SCHISTOSOMA MANSONI, S. JAPONICUM: CHARACTERIZATION OF HOST ATTRACTION AND ATTACHMENT, WITH EVALUATION OF A NOVEL ENVIRONMENTAL SURVEILLANCE DEVICE

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DEDICATION

This dissertation is dedicated to my loving and supportive parents and brother, who have always encouraged me to reach higher, but to never lose sight of the ground.

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ABSTRACT

The goal of this study was to develop a passive, low-cost, low-technological, rapid surveillance method for detecting schistosome cercariae in schistosomiasis endemic regions, compared to other available methods. Schistosomiasis is a parasitic disease that is linked to abdominal pain, enlarged liver, and multiple organ failure, yet it is highly preventable as individuals are infected through dermal contact with contaminated waters. In areas where transmission mitigation efforts have been successful, a combination of control initiatives were employed, such as mass drug treatment, hygiene improvement, and monitoring of transmission foci. The environmental detection methods currently in use are labor- and time- intensive or cost-prohibitive in rural developing areas with high transmission rates and very limited resources.

Three aims were formulated in order to develop and evaluate the device. The first aim was to examine the chemotactic response of *Schistosoma mansoni* and *S. japonicum* cercariae to media containing oleic acid (OA) and explore how cercarial age plays a role in that response. The second aim was to apply the findings from the first aim to the development of an environmental surveillance device for schistosome cercariae (ESDSC) and to laboratory optimize the device to maximize cercarial capture. After examining the performance of the device, the third aim was to compare the ESDSC to other environmental surveillance methods that are currently available for the detection of schistosome cercariae through a review of the literature.

While both *S. mansoni* and *S. japonicum* cercariae exhibited highest attachment to media when freshly shed, they preferentially attached to different media types: *S. mansoni* to beeswax with 0.3 g/mL OA and *S. japonicum* to plain beeswax. An ESDSC

was designed from inexpensive, easily sourced materials (acrylic and aluminum sheets) and a *S. mansoni*-specific ESDSC was laboratory tested by utilizing beeswax with 0.3 g/mL of OA to attract cercariae. The cercarial capture capability of the ESDSC was tested in different orientations, depths, distances (from origin of cercariae), and with or without the application of a heat source. The highest median cercarial capture rate of 3.3% was observed with the device in a horizontal orientation, submerged, and within 25 cm of the cercarial point of origin. There were no instances in which the ESDSC failed to capture any cercariae, even at a distance of 70 cm.

Through a review of the literature, the ESDSC was compared to other environmental surveillance methods based on five attributes: time and labor, technical training, cost, infrastructure and capital investment, and sensitivity. Compared to the other surveillance methods, the ESDSC requires minimal time and labor, technical training, cost and resources; however, it exhibits only a moderate level of sensitivity. The value of the device will have greatest realization in endemic regions where health and economic resources are limited. What may work in one endemic region may not work best in another when factors such as availability of infrastructure, resources, and skilled technicians are considered. The ESDSC has the potential to function as a preliminary screening to detect contaminated sites so that complex and costly surveillance methods can be targeted more efficiently.

INTRODUCTION

1. Explanation of the Problem

Schistosomiasis is a significant parasitic disease that causes devastation to human health. An estimated 218 million people worldwide received preventative treatment for schistosomiasis in 2015 and over 66 million people were treated for the disease [1]. Schistosomiasis is a high morbidity disease that progressively worsens with repeated infections [2, 3] and is linked to severe anemia, reduced cognitive function, and chronic inflammation, disproportionately affecting impoverished people in underdeveloped nations [4, 5]. In sub-Saharan Africa, where at least 90% of the infections occur, *S. mansoni* and *S. haematobium* are responsible for the burden of the disease [6]. The population at risk is currently estimated to be nearly 800 million, but is growing due to water resource development projects [7, 8] that increase potential habitats for the intermediate host of the parasite. Poor sanitation is the leading culprit as to why so much of the world's population is still at risk of contracting the disease, but a lack of infrastructure makes transmission control challenging.

The disease is caused by a free-swimming parasite that infects by penetrating through the dermis of its host. The human infective form, the cercariae, attach, creep across, and penetrate the dermis of the primary host [9, 10]. Factors that stimulate cercarial attraction include both biochemical cues and temperature gradients [11-14].

Mitigation of the disease and infection transmission requires the implementation of a combination of control measures [15, 16]. Reducing transmission through snail population control, mass drug treatment, sanitation improvement, and education have been successful in some regions but current epidemiological surveys indicate that

schistosomiasis is reemerging in places where it was once under control due to snail population increases after flooding events as well as a scaleback in funding to disease control efforts [17]. The WHO mainly promotes periodic distribution of the drug, praziquantel, based on the prevalence of schistosomiasis among school-aged children. The long-term use of praziquantel has been shown to be relatively safe and effective [18-20] but this method of transmission control is problematic. Praziquantel does not protect against infection with schistosome cercariae and has limited activity against juvenile worms [21]. Additionally, only 43% of school-aged children and 11% of adults received praziquantel treatment in 2015 [22], leaving a large proportion of the infected population to continue spreading the disease.

As part of the mass drug treatment initiative, surveying the population for prevalence of disease is routinely performed [6]. While this is necessary to target treatment of individuals for the disease, prevention strategies should also be employed. One of the most effective methods in breaking the transmission cycle is to provide access to clean water. An important component of this is the ability to quickly identify unsafe sources so that access can be limited and the source can be treated. Several antiquated methods of environmental surveillance have been in use for decades, including the sentinel mouse model, malacology (study of snails), and water filtration or centrifugation. These methods can be very time consuming and lack sensitivity in positively identifying contaminated bodies of water [23-26]. Newer, molecular methods are highly sensitive but require a high level of technical expertise and is cost-prohibitive in endemic rural areas [27]. The development of a baited trap has been previously explored but is not currently in use [28, 29]. The basic principle behind the design of the trap is to leverage

factors that attract cercariae to attach, trap them on media during attachment and penetration attempts, and then examine the media under a microscope. Routine surveillance of contaminated bodies of water exists and surveillance initiatives are in place, however, global rates have remained relatively unchanged. This indicates that it may be necessary to increase the efficiency of how transmission sites are currently being monitored.

2. Specific Aims and Hypotheses

Three aims and their associated hypotheses are the focus of this dissertation and represent three separate manuscripts appended to this document:

2.1 Specific Aim 1: Determine the effect of oleic acid (OA) and cercarial age on the attraction and attachment of *S. mansoni* and *S. japonicum* cercariae (Appendix A). In a previously published study, *S. mansoni* cercariae were observed to have significantly higher ($p < 0.001$) attachment to media containing 0.3 g/mL of OA in beeswax media, compared to media containing 0.9 and 1.8 g/mL of OA (7.5%, 4.2%, and 3.7%, respectively). Freshly shed and 5-hours post-shed *S. mansoni* cercariae were observed to attach to media at a higher percentage than cercariae that were 10-hours post-shed (3.03%, 2.57%, and 1.04%, respectively) [30]. It is not known whether his combination of factors has the same effect on *S. japonicum*. This aim was achieved by exposing *S. mansoni* and *S. japonicum* cercariae to various concentrations of OA (0.15, 0.3, 0.9 g/mL) in beeswax at three cercarial age points (freshly shed, 5-hours post-shed, and 10-hours post-shed). It was hypothesized that each of the two species of *Schistosoma* cercariae will be differentially attracted to the various concentrations of OA and freshly

shed cercariae will exhibit higher attachment to media than aged cercariae. The findings will provide us with a basis by which to design and develop an environmental surveillance device for schistosome cercariae (ESDSC).

2.2 Specific Aim 2: Develop a *S. mansoni*-specific ESDSC and evaluate positional attributes of the device to optimize cercarial capture (Appendix B). To accomplish this aim, the combination of factors (media type and age of cercariae) yielding the highest percent attachment of *S. mansoni* cercariae (Specific Aim 1, Appendix A) was applied to the design and operation of the ESDSC. Positional attributes of the device: distance from cercarial origin, orientation, and depth were evaluated for *S. mansoni* cercarial capture. Additionally, application of a heat source (in the form of a reusable hand-warmer) within the device was assessed after the optimization of the positional attributes. The ESDSC was evaluated in a 114-liter aquarium with a built-in ramp. Upon completion of this aim, the device will be ready for field evaluation and comparison against environmental surveillance methods that are currently being used in *S. mansoni* endemic areas.

2.3 Specific Aim 3: Conduct a review of the literature to examine how the ESDSC compares to other environmental surveillance methods that detect schistosome cercariae in water (Appendix C). To achieve this aim, peer-reviewed, primary research articles were identified by searching through an electronic database. Only articles that evaluated environmental surveillance methods that detect cercariae in water were reviewed. The surveillance methods were assessed on five attributes in order to determine the utility of the ESDSC. Research questions were: 1) What environmental surveillance methods are being used to detect schistosome cercariae? 2) How does the

ESDSC compare, in relation to these other identified methods? 3) Can the ESDSC enhance, supplement, or replace these other environmental surveillance methods? It is hypothesized that the ESDSC will provide a passive, low-cost, low-technological, and rapid surveillance method for the early detection of *S. mansoni*-contaminated waters that is appropriate, given the resource limitations in endemic areas.

3. Dissertation Format

This dissertation contains three manuscripts ready for publication submission. Appendix A contains the manuscript "Factors that may influence the parasite-host attraction of *Schistosoma mansoni* and *S. japonicum*: assessment of oleic acid and cercarial age", which explores the chemotactic effect of oleic acid on the attraction and attachment of *S. mansoni* and *S. japonicum* cercariae, taking into account cercarial age. This manuscript will be submitted to the International Journal of Parasitology. Appendix B contains "Development of an environmental surveillance device for *Schistosoma mansoni* cercariae", which describes the development of a *S. mansoni*-specific environmental surveillance device for schistosome cercariae (ESDSC) and how it was evaluated to optimize cercarial capture. The manuscript in Appendix B will be submitted to the journal, Environmental Science and Technology. Appendix C contains the manuscript, "Comparison of Environmental Surveillance Methods for Schistosome Cercariae through a Review of the Literature", which will be submitted to Parasitology International. This manuscript reviews peer-reviewed literature pertaining to the evaluation of environmental surveillance methods for schistosome cercariae, to ascertain

how the development of the ESDSC enhances methods that are currently in use in endemic areas.

I am responsible for the data collection, analysis, and writing of the manuscripts in all appendices.

4. Literature Review

4.1 Schistosomiasis: An estimated 218 million people worldwide received preventative treatment for schistosomiasis in 2015 and over 66 million people were treated for the disease [1]. The parasite is endemic in 78 countries, including: Africa, China, Indonesia, the Middle East, and the Caribbean. There are five species that predominantly affect humans across these endemic areas: *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongi*, and *S. intercalatum*, with the first three species accounting for the majority of the cases [31]. Infection with *S. mansoni* and *S. japonicum* cercariae result in intestinal schistosomiasis and *S. haematobium* manifests as the urogenital form of the disease.

The disease is caused by a free-swimming parasite that infects by penetrating through the dermis of its host. Individuals become exposed to the parasite during contact with contaminated freshwater, primarily through fishing, agricultural, and recreational activities. Symptoms of this disease do not typically present until one to two months after initial infection by the parasite. Schistosomiasis is a high morbidity disease that progressively worsens with repeated infections [2, 3] and is linked to severe anemia, reduced cognitive function, and chronic inflammation, disproportionately affecting impoverished people and underdeveloped nations [4, 5].

4.2 Global Public Health Impact: The population at risk, currently estimated at nearly 800 million, is growing due to the damming of water, irrigation canal construction, and other water resource development projects [7, 8]. In sub-Saharan Africa, where at least 90% of the infections occur, *S. mansoni* and *S. haematobium* are responsible for the burden of the disease [6]. Decreases in infections have been reported throughout Asia and South America, but total global cases have remained relatively unchanged. This phenomenon is likely due to the high prevalence of disease in sub-Saharan Africa, where the population has grown by 70% over the last 25 years [15]. Despite implementation of broad control measures resulting in a reduction of cases, current epidemiological surveys indicate that schistosomiasis is re-emerging in places where it was once under control [17]. Political climate, increased mobility of humans and animals, and inadequacies in disease surveillance tools are each factors contributing to this re-emergence [32].

4.2.a Co-morbidity: Chronic urinary schistosomiasis causing lesions and mucosal bleeding in female genitalia is known as female urogenital schistosomiasis (FUS) and affects roughly 55-65% of the population infected with *S. haematobium* [33, 34]. Presence of these open sores can increase the chances of contracting an infectious disease through sexual contact [35]. FUS as a risk factor for HIV was first suggested in 1995 [36] and multiple studies supporting this association have been published since. A cross-sectional study conducted by Kjetland et al. on women living in a Zimbabwean community showed that among those that had laboratory diagnosed genital schistosomiasis, 41% were also HIV positive, as opposed to 26% in the urogenital schistosomiasis negative group (OR=2.1; p-value = 0.008) [37]. In another cross-sectional study conducted in Tanzania, women with urinary schistosomiasis had a HIV

prevalence rate of 17%, while women without urinary schistosomiasis had a HIV prevalence rate of 5.9%. FUS was shown to be associated with HIV infection rates (OR= 4.0) and younger age (29 years and younger; OR=5.5) [38]. Human Papilloma Virus (HPV) infections, and by extension, cervical cancers, have also been shown to be associated with FUS. In sub-Saharan Africa, a study found that 18% of women with FUS and cervical cancer were under the age of 25, whereas only 5% of women with cervical cancer and without FUS fell into this age category [39]. This may be exacerbated by the finding that FUS appears to develop during puberty [40]. Cancer of the cervix is not the only cancer to be associated with *S. haematobium* infections. The association between *S. haematobium* infections and bladder cancer was first described in the early 1900s and is now widely accepted [41].

4.3 Transmission: Schistosome cercariae, which are the mammalian infective stage, attach, creep across, and penetrate the dermis of the primary host [9, 10]. Penetration through intact skin is facilitated by serine proteases, or cercarial elastase [42], that aid in digestion of connective tissues by breaking down elastin and hydrolyzing keratin. Factors that stimulate cercarial attraction include both biochemical cues and temperature gradients [11-14]. Cercariae undergo transformation and maturation into worms over an eight to 10 day period and travel through tissue to the liver where they develop into sexually dimorphic adult worms, which occurs over two to three weeks. The female worm is held within the gynecophoral canal of the male worm and they migrate to their final species-specific residence vein. After about 30 days, the female worm starts producing eggs and continues to do so for the entirety of her life. Transmission occurs when eggs exit the body through excrement and contaminate bodies

of water that are shared by the appropriate intermediate snail host and the primary mammalian host. Miracidia emerge from the eggs when in water and this free-swimming intermediate life-stage search for an appropriate snail host to infect. Miracidia develop into sporocysts and daughter sporocysts in the snail, undergoing asexual division and maturing to the mammalian infective form, cercariae (Figure 1).

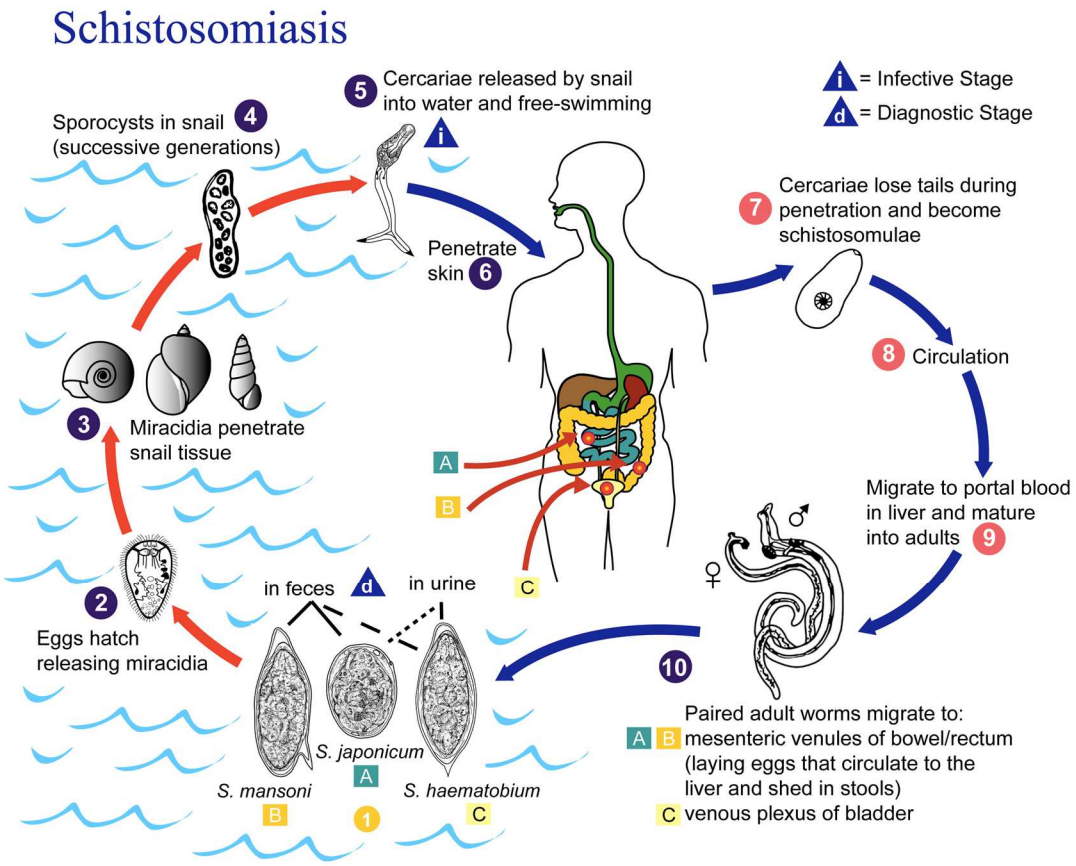


Figure 1. Two-host life cycle of *Schistosoma sp.* from egg to reproductive adult worms (DPDx-CDC Schistosomiasis 2012).

