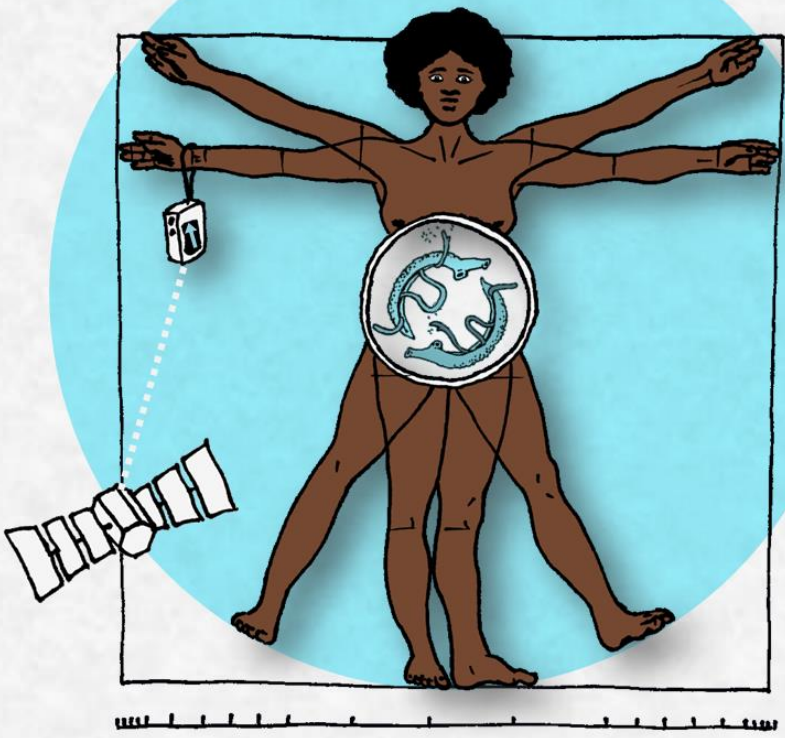
Schistosoma mansoni and *Schistosoma haematobium* infection and morbidity in a co-endemic focus



Integrated study of epidemiological, micro-geographical and immunological patterns

Lynn Meurs

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ISBN: 978-90-5335-910-5

The work presented in this thesis was performed at the Unit of Medical Helminthology, Department of Biomedical Sciences, Institute of Tropical Medicine in Antwerp, Belgium, at the 'Centre de Santé' in Richard-Toll, Senegal, at the Leiden Parasite Immunology Group, Department of Parasitology, Leiden University Medical Center, the Netherlands, and at the Medical Research Unit, Albert Schweitzer Hospital in Lambaréné, Gabon.

The studies described in this thesis were financially supported by the Sixth Framework Program (FP6) of the European Union.

Cover: By Lynn Meurs, based on the drawing of the Vitruvian man by Leonardo da Vinci ('Le proporzioni del corpo umano secondo Vitruvio').

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op 9 oktober 2014
klokke 10.00 uur

door

Lynn Meurs
geboren te Bemmel (Nederland)
in 1983

Promotiecommissie

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Schistosoma mansoni en *Schistosoma haematobium* infectie en morbiditeit in een co-endemische focus:

Geïntegreerde studie van epidemiologische, micro-geografische en immunologische patronen

Dissertation for the degree of Doctor in Biomedical Sciences at the University of Antwerp to be defended by

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9 oktober 2014, Leiden

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“The commonality between science and art is in trying to see profoundly - to develop strategies of seeing and showing”

Edward Tufte

Zachry, Thralls. An interview with Edward R. Tufte. Technical Communication Quarterly 2004; 13(4):447-62

