtered, aerated water, approximately 30-40 *S. mansoni* infected *B. glabrata* snails were placed. The water was enough to cover the snails. The cercariae were shed from the snails, by exposing the snails to a 120V 60W light bulb for up to 2 hours. The water from each beaker was filtered through a glass funnel (*VWR catalog number #89428-960*) containing a 47-uM pore sized metal screen (*VWR catalog number # 89428-966*). Once the cercariae were transferred to a pre-chilled 50 ml. falcon tube placed on ice, the cercariae were allowed to settle at the bottom of the falcon tube. The supernatant was discarded, and 5-10 ml. solution with the pellet was left at the bottom of the tube.

4.2.3. Transformation of Cercariae To Schistosomules, Separation and Collection of The Somules

In order to produce schistosomules the cercarial solution was vortexed 6-7 times for 1 minute followed by 1 minute on ice thus manually separating the tails from the cercarial bodies. Percoll density gradient centrifugation was then used to separate the tails from the cercarial bodies. 40 ml. of a 60% Percoll solution were prepared from 24 ml. percoll (*Fisher Scientific catalog number # 45001754*), 4 ml. of 10X MEM Vitamin solution (*Life Technologies catalog number #11430030*), 1 ml of 85% (weight/volume) sodium chloride in 1M HEPES, 1.5 ml. of 100X antibacterial-antimycotic solution (*Thermo Fisher Scientific catalog number #15240062*) and 9.5 ml. of molecular grade water. The vortexed cercarial

solution was pipetted on top of the percoll gradient solution. Centrifugation was carried out at 900g for 45 minutes at 4°CPost centrifugation, the tails accumulated at the top of the percoll were discarded, and the somules collected as a prominent pellet at the bottom of the tube. The somules were washed once with sterile Dulbecco's phosphate buffered solution (DPBS) (*Thermo Fisher Scientific catalog number #14190250*) and centrifuged at 20 minutes at 500g at 4°C. The recovered pellet containing the purified somules was resuspended in freshly made, sterile Basch media, and incubated at 37°C , 5% CO_2 overnight in an incubator.

4.2.4. Composition of Basch Media

The protocol for Basch media was provided by the Caffrey lab at the Skaggs School of Pharmacy and Pharmaceutical Science, University of California San Diego, USA. The media was made in the following way: - 9.23 gms. of MEM with Earl's Salt, without L-Glutamate, phenol red, sodium bicarbonate (*JRC catalog number # 56-119-000*) was dissolved in 1L of distilled water, followed by 1000 mgms. of glucose (*Fisher Scientific catalog number # D-16-3*) and 2400 mgms. of HEPES (*Fisher Scientific catalog number # 6003H*). 1000 milligrams of lactalbumin hydrolysate (*BRL Catalog number # 11800-025*) was added to the mixture and it was allowed to dissolve for 15-20 minutes on a magnetic shaker. Once dissolved, 2.2 gms of sodium bicarbonate (*Corning Cellgro catalog number # 90-009-PB*) was added to the media. 500 uls. of 1 mM hypoxanthine (*Sigma catalog number # H-9377*), 500 uls. of 1 mM hydrocortisone (*Sigma catalog number # H-0888*), 500 uls. of 1 mM triiodothyronine (*Sigma catalog number # T-5516*) and 900 uls. of bovine pancreas insulin (*Sigma catalog number # I-5500*) was added to the mixture, after which the pH was adjusted to 7.3-7.4. 50 uls. of 1X Schneider's media (*Invitrogen catalog number # 11720-*

034) and 5 ml of 100X MEM Vitamins (*Invitrogen catalog number # 11120-052*) were added and the pH was again calibrated to 7.3-7.4. The entire media was filter sterilized and to the sterile Basch media, 500 uls. of Penicillin-Streptomycin and 50 uls. of 5% re filtered, heat inactivated fetal bovine serum (*JRS catalog number # 43640-100*) was added. Aliquots of 50 ml were made and stored at 4°C.