In the environment, cercariae are diurnally shed, with emergence from the snail host being stimulated by sunlight and reports of highest concentrations occurring between the hours of mid-morning and early afternoon [43-46]. Age of the cercariae may also influence the success of finding a host as cercariae typically only live 24-36 hours under

laboratory conditions and less than 24 hours in the environment [47, 48], dying when their glycogen energy stores have been depleted.

4.4 Mitigation Initiatives

4.4.a Snail Control: Chemical-based molluscicide of contaminated waters to control the population of intermediate snail hosts has been a long-established strategy for reducing transmission of the disease [49]. Niclosamide was favored over the use of other compounds because it exhibited low toxicity to humans and livestock and is potent at low concentrations again snails, their eggs, and even schistosome cercariae [50]. However, with the inception of the mass distribution of chemotherapy agents as a form of treating at-risk populations, the WHO, due to the potential risks of chronic toxicity, no longer recommended regular application of niclosamide application. Natural (and in some cases, local) plant-derived molluscicides have been explored in more recent years with promising results [51-53]. Molluscicide, alone, has shown mixed results, wherein snail populations experience a temporary reduction in number, but without constant treatment, they rebound very quickly [54]. Altering the environment of the snail intermediate host by lining man-made habitats with cement or through the introduction of natural predators have also been explored as environmentally friendly and sustainable methods of snail population control [55-57]. Control measures focusing on the snail intermediate host have shown highest efficacy when used in conjunction with other controls [15, 16].

4.4.b Hygiene and Education: Improvement of public health infrastructure, such as access to clean water and proper disposal of human and animal waste usually requires governmental investment. Increasing access to sanitary infrastructure and clean water has been credited with reducing morbidity by 69-77% [58] and is associated with lower

odds of schistosomiasis [59, 60]. Water treatment and storage have also been shown to lower exposure to schistosome cercariae [61, 62]. Piped water decreases the necessity for contact with potentially contaminated waters for domestic purposes may not impact recreational contact exposures. The construction and usage of sanitary latrines reduce the likelihood of infected human excrement contaminating waterways with frequent human activity. Though latrines are made available, they must be used in order for the community to see benefits. Adjusting personal habits and cultural customs can be a challenge; therefore, improving community awareness of the disease and knowledge about the modes of transmission is a crucial component in reducing the prevalence of schistosomiasis, especially in children under the age of 14 [63, 64].

4.4.c Mass Drug Treatment: Periodic distribution of the drug, praziquantel, to at-risk populations (regardless of confirmed infection status) has been promoted by the WHO for decades and administration is based upon the prevalence of schistosomiasis among school-aged children. The long-term use of praziquantel has been shown to be relatively safe and effective [18-20] but there are concerns of drug resistance by adult worms and decreased protective immunity [65]. Several challenges have been encountered with reliance of mass drug treatment as the only source of infection and transmission control. Praziquantel does not protect against infection with schistosome cercariae and has limited activity against juvenile worms [21]. Additionally, only 43% of school-aged children and 11% of adults received praziquantel treatment in 2015 [22] and political unrest increases vulnerable populations. There has been increasing pressure by the WHO to administer the drug to pre-school aged children in order to address very early infections that detrimentally affect early childhood development, but the

praziquantel pill is very large and has a bitter taste. Development of a child formulation that retains the efficacy of the drug while making it palatable is currently under investigation [66, 67].

4.4.d Vaccine Development: Presently, a vaccine for schistosomiasis does not exist for humans and very few candidates (especially for *S. mansoni* and *S. haematobium*) have been targeted [68]. Co-infection with the two major human relevant schistosomes in sub-Saharan Africa is common so an ideal vaccine candidate would need to address both species [69]. *S. japonicum* is unique in that it not only infects humans but also non-human mammalian reservoirs, such as cattle and buffalo. Due to this zoonotic aspect, vaccines for livestock have been developed as a means of infection and transmission control. Field studies with vaccine candidates have shown variable efficacies but appears to be well-tolerated by animals, resulting in little to no adverse effects [70, 71].

4.5 Surveillance

4.5.a Population Surveillance: As part of the mass drug treatment initiative, surveying the population for prevalence of disease is routinely performed [6]. Detection of schistosomiasis infection in human is accomplished through the collection and microscopic examination of stool samples, or through serological tests on blood or urine. School-aged children are usually the subjects of surveillance programs and as such, stool and urine collection are the preferred methods for diagnoses. This can result in missed diagnosis if adult mating schistosome worms are not present in the body, since the observation of eggs indicates infection. This method touts high specificity (with a well-trained eye) but sensitivity is dependent on the intensity of the infection as well as the number of samples that are examined [72]. Severity of disease can also be estimated

through this method by quantifying the egg burden in the sample [31]. Urine antigen tests that detect antigens that are released by viable adult worms in the body have also been developed to detect schistosomiasis infection in populations, but again, missed diagnosis can occur during early infection [73]. Serological tests on blood samples can detect infections earlier than the examination of stool or urine, but is more challenging to acquire, especially in the school-aged children. While this method is sensitive, cross-reactivity with antigens from other helminthes have been reported [74]. A less technical method for determining broad prevalence of infection includes administering of questionnaires regarding the observation of blood in urine or stool, which has shown to be a rapid, inexpensive, and reliable tool [75].

4.5.b Environmental Surveillance: One of the most effective methods in breaking the transmission cycle of schistosomiasis is to provide access to clean water and proper disposal of human and animal waste. An important component of access to clean water is the ability to quickly identify unsafe sources and disseminate that information to surrounding communities. Several antiquated methods of environmental surveillance have been in use for decades, including the sentinel mouse model, malacology (study of snails), and water filtration or centrifugation. The gold standard method uses a rodent exposure model, wherein caged sentinel mice are immersed, such that only their legs and belly are submerged. The cage is left in the water for 2-4 hours per day for 2-4 consecutive days and then removed. Rodents are reared for 6-14 weeks (allowing any cercariae to mature into worms) and then euthanized and dissected to identify presence of *Schistosoma* worms [24]. This is the only method that detects infective cercariae rather than just presence or absence. Snail collection, and either crushing to look for developing

parasites or holding to look for cercarial shedding, is a low-cost, low-technological method for determining transmission foci. Finally, differential filtration or centrifugation of large volumes of water and subsequent staining and examination under a microscope has also been used in rural endemic regions[26, 76-78]. Newer and more sensitive molecular methods have also been used to survey bodies of water for presence of cercariae. Instead of examining a filtered water sample under the microscope, polymerase chain reaction (PCR) and qualitative PCR (qPCR) can be performed to detect schistosome cercariae. These laboratory-based methods are sensitive and quantitative (qPCR) [79].

4.5.c Emerging Technologies: In recent years, newer technologies and research findings have emerged that have the potential to be coupled with techniques currently in use. Researchers have explored the use of new methodologies to screen the sera of sentinel mice for early biomarkers of infection or empty snail shells for trace evidence of schistosome infection, which significantly decreased the required holding time [80-84]. Another new development is the engineering of a biosensor to detect schistosome cercariae [85]. The technology is based on recognizing the enzymatic activity of elastase, which is secreted by cercariae during the process of skin invasion. *S. mansoni*-specific elastase on the biosensor leads to a colorimetric change, indicating presence of cercariae. The biosensor alone would not be used in the field, but could be used in conjunction with a baited trap, wherein cercariae are attracted to the trap and attempt to invade the media, leading to secretion of elastase. The development of the biosensor has great potential for rapid field-speciation of trapped cercariae.

PRESENT STUDY

The goal of this study was to develop a passive, low-cost, low-technological, rapid surveillance method for detecting schistosome cercariae in the environment as well as determine its value, compared to other available methods. Three aims were formulated in order to develop and evaluate the device. The first aim was to determine what concentration of OA in beeswax would result in the highest attachment of cercariae and at what cercarial age this occurred (Appendix A). Schistosome cercarial attraction has been previously shown to be driven in part by biochemical prompts such as L-arginine, skin ceramides, and essential fatty acids [13, 86, 87]. OA is an unsaturated free fatty acid that is highly abundant (~47% composition of total fatty acid content) in mammalian skin [88], which is why it was chosen as a chemoattractant for this study. The second aim harnessed the information learned in the first aim to the development of the ESDSC, which was laboratory optimized under controlled conditions (Appendix B). After examining the performance of the device in the laboratory, the third aim was to compare the ESDSC to other environmental surveillance methods that are currently available for the detection of schistosome cercariae, through a review of the literature (Appendix C). The following sections provide a summary of how the study was conducted and the main findings related to each of the three aims.

Methods

The goal of this study was addressed and achieved through the development and execution of three methods. First, the chemotactic response of *S. mansoni* and *S. japonicum* cercariae to different concentrations of OA in beeswax was assessed, as well

as how the age of the cercariae affects attraction and attachment to media. Second, the combination of factors (concentration of OA in beeswax and cercarial age) resulting in the highest attachment percentage of *S. mansoni* cercariae informed the development and operation of the ESDSC. The ESDSC was optimized by examining the cercarial capture percentage based on the distance from cercarial origin, orientation, and depth of the device. Additionally, application of a heat source within the device was assessed. Third, a review of the literature was performed to identify environmental surveillance methods that are available and to compare those identified methods to the ESDSC on five attributes, in order to put the relevance of the device in context.

Chemotactic Response and Age of Cercariae: Infected 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. *Biomphalaria glabrata* snails (infected with *S. mansoni* miracidia) and *Oncomelania hupensis* snails (infected with *S. japonicum* miracidia) were reared using standard methods (NIAID Schistosomiasis Resource Center 2015). Exposure media were prepared by spreading beeswax and beeswax with OA (0.15, 0.3, and 0.9 g/mL) on microscope slides [30].

In a 50 mL beaker, 100 – 150 cercariae in 20 mL of water were exposed to vertically oriented exposure slides (one per beaker) for two hours. *S. mansoni* cercariae typically swim up and fall down in a figure-eight pattern [89] in the water column, while *S. japonicum* cercariae float at the water surface (visual observation); as such, the vertical placement of the slide was applied to minimize accidental bumping. After the exposure period, slides were stained with iodine and examined under a microscope. The number of

detached cercariae heads and whole cercariae were counted. The experimental methods, as described above, were repeated for three age points: freshly shed, 5-hours post-shed, and 10-hours post-shed cercariae. Media type and age of cercariae were evaluated for their effect on percent cercarial attachment. The Kruskal-Wallis test (alpha level set at $p < 0.05$) and Mann-Whitney post-hoc test with Bonferroni correction was applied to evaluate differences between each of the groups within the two variables (media type and age of cercariae).

Development and Optimization of the ESDSC: A hollow acrylic box was constructed to allow for the insertion of a small reusable hand-warmer. Metal microscope slide holders were glued onto the long sides of the box, capable of holding five standard-sized slides on each side (Figure 2A). All experiments were conducted in a 114-liter fish aquarium with a built-in 24° angled ramp to simulate an embankment. The ramp decreased the water capacity of the aquarium to 57.6 L when filled and a spigot was installed at the "deep" end of the aquarium to allow for drainage (Figure 2B). Based on the findings in Specific Aim 1 (Appendix A), freshly-shed *S. mansoni* cercariae exhibited highest percent attachment to beeswax media containing 0.3 g/mL of OA and as such, was the media type used in the *S. mansoni*-specific ESDSC. Three thousand *S. mansoni* cercariae were used in each experiment. The Kruskal-Wallis test with Mann-Whitney post-hoc test (non-parametric data) and the t-test (parametric data) were applied to evaluate the differences between groups within variables (significance level set to $p < 0.05$). Percent cercarial attachment with regard to device distance from cercarial point of origin, device orientation, device depth, and application of heat (in device) were examined.

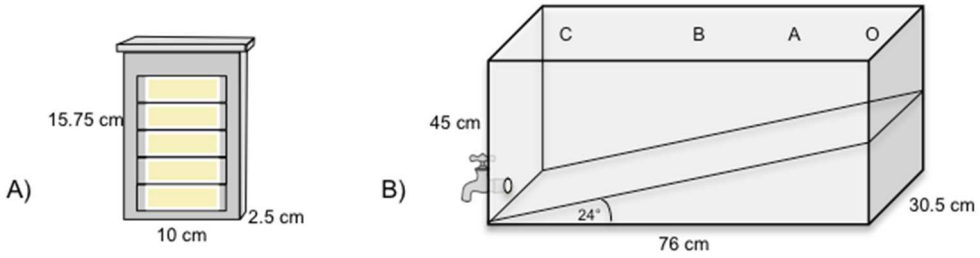


Figure 2. (A) Environmental Surveillance Device for Schistosome Cercariae (ESDSC) prototype with removable lid for insertion of reusable hand-warmer (not to scale). (B) Aquarium in which the ESDSC was tested for cercarial capture (not to scale). Position O was the cercarial origin. Position A was 24.5 cm away, position B was 43.5 cm away, and position C was 70 cm away from position O.

To evaluate the effect of distance from cercarial origin and device orientation, the ESDSC (containing the activated hand-warmer) was placed at three distances along the aquarium, at the surface of the water. Position A was closest to the origin of *S. mansoni* cercariae release, position B was in the middle of the aquarium, and position C was farthest (Figure 2B), at distances of 24.5, 43.5, and 70 cm away, respectively. At each of the three distances, the ESDSC was tested in two orientations: horizontal and vertical (or perpendicular) to the surface of the water. The ESDSC was left in the aquarium for a 2-hour time period before being retrieved. The slides were removed from the slide holder, stained with iodine, and examined under a microscope. The number of whole cercariae, detached heads, and detached tails were recorded.

To evaluate the effect of depth of the device, the orientation in which the highest percentage of cercariae was captured was held constant. The ESDSC (containing the activated hand-warmer) was submerged (just hovering over the ramp) at the three distances along the aquarium. The depths at which the ESDSC was placed were 14, 23, and 34 cm from the surface of the water at positions A, B, and C, respectively. Again, the ESDSC was left in the aquarium for a 2-hour period. Slides were processed as previously described. These data were compared to the corresponding surface data.

To evaluate the effect of heat on cercarial capture, the combination of orientation, distance from cercarial origin, and depth at which the highest cercarial attachment was observed was held constant. The ESDSC without the hand-warmer was exposed to cercariae for a 2-hour time period, removed from the aquarium, and the slides were stained and examined. These data were compared to the corresponding data from the ESDSC with the activated hand-warmer in the cavity.

A "worst-case" and "best-case" system was developed to represent a range of cercarial attachment values since it was unknown whether an observed detached head and detached tail were from the same whole cercariae. The "worst-case" value assumes that detached heads and tails are paired and the "best-case" value assumes that detached heads and tails came from different cercariae. Data analyses were performed on both the worst- and best-case values.

Comparison of ESDSC to Other Environmental Surveillance Methods through Review of Literature: General queries using search terms "*Schistosoma mansoni*", "*Schistosoma japonicum*", and "*Schistosoma haematobium*" were performed in PubMed, Scopus, and Web of Science to get an idea of the extent of the body of knowledge regarding the three main human-relevant species of schistosome parasite. The Boolean phrase, "(((((detection) OR surveillance) OR trap) OR cercariometry) AND Schistosoma) AND cercariae", was typed into the search bar on the three scholarly databases mentioned previously. PubMed yielded the highest number of hits, a total of 338 publications from years 1946 to 2017. The results were filtered to exclude scholarly articles that were not written in English, leaving 292 journal articles. The titles and abstracts of these publications were reviewed to determine their eligibility to be included

in this review of the literature. Only primary research articles that reported on detection or capture of schistosome cercariae in a sample (spiked or otherwise) or body of water were included. These inclusionary criteria narrowed the number of pertinent articles to 20, two of which described methods of detecting avian schistosomes [90, 91]. Two review articles [92, 93], summarizing the molecular approaches for monitoring transmission sites as well as cercariometric methods, were excluded. The reference lists of the 20 articles were reviewed in order to identify publications that may have been missed by the Boolean search, which revealed an additional five papers. The surveillance methods discussed in these 25 articles were assessed on the following properties: time and labor, sensitivity, cost and infrastructure and capital investment, and technical training.

Results

Chemotactic Response and Age of Cercariae: Median *S. mansoni* cercarial attachment was lowest to plain beeswax media and highest to 0.3 g/mL OA, regardless of whether only cercariae heads or head and whole cercariae were counted (Table 1). Median *S. japonicum* cercarial attachment on plain beeswax was statistically significantly higher than on beeswax with 0.3 and 0.9 g/mL of OA ($p < 0.003$) (Table 1).