Voor opa

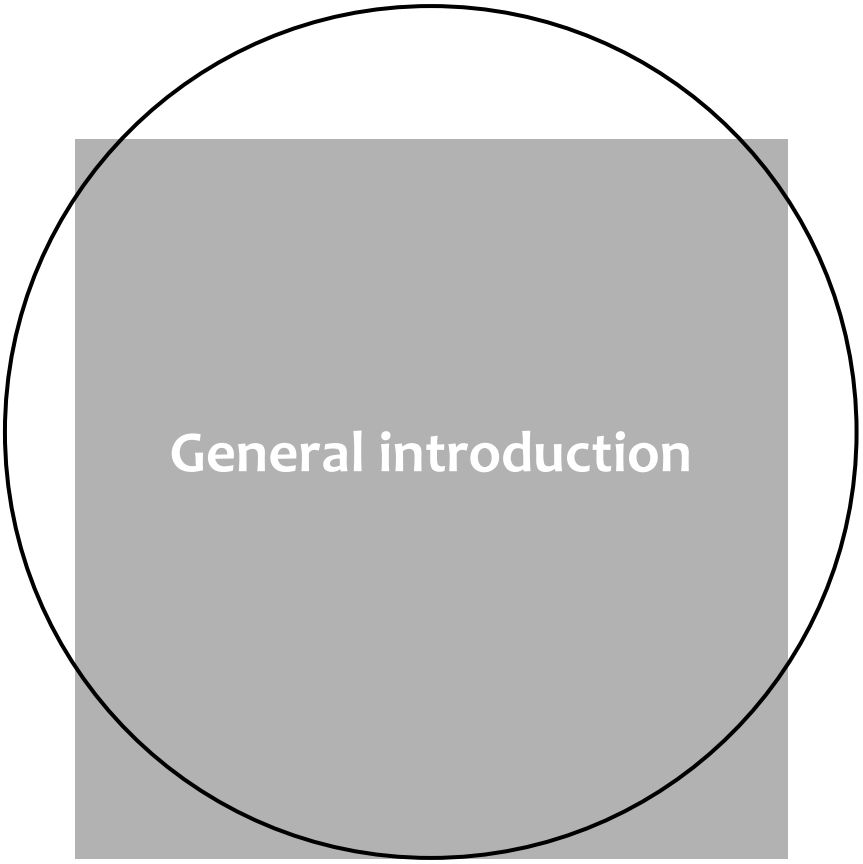
Contents

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
APC	Antigen-presenting cell
AWA	Adult worm antigen (AWAm for *S. mansoni*; AWAh for *S. haematobium*)
BMI	Body mass index
CD	Cluster of differentiation
CI	Confidence interval
CTL	Cytotoxic CD8 T lymphocyte
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ep10ml	Eggs per 10ml of urine
epg	Eggs per gram of feces
FSL-1	A synthetic diacylated lipoprotein mimicking the N-terminal part of LP44 from *Mycoplasma salivarium*
GIS	Geographic information system(s)
GM	Geometric mean
GPS	Global positioning system
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IP	(Liver) image pattern (or IFN-γ inducible protein)
ITM	Institute of Tropical Medicine
LPS	Lipopolysaccharide (derived from *Escherichia coli*)
LUMC	Leiden University Medical Centre
MDA	Mass drug administration

Mda5	Melanoma differentiation-associated protein 5
n	Number of subjects
N/A	Not applicable
NK cell	Natural killer cell
nMDS	Nonmetric multidimensional scaling
NTD	Neglected tropical disease
OR	Odds ratio
Pam3	Pam3CSK4 (a synthetic bacterial triacylated lipopeptide)
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHA	Phyto-hemagglutinin
Poly(I:C)	Polyinosinic-polycytidylic acid (a synthetic analogue of viral dsRNA)
PRR	Pattern recognition receptor
RDA	Redundancy analysis
Ref.	Reference category
RPMI	Roswell Park Memorial Institute medium
RR	Relative risk
SEA	Soluble egg antigen (SEAm for *S. mansoni*; SEAh for *S. haematobium*)
SCHISTOINIR	Innate immune responses and immunoregulation in schistosomiasis
Sh	*Schistosoma haematobium*
Sm	*Schistosoma mansoni*
Spp.	Species
STH	Soil-transmitted helminth
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T (cell)
WHO	World Health Organization

Chapter 1



Schistosomiasis is a parasitic disease caused by blood flukes of the genus *Schistosoma* (class of trematodes and phylum of Platyhelminthes (flatworms)). It is also referred to as bilharzia(sis) or snail fever. The word *Schistosoma* is derived from the two Greek words 'σχιστος', meaning 'split' and 'σωμα' meaning 'body'. Split body refers to the adult male schistosome, having a flattened body with lateral folds that form a ventral groove, the gynecophoric canal.