4.2.5. Testing Drugs on Schistosomules In A 24 Welled Plate

Freshly isolated *S. mansoni* somules (approximately 200 somules per 500µls /well) were added to 6 welled cell culture, non-pyrogenic, polystyrene plates (*Corning Incorporated catalog number #3506*) containing Basch Media To the somules, a 1:1 dilution of the drugs (diluted in Basch media) of varying concentrations per well i.e. 0.1 uM, 1.0 uM, 5 uM, 10 uM, 50 uM and 100 uM, were added and were incubated for 30 minutes at 37°C, 5% CO_2. Motility of the somules were recorded for 5 minutes, at the rate of 10 somules per view through a 10X objective lens of a compound microscope (*Nikon Eclipse TS100*), equipped with a digital camera (*Zoom Nikkor ED Glass 5.0 Mega pixels 8X zoom*, *electric view finder*). Serotonin hydrochloride (*Sigma Aldrich catalog number # H9523*) was used as a positive myo-excitatory control as it can stimulate movement and carbamoyl choline chloride (*Sigma Aldrich catalog number # C4382*) was used as negative control, as it can inhibit movement. Both products were used at the following concentrations: - 0.1uM, 1uM, 5uM, 10uM and 100uM. The videos were uploaded to Iowa State University's Cybox, and they were analyzed by Dr. John Chan. The WrMTrck plugin of Image J was used to quantify the effect of the drug on the somule's movement.

4.2.6. Isolation of Adult Schistosoma mansoni Worms from Swiss Webster Mice

The mice exposed to *Schistosoma mansoni* cercaria were euthanized at 6-8 weeks post infection. The mice were administered CO_2 in a carbon dioxide chamber, and then sacrificed by cervical dislocation. The mice were perfused with 25mM of sodium citrate solution (*Fisher Scientific catalogue number S279-500*) and the adult worms were harvested from the mesenteric veins of the mice. The worms were incubated at 37°C, 5 % Co_2 overnight, and the motility of the adult worms were analyzed by Dr. John Chan.

CHAPTER 5 : PHARMACOLOGICAL PROFILING AN ABUNDANTLY EXPRESSED SCHISTOSOME SEROTONERGIC GPCR IDENTIFIES NUCIFERINE AS A POTENT ANTAGONIST

Modified from a manuscript published in International Journal for Parasitology : Drugs and Drug Resistance. (2016).

John D. Chan [a], Sreemoyee Acharya [b], Timothy A. Day [b], Jonathan S. Marchant [a,c.]

[a] Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota, 55455, USA.

[b] Department of Biomedical Sciences, Iowa State University, Ames, Iowa, 50011, USA.

[c] Stem Cell Institute, University of Minnesota, Minneapolis, Minnesota, 55455, USA.

5.1. Summary

Schistosomiasis is the second most dangerous neglected tropical disease, after malaria and is caused by water borne parasitic worms called schistosomes. *S.mansoni* is responsible for causing intestinal schistosomiasis, which is presently prevalent in more than 149 countries. According to a report filed by the World Health Organization (WHO) in 2016, schistosomiasis is responsible for approximately 200,000 deaths per year in sub-Saharan Africa alone. The lone drug responsible for treating patients is praziquantel, however with the recent reports of increasing drug resistance, it is highly important that more effective drugs be designed and manufactured. Potential anti schistosomal drugs were screened using a novel cAMP biosensor assay, and four structurally related aporphines – nuciferine, D-glaucine, boldine, bulbocalpine- were found to be highly effective in preventing motility in adult worms. Of the 4 drugs, nuciferine was found to be most effective. Like the previous published paper, my contribution towards this paper was in the Materials and Methods section, where I isolated *Schistosoma mansoni* cercariae from infected *Bioamphalaria*

glabrata snails. I transformed the cercariae into schistosomules, purified them and tested a set of chemical compounds of varying concentrations on them, to study their motility under the effect of the drugs. I also carried out the euthanization of the *Schistosoma mansoni* infected Swiss Webster mice, and assisted Dr. John Chan in isolating and purifying the adult worms from the mesenteric veins of the mice.

5.2. Materials and Methods

5.2.1. Experimental Animals

The NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (BRI, Rockville, MD) provided the *S. mansoni* infected *Biomphalaria glabrata* snails, through NIH-NIAID Contract HHSN272201000005I for distribution through BEI Resources (*Catalogue number #NR-21961)*. The infected snails were fed fresh lettuce leaves and maintained at 26°C-28°C in charcoal-filtered, aerated water. Swiss Webster mice exposed to *Schistosoma mansoni* cercaria at 5-7 weeks old were obtained from BEI Resources (*Catalogue number # NR-34792*) and maintained at the laboratories animal resources (LAR) facilities at Iowa State University College of Veterinary Medicine. The protocol of animal work was approved by the LAR facility.