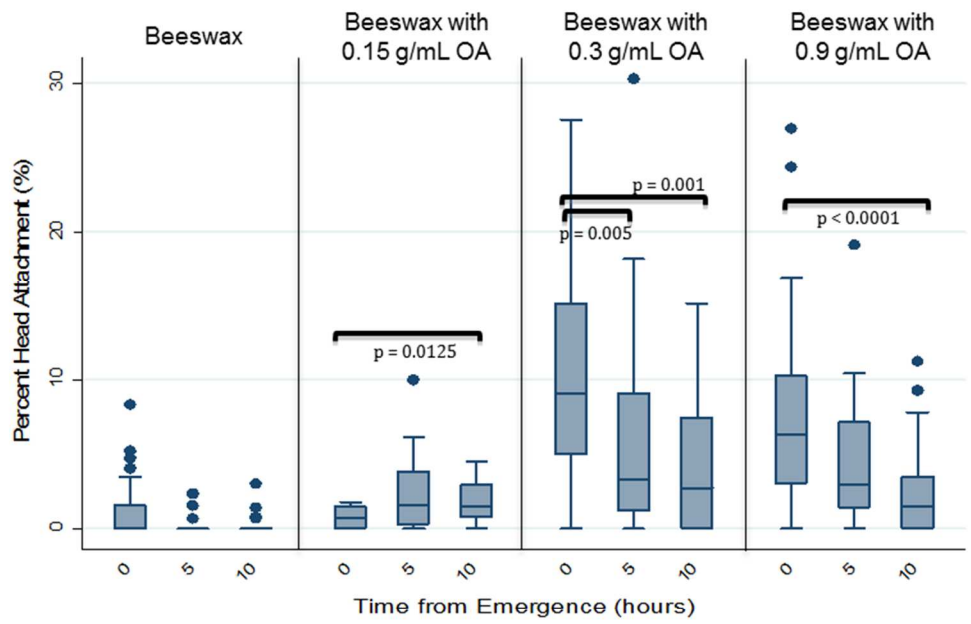
Table 1. Descriptive statistics for percentage of Schistosoma mansoni and S. japonicum cercariae attached to different media types.

			Cercariae Head Only (% attached)		Head and Whole Cercariae (% attached)	
	Media Type	n	Max	Median	Max	Median
S. mansoni	Beeswax	110	8.33	0	9.17	0
	Beeswax with oleic acid (0.15 g/mL)	60	10	1.47[a]	12.31	1.52[a]
	Beeswax with oleic acid (0.3 g/mL)	110	30.3	5.72[a,b]	30.3	6.16[a,b]
	Beeswax with oleic acid (0.9 g/mL)	110	26.95	3.64[a,b]	27.83	3.91[a,b]
S. japonicum	Beeswax	30	58	5.06	58.25	5.23
	Beeswax with oleic acid (0.15 g/mL)	30	29	2.07	29.03	2.07
	Beeswax with oleic acid (0.3 g/mL)	29	19.57	1[c]	19.59	1.03[c]
	Beeswax with oleic acid (0.9 g/mL)	29	22	1.23[c]	22.12	1.25[c]

Note: Minimum values were zero for all media types tested.
a Statistically significantly different from beeswax media, $p < 0.0001$
b Statistically significantly different from beeswax with oleic acid (0.15 g/mL), $p < 0.0001$
c Statistically significantly different from beeswax media, $p < 0.003$

Looking at age as a factor of cercarial attachment, both *S. mansoni* and *S. japonicum* exhibited a statistically significant decrease in attachment when cercariae were exposed to media at 10-hours post-shed, compared to freshly-shed cercariae. To further explore the chemotactic response of *S. mansoni* and *S. japonicum* cercariae, the data were stratified first by media type and then by age to determine the combination of the two factors that resulted in the highest attachment (Figures 3 & 4). On beeswax media with 0.3 and 0.9 g/mL OA, freshly shed *S. mansoni* cercariae exhibited significantly higher attachment than cercariae that were 10-hours post-shed (Figure 3). For *S. japonicum* cercariae, there were no statistically significant differences in attachment percentages between age points within each media type; however, median attachment values for freshly shed cercariae were highest and generally decreased as the cercariae aged throughout the day (Figure 4).

A



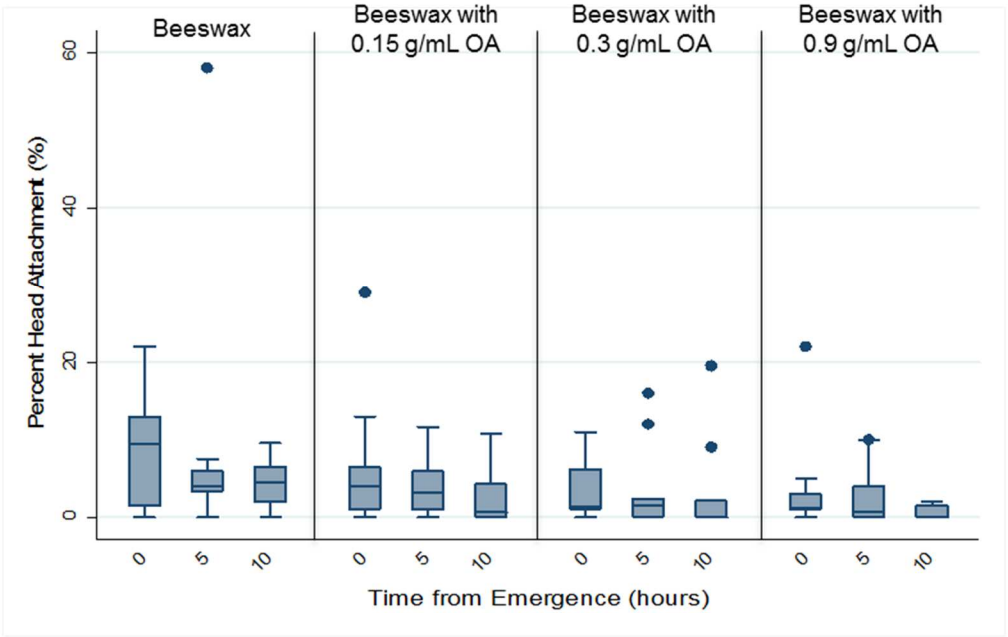
B

	Media	Age	n	Cercariae Head Only (% attached) Max	Cercariae Head Only (% attached) Median	Head and Whole Cercariae (%attached) Max	Head and Whole Cercariae (%attached) Median
S. mansoni	Beeswax	Fresh	50	0	0	9.17	0.3
		5 h	30	2.38	0	2.38	0
		10 h	30	3.03	0	3.03	0
	Beeswax with oleic acid (0.15 g/mL)	Fresh	20	1.79	0.74[b]	3.57	1.52
		5 h	20	10	1.54	12.31	1.54
		10 h	20	4.55	1.52	5.3	1.89
	Beeswax with oleic acid (0.3 g/mL)	Fresh	50	27.52	9.1[a,b]	29.36	10.05[a,b]
		5 h	30	30.3	3.33	30.3	3.96
		10 h	30	15.2	2.75	18.18	2.75
	Beeswax with oleic acid (0.9 g/mL)	Fresh	50	26.96	6.29[b]	27.83	6.39[b]
		5 h	30	19.13	3	19.13	3.8[c]
		10 h	30	11.25	1.52	12.5	1.9

Note: Minimum values were zero for all media types tested.
a Statistically significantly different from 5-hours post-shed, p = 0.005
b Statistically significantly different from 10-hours post-shed, p < 0.0125
c Statistically significantly different from 10-hours post-shed, p = 0.0131

Figure 3. (A) *Schistosoma mansoni* cercarial attachment stratified by media type (beeswax, 0.15 g/mL oleic acid (OA) in beeswax, 0.3 g/mL OA in beeswax, and 0.9 g/mL OA in beeswax) and age of cercariae (0-hours, 5-hours, and 10-hours from time of emergence). (B) Descriptive statistics for percentage of *S. mansoni* cercariae attached to different media types, stratified by age of cercariae.

A



B

				Cercariae Head Only (% attached)		Head and Whole Cercariae (%attached)		
	Media	Age	n	Max	Median	Min	Max	Median
S. japonicum	Beeswax	Fresh	10	22	9.5	0.05	22.23	9.63
		5 h	10	58	4	0.4	58.25	4.08
		10 h	10	9.52	4.48	0.01	9.83	4.64
	Beeswax with oleic acid (0.15 g/mL)	Fresh	10	29	4	0	29.03	4.01
		5 h	10	11.67	3.16	0	11.67	3.2
		10 h	10	10.74	0.63	0	10.75	0.63
	Beeswax with oleic acid (0.3 g/mL)	Fresh	9	11	1.43	0	11.01	1.44
		5 h	10	16	1.61	0	16.11	1.61
		10 h	10	19.57	0	0	19.59	0
	Beeswax with oleic acid (0.9 g/mL)	Fresh	9	22	1.25	0	22.12	1.26
		5 h	10	10	0.71	0	10.07	0.71
		10 h	10	2.06	0	0	2.06	0

Note: Minimum values were zero for all media types tested, cercariae head only

Figure 4.(A) *Schistosoma japonicum* cercarial attachment stratified by media type (beeswax, 0.15 g/mL oleic acid (OA) in beeswax, 0.3 g/mL OA in beeswax, and 0.9 g/mL OA in beeswax) and age of cercariae (0-hours, 5-hours, and 10-hours from time of emergence). (B) Descriptive statistics for percentage of *S. japonicum* cercariae attached to different media types, stratified by age of cercariae.

Development and Optimization of the ESDSC: The ESDSC was tested at the surface of the water, in two orientations, at three positions along the length of the

aquarium. Median cercarial capture values were typically highest at position A (Table 2), however the differences were not significant. Cercarial capture was significantly higher in the horizontal orientation than the vertical orientation at every position tested ($p < 0.05$). The highest median cercarial capture percentage of 2.63% was observed at position A with the device in the horizontal orientation. In the horizontal orientation, the ESDSC captured a significantly ($p < 0.05$) higher number of *S. mansoni* cercariae on the top of the device (facing the surface of the water) than on the bottom of the device (facing the bottom of the aquarium) (data not shown).

Table 2. *Schistosoma mansoni* cercarial capture (starting number of 3000 cercariae) on the Environmental Surveillance Device for Schistosome Cercariae, testing distance from cercariae point of origin and orientation of device at the surface of the water.

		Worst-case (% attached)			Best-case (% attached)		
Distance from Cercariae Origination	Orientation	Median	Min	Max	Median	Min	Max
Position A (Closest)	Vertical	0.47	0.17	0.97	0.7	0.27	1.5
	Horizontal	1.53[a]	0.87	4.07	2.63[a]	1.6	5.93
Position B (Middle)	Vertical	0.27	0.03	0.57	0.33	0.03	0.9
	Horizontal	0.97[a]	0.47	1.4	1.5[a]	0.67	2.17
Position C- (Farthest)	Vertical	0.47	0.23	0.7	0.7	0.27	1.07
	Horizontal	0.9[a]	0.57	1.67	1.6[a]	0.87	2.73

Note: Worst case values assume that detached heads and tails are paired, equaling one whole cercariae and best case values assume that detached heads and tails are from different cercariae.
a Statistically significantly different from vertical orientation at that position, $p < 0.05$

Since the horizontal orientation of the ESDSC yielded highest cercarial capture at every position tested, it was held constant while depth of the device was examined. The device in position A exhibited a significantly higher cercarial capture compared to positions B and C (best-case value). The worst-case value for cercarial capture percentage was not statistically significantly different between positions along the aquarium ($p = 0.053$). The ESDSC performed significantly better, in regards to cercarial capture, when it was submerged rather than placed at the surface of the water ($p < 0.05$ for both worst- and best-case values). The highest median cercarial capture percentage of

3.3% was observed at position A with the ESDSC submerged in the horizontal orientation (Table 3).

Table 3. *Schistosoma mansoni* cercarial capture (3000 starting) on Environmental Surveillance Device for Schistosome Cercariae, testing distance from cercariae point of origin and depth, with device in horizontal orientation.

		Worst-Case (% attached)			Best-Case (% attached)		
Distance from Cercariae Origination	Water level	Median	Min	Max	Median	Min	Max
Position A (Closest)	Surface	1.53	0.87	4.07	2.63	1.6	5.93
	14 cm from Surface	2.1	1.5	2.57	3.3	2.27	4.4
Position B (Middle)	Surface	0.97	0.47	1.4	1.5	0.67	2.17
	23 cm from Surface	1.33	0.8	2.27	2.4	1.4	3.23
Position C- (Farthest)	Surface	0.9	0.57	1.67	1.6	0.87	2.73
	34 cm from Surface	1.37	0.97	1.8	2.13	1.47	2.9

Note1: Worst case values assume that detached heads and tails are paired, equaling one whole cercariae and best case values assume that detached heads and tails are from different cercariae.
Note2: The device in position A performed statistically significantly better than in positions B and C ($p = 0.019$ and $p = 0.038$, respectively; best-case value) and also when the device was submerged, rather than at the water surface ($p < 0.033$; worst- and best-case))

To evaluate the application of heat, the ESDSC was submerged horizontally in position A without insertion of the activated hand-warmer. These data were compared to the data collected from the device with insertion of the hand-warmer. The best-case mean value for percentage of cercariae captured by the device was higher with the application of heat; however, this difference was not statistically significant ($p > 0.28$, Table 4).

Table 4. *Schistosoma mansoni* cercarial capture (3000 starting) on Environmental Surveillance Device for Schistosome Cercariae, testing the application of heat.

	Worst-case (% attached)			Best-case (% attached)		
Application of Heat	Mean	Min	Max	Mean	Min	Max
Hand-Warmer	2.05	1.5	2.57	3.37	2.27	4.4
No Hand-Warmer	2.09	1.6	2.57	2.87	1.93	3.6

Note: Worst case values assume that detached heads and tails are paired, equaling one whole cercariae and best case values assume that detached heads and tails are from different cercariae.

Comparison of ESDSC to Other Environmental Surveillance Methods through Review of Literature: The average number of articles returned for *S. mansoni*, *S.*

japonicum, and *S. haematobium* were 14,813, 3,948, and 3,325, respectively. Table 5 summarizes the properties of the 25 articles and the six surveillance methods that were identified through the review of the literature.

Table 5. Summary information of the 25 peer-reviewed primary research articles that were included in the review of the literature.

			Surveillance Method(s) Evaluated					
Year	**Author**	**Country**	Snail Shedding/ Crushing	Water Filtration/ Centrifugation	Sentinel Rodent	PCR (snail or water)/ ELISA	Baited Trap	C-6 Film***
1971	Butler et al.	USA	X	X				
1982	Kloos et al.	Egypt		X				
1984	Prentice	Kenya		X				
1989	Ouma et al.	Kenya	X	X				
1993	Shiff et al.	USA					X	
1996	Yousif et al.	Egypt	X	X				
1996	Yousif et al.	Egypt		X	X			
1998	Hamburger et al.	Israel				X		
1998	Hamburger et al.	Israel				X		
1999	Cai et al.	China			X			X
2000	Graczyk & Shiff *	USA					X	
2002	Ahmed et al.	Sudan					X	
2005	Driscoll et al.	USA				X		
2006	Melo et al.	Brazil	X			X		
2008	Hung & Remais	USA				X		
2011	Carlton et al.	China	X					
2011	Worrell et al.	USA			X	X		
2013	Allan et al.	United Kingdom	X			X		
2013	Lee et al.	USA					X	
2015	Jothikumar et al. *	USA				X		
1965	Barrett & Ellison**	Rhodesia		X				
1983	Blumenthal & Jewsbury**	UK		X				
1989	Hamburger et al.**	Israel	X			X		
1997	Hanelt et al.**	USA				X		
2004	Hamburger et al.**	Israel	X			X		

* Research performed on avian schistosomes
** Additional publications identified through the review of references
*** Proprietary information on the make-up of C-6 film

Discussion

Chemotactic Response and Age of Cercariae: The chemotactic response in *S. japonicum* cercariae appears to differ from that of *S. mansoni*, as *S. japonicum* exhibited highest attachment to plain beeswax, while presence of OA (regardless of concentration) resulted in a significantly higher attachment in *S. mansoni* cercariae compared to plain

beeswax. Both *S. mansoni* and *S. japonicum* cercariae exhibited highest attachment to media when they were freshly shed rather than aged.

Cercariae of *S. mansoni* and *S. haematobium* have previously been shown to respond to skin lipids [13, 87, 94-96], while *S. japonicum* cercariae appear to be stimulated by skin surface lipids only after they have found a suitable surface to penetrate [97]. Cercarial stimulatory attractants have been applied to the development of baited traps for the detection of free-swimming *S. mansoni* cercariae [28, 29]. In these studies, slides were horizontally oriented and it is unclear as to whether cercarial counts included whole cercariae as well as detached heads and detached tails. In the present study, for both *S. mansoni* and *S. japonicum* cercariae, a stringent and conservative methodology for stimulating and counting cercariae was employed. The focus of this aim was to explore the factors of the parasite-host relationship. As such, media was placed in a vertical position to counter the swimming/floating pattern of the cercariae and only cercarial heads were counted towards our assessment of PCA. The concentration of OA in beeswax (0.3 g/mL) that led to the highest attachment of *S. mansoni* cercariae is equivalent to about 25% OA, which is lower in concentration to what is typically present in mammalian skin (~47%). Attachment of *S. mansoni* cercariae decreased when exposed to media containing 0.9 g/mL of OA, likely because the concentration was higher than physiologically relevant concentrations in the definitive host skin composition.