Schistosomes were first discovered by Theodor Bilharz in Egypt in 1851. He found different types of *Schistosoma* eggs in the feces of the same patient. In 1903, Patrick Manson proposed that these eggs were from different *Schistosoma* species which are now known as *Schistosoma mansoni* and *S. haematobium* (Blair, 1965) (Table 1.1).

Table 1.1. Comparison of principal features of *S. mansoni* and *S. haematobium*.

	S. mansoni	*S. haematobium*
Intermediate host snail	 *Biomphalaria*	 *Bulinus*
Definitive host	Human (also primates and rodents)	Human
Location adult worm	Veins of the mesenteric plexus	Veins of the pelvis
Egg excretion via	Feces	Urine
Egg morphology	 Lateral spine	 Terminal spine
Organ-specific chronic disease	Intestinal and hepatic	Urinary and genital
Geographic distribution	Africa, Middle East, Caribbean and South America	Africa and Middle East

Geographic distribution

With 200 to 250 million people infected, schistosomiasis is one of the most prevalent parasitic diseases worldwide (Chitsulo et al., 2000; Steinmann et al., 2006; WHO, 2010). Figure 1.1 shows the global distribution of the five *Schistosoma* spp. that are able to infect man: *S. mansoni*, *S. haematobium*, *S. intercalatum*, *S. japonicum*, and *S. mekongi* (Gryseels et al., 2006). Circa 90% of *Schistosoma*-infected people live in Africa (Steinmann et al., 2006). In this thesis, we focus on the two major *Schistosoma* spp., *S. mansoni* and *S. haematobium*. The global distribution map of *Schistosoma* shows a large overlap of *Schistosoma mansoni*- and *Schistosoma haematobium*-endemic areas (Figure 1.1).

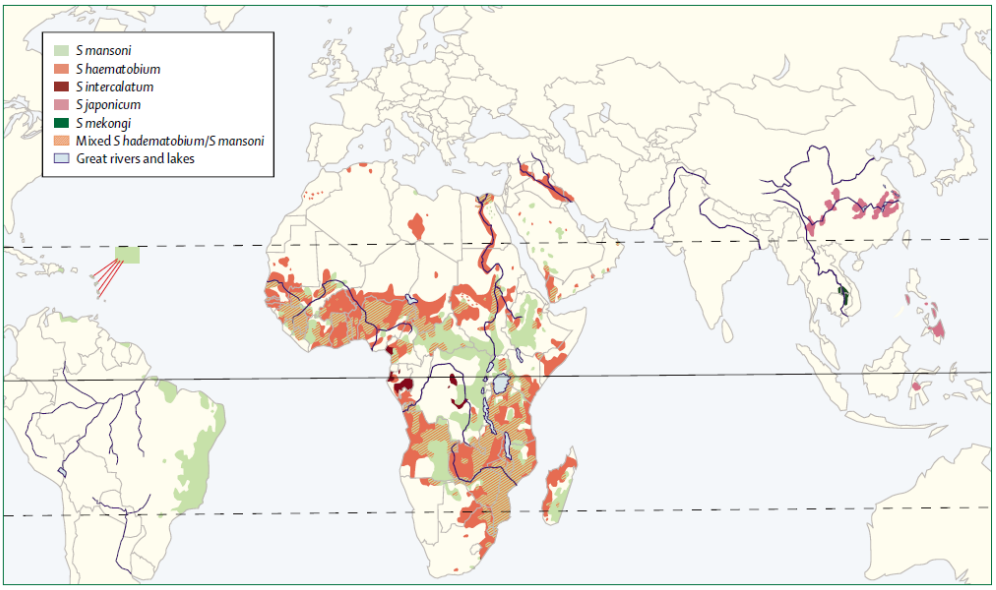


Figure 1.1. Global distribution of schistosomiasis.

Reprinted from The Lancet, Vol. 368, Gryseels et al., 'Human schistosomiasis', pages 1106-18, 2006, with permission from Elsevier and WHO (Doumenge et al., 1987).