5.2.2. Cercarial Shedding of Snails

In a 250 ml. glass beaker containing 40 ml. of charcoal-filtered, aerated water, approximately 30-40 *S. mansoni* infected *B. glabrata* snails were placed. The water was enough to cover the snails. The cercariae were shed from the snails, by exposing the snails to a 120V 60W light bulb for up to 2 hours. The water from each beaker was filtered through a glass funnel (*VWR catalog number #89428-960*) containing a 47-uM pore sized metal screen (*VWR catalog number # 89428-966*). Once the cercariae were transferred to a pre-chilled 50 ml. falcon tube placed on ice, the cercariae were allowed to settle at the bottom of the falcon

tube. The supernatant was discarded, and 5-10 ml. solution with the pellet was left at the bottom of the tube.

5.2.3. Transformation of Cercariae To Schistosomules, Separation and Collection of The Somules

In order to produce schistosomules the cercarial solution was vortexed 6-7 times for 1 minute followed by 1 minute on ice thus manually separating the tails from the cercarial bodies. Percoll density gradient centrifugation was then used to separate the tails from the cercarial bodies. 40 ml. of a 60% Percoll solution were prepared from 24 ml. percoll (*Fisher Scientific catalog number # 45001754*), 4 ml. of 10X MEM Vitamin solution (*Life Technologies catalog number #11430030*), 1 ml of 85% (weight/volume) sodium chloride in 1M HEPES, 1.5 ml. of 100X antibacterial-antimycotic solution (*Thermo Fisher Scientific catalog number #15240062*) and 9.5 ml. of molecular grade water. The vortexed cercarial solution was pipetted on top of the percoll gradient solution. Centrifugation was carried out at 900g for 45 minutes at 4°C.

Post centrifugation, the tails accumulated at the top of the percoll were discarded, and the somules collected as a prominent pellet at the bottom of the tube. The somules were washed once with sterile Dulbecco's phosphate buffered solution (DPBS) (*Thermo Fisher Scientific catalog number #14190250*) and centrifuged at 20 minutes at 500g at 4°C. The recovered pellet containing the purified somules was resuspended in freshly made, sterile Basch media, and incubated at 37°C , 5% CO_2 overnight in an incubator.

5.2.4. Composition Of Basch Media

The protocol for Basch media was provided by the Caffrey lab at the Skaggs School of Pharmacy and Pharmaceutical Science, University of California San Diego, USA. The media was made in the following way: - 9.23 gms. of MEM with Earl's Salt, without L-Glutamate, phenol red, sodium bicarbonate (*JRC catalog number # 56-119-000*) was dissolved in 1L of distilled water, followed by 1000 mgms. of glucose (*Fisher Scientific catalog number # D-16-3*) and 2400 mgms. of HEPES (*Fisher Scientific catalog number # 6003H*). 1000 milligrams of lactalbumin hydrolysate (*BRL Catalog number # 11800-025*) was added to the mixture and it was allowed to dissolve for 15-20 minutes on a magnetic shaker. Once dissolved, 2.2 gms of sodium bicarbonate (*Corning Cellgro catalog number # 90-009-PB*) was added to the media. 500 uls. of 1 mM hypoxanthine (*Sigma catalog number # H-9377*), 500 uls. of 1 mM hydrocortisone (*Sigma catalog number # H-0888*), 500 uls. of 1 mM triiodothyronine (*Sigma catalog number # T-5516*) and 900 uls. of bovine pancreas insulin (*Sigma catalog number # I-5500*) was added to the mixture, after which the pH was adjusted to 7.3-7.4. 50 uls. of 1X Schneider's media (*Invitrogen catalog number # 11720-034*) and 5 ml of 100X MEM Vitamins (*Invitrogen catalog number # 11120-052*) were added and the pH was again calibrated to 7.3-7.4. The entire media was filter sterilized and to the sterile Basch media, 500 uls. of Penicillin-Streptomycin and 50 uls. of 5% re filtered, heat inactivated fetal bovine serum (*JRS catalog number # 43640-100*) was added. Aliquots of 50 ml were made and stored at 4°C.