Age likely plays a substantial role in host infection success rate as cercariae do not feed, depleting their glycogen stores in host-finding [47]. In contrast to the swimming pattern of *S. mansoni* cercariae, *S. japonicum* cercariae float at the surface of

the water, which increases the chance of coming in contact with a host (as the surface of the water is being broken), while minimizing the amount of energy that must be spent [98]. In the present study, *S. mansoni* cercariae exhibited significantly higher attachment to media when they were freshly shed, compared to when they were 10-hours post-shed. These findings echo those of a study conducted by Whitfield et al. [99], wherein the cercarial infectivity of *S. mansoni* was found to be highest within 1-to-9 hours post-emergence. *S. japonicum* cercariae exhibited highest attachment percentages to media when they were freshly shed (beeswax and beeswax with 0.15 g/mL OA) and 5-hours post-shed (beeswax with 0.3 and 0.9 g/mL OA).

The findings from this first aim were the basis for the development of the ESDSC. The media types that elicited the highest attachment in *S. mansoni* and *S. japonicum* cercariae could be harnessed to attract the parasite and the in-field deployment of the device would ideally occur in mid-morning to increase chances of being exposed to freshly shed cercariae since they emerge from snails in a diurnal pattern.

Development and Optimization of the ESDSC: With an approximate starting number of 3000 cercariae in the exposure aquarium, the highest median cercarial capture percentage of 3.3% was achieved with a horizontally-oriented, heated ESDSC that was deployed near the cercarial point of origin and submerged in the water. It should be noted that there were no instances of the device failing to capture any *S. mansoni* cercariae, even at the farthest position tested. This indicates that under similar conditions in the field (similar distance and cercarial concentration), the ESDSC would likely detect *S. mansoni* cercariae if present. In the horizontal position, the ESDSC captured more cercariae on the top of the device compared to the bottom. This may indicate that the

device captured "sinking" cercariae or captured cercariae as they were in the falling sequence of their swimming pattern.

Biomphalaria spp., the intermediate snail host for *S. mansoni*, are aquatic snails that favor habitats with ample aquatic vegetation or rotting plant material and shade from plants that afford protection from extreme sun exposure [100]. Additionally, the source of *S. mansoni* miracidia that infect the snail host is from fecal excretions of infected mammals. These would typically be deposited along the edge of a body of water. Therefore, it is hypothesized that the majority of infected snails reside along the land-water interface. The built-in ramp in the aquarium functioned as a simulated embankment that would likely be found in the endemic environmental setting. As expected, capture of *S. mansoni* cercariae was highest when the device was placed close to the origination point. Given these findings, the ESDSC can be appropriately deployed during field evaluation.

While the application of heat did not appear to significantly increase cercarial capture, the presence of the activated hand-warmer in the device resulted in a higher median cercarial attachment percentage. The particular hand-warmer used in this aim was chosen for a number of reasons, including: the ability to use it underwater, the safety of the material contained in the hand-warmer, the reusability of the product (through boiling), and the potential for its use beyond the ESDSC. The manufacturer reports that the surface of the hand-warmer typically reaches a temperature of 46-49°C and lasts 50-60 minutes, after which the products gradually cools off. It is unknown how much, if any, heat from the hand-warmer was able to penetrate through the ESDSC to be detected by cercariae, as this temperature was not measured. Future studies will include

measuring the surface temperature of the slide, as well as exploration of comparable hand-warmers that reach a higher surface temperature and retain heat for a longer period of time.

In similar studies, wherein baited traps were tested for cercarial retrieval, Shiff et al. [28] and Ahmed et al. [29] exposed a wide range of cercarial concentrations to a trap, constructed of glass slides with linoleic acid as the stimulant, arranged on a triangular stand (25 and 35 cm tall). In the Shiff et al. study, concentrations of 30 and 60 cercariae per liter of water (total of 1,800 and 3,600 cercariae, respectively) were exposed to a baited trap with 28 slides. The average number of cercariae collected on each slide was reported to be 1.5 and 2.8 (30 and 60 cercariae/L concentrations, respectively), which would equate to a calculated 2.3% and 2.2% recovery rate per stand set-up. In the Ahmed et al. study, concentrations of 10 and 25 cercariae per liter of water (total of 12,000 and 30,000 cercariae, respectively) were exposed to five baited traps, each with 35 slides. The average number of cercariae collected on each slide was reported to be 2.5 and 7.2 (10 and 25 cercariae/L concentrations, respectively), which would equate to a calculated 0.73% and 0.84% recovery rate per stand set-up. The ESDSC, which holds 10 slides in a compact and slim design, had a calculated average of 10 cercariae captured per slide. The performance of the device suggests that it has the potential to effectively capture *S. mansoni* cercariae, even at low concentrations.

Several limitations regarding the generalizability and utility of the device exist. The ESDSC does not provide quantitative results, but rather functions as a presence/absence indicator of schistosome cercariae. Additionally, the device itself does not provide information on the species of the schistosome that is captured on the media,

as it is possible for multiple species of schistosome, both human-pathogenic and non, to be co-localized in a geographic area. Microscopic examination of the slides by a skilled technician who is capable of identifying species by morphological differences would be necessary. Furthermore, the media used to attract cercariae may have a limited shelf life due to the incorporation of OA, which is photosensitive. Slides would need to be obtained or made within a few days of being used and kept in the dark until then.

Comparison of ESDSC to Other Environmental Surveillance Methods through Review of Literature: Six categories of environmental surveillance methods for schistosome cercariae were identified in the review of the literature: snail shedding or crushing, water filtration or centrifugation, sentinel mouse, PCR or ELISA, baited trap and C-6 film. Cai et al. [101] described the method of using C-6 film to touch the surface of the water in order to adsorb *S. japonicum* cercariae and subsequently examine the surface of the film under a microscope. This method, however, is only applicable with *S. japonicum* cercariae as it leverages the unique swimming behavior (floating on the surface of the water), which differs from the other species of human-relevant schistosomes. The ESDSC falls under the "baited trap" category. These six surveillance methods were evaluated on five attributes: time and labor, technical training, cost, infrastructure and capital investment, and sensitivity.

Time and Labor

The identification of bodies of water that harbor the parasite is time sensitive when implementing controls to break the transmission cycle. Ideally, an environmental surveillance method should be able to provide results *in situ*. The level of labor associated with testing a body of water is also a factor that should be considered. The

gold standard sentinel mouse method is by far the most time- and labor-intensive method of surveillance. Alternatively, the collection of snails requires minimal labor expenditure but it could potentially take five weeks before adult cercariae emerge [102]. Snail-crushing to look for developing sporocysts can be performed, but visualization can depend on how long ago the snail was infected with miracidia [103]. Water collection followed by filtration or centrifugation can be accomplished within a day, however, the labor associated with it can vary depending on the turbidity of the water. The higher the level of debris, the slower the water will filter and more frequently the filters will become clogged [92]. Centrifugation of water samples tends to take less time than filtration [24] but the equipment can be heavy to transport to sites and it requires a power source [104]. Following filtration or centrifugation of a water sample, the filter can be examined for the presence of cercariae or processed for DNA extraction to look for evidence of cercariae by PCR amplification [105-107]. More recently, DNA extracted from collected snails has been the subject of PCR analysis, rather than looking for cercariae in the water. This eliminates the need to hold a snail until it sheds and can actually detect infection as early as one day post-infection with miracidia [108, 109]. The extraction of DNA and PCR process can be completed within one day, and while it requires some level of skill, the labor demand is relatively low. Finally, the baited trap method, which the ESDSC is categorized in, can provide results within a few hours and requires little labor beyond deploying the device, retrieving it, and examining the slides using a microscope [28-30, 91].

Technical Training

Molecular methods of environmental surveillance require the highest level of technical training. Technicians work with very small volumes and sophisticated equipment, and the interpretation of results requires theoretical understanding of the methods employed. While the sentinel mouse method requires very little high-technological equipment, it does require hands-on perfusion training and an experienced eye to identify worms during dissection, as they are only 7 – 20 mm long and can be easily missed. Water filtration and centrifugation require a moderate amount of training on equipment operation, which occasionally malfunction due to the nature of field work [76]. Because of this, the technician must have the knowledge to troubleshoot the repair on-site. The methods that require the least amount of technical training are snail collection and the use of a baited trap. Holding snails and waiting for cercarial shedding requires no specialized equipment, nor technical training. The drawback of this method lies in the high potential of a long lag time to receive confirmation. The baited trap only requires training on the use of a microscope, as it utilizes a passive method of attracting and trapping cercariae. In addition, it is possible to stain the slides and examine them at a later time, as the integrity of the slide is maintained for weeks after staining [91, 110].

Cost, Infrastructure and Capital Investment

As many of the endemic schistosomiasis areas are rural villages in developing countries; cost, infrastructure and capital investment are relevant factors. Some of the methods are more cost-prohibitive than others, namely the sentinel mouse and molecular methods. The sentinel mouse method is estimated to cost upwards of $100 per mouse [23] which doesn't account for the capital investment in either building or converting

existing infrastructure to house and care for animals during incubation. PCR, on the other hand, is getting less expensive as reagents become more affordable [23, 27]; but like the sentinel mouse method, the capital investment required to run analyses is very expensive, and the equipment must be maintained and calibrated, which introduces recurring costs besides replacing reagents. Water filtration and centrifugation require an initial investment of purchasing the equipment but beyond that, the costs associated with running the equipment are minimal and sample examination merely requires a microscope. Collection of snails and use of a baited trap are the least costly. The harvesting of snails requires a net and holding receptacles, and no specialized holding facility. The ESDSC utilizes materials that are easily sourced; glass slides, and media, which are all inexpensive. Glass slides are reusable as the used media (beeswax and oleic acid) can be melted off, discarded, and replaced. It is estimated that the cost to make one ESDSC device is less than $10 and requires little to no maintenance.

Sensitivity

Snail collection and sentinel mice are the least sensitive of the surveillance methods. The prevalence of schistosome infection in snails varies by season, but is typically lower than 10% [111, 112]. Surveying for infected snails provides a gross underestimate of the number of potential transmission sites [103, 113, 114], with a reported sensitivity of 3% [115]. The use of sentinel mice not only provides information regarding the presence of cercariae, but also whether those cercariae are infective. This method, like the holding of snails, has been shown to suffer from low sensitivity [23-25]. Water filtration and centrifugation in the laboratory setting with spiked samples have shown cercarial recovery rates ranging from 51-80% [77, 78], however, Ouma et al. [26]

found that in the field, samples yielded either infected snails or cercariae (through filtration) and there was no significant difference between the two methods (snail collection and water filtration) in detecting transmission sites. The ESDSC exhibited a lower cercarial capture percentage, which could translate to a higher chance of missed identification of contaminated water. In regards to the use of the device as a presence/absence method, the ESDSC detected presence of cercariae in 100% of experiments under laboratory-controlled settings, even when the device was placed at a distance of nearly one meter away from the point of cercarial origin (Lee et al., manuscript in preparation).

PCR itself is a very sensitive method, able to detect one cercariae in five liters of water and a minute amount of target DNA (10^{-6} ng) [116]. This method, however, must be coupled with either the water filtration or centrifugation method, which, as previously discussed, can have variable outcomes. Hung and Remais [107] were able to positively identify 93% of filtered cercariae-spiked samples using PCR, but the replicates were highly variable, likely due to losses through the filtration step or interference from debris in the water. Alternatively, PCR can also detect prepatent infections in snails with high sensitivity, even when the sample is diluted with DNA from uninfected snails [108].

Limitations

There were a number of methodological limitations that occurred in both of the first two aims. Though the method of cercarial estimation and transfer utilized in this study is standard practice, it is difficult to quantify the viability of the cercariae after they have been handled or pipetted, and there was assumed equal aliquoting of cercariae between experiments. Additionally, the water used in the experiments was still and

extremely clean, free of any debris or contaminants that would normally be found in the environment. Ambient laboratory conditions such as irradiance changes [89] and temperature are factors that may have influenced the attachment of cercariae to media. All overhead laboratory lights were turned on to limit the variability between experiments and there were no extreme temperature variations during the collection of data (average temperature of 23.1°C ± 0.98).

In the first aim of the study, there were challenges with *O. hupensis* snail rearing and subsequent shedding of *S. japonicum* cercariae in high enough numbers (≥ 100) to conduct experiments. In the U.S., these reagents are shipped from a single source, which posed a supply issue when the laboratory moved to a different location and the snails experienced a sudden change in water source and quality that reduced the snail population by half and severely affected the health of the surviving snails. As a result, the sample size for the *S. japonicum* study was limited and it was not possible to conduct the evaluation of a *S. japonicum*-specific ESDSC.

In the second aim of the study, significant differences in cercarial capture were demonstrated with the exploration of the positional attributes of the ESDSC, despite the relatively small sample size (five replicates for each stratification). Optimization of the device would benefit from additional data collection. As mentioned previously, water quality and flow were controlled in the laboratory setting and it is unknown how the performance of the ESDSC may have been affected. The application of heat in the device was evaluated; however, it is unknown how much, if any, heat from the hand-warmer was able to penetrate through the ESDSC to be detected by cercariae. Future

studies should include exploration of comparable hand-warmers that reach a higher surface temperature and retain heat for a longer period of time.

In determining the eligibility of articles for the third aim of the study, 46 were excluded because they were written in a foreign language. A majority of those articles were published in Chinese and likely described environmental surveillance methods for the detection of *S. japonicum* cercariae. Due to the geographic distribution of human-relevant schistosomes, field evaluation of surveillance techniques requires extensive research collaboration with local parties in endemic countries. With any research, biases in reporting can occur, but given the political and economic instability many of these regions experience, there may be additional temptation to do so for personal or community benefit.. The body of literature addressing the matter of environmental surveillance is severely lacking, which made conducting a critical comparison of methods particularly difficult. In addition, the sensitivity of the ESDSC was based on laboratory-controlled experiments, while some (but not all) of the other methods reported sensitivities from field evaluation experiments, making it difficult to make direct comparisons.

Conclusion

The goal of this study was to develop a passive, low-cost, low-technological, rapid device to detect schistosome cercariae in bodies of water and to compare this device to other environmental surveillance methods through the review of published literature. This goal was accomplished in three parts: 1) Explore the use of beeswax with OA to attract schistosome cercariae and determine the combination of media type and cercarial

age that results in highest cercarial attachment in *S. mansoni* and *S. japonicum* cercariae, 2) Apply the combination of media type and age with the highest cercarial attachment in *S. mansoni* to the development of an ESDSC and laboratory optimize the device with regard to positional attributes, and 3) Review published literature to identify other environmental surveillance methods that detect schistosome cercariae in order to assess how the ESDSC compares.

The development of the ESDSC is an important addition to the arsenal of public health tools aimed at disease prevention through the monitoring and surveillance of suspected transmission foci; access to positively identified contaminated bodies of water could be restricted in order to prevent further contraction of infections and mitigation efforts could be applied. The value of the device will have greatest realization in endemic regions where health and economic resources are limited; what may work in one endemic region may not be what works best in another. The availability of infrastructure, resources, and skilled technicians favor the use of molecular methods to survey bodies of water that may be contaminated. The ESDSC could also function as preliminary screening to detect contaminated sites so that complex and costly surveillance methods are more targeted. An effective approach to combat the transmission of schistosomiasis is one that is multi-faceted and should include improvements to sanitary conditions, mass drug treatment of at-risk populations, and community education and engagement, in addition to environmental surveillance

The findings in the first part of the study expand on what is currently known about the chemotactic response of schistosome cercariae, especially that of *S. japonicum*, for which there are limited data. As shown in the review of the literature, there is ground

to be made up in research on *S. japonicum* and *S. haematobium*. Future work includes increasing the sample size for *S. japonicum* and conducting a similar study on *S. haematobium*. Furthermore, as the data for *S. mansoni* was applied to the development of an ESDSC specific to the response of *S. mansoni* cercariae, the data from such studies will inform the development of two additional species-specific devices. Laboratory-optimization followed by field-verification against currently employed environmental surveillance methods is necessary to understand how the ESDSC truly compares.