Within endemic regions, schistosomiasis occurs in focal pockets and is closely linked to the presence of water bodies that harbor compatible freshwater snails (Cook and Zumla, 2009). Natural streams, ponds and lakes are typical sources of infection, but over the past few decades, man-made reservoirs and irrigation systems have contributed to the spread of schistosomiasis (Gryseels et al., 2006; Steinmann et al., 2006), e.g. in the north of Senegal. In such pockets of transmission, all three ingredients are present to drive the complex life cycles of *S. mansoni* and/or *S. haematobium* (Figure 1.2):

- Contamination of surface water by excreta,
- Presence of specific freshwater snails as intermediate hosts, and
- Human water contact (e.g. agricultural, domestic, recreational).

Schistosoma life cycle

Figure 1.2 demonstrates the life cycle of *S. mansoni* and *S. haematobium*. Juvenile worms mature in the hepatic sinuses of the human portal system. The human host is the main definitive host for *S. mansoni*, and the only definitive host for *S. haematobium*. When the worms approach maturity they form male-female pairs. The 10-20 mm long male embraces the longer, cylindrical female in its gynecophoric canal. Subsequently, the male uses its ventral sucker to travel along the blood vessel wall and against the

blood flow of the portal vein to the oviposition site. The adult *S. mansoni* male carries the female to the mesenteric plexus whereas the *S. haematobium* couple continues its way to the veins of the pelvis (Mahmoud, 2001). In these sites, where each couple produces hundreds of eggs a day, they usually survive for 3-5 years but their lifespan can be as long as 30 years (Jordan et al., 1993). Roughly half of the eggs traverse the blood vessel wall to reach the lumen of the intestine (*S. mansoni*) or bladder (*S. haematobium*). These eggs are excreted via the feces or urine, respectively. The other half of the eggs get trapped in host tissues, and can cause disease (King, 2011).