5.2.5. Testing Drugs on Schistosomules

In A 24 Welled PlateFreshly isolated *S. mansoni* somules (approximately 200 somules per 500µls /well) were added to 6 welled cell culture, non-pyrogenic, polystyrene

plates (*Corning Incorporated catalog number #3506*) containing Basch Media To the somules, a 1:1 dilution of the drugs (diluted in Basch media) of varying concentrations per well i.e. 0.1 uM, 1.0 uM, 5 uM, 10 uM, 50 uM and 100 uM, were added and were incubated for 30 minutes at 37°C, 5% CO_2. Motility of the somules were recorded for 5 minutes, at the rate of 10 somules per view through a 10X objective lens of a compound microscope (*Nikon Eclipse TS100*), equipped with a digital camera (*Zoom Nikkor ED Glass 5.0 Mega pixels 8X zoom*, *electric view finder*). Serotonin hydrochloride (*Sigma Aldrich catalog number # H9523*) was used as a positive myo-excitatory control as it can stimulate movement and carbamoyl choline chloride (*Sigma Aldrich catalog number # C4382*) was used as negative control, as it can inhibit movement. Both products were used at the following concentrations: - 0.1uM, 1uM, 5uM, 10uM and 100uM. The videos were uploaded to Iowa State University's Cybox, and they were analyzed by Dr. John Chan. The WrMTrck plugin of Image J was used to quantify the effect of the drug on the somule's movement.

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CHAPTER 6. PUBLICATIONS

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CHAPTER 7 : GENERAL CONCLUSION

Schistosomiasis is an endemic neglected tropical disease (NTD), second only to malaria as the most devastating parasitic disease in the world [8-11].According to the reports filed by World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC), more than one billion people, mostly from low-income and middle-income populations, are affected by NTDs in at least 149 countries [1-6]. The fresh water parasitic worm belonging to the phyla platyhelminthes, *Schistosoma mansoni* causes intestinal schistosomiasis, and is responsible for high morbidity and mortality rates [9]. The lone drug responsible for treating this disease is praziquantel, however due to several problems associated with the usage of the drug such as its effectiveness only against adult worms, as well as emerging reports of drug resistance, it is extremely important to look into more efficient chemotherapeutic agents to treat schistosomiasis [66].

The transmission of schistosomiasis largely depends on the motility of the parasite at different stages of its life cycle. When infected people expel schistosomal eggs into the fresh water through their excretory systems, the eggs hatch to produce ciliated and highly active miracidia. Once the miracidia penetrates the intermediate invertebrate snail host, it undergoes asexual reproduction to produce sporocysts, which eventually differentiates into free swimming, non-feeding, highly infectious larval forms called cercariae [22, 24, 173]. These highly mobile parasites survive in water for approximately 2 days or 48 hours, relying on its store of glycogen to provide energy [151]. In fact, the cercarial tails are found to possess more glycogen than cercarial bodies, and the glycogen undergoes glycogenolysis to provide glucose which then proceeds to produce ATP via glycolysis [151]. The main purpose of the cercariae it to seek out a suitable mammalian host to infect, thus bridging the gap

between the intermediate host and the definitive host [4, 28]. Once the human host is infected, the cercariae sheds its tail and transforms into schistosomules, which then travel through the circulatory system to the superior mesenteric channels, where they proceed to mature into adult worms [4]. The adult worms mate and produce a large quantity of eggs (100-300 eggs/day), most of which are excreted through the host's excretory system, and the remaining are permanently lodged within the liver and small intestine of the human body [4, 39, 96]. The pathology of the disease is caused by the response of the host's immune system to the eggs, and not just by the presence of the juvenile or adult parasitic worms in the body [4, 8, 13, 24, 40]. Schistosomiasis relies on the motility of the cercariae before human infection, and the movement of the schistosomules through the human body post-infection. For my doctoral dissertation, I have focused on the motility aspect of the *S.mansoni* worms pre and post infection, with the hope of identifying possible targets for designing potential chemotherapeutic agents, to prevent schistosomal movement, thus inhibiting the spread of the disease.

My research is broadly divided into two categories— the first part of my dissertation deals with the development of a sensitive, simple, cheap and easily accessible biological assay to study the movement of *S.mansoni* somules as they travel through the host circulatory system. In recent times, microfluidic devices or "Lab-on-a-chip" technology have become increasingly popular in biological applications such as electrophysiology, pharmaceutical analysis, screening of drugs, analysis of cells and cellular materials, separation and screening of biomolecules, diagnostics, and tissue engineering [92]. In fact, microfluidics has been used to successfully study the movement of *C. elegans* [93, 120]. This assay has many significant advantages such as the integration of an entire lab on a single small chip; reduced

chemical wastage, lowered costs, reduced reaction times, faster screening of novel compounds, simpler, quicker, portable, affordable manufacture as well as the ability to study several experiments simultaneously [96, 119, 137]. Here we have demonstrated specially manufactured microfluidic chips to study the movement and behavior of *S. mansoni* somules as they move through the sinusoidal channels designed on the chips, thus mimicking the host circulatory system. To quantitatively analyze somule motility, the Njus-Pandey research group at Iowa State University created a custom software program using two parameters (1) centroid distance to measure the activity of the somule at one point, and (2) track distance to measure the distance covered by the worm from one position to the other. Of the 11 chosen neuropeptides, 3 peptides were chosen to study the effect of each NP on somule motility. The 3 peptides were AFVRL-amide, GFVRI-amide and YIRF-amide.