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Appendix A - Factors that may influence the parasite-host attraction of *Schistosoma mansoni and S. japonicum*: assessment of oleic acid and cercarial age

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Abstract

Host-finding by schistosome cercariae is a process that involves the orchestration of host, parasite, and environmental factors. Building on previously published work, we sought to explore the attraction potential of various concentrations of oleic acid (OA) in beeswax media on *Schistosoma mansoni* and *S. japonicum* cercariae and the role that age of the cercariae plays. Cercariae that were freshly shed, 5-hours post-shed, or 10-hours post-shed were exposed to plain beeswax and three concentrations of OA (0.15, 0.3, and 0.9 g/mL) in beeswax. *S. mansoni* cercariae that were exposed to beeswax containing OA all exhibited statistically significant higher attachment to the media than to beeswax alone ($p < 0.0001$). Median cercarial attachment was highest to beeswax media containing 0.3 g/mL of OA (5.72%; maximum attachment of 30.3%). In contrast, median *S. japonicum* cercariae attachment was highest to beeswax without any OA (5.06%; maximum attachment of 58%) and cercariae attachment decreased as concentration of OA in beeswax increased. For both *S. mansoni* and *S. japonicum*, cercariae exhibited highest attachment to media when freshly shed. Stratification of percent cercarial attachment by media type and age indicate that in this study, optimal attachment for *S. mansoni* was achieved with freshly shed cercariae exposed to media containing 0.3 g/mL of OA ($p = 0.005$) and for *S. japonicum*, was achieved with freshly shed cercariae exposed to plain beeswax. These research findings will be applied to the development of species-specific environmental detection devices for human-relevant schistosome cercariae.

Keywords: *Schistosoma mansoni*, *Schistosoma japonicum*, cercariae, attraction properties, oleic acid, cercarial age, environmental detection

1. Introduction

In 2015, at least 218 million people required prophylactic medication to prevent schistosomiasis and over 66 million people received treatment for the infection (WHO, 2017). While the global schistosomiasis mortality rate is low, symptoms of the disease can be severe and debilitating, worsening with repeat infections (van der Werf et al., 2003). Agricultural workers, fishermen, and children are most at risk of becoming infected by the parasite that causes the disease. Infected children exhibit severe anemia, stunted growth, and decreased ability to learn (Abdelwahab et al., 1993), which can all be major contributors to the cycle of poverty in developing nations. The three main human-relevant species of the parasite are: *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*.

Cercariae, the free-swimming mammalian-infective life stage of the *Schistosoma* spp. parasite, emerge from the snail and infect hosts by attaching to, creeping across, and penetrating the dermis. Factors that stimulate cercarial attraction include both biochemical cues and temperature gradients (Cohen et al., 1980; Fusco et al., 1993; Granzer and Haas, 1986; Haeberlein and Haas, 2008). In the environment, cercariae are diurnally shed, with emergence from the snail host being stimulated by sunlight and reports of highest concentrations occurring between the hours of mid-morning and early afternoon (Ahmed et al., 2006; Asch, 1972; Giovannola, 1936; Rowan, 1958). Age of the cercariae may also influence the success of finding a host as cercariae typically only live 24-36 hours under laboratory conditions and less than 24 hours in the environment (Faust and Huffman, 1934; Lawson and Wilson, 1980), dying when their glycogen energy stores have been depleted.

Oleic acid (OA), a highly abundant unsaturated free fatty acid found in human skin, was previously demonstrated to stimulate *S. mansoni* cercariae to attach to contact media (beeswax)(Lee et al., 2013). Presence of OA at a concentration of 0.3 g/mL in beeswax significantly increased the percent cercarial attachment (PCA) as compared to plain beeswax. Three increasing concentrations of OA in beeswax were tested for its stimulating properties, but as OA concentrations increased above 0.3 g/mL, PCA decreased. Age of cercariae was also a factor that significantly affected PCA on contact media, with maximum cercarial attachment occurring between 0- and 5-hours post-shed cercariae (Lee et al., 2013). Building on previously published work, the goal of this study was to further elucidate the factors that drive parasite-to-host attraction in *S. mansoni* and to explore whether the factors that influence *S. mansoni* cercariae attraction also apply to *S. japonicum* cercariae. We hypothesized that each species of *Schistosoma* cercariae will be differentially attracted to the various concentrations of OA and freshly shed cercariae will exhibit higher attachment to media than aged cercariae.

2. Materials and methods

2.1. Infected Snails

Infected 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. *Biomphalaria glabrata* snails (infected with *S. mansoni* miracidia) and *Oncomelania hupensis* snails (infected with *S. japonicum* miracidia) were reared using standard methods (NIAID Schistosomiasis Resource Center 2015). Snails were checked for patency (cercariae shedding) and positive snails were separated from pre-patent snails. Patent snails were

kept in containers that were shielded from the light in order to maintain their infection and minimize cercarial shedding. The ambient laboratory temperature averaged 23.1°C (± 0.98).

2.2. Estimating Schistosoma mansoni Cercarial Concentrations

Patent *B. glabrata* snails were gently rinsed with aged deionized water (DI water) using a plastic squirt bottle before being transferred into a separate beaker containing aged DI water. The beaker was placed under a light source for up to two hours to induce cercarial shedding. Snails were transferred back into their original containers prior to cercarial enumeration. To ensure homogeneity, the suspension of cercariae was gently agitated with a pipette tip before a 1 mL aliquot was drawn and placed into a glass crucible. Lugol's iodine was used to kill and stain cercariae for ease of counting under the microscope. This process was repeated three more times and an average concentration of cercariae per mL was calculated. Experiments were initiated at the same time on all days to reduce variability and to allow for cercarial aging throughout the day. One hundred to 150 cercariae in 20 mL of aged DI water were used in each experiment.

2.3. Schistosoma japonicum Cercariae Collection

Patent *O. hupensis* snails were rinsed with aged DI water before being transferred into a dry petri dish along with a small wad of dampened paper towel. The dampened paper towel provides a little bit of moisture to the snails as they are drying. The snails were covered with the petri dish lid to prevent escape and allowed to sit over 15 days. On the day that *S. japonicum* cercariae were to be shed, the snails were placed in a beaker with aged DI water and exposed to a light source for up to two hours to induce cercarial shedding. *O. hupensis* snails were removed from the shedding receptacle prior to

cercarial transfer. As *S. japonicum* cercariae float on the surface of the water, a drawn Pasteur pipette was used to count and transfer each cercariae into the beaker in which the experiment would be conducted. One hundred cercariae in 20 mL of aged DI water were used for each experiment.

2.4. Preparation of Exposure Slides- Beeswax with Oleic Acid

Beeswax-coated slides were determined to be the optimal medium for microscopic visualization and slides were prepared according to previously published literature (Lee et al., 2013). Oleic acid was mixed with beeswax at concentrations of 0.15, 0.3, and 0.9 g/mL.

2.5. Oleic Acid as a Stimulant of Attraction

A known concentration of cercariae (100 – 150 cercariae) was transferred from the original shedding receptacle to a 50mL beaker containing aged DI water, making up a total volume of 20mL. Exposure slides (one per beaker) were vertically placed on the inside edge of each beaker. *S. mansoni* cercariae typically swim up and fall down in a figure-eight pattern (Brachs and Haas, 2008) in the water column, while *S. japonicum* cercariae float at the water surface (visual observation); as such, the vertical placement of the slide was applied as an attempt to minimize accidental bumping. Cercariae were exposed to the media for 2 hours at ambient lab temperature (~23°C) and light conditions. Slides were collected and gently dipped into beakers containing water to remove superficially attached cercariae. The slides were placed in a glass slide-staining dish filled with iodine to kill and stain cercariae for microscopic viewing.

2.6. Age of Cercariae as a Factor in Attachment

The experimental methods, as described above, were repeated for three cercarial age points: freshly shed cercariae, 5-hours post-shed cercariae, and 10-hours post-shed cercariae. Cercariae were collected at the start of the day and once shedding snails were removed, no new cercariae would be introduced into the starting population. The cercariae used for each age point originated from the starting population that was collected on the morning of the day of the experiment.

2.7. Data Analyses

All data analyses were conducted using STATA v12 (StataCorp LLP, College Station, TX). Media type and age of cercariae were evaluated for their effect on percent cercarial attachment. The normality of the data was assessed using the Shapiro-Wilks test and histograms. The data were determined to be nonparametric and as such, the Kruskal-Wallis test (alpha level set at $p < 0.05$) and Mann-Whitney post-hoc test with Bonferroni correction was applied to evaluate the differences between each of the groups within the two variables (media type and age of cercariae).

3. Results

3.1. Chemotactic Response

As in our previously published study (Lee et al., 2013), *S. mansoni* cercariae were exposed to plain beeswax, 0.3 g/mL OA in beeswax, and 0.9 g/mL OA in beeswax. Additionally, in the current study, a lower concentration of OA in beeswax, 0.15 g/mL, was tested to determine whether it would result in higher attachment of cercariae to media. In comparing the data from our previously published study against the newly

collected data, both data sets exhibited similar trends regarding the relationship between percent cercarial attachment (PCA) and media, and PCA and age of cercariae.

As shown in Table 1, for *S. mansoni*, the median PCA were 0%, 1.47%, 5.72%, and 3.64% for beeswax with 0, 0.15, 0.3, and 0.9 g/mL OA, respectively. These values were statistically significantly higher than the PCA on plain beeswax for all concentrations of OA tested ($p < 0.0001$). The PCA on beeswax with 0.3 and 0.9 g/mL OA were also higher than that of 0.15 g/mL of OA in beeswax ($p < 0.0001$). The same experiments were conducted on *S. japonicum* cercariae to explore whether OA could elicit a chemotactic response. The median PCA of *S. japonicum* to plain beeswax, 0.15, 0.3, and 0.9 g/mL OA were 5.06%, 2.07%, 1%, and 1.23%, respectively (Table 1). The PCA on plain beeswax was statistically significantly higher than on beeswax with 0.3 and 0.9 g/mL of OA ($p < 0.003$). The difference between PCA on plain beeswax and 0.15 g/mL OA in beeswax, the lowest concentration of OA tested, was not statistically significant. For this study, as with our previous study, we were most interested in counting the number of cercarial heads that were attached to the media since that was considered a true attempt at attachment and penetration. Additional analysis revealed that when the number of whole cercariae were included in the head-only count, it did not make a difference, statistically, and only minimally increased maximum and median PCA values.

Table 1. Descriptive statistics for percentage of *Schistosoma mansoni* and *S. japonicum* cercariae attached to different media types.

			Cercariae Head Only (% attached)		Head and Whole Cercariae (% attached)	
	Media Type	n	Max	Median	Max	Median
S. manso	Beeswax	110	8.33	0	9.17	0
	Beeswax with oleic acid (0.15 g/mL)	60	10	1.47[a]	12.31	1.52[a]

	Beeswax with oleic acid (0.3 g/mL)	110	30.3	5.72[a,b]	30.3	6.16[a,b]
	Beeswax with oleic acid (0.9 g/mL)	110	26.95	3.64[a,b]	27.83	3.91[a,b]
S. japonicum	Beeswax	30	58	5.06	58.25	5.23
	Beeswax with oleic acid (0.15 g/mL)	30	29	2.07	29.03	2.07
	Beeswax with oleic acid (0.3 g/mL)	29	19.57	1[c]	19.59	1.03[c]
	Beeswax with oleic acid (0.9 g/mL)	29	22	1.23[c]	22.12	1.25[c]

Note: Minimum values were zero for all media types tested.
a Statistically significantly different from beeswax media, $p < 0.0001$
b Statistically significantly different from beeswax with oleic acid (0.15 g/mL), $p < 0.0001$
c Statistically significantly different from beeswax media, $p < 0.003$

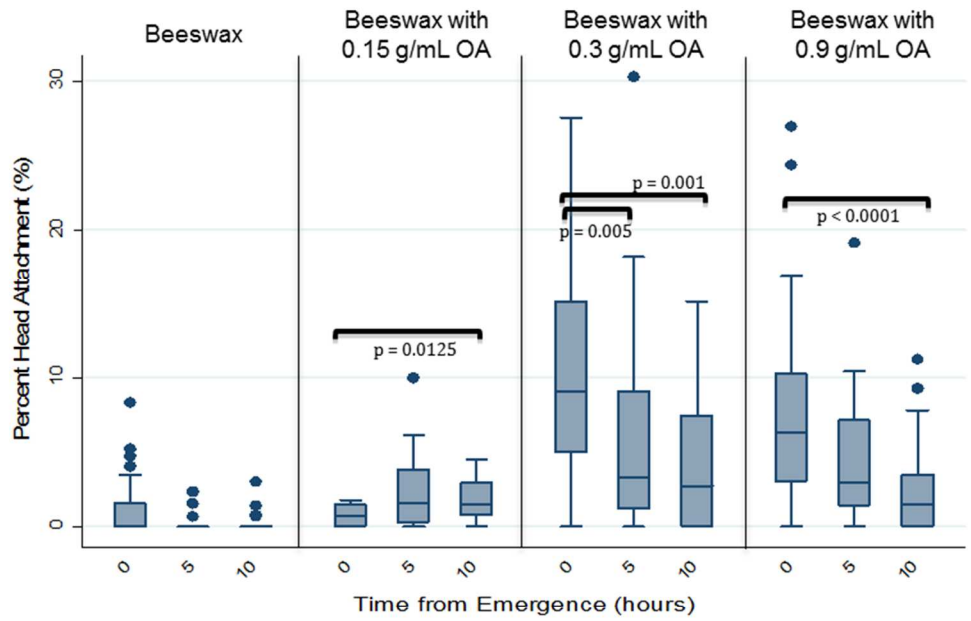
3.2. Age of Cercariae and Chemotactic Response

For *S. mansoni*, combining all media types, the median values for PCA of the three age groups tested were 3.03%, 1.49% and 0.83% for freshly shed, 5-hours, and 10-hours post-shed, respectively (data not shown, before media-type stratification). There was a statistically significant difference in percent attachment between freshly shed cercariae and the other two age points tested, but no difference between 5-hours and 10-hours. For *S. japonicum*, combining all media types, percent attachments for freshly shed, 5-hours and 10-hours post-shed cercariae were 2.61%, 2.18%, and 1.27%, respectively (data not shown, before media-type stratification). The PCA of freshly shed and 5-hours post-shed cercariae were not statistically significantly different, but there was a statistically significant difference between freshly shed and 10-hours post-shed cercariae.

Percent cercarial attachment was stratified first by media type and then by age to determine the combination of the two factors that yielded the highest attachment (Figures 1 & 2). Statistically significant differences in PCA between age points existed in all media types with OA, regardless of concentration, for *S. mansoni* cercariae (Figure 1). On beeswax with 0.3g/mL of OA, the PCA for freshly shed cercariae was statistically significantly higher than for 5-hours and 10-hours post-shed cercariae ($p = 0.005$ and $p = 0.001$, respectively). On media containing 0.15 g/mL OA in beeswax, freshly shed

cercariae exhibited lower attachment percentage than 10-hours post-shed cercariae (p = 0.0125). For *S. japonicum* cercariae, there were no statistically significant differences in PCA between the age points within each media type; however, median PCA values for freshly shed cercariae were highest and generally decreased as the cercariae aged throughout the day (Figure 2).

A



B

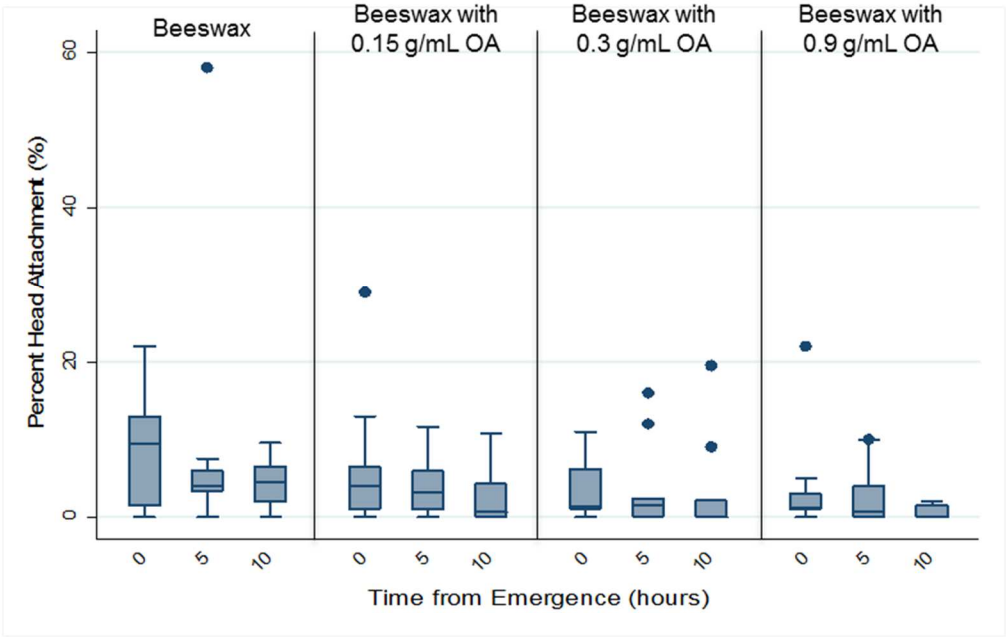
				Cercariae Head Only (% attached)		Head and Whole Cercariae (%attached)	
	Media	Age	n	Max	Median	Max	Median
S. mansoni	Beeswax	Fresh	50	0	0	9.17	0.3
		5 h	30	2.38	0	2.38	0
		10 h	30	3.03	0	3.03	0
	Beeswax with oleic acid (0.15 g/mL)	Fresh	20	1.79	0.74[b]	3.57	1.52
		5 h	20	10	1.54	12.31	1.54
		10 h	20	4.55	1.52	5.3	1.89
	Beeswax with oleic acid (0.3 g/mL)	Fresh	50	27.52	9.1[a,b]	29.36	10.05[a,b]
		5 h	30	30.3	3.33	30.3	3.96
		10 h	30	15.2	2.75	18.18	2.75
	Beeswax with oleic acid (0.9 g/mL)	Fresh	50	26.96	6.29[b]	27.83	6.39[b]
		5 h	30	19.13	3	19.13	3.8[c]
		10 h	30	11.25	1.52	12.5	1.9

Note: Minimum values were zero for all media types tested.

a Statistically significantly different from 5-hours post-shed, p = 0.005
b Statistically significantly different from 10-hours post-shed, p < 0.0125
c Statistically significantly different from 10-hours post-shed, p = 0.0131

Figure 1. (A) *Schistosoma mansoni* cercarial attachment stratified by media type (beeswax, 0.15 g/mL oleic acid (OA) in beeswax, 0.3 g/mL OA in beeswax, and 0.9 g/mL OA in beeswax) and age of cercariae (0-hours, 5-hours, and 10-hours from time of emergence). (B) Descriptive statistics for percentage of *S. mansoni* cercariae attached to different media types, stratified by age of cercariae.