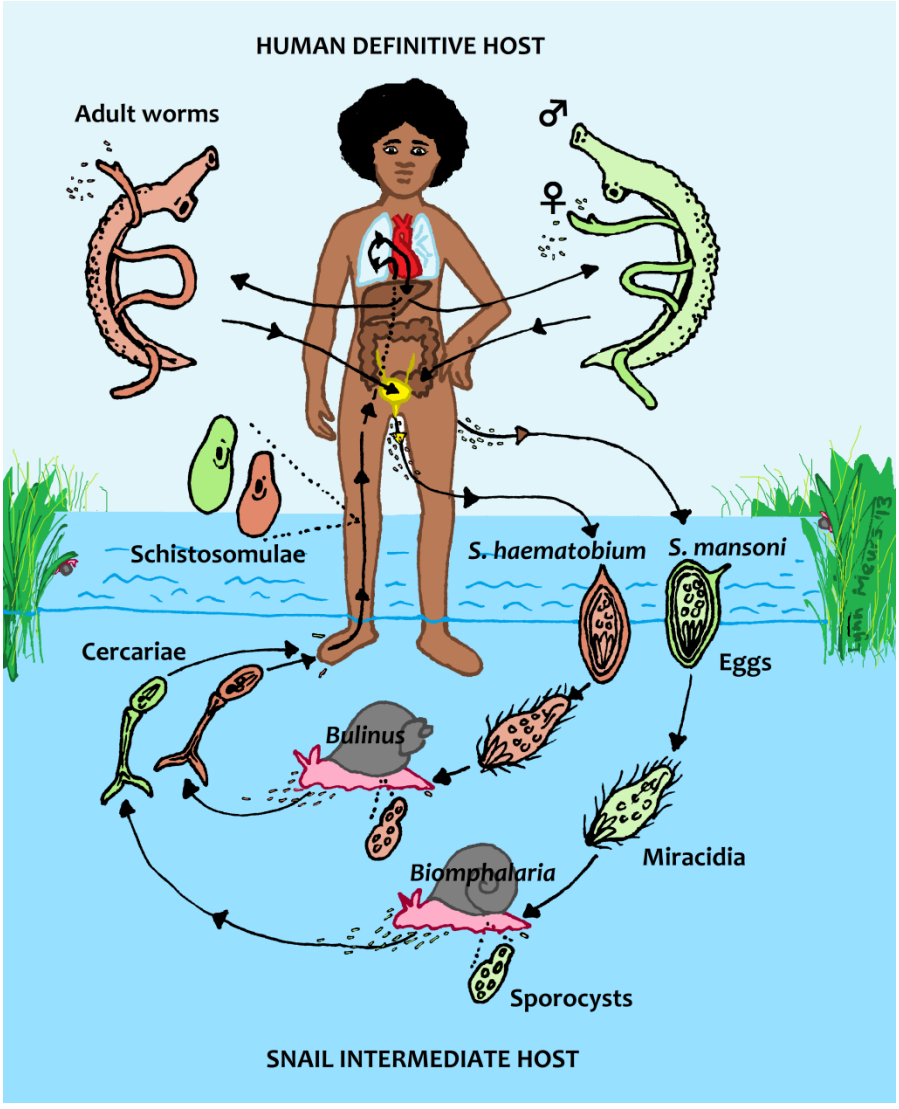


Figure 1.2. Life cycle of *S. mansoni* and *S. haematobium*.

Under conditions of poor sanitation and hygiene, excreted eggs can reach freshwater. The egg hatches in the water and releases the free-living larval stage, i.e. the ciliated miracidium. The miracidium searches for a compatible intermediate host snail. After penetrating the snail, it will transform into a sporocyst, multiply asexually and then produce hundreds of thousands of cercarial larvae. These cercariae are then shed into the water following a circadian rhythm - most are shed around noon. Subsequently, the cercariae swim with their bifurcated tails in search for the (intact) skin of the human host (Mahmoud, 2001).

While penetrating the skin, the cercariae transform into schistosomulae. They then enter the vascular system where they start to feed on blood. The blood stream carries them via the right heart to the lungs, to the left heart, to the systemic circulation, and then to the hepatic portal system, where the juveniles continue a new cycle (Mahmoud, 2001).

Epidemiology

Within endemic areas, schistosomiasis is characterized by a focal geographical distribution, even on the small scale (Pinot de Moira et al., 2007). This has been related to spatiotemporal variations in snail populations, cercarial densities, and patterns of human water contact, but other factors are probably involved too.

Another typical feature of schistosomiasis is the convex age-infection curve: prevalences and intensities of infection almost invariably increase from an early age to a peak around adolescence and decline thereafter (Woolhouse, 1994; Gryseels et al., 2006; Cook and Zumla, 2009). The mechanisms behind this phenomenon have not yet been fully elucidated. They may be related to the following three hypotheses that have been proposed (Dalton and Pole, 1978; Gryseels, 1994; Polman et al., 2002):

- **Water contact** increases with age in children up to adolescence, and decreases in adults.
- Cumulative exposure to *Schistosoma* antigens contributes to a **slow acquisition of protective immunity** with age. Around adolescence, the level of immunity is generally sufficient to counteract infection.
- **Innate age-related factors** such as changes in skin properties, metabolism and growth, hormonal factors, and innate immunity may predispose adolescents for infection.

The exact age at which *Schistosoma* infection peaks may vary from 10 to more than 20 years (Woolhouse, 1998). Anderson and May (1985b) proposed that this variation may be due to differences in the level of transmission. In populations subject to high levels of transmission, peak infection levels are generally higher and occur at a younger age, while in populations with low levels of transmission, peak infection levels are lower and occur at an older age. This pattern arose from a statistical model assuming that

protective immunity is gradually acquired as a function of cumulative exposure to parasite antigens. Thus, levels of infection will rise more rapidly in populations with high transmission. However, acquired immunity will also develop more rapidly in these populations and so levels of infection will begin to decline at a younger age. The result is a negative correlation between peak levels of infection and the peak age: the so-called 'peak shift' hypothesis.

The strong geographical and age trends generally contribute to an uneven distribution of schistosomiasis in a given population. For this reason, infection intensities (see 'Diagnosis') are generally summarized as geometric instead of simple arithmetic means on population level.