With the first set of experiments conducted in 6 welled plates, it was observed that the motility of the somules increased with an increase in concentration of GFVRI-amide as well as AFVRL-amide and there was a substantial decrease in somule motility with an increase in the concentration of YIRF-amide. The results we obtained from the GFVRI-amide treated somules did not correspond with previously published data, which had stated that GFVRI-amide has an inhibitory effect on *S. mansoni* adults and somules [80]. In addition, the data we obtained from the YIRF-treated somules did not match with those published in 2011, which stated that the peptide had an inhibitory effect on the worms [80]. However our results did agree with those published earlier in 1997 by *Day et al*, which suggested that YIRF-amide had a myoexcitatory effect in somules [115].

In the second state of experiments, we introduced the drug treated somules to microfluidic channels, and the movement was recorded for 5 minutes. Using Dr. Njus' custom software program, the video recordings were analyzed, and it was confirmed that the schistosomules treated with 100μM of AFVRL-amide displayed maximum centroid distance and those treated with 100μM of GFVRI- amide displayed minimum centroid distance, when compared to the controls. In other words, AFVRL-amide caused the worms to undergo high levels of contraction and expansion at one point, whereas GFVRI- amide caused the somules to demonstrate minimum expansion and contraction. This result corresponds with the data published by McVeigh *et al*. in 2011 [80].

On the other hand, somules treated with 100μM of YIRF-amide demonstrated the maximum track distance i.e. these schistosomules covered the maximum distance from one point to the second point. The somules treated with 100μM of GFVRI-amide demonstrated the least track distance i.e. the schistosomules covered minimum distance between two points. These results introduced another novel hypothesis i.e. contractile activity of worms was inversely proportional to directional motility of the worms. In other words, the more a parasite moves at one point, less is the distance it can travel. To prove this hypothesis, we tested somules with increasing concentrations of 5-HT and it was observed that centroid distance of the somules increased whereas the track distance decreased. This conclusively proves that contractile activity was inversely proportional to directional motility. It was proposed that the hyperactivity of the 5-HT treated somules caused a depletion in the energy stores, and this lack of energy prevented the somule from performing other activities, like migration. So, although the viability of the somule is dependent upon 5-HT, exposure to higher concentrations of 5-HT would destroy the somule.

In order to improve the microfluidic chip, it was of great importance to choose a suitable media, through which the schistosomules could move ahead easily, with minimum resistance. Of all the different types of media tested, we believe that collagen was the ideal media to use in microfluidic assays. Although this microfluidic assay seems promising for future research, it is not a perfect device, and requires improvement. But we believe it holds potential for studying the movement of parasites with ease and without the expenditure of too much money.

The second part of my dissertation deals with the qualitative and quantitative proteomic analysis of the cercarial tails and cercarial bodies. The world of proteomics allows large-scale experimental analysis of proteins of a cell line, tissue or organism as well as an in-depth study of cellular systems [94]. The popularity and advances in this field is largely due to improvements in mass spectrometry, the development of databases and the creation of new computer algorithms [94]. For the field of schistosomiasis, the genomes of *S. japonicum*, *S. mansoni* and that of the intermediate snail host, *Bioamphalaria glabrata*, have been analyzed extensively and documented [22, 66, 95, 140]. A vast transcriptome database containing resources for peptide sequences for *S. mansoni* was published in 2003, thus providing clues for research in parasite cell biology [141]. Since the cercarial tail plays a significant role in identifying and penetrating a host, we aimed to investigate and analyze cercarial tail proteins in detail, with the hope of identifying specific proteins as potential targets for designing chemotherapeutic drugs.