A



B

				Cercariae Head Only (% attached)		Head and Whole Cercariae (%attached)		
	Media	Age	n	Max	Median	Min	Max	Median
S. japonicum	Beeswax	Fresh	10	22	9.5	0.05	22.23	9.63
		5 h	10	58	4	0.4	58.25	4.08
		10 h	10	9.52	4.48	0.01	9.83	4.64
	Beeswax with oleic acid (0.15 g/mL)	Fresh	10	29	4	0	29.03	4.01
		5 h	10	11.67	3.16	0	11.67	3.2
		10 h	10	10.74	0.63	0	10.75	0.63
	Beeswax with oleic acid (0.3 g/mL)	Fresh	9	11	1.43	0	11.01	1.44
		5 h	10	16	1.61	0	16.11	1.61
		10 h	10	19.57	0	0	19.59	0
	Beeswax with oleic acid (0.9 g/mL)	Fresh	9	22	1.25	0	22.12	1.26
		5 h	10	10	0.71	0	10.07	0.71
		10 h	10	2.06	0	0	2.06	0

Note: Minimum values were zero for all media types tested, cercariae head only

Figure 2.(A) *Schistosoma japonicum* cercarial attachment stratified by media type (beeswax, 0.15 g/mL oleic acid (OA) in beeswax, 0.3 g/mL OA in beeswax, and 0.9 g/mL OA in beeswax) and age of cercariae

(0-hours, 5-hours, and 10-hours from time of emergence). (B) Descriptive statistics for percentage of *S. japonicum* cercariae attached to different media types, stratified by age of cercariae.

4. Discussion

Building from previous work, this study aimed to evaluate implications of lower OA concentrations in beeswax on *S. mansoni* cercarial attraction and attachment. Further, these factors were applied and evaluated for S. *japonicum*. The chemotactic response in *S. japonicum* cercariae appears to differ from that of *S. mansoni*, as the highest PCA for *S. japonicum* was observed on plain beeswax, while presence of OA (regardless of concentration) in beeswax resulted in a significant increase in *S. mansoni* cercarial attachment. Our data also suggest that cercarial age plays a role in the attachment of *S. mansoni* cercariae, with freshly shed cercariae exhibiting statistically significantly higher attachment than aged cercariae. In the case of *S. japonicum* cercariae, the trend indicates that freshly shed cercariae also appear to attach at a higher percentage than older cercariae, but there was no statistically significant difference among the age points tested.

Cercariae of *S. mansoni* and *S. haematobium* have previously been shown to respond to skin lipids, specifically the fraction containing unsaturated free fatty acids (Austin et al., 1972; Haas et al., 2008; Haas and Schmitt, 1982; Haeberlein and Haas, 2008; Shiff et al., 1972). As a follow-up to these findings, cercarial stimulatory attractants have been applied to the development of baited traps for the detection of free-swimming *S. mansoni* cercariae (Ahmed et al., 2002; Shiff et al., 1993). In these studies, the traps were oriented such that the slides with exposure media were in the horizontal

position and it is unclear as to whether cercarial counts included whole cercariae as well as detached heads and detached tails. In the present study, for both *S. mansoni* and *S. japonicum* cercariae, we were stringent and conservative with our methods of stimulating and counting cercariae. The focus of this research was to explore the factors of the parasite-host relationship, not to maximize cercarial retrieval. As such, media was placed in a vertical position to counter the swimming/floating pattern of the cercariae and only cercarial heads were counted towards our assessment of PCA. In our previous study, *S. mansoni* cercariae attached to beeswax media containing 0.3 g/mL of OA at the highest percentage and PCA decreased as the concentration of OA increased. In this study, we found that decreasing the concentration of OA to 0.15 g/mL of beeswax did not result in higher PCA.

Historically, the majority of research conducted on schistosome cercariae has been on *S. mansoni* due to the ease of rearing and maintenance of *Biomphalaria* spp. snails. There remains a large knowledge gap in what is known about the attraction and penetration behaviors of *S. japonicum* cercariae. Haas et al. (1987) reported that *S. japonicum* cercariae were not found to be stimulated to swim toward a host through chemical or thermal cues; however, once cercariae were able to find and cling onto a surface, the act of penetrating appeared to be stimulated by skin surface lipids. In our study, the PCA on media containing OA was lower than on plain beeswax. There was a statistically significant difference between PCA on plain beeswax and PCA on beeswax with 0.3 and 0.9 g/mL of OA. This appears to support findings by Haas et al., that attraction may not be elicited by a chemical stimulus. As seen in Table 1, the sample size for the *S. japonicum* experiments was much smaller than that of *S. mansoni* and is a

testament to the difficulty in working with this organism and the intermediate snail host, *Oncomelania* spp. It is possible that with increasing the sample size, we could start to see greater significant differences emerge in PCA to plain beeswax versus beeswax with OA.

Age of the cercariae likely plays a substantial role in host infection success rate. Cercariae do not feed and as they expend energy in swimming, their glycogen stores are depleted (Lawson and Wilson, 1980). In contrast to the swimming pattern of *S. mansoni* cercariae, *S. japonicum* cercariae float at the surface of the water, which increases the chance of coming in contact with a host (as the surface of the water is being broken), while minimizing the amount of energy that must be spent (He et al., 2005). In the present study, *S. mansoni* cercariae exhibited a significantly higher attachment percentage to media when they were freshly shed, compared to 10-hours aged cercariae, on beeswax with 0.3 and 0.9 g/mL OA. These findings echo those of a study conducted by Whitfield et al. (2003), wherein the cercarial infectivity of *S. mansoni* was found to be highest within 1-to-9 hours post-emergence. *S. japonicum* cercariae exhibited highest attachment percentages to media when they were freshly shed (beeswax and beeswax with 0.15 g/mL OA) and 5-hours post-shed (beeswax with 0.3 and 0.9 g/mL OA); however, tests reveal that these differences were not statistically significant. Again, as PCA was stratified by both media and age, the sample size in each group decreased, which likely reduced statistical power. Using the knowledge that: 1) the attachment of cercariae appears to be highest from the period when they are freshly shed to 5-hours post-shed, and 2) that cercariae emerge from snails in a diurnal pattern, we can leverage these two factors to maximize cercarial capture for both species.

While the statistical analysis may have been impacted by the small sample size for *S. japonicum* experiments, the behavior of both species of *Schistosoma* cercariae may have been affected by the laboratory conditions under which the experiments were performed. Irradiance changes (Brachs and Haas, 2008) and ambient temperature are factors that may have influenced the attachment of cercariae to media. All overhead laboratory lights were turned on to limit the variability between experiments. Changes in ambient temperature were more difficult to control, due to the open-bay configuration of the space; however, the laboratory temperature is thermostat-controlled and no extreme temperature variations were observed during the collection of data (average temperature of 23.1°C ± 0.98). Additional limitations of this study include: assumed equal aliquoting of cercariae, assumed viability of cercariae, and potential dislodging of attached cercariae from slides.

The knowledge gained from this research is important for the advancement of public health initiatives that are aimed at prevention of the disease through monitoring and identifying contaminated bodies of water. The development of a passive, low-cost, low-technological device informed by the findings in our research would hold the greatest value in endemic regions where health and economic infrastructure are severely lacking.

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Appendix B - Development of an Environmental Surveillance Device for *Schistosoma mansoni* Cercariae

Paper prepared for submission to Environmental Science and Technology

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Abstract

Schistosomiasis is a preventable disease as it is contracted through contact with water that is contaminated with cercariae. Access to clean water is the most effective method to prevent transmission of the disease but lack of infrastructure makes control difficult. Current methods of environmental detection of the parasite in bodies of water are time- and labor-intensive, requiring resources that are unavailable in endemic rural regions. The aim of this study is to develop and optimize a passive, low-technological, low-cost environmental surveillance device for schistosome cercariae (ESDSC). Freshly shed *S. mansoni* cercariae were exposed to the ESDSC in different orientations, depths, and at different distances. All experiments were performed in an aquarium and the cercarial capture efficiency was recorded. The highest median cercarial capture of 3.3% ($p < 0.033$) was achieved with a horizontally oriented ESDSC, deployed near the cercarial point of origin and submerged 14 cm from the surface of the water. Heat was also explored as a factor of the device but there was no significant difference in cercarial capture with and without the inclusion of a heat source. A device for the detection of *S. mansoni* cercariae in water has been developed and laboratory optimized in this study. .

Keywords: *Schistosoma mansoni*, environmental surveillance, baited trap, cercarial capture, device development.

1. Introduction

Schistosomiasis is a significant parasitic disease that causes devastation to human health; yet, it is highly preventable as individuals are infected through dermal contact with contaminated waters. Poor sanitation is the leading factor as to why so much of the world's population is still at risk of contracting the disease, but a lack of infrastructure makes transmission control difficult. Targeted mass treatment of communities with praziquantel has been shown to be extremely effective in some regions [1-3], while not in others [4], suggesting that *Schistosoma* worms are becoming resistant to the drug [5, 6].

Arguably, the most effective method in breaking the transmission cycle is to provide access to clean water and proper disposal of human and animal waste. An important component of access to clean water is the ability to quickly identify unsafe sources and disseminate that information to surrounding communities. Current methods of environmental detection of the parasite are time- and labor-intensive, requiring resources that can be difficult to access in schistosomiasis endemic areas. The gold standard method uses a rodent exposure model, wherein caged sentinel mice are immersed, such that only their legs and belly are submerged. This method can take 2 – 3 months as rodents are reared for 6-14 weeks (allowing any cercariae to mature into worms) and then sacrificed to identify presence of *Schistosoma* worms [7]. Alternatively, water samples from bodies of water can be tested through polymerase chain reaction (PCR) and qualitative PCR (qPCR) to detect *Schistosoma* cercariae. These laboratory-based methods are sensitive and quantitative (qPCR), capable of detecting a minimum of one cercariae [8]. Another method that has been explored is detection of infection in the snail intermediate host, which is accomplished by either crushing the snail to look for

developing parasites or holding snails over a period of days to weeks to look for cercarial shedding. Finally, a simple differential filtration technique has been explored in which large volumes of water are collected and passed through a series of filters, varying in pore-size and subsequently stained and examined under the microscope for evidence of cercariae [9].

Baited traps have also been explored wherein knowledge regarding biochemical cues have been applied to attract cercariae [10, 11], but these methods have not garnered widespread use. Temperature gradients have also been shown to induce cercarial attraction [12, 13] but have not been explored in application to such a baited trap. In our previous study, we investigated various concentrations of oleic acid (OA) in beeswax as a medium for attracting cercariae and found that among the concentrations tested, freshly shed *S. mansoni* cercariae exhibited highest attraction to 0.3 g/mL of OA in beeswax [14]. In that study, cercariae were exposed to media in a 50 mL beaker (equivalent cercarial concentration of 5,000 – 7,500 cercariae per L). The aim of this study is to implement our previous findings to the development of a passive, low-technological, low-cost, rapid device capable of detecting *S. mansoni* cercariae, and to optimize cercarial capture through exploiting positional attributes.

2. Materials and methods

2.1. Environmental Surveillance Device for Schistosome Cercariae (ESDSC)-Prototype Development

An acrylic box with dimensions of 15.75 cm x 10 cm x 2.5 cm was constructed to allow for the insertion of a reusable hand-warmer within its cavity. Metal microscope

slide holders were glued onto the long sides of the box, capable of holding five slides (7.5 cm x 2.5 cm standard sized slides) on each side (Figure 1). The reusable hand-warmers were purchased from HotSnapZ (Stratikore, Inc Company, La Porte, IN).

2.2. Aquarium Design

A 114-liter fish aquarium (dimensions approximately 76 cm x 45 cm x 30.5 cm) was used to perform all experiments in a controlled laboratory setting. A 24° angled ramp was installed to simulate an embankment. The ramp decreased the water capacity of the aquarium to 57.6 L when filled. A spigot was also installed at the "deep" end of the aquarium to allow for drainage after each experiment (Figure 1).

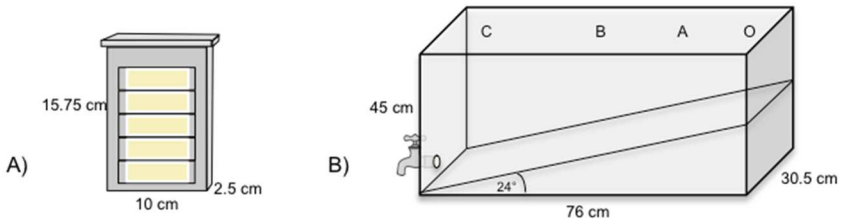

Figure 1. (A) Environmental Surveillance Device for Schistosome Cercariae (ESDSC) prototype with removable lid for insertion of reusable hand-warmer (not to scale). (B) Aquarium in which the ESDSC was tested for cercarial capture (not to scale). Position O was the cercarial origin. Position A was 24.5 cm away, position B was 43.5 cm away, and position C was 70 cm away from position O.

2.3. Schistosoma mansoni Cercariae Collection and Exposure Slides

Infected 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 methods for snail rearing, and cercariae collection and concentration estimation from Lee et al. [14] were followed. Exposure slides were prepared using the method described in the aforementioned study. Briefly, a thin layer of melted beeswax with 0.3 g/mL of OA was spread across a glass slide with a metal spatula and allowed to dry for at least three hours

before being used in experiments. Slides were kept covered to prevent gravitational settling of dust on the media. Three thousand cercariae were used in each experiment. Therefore, the concentration of cercariae in the aquarium was 52 cercariae per liter of water (for comparison, the concentration of cercariae in the beaker study (Lee et al., 2013) was equivalent to 5,000 – 7,500 cercariae per liter).

2.4. Data Analyses

All data were analyzed using STATA v12 (StataCorp LLP, College Station, TX). The normality of the data was assessed using the Shapiro-Wilks test and histograms. The Kruskal-Wallis test with Mann-Whitney post-hoc test (non-parametric data) and the t-test (parametric data) were applied to evaluate the differences between each of the groups. The alpha level for statistical significance was set to $p < 0.05$. Differences in percent cercarial attachment with regard to device distance from cercarial point of origin, device orientation, device depth, and application of heat (in device) were examined. In the case of the horizontally oriented ESDSC, the cercarial capture percentage on the top of the device was also compared to that of the bottom of the device. Due to the uncertainty surrounding whether an observed detached cercariae head and detached cercariae tail were from the same whole cercariae, a range of percent cercarial attachment values was recorded. The "worst-case" value assumes that detached heads and tails are paired and the "best-case" value assumes that detached heads and tails came from different cercariae. Data analyses were performed on both the worst- and best-case values.

2.5. Experimental Conditions

2.5.1. Distance from Cercarial Point of Origin and Orientation of ESDSC

The ESDSC (containing the activated hand-warmer) was placed at three distances along the aquarium, at the surface of the water. Position A was closest to the origin of *S. mansoni* cercariae release, position B was in the middle of the aquarium, and position C was farthest (Figure 1), at distances of 24.5, 43.5, and 70 cm away, respectively. At each of the three distances, the ESDSC was tested in two orientations: horizontal and vertical (or perpendicular) to the surface of the water. The device was tested for cercarial capture at each distance and orientation five times. The ESDSC was left in the aquarium for a 2-hour time period before being retrieved. The slides were removed from the slide holder, stained with Lugol's iodine, and examined under a light microscope. The number of whole cercariae, detached heads, and detached tails were recorded.

2.5.2. Depth of ESDSC

Data from section *2.5.1. Distance from Cercarial Point of Origin and Orientation of ESDSC* were analyzed to determine the optimal orientation or distance at which the highest percentage of cercariae were captured. The ESDSC (containing the activated hand-warmer) was placed in the orientation that resulted in the highest percentage of captured cercariae at the three distances along the aquarium, but this time it was submerged, just hovering over the ramp at each location. The depths at which the ESDSC was placed were 14, 23, and 34 cm from the surface of the water at positions A, B, and C, respectively. Again, the ESDSC was left in the aquarium for a 2-hour period. The slides were removed, stained, and examined. A total of five data points were collected at each

submerged position along the aquarium. These data were compared to the corresponding surface data.