Acute morbidity

Acute disease is the result of a first encounter with schistosomes. It is exceptional in endemic areas where people are chronically exposed. Travelers or migrants mostly present with acute schistosomiasis. Cercariae that penetrate the skin can cause a temporary dermatitis, also known as swimmer's itch. In other climate zones, animal trematodes can cause similar symptoms. Several weeks upon infection with *S. mansoni* or *S. japonicum*, Katayama fever suddenly follows (WHO, 1994). This is a systemic hypersensitivity reaction against migrating schistosomulae and gives rise to symptoms such as fever, fatigue, myalgia, malaise, and non-productive cough. Later on, abdominal symptoms can develop (Gryseels et al., 2006).

Chronic morbidity

Even though morbidity only develops in a minority of chronically infected people (King, 2001), this group is still considerably large (King et al., 2005; Hotez et al., 2009). In contrast to acute disease, chronic schistosomiasis is a major public health concern. Organ-specific morbidity results from trapped *Schistosoma* eggs (see 'Schistosoma life cycle'). Trapped in host tissue, the eggs provoke inflammatory and granulomatous host immune responses which are progressively replaced by fibrotic deposits (Gryseels et al., 2006). As the location of egg-producing adult worms differs between the two species, *S. mansoni* and *S. haematobium* infections affect different organs (see below).

Intestinal and hepatic schistosomiasis

Adult *S. mansoni* worms are located in the veins of the mesenteric plexus. Their eggs migrating through the intestinal wall cause lesions mainly in the large bowel and rectum, i.e. intestinal schistosomiasis. This can lead to nonspecific abdominal symptoms (Gryseels et al., 2006). Eggs that are swept away back to the liver by the blood stream initially give rise to inflammatory hepatic schistosomiasis and hepatosplenomegaly (Wilson et al., 2011). These early pathologies are mostly found in

children and adolescents and probably vary according to the intensity of infection. Egg-induced hepatic lesions are gradually replaced by fibrotic deposits. After 5-15 years of cumulative exposure, this results in hepatic fibrosis, the late manifestation of *S. mansoni*. Fibrosis can lead to progressive occlusion of the portal veins, portal hypertension, splenomegaly, collateral venous circulation, porto-caval shunting, gastro-intestinal varices, and ascites. In some individuals, portal hypertension eventually causes gastro-esophageal varices to erupt. This is the most serious complication of *S. mansoni* infection and often fatal (Gryseels et al., 2006).

Urinary schistosomiasis

Adult *S. haematobium* worms are located in the veins of the pelvis. Their eggs are deposited in the wall of the urinary and genital tract. In urinary schistosomiasis, egg-induced immune responses lead to inflammation, ulceration and pseudopolyposis of bladder and ureteral walls. In children this is characteristically accompanied by hematuria. In addition, symptoms such as dysuria, increased urinary frequency and proteinuria can become apparent. Chronic lesions may evolve to fibrotic and sandy patches with severe sequelae such as hydroureter, hydronephrosis, and squamous cell bladder cancer (Smith and Christie, 1986; King, 2002; Gryseels et al., 2006).

In females, *S. haematobium* eggs affect the cervix, Fallopian tubes and vagina, i.e. female genital schistosomiasis. This is clinically characterized by sandy patches, pathological vessel morphology and contact bleeding. The lesions can lead to reduced fertility, genital itch, and abnormal discharge (Gryseels et al., 2006; Kjetland et al., 2012). In males, eggs affect the seminal vesicles and prostate. Hemospermia is a common symptom (Gryseels et al., 2006; Ramarakoto et al., 2008).

Nonspecific morbidity and disease interactions

In addition to the aforementioned organ-specific morbidity, chronic *Schistosoma* infection is thought to indirectly contribute to nonspecific and multifactorial morbidity. The process of chronic inflammation is likely to affect the host's metabolism and thus impair child development and nutritional status (King, 2011). Moreover, *Schistosoma*-induced lesions may lead to chronic blood loss, and indirectly contribute to anemia, malnutrition, and physical and cognitive impairment.

Like schistosomiasis, other neglected tropical disease (NTDs), HIV, tuberculosis, and malaria are related to poverty. Hence, they frequently co-occur in the same disadvantaged populations. Some studies have demonstrated that schistosomiasis is likely to increase the transmission of these diseases or to worsen disease outcomes (Sangweme et al., 2010; Abruzzi and Fried, 2011). This possibly happens through lesions induced in local tissues (see above), or through its effect on host immunological balances (see below) (Wilson and Dunne, 2012; Secor, 2012; Salgame et al., 2013).