There are several factors responsible for the hyperactivity of cercarial tails e.g. the presence of diagonal muscles, longitudinal muscles and circular muscles in the cercarial body; striated muscles in the tail and longitudinal as well as circular muscles in the furcae

[27] . Other factors include gravity, light, temperature, water turbulence and chemoattractant gradients of skin and blood of the host [154, 156, 157]. Once the cercariae penetrates into the human skin, the secretion of the acetabular glands occur in response to the host cues and chemical host signals, thus allowing the parasite to penetrate into the host skin [158, 159]. Once inside the host, the cercariae transform into schistosomules by the removal of the cercarial tails. The somules then travel through the portal system, pulmonary system and the hepatic portal system of the host body, eventually developing into adult worms and lay eggs [166]. The symptoms of the disease are a result of the accumulation of the parasitic eggs within the host's internal organs.

Mass spectrometric analysis of the proteins isolated and purified from the cercarial tails as well as the cercarial bodies, using the parasitic genome database (www.gene.db) revealed the presence of 791 peptide hits in the cercarial tail and 645 peptide hits in the cercarial body, with an overlap of 491 proteins. With the help of gene ontology (GO) analysis, the peptide hits were divided into three primary groups – those based on molecular function , those based on biological function and finally those based on cellular location. The peptide hits in the cercarial tail had 368 molecular functions, 324 biological functions and 134 subcellular locations, whereas those in the cercarial body had 315 molecular functions, 270 biological functions and 128 subcellular locations. For the cercarial tails the top molecular functions were ATP binding , metal ion binding and calcium ion binding ; the top biological functions were ubiquitin dependent protein catabolic process, signal transduction and tricarboxylic acid cycle and the top subcellular locations for the peptide hits were in the cytoplasm , the integral component of the membrane , and the nucleus. In the case of the cercarial bodies, the top molecular

functions were ATP binding, calcium ion binding and metal ion binding ; the top biological functions were mitochondrial electron transport, protein finding, and tricarboxylic acid cycle and the top ten subcellular locations for the peptide hits were in the cytoplasm , the integral component of the membrane and the nucleus. The molecular functions of the peptide hits in both the tails and the bodies i.e. ATP, metal and calcium binding—explained the aggressive activity of the cercariae pre-infection. In fact, the presence of more ATP binding peptide hits in the tail when compared to those present in the body is a significant result, because the tails are more active than the bodies especially since the entire responsibility of identifying and penetrating a mammalian host is undertaken by the cercarial tails.

One of the top biological functions of the peptide hits in both cercarial bodies and cercarial tails is the tricarboxylic acid (TCA cycle). As mentioned before the cercarial stage of *S.mansoni* relies on its glycogen store for its supply of energy. Once the glycogen store is exhausted, the parasite is unable to survive due to lack of any glycolytic activity. The process in which glycogen is converted to glucose is defined as glycogenolysis, in which glycogen is cleaved by glycogen phosphorylase to provide glucose -1- phosphate, which in turn is converted to glucose-6-phosphate by phosphoglucomutase. The glucose-6-phosphate undergoes glycolysis to provide energy in the form of ATP, and this fuels the movement of the muscular cells of the cercariae. The pyruvate is produced as a by-product of glycolysis, which is oxidized during the tricarboxylic acid cycle into carbon dioxide and water. The total number of ATP molecules produced during glycolysis is 8, and in the TCA cycle, is 24. These ATP molecules provide energy which in turn can make the cercariae hyperactive. Since

glycogenolysis and glycolysis occur in the cytoplasm of the cell, it makes sense that the top subcellular location for the peptide hits in both cercarial bodies and cercarial tails is the cytoplasm. In summary, we attempted to present a completed proteomic analysis and an extensive comparison of the *S. mansoni* cercarial tails and cercarial bodies.

Schistosomiasis is a debilitating disease which is responsible for the loss of 1.53 million disability-adjusted life years (DALYs) and up to 280,000 deaths annually in sub-Saharan Africa alone [14-16]. It affects people from all stages of life---from a child to a senior citizen. Female urogenital schistosomiasis caused by *S. haematobium* which could lead to cysts or even cervical cancer, is a field which requires a lot of attention, but sadly it is often neglected. According to the reports published by the World Health Organization (WHO), in 2017 alone, 220.8 million people required preventive treatment out of which more than 102.3 million people were reported to be treated. Although a lot of scientific research is being carried out all over the world, with the sole aim of eliminating the disease, there is still a long way to go. The lack of funding for research is one of the main culprits in this war against parasitic diseases.

With my dissertation, I would like to take a separate route for treating the parasites i.e. preventing its motility or paralyzing the worm at different stages of the life cycle. For my future career, I aim to investigate host-parasite interactions, possibly by selecting targets which could interrupt the life cycle of schistosomes, and thus hopefully reduce the chances of contracting the disease.