2.5.3. Application of Heat

Data from section *2.5.2. Depth of ESDSC* were analyzed to determine whether depth was a significant contributor to cercarial capture. The combination of distance, orientation, and depth at which the highest cercarial attachment was observed was used to test heat as a factor of cercarial attraction and attachment. The ESDSC without the hand-warmer was exposed to cercariae for a 2-hour time period, removed from the aquarium, and the slides were stained and examined. The ESDSC without the hand-warmer was tested in this spatial configuration five times and compared to corresponding data from the ESDSC with the hand-warmer in the cavity.

3. Results

3.1. Distance from Cercarial Point of Origin and Orientation of ESDSC

Median cercarial capture values were, for the most part, highest at position A, closest to the origination point of the cercariae (Table 1). The difference in the percentage of cercariae captured at each position was not statistically significant; however, cercarial capture was significantly different, dependent on the orientation of the device for both the worst- and best-case values ($p < 0.001$). The horizontal orientation of the device consistently yielded significantly higher median values than the vertical orientation for cercarial capture at each position tested ($p < 0.05$). The highest median cercarial capture percentage of 2.63% was observed at position A with the device in the horizontal orientation. In the horizontal orientation, the ESDSC captured a significantly

($p < 0.05$) higher number of *S. mansoni* cercariae on the top of the device (facing the surface of the water) than on the bottom of the device (facing the bottom of the aquarium) (data not shown).

Table 1. *Schistosoma mansoni* cercarial capture (starting number of 3000 cercariae) on the Environmental Surveillance Device for Schistosome Cercariae, testing distance from cercariae point of origin and orientation of device at the surface of the water.

		Worst-case (% attached)			Best-case (% attached)		
Distance from Cercariae Origination	Orientation	Median	Min	Max	Median	Min	Max
Position A (Closest)	Vertical	0.47	0.17	0.97	0.7	0.27	1.5
	Horizontal	1.53[a]	0.87	4.07	2.63[a]	1.6	5.93
Position B (Middle)	Vertical	0.27	0.03	0.57	0.33	0.03	0.9
	Horizontal	0.97[a]	0.47	1.4	1.5[a]	0.67	2.17
Position C (Farthest)	Vertical	0.47	0.23	0.7	0.7	0.27	1.07
	Horizontal	0.9[a]	0.57	1.67	1.6[a]	0.87	2.73

Note: Worst case values assume that detached heads and tails are paired, equaling one whole cercariae and best case values assume that detached heads and tails are from different cercariae.
a Statistically significantly different from vertical orientation at that position, $p < 0.05$

3.2. Depth of ESDSC

The horizontal orientation of the ESDSC was held constant while depth of the device as a factor of cercarial capture was examined. The median cercarial capture percentage values at each position along the aquarium and each water level are shown in Table 2. There was a statistically significant difference in the best-case value for cercarial capture between the three positions ($p < 0.05$), with position A exhibiting the highest percentage of cercariae attached to media on the device when compared to positions B and C. The worst-case value for cercarial capture percentage was not statistically significantly different between positions along the aquarium ($p = 0.053$). The cercarial capture percentage of the ESDSC was statistically significantly higher when the device was submerged, compared to water-surface placement ($p < 0.05$ for both worst-

and best-case values). The highest median cercarial capture percentage of 3.3% was observed at position A with the ESDSC submerged in the horizontal orientation.

Table 2. *Schistosoma mansoni* cercarial capture (3000 starting) on Environmental Surveillance Device for Schistosome Cercariae, testing distance from cercariae point of origin and depth, with device in horizontal orientation.

		Worst-Case (% attached)			Best-Case (% attached)		
Distance from Cercariae Origination	Water level	Median	Min	Max	Median	Min	Max
Position A (Closest)	Surface	1.53	0.87	4.07	2.63	1.6	5.93
	14 cm from Surface	2.1	1.5	2.57	3.3	2.27	4.4
Position B (Middle)	Surface	0.97	0.47	1.4	1.5	0.67	2.17
	23 cm from Surface	1.33	0.8	2.27	2.4	1.4	3.23
Position C (Farthest)	Surface	0.9	0.57	1.67	1.6	0.87	2.73
	34 cm from Surface	1.37	0.97	1.8	2.13	1.47	2.9

Note1: Worst case values assume that detached heads and tails are paired, equaling one whole cercariae and best case values assume that detached heads and tails are from different cercariae.
Note2: The device in position A performed statistically significantly better than in positions B and C ($p = 0.019$ and $p = 0.038$, respectively; best-case value) and also when the device was submerged, rather than at the water surface ($p < 0.033$; worst- and best-case))

3.3. Application of Heat

The ESDSC was submerged horizontally in position A while the application of heat was evaluated for cercarial capture. The best-case mean value for percentage of cercariae captured by the device was higher with the application of heat; however, this difference was not statistically significant ($p > 0.28$, Table 3).

Table 3. *Schistosoma mansoni* cercarial capture (3000 starting) on Environmental Surveillance Device for Schistosome Cercariae, testing the application of heat.

	Worst-case (% attached)			Best-case (% attached)		
Application of Heat	Mean	Min	Max	Mean	Min	Max
Hand-Warmer	2.05	1.5	2.57	3.37	2.27	4.4
No Hand-Warmer	2.09	1.6	2.57	2.87	1.93	3.6

Note: Worst case values assume that detached heads and tails are paired, equaling one whole cercariae and best case values assume that detached heads and tails are from different cercariae.

4. Discussion

The aim of this study was to develop a device to attract and trap *S. mansoni* cercariae and explore attributes of such a device to maximize cercarial capture. With an approximate starting number of 3000 cercariae in the exposure aquarium, the highest median cercarial capture percentage of 3.3% was achieved with a horizontally-oriented, heated ESDSC that was deployed near the cercarial point of origin and submerged in the water. While the highest cercarial capture was observed with a heated device, it was not significantly higher than that of the un-heated ESDSC. It should be noted that there were no instances of the device failing to capture any *S. mansoni* cercariae, even at the farthest position tested. This indicates that under similar conditions in the field (similar distance and cercarial concentration), the ESDSC would likely detect *S. mansoni* cercariae if present. In the horizontal position, the ESDSC captured more cercariae on the top of the device compared to the bottom. This may indicate that the device captured "sinking" cercariae or captured cercariae as they were in the falling sequence of their swimming pattern.

The built-in ramp was intended to simulate an embankment that would likely be found in the endemic environmental setting. *Biomphalaria spp*., the intermediate snail host for *S. mansoni*, are aquatic snails that favor habitats with ample aquatic vegetation or rotting plant material and shade from plants that afford protection from extreme sun exposure [15]. Additionally, the source of *S. mansoni* miracidia that infect the snail host is from fecal excretions of infected mammals. These would typically be deposited along the edge of a body of water. Therefore, we hypothesize that the majority of infected snails reside along the land-water interface. In our study, the device placed close to

where the cercariae originated exhibited the highest cercarial capture, and were detectable at the farthest position, at a distance of 70 cm. Given these findings, we can appropriately deploy the ESDSC during field evaluation.

While cercarial capture was not significantly different between the ESDSC with heat and without, the application of heat yielded higher percentages of cercariae found on media. The particular hand-warmer used in our study was chosen for a number of reasons, including: the ability to use it underwater, the safety of the material contained in the hand-warmer, the reusability of the product (through boiling in water), and the potential for its use beyond the ESDSC. The manufacturer reports that the surface of the hand-warmer typically reaches a temperature of 46-49°C and lasts 50-60 minutes, after which the products gradually cools off. It is unknown how much, if any, heat from the hand-warmer was able to penetrate through the ESDSC to be detected by cercariae, as this temperature was not measured. Future studies should include measuring the surface temperature of the slide, as well as exploration of comparable hand-warmers that reach a higher surface temperature and retain heat for a longer period of time.

Freshly shed *S. mansoni* cercariae were previously shown to have a chemotactic response to beeswax media containing 0.3 g/mL of oleic acid [14]. In our 2013 study, cercariae, at a concentration of 5,000 - 7,500 per liter of water, were exposed to media in a small beaker containing 20 mL of water; the highest median cercarial attachment was 10.05%. In our current study, the concentration of cercariae used in each experiment was significantly lower, at 52 per liter of water. Despite this 100-fold lower cercarial concentration (in comparison to the beaker study), the highest median cercarial capture was 3.3%, only three times lower than that of the previous study. In similar studies,

wherein baited traps were tested for cercarial retrieval, Shiff et al. [10] and Ahmed et al. [11] exposed a wide range of cercarial concentrations to a trap, constructed of glass slides with linoleic acid as the stimulant, arranged on a triangular stand (25 and 35 cm tall). In the Shiff et al. study, concentrations of 30 and 60 cercariae per liter of water (total of 1,800 and 3,600 cercariae, respectively) were exposed to a baited trap with 28 slides. The average number of cercariae collected on each slide was reported to be 1.5 and 2.8 (30 and 60 cercariae/L concentrations, respectively), which would equate to a calculated 2.3% and 2.2% recovery rate per stand set-up. In the Ahmed et al. study, concentrations of 10 and 25 cercariae per liter of water (total of 12,000 and 30,000 cercariae, respectively) were exposed to five baited traps, each with 35 slides. The average number of cercariae collected on each slide was reported to be 2.5 and 7.2 (10 and 25 cercariae/L concentrations, respectively), which would equate to a calculated 0.73% and 0.84% recovery rate per stand set-up. In our current study, we report a calculated average of 10 cercariae per slide on the ESDSC, which holds 10 slides in a compact and slim design. The performance of our device suggests that it has the potential to effectively capture *S. mansoni* cercariae, even at low concentrations.

The goal in developing the ESDSC was to design a device that would not only effectively detect presence of *S. mansoni* cercariae, but also be simplistic, using easily-sourced materials. Additionally, the device would ideally require little maintenance, repair, and training, as well as minimal technical experience and scientific infrastructure. The methods of environmental detection that are currently in practice are time- and labor-intensive or require highly trained individuals and expensive equipment. Challenges with the gold standard sentinel mouse method of screening bodies of water for infective

schistosome cercariae include frequent loss of mice, not only to drowning but also in the animal holding facility after being exposed to potentially infected bodies of water. In recent years, researchers have explored the use of new technologies to screen the sera of sentinel mice for infection, which significantly decreased the required holding time [16, 17]. However, the dilemma of scientific infrastructure inadequacy persists with the use of this method. Detection of cercariae in water by PCR (which requires the collection, filtration, and elution of large volumes of water) has been shown to have a 93% efficacy rate, but there are complications with environmental inhibition of amplification products due to high concentrations of non-target DNA [18] and equipment is expensive to purchase as well as maintain. Researchers have found that surveying the disease by means of detecting snail infection through crushing provided a major underestimate (sensitivity of 3%) of the presence of infection in humans [19]. In the study, snails were collected, crushed, and examined for presence of infection (cercariae) under a dissecting microscope. Researchers found only one snail that positively screened for the infection out of over 7,000 collected. Detecting the infection in snails is highly reliant on the developmental stage of the parasite found within the intermediate host and does not directly elucidate the exposure risk to the primary host. The method of holding snails and waiting for cercarial shedding requires no specialized equipment, nor technical training, however, researchers have found that the snail infection rates are extremely low [20], as with the method of snail crushing. Finally, while filtering water to concentrate cercariae requires virtually no expertise, limitations with this approach include the necessity in collecting and transporting large, potentially contaminated volumes of water, as well as the presence of debris, which can quickly clog the filters [21]. This method, as with the

ESDSC, however, only informs of the presence of cercariae and not infectivity, whereas the gold standard mouse model confirms that there are cercariae capable of infecting a host.

In our study, we were able to statistically show that a combination of attributes of the ESDSC could lead to higher cercarial capture, despite our relatively small sample size (five replicates for each stratification). Optimization of the device would benefit from additional data collection. The water used in our experiments was extremely clean, free of any debris, contaminants, or other species of cercariae that may found in the environment. In *S. mansoni* endemic regions, *S. haematobium* and non-human pathogenic schistosomes may be co-localized and it is unknown how the ESDSC would perform under those conditions and whether other species of schistosome cercariae would be detected. Additionally, the water in the aquarium was still, devoid of any current that may have affected cercarial swimming pattern. These variables were controlled in our lab setting, which may have influencedthe performance of the ESDSC. Though the method of cercarial estimation and transfer utilized in this study is standard practice, it is difficult to quantify the viability of the cercariae after they have been handled or pipetted. The aquarium aspect of our experimental design made visualization of the cercariae (to check for live/swimming cercariae) virtually impossible.

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Appendix C - Review and Comparison of Environmental Surveillance Methods for Schistosome Cercariae.

Paper prepared for submission to Parasitology International

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Abstract

It is estimated that nearly 800 million people are at risk of becoming infected with human-relevant schistosomes through contact with contaminated water and that number is growing due to the damming of water, irrigation canal construction, and other water resource development projects that increase potential habitats for the intermediate host of the parasite. Access to and protection of clean water are of utmost importance in limiting exposure to the parasite. For this reason, bodies of water with high human activity should be monitored regularly to identify transmission foci. We have developed an environmental surveillance device for schistosome cercariae (ESDSC) and through a review of the literature, compared our device to other available surveillance methods. The surveillance methods were compared and contrasted on five attributes: time and labor, technical training, cost, infrastructure and capital investment, and sensitivity. A total of 20 peer-reviewed, primary research publications were included in the review of the literature. Six surveillance methods were identified and compared: snail shedding or crushing, water filtration or centrifugation, sentinel mouse, molecular methods (PCR or ELISA), baited trap and C-6 film. Compared to the gold standard sentinel mouse method, the ESDSC provides rapid results and requires minimal labor and is far less cost-prohibitive in rural regions. While molecular methods of cercarial detection are more sensitive and results can be obtained within 24 hours, the method requires technical training and infrastructure that is often economically and geographically unavailable. The ESDSC is a rapid, low-cost, low-technological option for the early detection of transmission sites so that complex and costly surveillance methods can be more effectively targeted.

Keywords: environmental surveillance methods, schistosome cercariae, literature review, methods comparison

Introduction

An estimated 218 million people worldwide received treatment for schistosomiasis in 2015 and over 66 million people were treated for the disease (WHO, 2017). While our schistosomiasis knowledge base continues to grow and research advancements made, the number of individuals affected by the parasite has remained relatively unchanged. However, it has been reported that the population at risk, currently estimated at nearly 800 million, is growing due in part to the damming of water, irrigation canal construction, and other water resource development projects that increase the reach of the intermediate host of the parasite (Sokolow et al., 2017; Steinmann et al., 2006). In sub-Saharan Africa, where at least 90% of the infections occur, only 43% of school-aged children and 11% of adults that required praziquantel treatment received it in 2015 (WHO, 2016). Schistosomiasis is a high morbidity disease that is linked to severe anemia, reduced cognitive function, and chronic inflammation, and disproportionately affects the impoverished and underdeveloped (King, 2011; Savioli et al., 2017). Transmission of the infection is perpetuated by a lack of investment in public health infrastructure such as sanitary waste disposal, but also in the continuation of cultural practices and beliefs (misconceptions regarding treatment and transmission) that put people at risk.

Individuals are exposed to the parasite through contact with contaminated water. The obligate intermediate snail host has been targeted by transmission control strategies, namely the application of molluscicides or the reintroduction of natural predators (Sokolow et al., 2015). These control measures have shown highest efficacy when used in conjunction with other controls (Engels et al., 2002). Targeted mass-treatment

programs, mostly of school-aged children, with praziquantel is extremely effective in some regions (Chen, 2005; Simarro et al., 1991; Wiest et al., 1994), while not in others (Ross et al., 2015), which has prompted concerns of drug resistance (Ismail et al., 1999; Melman et al., 2009). Additionally, praziquantel does not protect against infection with schistosome cercariae and has limited activity against juvenile worms (Dong et al., 2010). Presently, a vaccine for schistosomiasis does not exist for humans and very few candidates have been identified for target (Tebeje et al., 2016) due to the parasite's host immune system evasion technique of molecular mimicry.

Development of sanitary infrastructure and clean water provision has been credited with a 69-77% reduction in schistosomiasis morbidity (Esrey et al., 1991). Access to and protection of clean water are of utmost importance in limiting exposure to the parasite. For this reason, bodies of water with high human activity (bathing, washing of clothes, fishing, playing) should be monitored regularly to identify transmission foci. We have developed an environmental surveillance device for schistosome cercariae (ESDSC) that has been laboratory-optimized to attract and trap *Schistosoma mansoni* cercariae (Lee et al., manuscript in preparation). To evaluate how our device compares to other available methods, we performed a review of the literature.

Methods and Results

To get an idea of the breadth of the body of knowledge regarding the three main human-relevant species of schistosome parasites as well as show the level of disparity in research conducted on *S. mansoni*, *S. japonicum*, and *S. haematobium*, a general query in PubMed, Scopus, and Web of Science was performed. The average number of articles

returned for *S. mansoni*, *S. japonicum*, and *S. haematobium* were 14,813, 3,948, and 3,325, respectively, with a marked increase in publications in the early-1970s (Figure 1).