Moreover, *Schistosoma* infections may reduce the effectiveness of immunization (Malhotra et al., 1999; King, 2011).

Immunology

Schistosomes live, grow, and reproduce in the blood circulation, where the host immune system is continuously on the look-out. In view of the fact that they can persist for up to 30 years, they have obviously found efficient ways to avoid host immune attack. Down-regulation of host immune responses is a prominent feature of chronic schistosomiasis, and assumed to be part of the parasite's arsenal of evasion strategies (Maizels, 1993). At the same time however, the host would benefit from this down-regulation which is likely to minimize egg-induced immunopathology (Burke et al., 2009). Immune down-regulation is thought to contribute to the fact that severe symptoms develop only in a minority (5-10%) of chronic *Schistosoma* infections (King, 2001). It has been hypothesized that severe disease may result from deregulated immune balances (see 'Adaptive immunity').

On the other hand, it seems that the host's immune system can eventually overcome immune evasion (at least in some people) and thus, reduce *Schistosoma* infection. There is longstanding epidemiological and clinical evidence that people living in endemic areas acquire some form of immunological protection after years of exposure (Jordan et al., 1993). The exact immunological pathways that underlie these phenomena have not been fully unraveled yet. An overview of general immunological principles is provided below.

Table 1.2. Comparison between the innate and adaptive human immune system.

	Innate	Adaptive
Main function	Initiate and shape adaptive response	Specific response and immunological memory
Onset of response	Rapid (hours)	Slow (days)
Specificity	Pathogen-associated molecular patterns	Antigen
Cell	Macrophage, dendritic cell, mast cell, natural killer cell, basophil, eosinophil, and neutrophil	B and T cells (lymphocytes)
Serum	Complement proteins, collectins, pentraxins	Antibodies

Innate immunity

Pathogens have a number of conserved molecular motifs, the so-called pathogen-associated molecular patterns (PAMPs). PAMPs are generally essential for the survival of the pathogen, and the host has evolved a limited number of receptors for these PAMPs, i.e. pattern-recognition receptors (PRRs) (Iwasaki and Medzhitov, 2004; Lee

and Kim, 2007). PRRs can be expressed in the cell cytosol or on the surface of immune cells (e.g. Toll-like receptors (TLRs) or C-type lectins), or they can be soluble (e.g. collectins and complement receptors). In general, engagement of PRRs leads to activation of the innate immune system (Table 1.2), either via intracellular signaling pathways (cell-based PRRs) or via extracellular activation cascades (soluble PRRs). Numerous PAMPs have been described for *Schistosoma* with corresponding PRRs. Importantly however, schistosomes also mimic molecular patterns that the host recognizes as its own (Maizels, 1993). Instead of activation of the innate system, such human patterns would lead to its inhibition, and thus contribute to parasite survival. Innate immune cells are the first to come into contact with any invading pathogen. Upon recognition, innate effector cells such as granulocytes, natural killer cells, and mast cells rapidly attack the pathogen. At the same time, innate antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), initiate, shape and regulate the subsequent and more specific adaptive immune response (Table 1.2). APCs express an array of different PRRs that can bind a wide range of potential pathogens. Upon recognition, they internalize the pathogen, and process and present the pathogen's antigens on their surface to cells of the adaptive immune system (Figure 1.3).