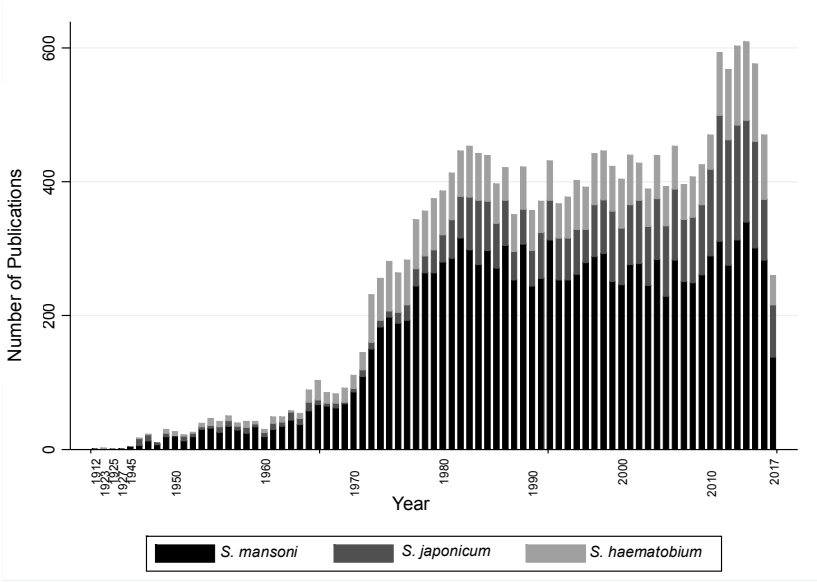

Figure 1. Graphical representation of the number of publications per year for each of the three main human-relevant schistosomes, using search terms: "*S. mansoni"*, "*S. japonicum"*, and "*S. haematobium"*. Note: publications with multiple species of schistosomes are represented in each of their respective categories for that year.

The Boolean phrase, "(((((detection) OR surveillance) OR trap) OR cercariometry) AND *Schistosoma*) AND cercariae", was typed into the search bar on the three scholarly databases mentioned previously and PubMed yielded the highest number of hits, a total of 338 publications from years 1946 to 2017. The results were filtered to exclude scholarly articles that were not written in English, leaving 292 journal articles. The titles and abstracts of these publications were reviewed to determine their eligibility to be included in this review of the literature. Human epidemiological studies, disease research, snail ecology studies, and case studies were excluded. Only primary research articles that reported on detection or capture of schistosome cercariae in a sample (spiked or otherwise) or body of water were included. These inclusionary criteria narrowed the number of pertinent articles to 20, two of which described methods of detecting avian

schistosomes (Graczyk and Shiff, 2000; Jothikumar et al., 2015). Two review articles (Abath et al., 2006; Aoki et al., 2003), summarizing the molecular approaches for monitoring transmission sites as well as cercariometric methods, were excluded. The reference lists of the 20 articles were reviewed in order to identify publications that may have been missed by the Boolean search, which revealed an additional five papers. A number of research articles were identified as describing techniques and technologies that could be enhanced by coupling with currently used surveillance techniques. Table 1 summarizes the properties of the articles that were included in the literature review. The surveillance methods discussed in these 20 articles were assessed on the following properties: time and labor, technical training, cost, infrastructure and capital investment, and sensitivity.

Table 1. Summary information of the 25 peer-reviewed primary research articles that were included in the review of the literature.

			Surveillance Method(s) Evaluated					
Year	**Author**	**Country**	Snail Shedding/ Crushing	Water Filtration/ Centrifugation	Sentinel Rodent	PCR (snail or water)/ ELISA	Baited Trap	C-6 Film***
1971	Butler et al.	USA	X	X				
1982	Kloos et al.	Egypt		X				
1984	Prentice	Kenya		X				
1989	Ouma et al.	Kenya	X	X				
1993	Shiff et al.	USA					X	
1996	Yousif et al.	Egypt	X	X				
1996	Yousif et al.	Egypt		X	X			
1998	Hamburger et al.	Israel				X		
1998	Hamburger et al.	Israel				X		
1999	Cai et al.	China			X			X
2000	Graczyk & Shiff *	USA					X	
2002	Ahmed et al.	Sudan					X	
2005	Driscoll et al.	USA				X		
2006	Melo et al.	Brazil	X			X		
2008	Hung & Remais	USA				X		
2011	Carlton et al.	China	X					
2011	Worrell et al.	USA			X	X		
2013	Allan et al.	United Kingdom	X			X		
2013	Lee et al.	USA					X	
2015	Jothikumar et al. *	USA				X		
1965	Barrett & Ellison**	Rhodesia		X				
1983	Blumenthal & Jewsbury**	UK		X				
1989	Hamburger et al.**	Israel	X			X		
1997	Hanelt et al.**	USA				X		
2004	Hamburger et al.**	Israel	X			X		

* Research performed on avian schistosomes
** Additional publications identified through the review of references
*** Proprietary information on the make-up of C-6 film

Discussion

The statistic that the WHO provides for the global number of people with schistosomiasis doesn't differentiate between species. Exposure risks and infection vary depending on where you live, due to the geographical distribution of the snail host as well as the level of transmission control that is utilized. It is estimated that 85% of those who are at risk of becoming infected are in Africa (Steinmann et al., 2006), where *S. mansoni* and *S. haematobium* are present (Figure 2; Weerakoon et al., 2015). This is partially reflected by the vast difference in the number of publications for each of the three main human-relevant species, but also likely due to the ease (or difficulty) of which the snails

are maintained. Fortunately, as technology, knowledge, and methods continue to improve, research of the to the two lesser-studied species, *S. japonicum* and *S. haematobium*, is increasing (Figure 1).

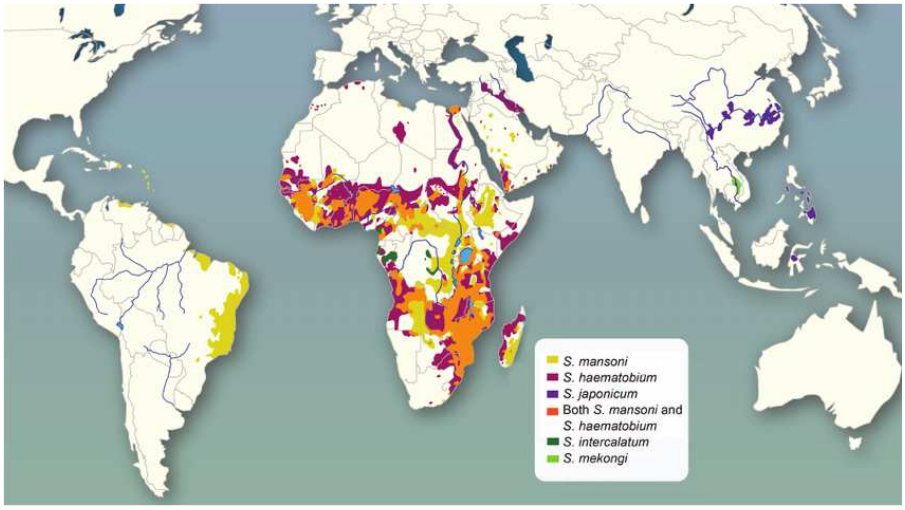

Figure 2. Global distribution of schistosomiasis in endemic areas, adapted from Weerakoon et al., 2015.

We have designed and laboratory-optimized a device for the capture of *S. mansoni* cercariae, the ESDSC (Lee et al., manuscript in preparation). Six categories of environmental surveillance methods for schistosome cercariae were identified in the review of the literature: snail shedding or crushing, water filtration or centrifugation, sentinel mouse, PCR or ELISA, baited trap and C-6 film (Table 1). The ESDSC falls under the "baited trap" category. These six surveillance methods were evaluated on five attributes: time and labor, technical training, cost, infrastructure and capital investment, and sensitivity, to determine what gaps the proposed ESDSC fills.

Time and Labor

Identifying bodies of water that harbor the parasite is time sensitive when implementing controls to break the transmission cycle. If a waterway is identified as being contaminated with schistosome cercariae, access to the area should be restricted

and individuals who use the body of water notified. The body of water and the population exposed will require treatment and subsequent re-testing. Ideally, an environmental surveillance method should provide results *in situ*. The level of labor associated with testing a body of water is also a factor that should be considered. The gold standard sentinel mouse method is by far the most time- and labor-intensive method of surveillance, requiring that mice be exposed to surface waters for four to five hours each day for at least two days. The mice are then housed for a minimum of six weeks (depending on the species of schistosome) to allow the infection to develop, after which the mice are sacrificed and perfused to look for presence of worms (Worrell et al., 2011). Alternatively, the collection of snails from suspected transmission foci requires minimal labor expenditure and possibly time, depending on the developmental stage of the parasite present. If snails are not patent at the time of collection, they are held for days and weeks to allow for shedding. Snail-crushing to look for developing sporocysts can be performed, instead of waiting for the snail to shed cercariae. Again, visualization depends on how long ago the snail was infected with miracidia (Sturrock et al., 1979). The minimum time to patency is estimated to be 5 weeks for wild-type snails (Hamburger et al., 2004). Water collection followed by filtration or centrifugation can be accomplished within a day, however, the labor associated with it varies depending on the turbidity of the water. The higher the level of debris, the slower the water will filter and more frequently the filters will become clogged, requiring labor to resolve it (Aoki et al., 2003). Additionally, with high turbidity, the water will need to be baled into the filtration setup in increments rather than all at once (Prentice, 1984). Centrifugation of water samples tends to take half the time to process (Yousif et al., 1996b) but the equipment

can be heavy to transport to sites and requires a power source (Barrett and Ellison, 1965). Following filtration or centrifugation of a water sample, the filter can be examined for the presence of cercariae or processed for DNA extraction to look for evidence of cercariae by PCR amplification (Driscoll et al., 2005; Hamburger et al., 1998b; Hung and Remais, 2008). More recently, rather than looking for cercariae in the water, DNA extracted from collected snails has been the subject of PCR analysis. This eliminates the need to hold a snail until it sheds and can actually detect infection as early as one day post-infection with miracidia (Hamburger et al., 1998a; Hanelt et al., 1997). The extraction of DNA and PCR process can be completed within one day, and while it requires some level of skill, the labor demand is relatively low. Enzyme-linked immunosorbent assays (ELISAs) were also explored as a method to detect early infection in snails. This proved to be a viable method, but the limit of the test was that it could only detect a prepatent infection two weeks after initial infection with miracidia (Hamburger et al., 1989b). Thus, PCR was pursued as a more robust approach. Cai et al. (1999) described the method of using C-6 film to touch the surface of the water in order to adsorb *S. japonicum* cercariae and subsequently examine the surface of the film under a microscope. This method requires little time and labor investment, however, it is only applicable with *S. japonicum* cercariae as they have a unique swimming behavior (floating on the surface of the water), which differs from the other species of human-relevant schistosomes. Finally, the baited trap method, which the ESDSC is categorized in, can provide results within a few hours and requires little labor beyond deploying the device, retrieving it, and examining the slides using a microscope (Ahmed et al., 2002; Graczyk and Shiff, 2000; Lee et al., 2013; Shiff et al., 1993).

Technical Training

Molecular methods of environmental surveillance require the highest level of technical training. Technicians work with very small volumes and sophisticated equipment, and the interpretation of results requires theoretical understanding. While the sentinel mouse method requires very little high-technological equipment, it does require hands-on perfusion training and an experienced eye to identify worms during dissection. The worms are only 7 – 20 mm long and can be easily missed by an untrained eye. The water filtration and centrifugation methods require equipment operation training, which is less technical than that of molecular methods; but since equipment occasionally malfunctions (Butler JM Jr, 1971), the technician will have to troubleshoot the repair on-site or else the sampling day is lost. The methods that require the least amount of technical training are malacological investigation and the use of a baited trap. Holding snails and waiting for cercarial shedding requires no specialized equipment, nor technical training. The drawback of this method lies in the high potential of a long lag time to receive confirmation. The baited trap requires training only on the use of a microscope, as it utilizes a passive method of attracting and trapping cercariae. In addition, it is possible to stain the slides and examine them at a later time, as the integrity of what is trapped on the media is maintained for weeks after staining (Graczyk and Shiff, 2000; Personal-observation).

Cost, Infrastructure and Capital Investment

As many of the endemic schistosomiasis areas are rural villages in developing countries; cost, infrastructure and capital investment are significant factors. Some of the methods are more cost-prohibitive than others, namely the sentinel mouse and molecular

methods. The sentinel mouse method is estimated to cost upwards of $100 per mouse (Worrell et al., 2011) which doesn't account for the capital investment in either building or converting existing infrastructure to house the animals such that they have a high chance of survival over the weeks that they are held. PCR, on the other hand, is getting less expensive as reagents become more affordable (Melo et al., 2006; Worrell et al., 2011); but like the sentinel mouse method, the capital investment required to run analyses is very expensive, and the equipment must be maintained and calibrated, which introduces recurring costs besides replacing reagents. Water filtration and centrifugation require an initial investment of purchasing the equipment but beyond that, the costs associated with running the equipment are minimal and sample examination merely requires a microscope. Collection of snails and use of a baited trap are the least costly methods. The harvesting of snails requires a net and holding receptacles, and no specialized holding facility. The ESDSC utilizes materials that are easily sourced, glass slides, and media, which are all inexpensive. In fact, the glass slides are reusable as the used media (beeswax and oleic acid) can be melted off, discarded, and replaced. It is estimated that the cost to make one ESDSC device is less than $10 and requires little to no maintenance.

Sensitivity

Snail collection and sentinel mice are the least sensitive of the surveillance methods. The prevalence of schistosome infection in snails varies by season, but is typically lower than 10% (Anderson and May, 1979; Born-Torrijos et al., 2014). Surveying for infected snails provides a gross underestimate of the number of potential transmission sites (Hamburger et al., 1989b; Sturrock et al., 1979; Yousif et al., 1996a),

with a reported sensitivity of 3% (Carlton et al., 2011). The use of sentinel mice not only provides information regarding the presence of cercariae, but also whether those cercariae are infective. This method, like malacological studies, has been shown to suffer from low sensitivity (Sturrock, 1973; Worrell et al., 2011; Yousif et al., 1996b). Water filtration and centrifugation in the lab setting with spiked samples have shown cercarial recovery rates ranging from 51-80% (Kloos et al., 1982; Prentice, 1984), however, Ouma et al. (1989) found that in the field, samples yielded either infected snails or cercariae (through filtration) and there was no significant difference between the two methods (snail collection and water filtration) in detecting transmission sites. PCR itself is a very sensitive method, able to detect one cercariae in five liters of water and a minute amount of target DNA (10^{-6} ng) in a sample (Hamburger et al., 1998c). This method, however, must be coupled with either the water filtration or centrifugation method, which, as previously discussed, can have variable outcomes. Hung and Remais (2008) were able to positively identify 93% of filtered cercariae-spiked samples using PCR, but the replicates were highly variable, likely due to losses through the filtration step. Alternatively, PCR can also detect prepatent infections in snails with high sensitivity, even when the sample is diluted with DNA from uninfected snails (Hamburger et al., 1998a). The baited trap method of surveillance is moderately sensitive, with reports of cercarial retrieval ranging from 30-100% (Ahmed et al., 2002; Shiff et al., 1993). In our own laboratory-controlled studies evaluating the performance of the ESDSC, we were able to detect presence of cercariae in 100% of our experiments, even when the device was placed at a distance of nearly one meter away from the point of cercarial origin (Lee et al., manuscript in preparation).

Enhancement of current surveillance methods

In reviewing the literature, articles describing research and findings that could potentially be coupled with techniques currently in use were identified. In recent years, researchers have explored the use of new technologies to screen the sera of sentinel mice for early biomarkers of infection or empty snail shells for trace evidence of schistosome infection, which significantly decreased the required holding time (Caldeira et al., 2004; Hamburger et al., 1989a; Hillyer and Gomez de Rios, 1979; Huang et al., 2016; Xu et al., 2017). Another new development is the engineering of a biosensor to detect schistosome cercariae (Webb et al., 2016). The technology is based on recognizing the enzymatic activity of elastase, which is secreted by cercariae during the process of skin invasion. *S. mansoni*-specific elastase on the biosensor leads to a colorimetric change, indicating presence of cercariae. The biosensor alone would not be used in the field, but could be used in conjunction with a baited trap, such as the ESDSC, wherein cercariae are attracted to the trap and attempt to invade the media, leading to secretion of elastase. The development of the biosensor has great potential for rapid field-speciation of trapped cercariae.

Conclusion

While each surveillance method can be assessed by the factors that affect it's efficiency, the environment and area in which it will be used also needs to be taken into account to determine the utility. What may work in one endemic area may not be ideal for another. The availability of infrastructure, skilled technicians and electricity will favor molecular methods and the use of sentinel mice, while the absence of these resources may limit the application of surveillance methods to snail collection and use of

baited traps. Keeping in mind that at least 90% of those requiring treatment for schistosomiasis live in rural Africa (WHO, 2017), it is likely that the most suitable method for surveying bodies of water are those that balance minimal input (time, labor and cost) and maximize output (results and sensitivity). The ESDSC is a viable option for the early detection of transmission sites so that surveillance methods that are more complex and involved can be targeted.

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