Adaptive immunity

In contrast to innate cells, individual adaptive immune cells such as T and B cells recognize only one specific antigen. Antigen-specific naïve CD4 T helper (Th) cells recognize the presented antigen, and co-stimulatory and cytokine signals from the APC prime the subsequent response of these T cells (Medzhitov and Janeway, Jr., 1997). Depending on the signal provided, this response can be polarized into two ways. The naïve T cell will proliferate and differentiate into either a Th1 or a Th2 cell. Th1 cells secrete cytokines such as IL-2, interferon (IFN)-γ and IL-12, while Th2 cells secrete IL-4, IL-5 and IL-13. Th1 cytokines stimulate Th1 and inhibit Th2 responses, while Th2 cytokines do the opposite. Th1 immunity is mainly cell-mediated and involves macrophages, neutrophils, natural killer cells and cytotoxic CD8 T cells. Th2 immunity on the other hand, mainly involves B cells, mast cells, and eosinophils. Th2 pathways induce B cells to produce antibodies. Later on in the course of events, immunoglobulin (Ig) class switching can occur (Figure 1.3). Theoretically, Th1 immunity is more effective against intracellular and Th2 immunity against extracellular infections. In chronic infections - analogous to their extracellular habitat - schistosomes skew the immune response towards Th2. *Schistosoma* infection also induces IgE class switching. IgE levels increase with age and decreasing levels of infection, suggesting that IgE may contribute to protective immunity in adults. However, also other Th2 as well as Th1 effector mechanisms may be involved in immunoprotection. As yet, it is poorly understood which phenotype or which effector mechanisms actually contribute to resistance to infection, or to pathogenesis (Wilson and Coulson, 2009).

Alternatively, naïve Th cells may differentiate into IL-10-producing regulatory T(reg) cells upon antigen recognition (Figure 1.3). Subsequent regulatory networks inhibit both Th1 and Th2 responses. Schistosomes are potent inducers of these regulatory mechanisms, and this greatly contributes to immune down-modulation. The down-regulated Th2 phenotype that is characteristic for chronic schistosomiasis is referred to as the modified Th2 response.

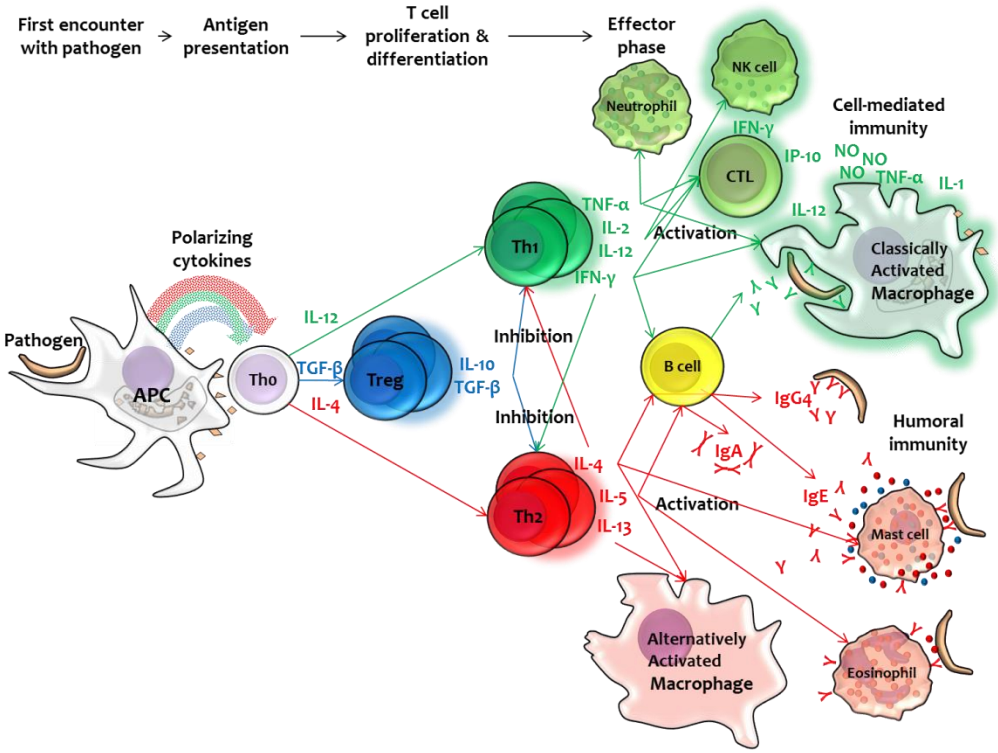


Figure 1.3. Schematic overview of classical induction of an adaptive immune response.

Schistosoma life stages are several orders of magnitude larger than the depicted pathogen. APC: Antigen-presenting cell; Tho: Naïve CD4 T helper lymphocyte; Treg: Regulatory T cell; CTL: Cytotoxic CD8 T lymphocyte; NK cell: Natural killer cell.

Diagnosis

The gold standard for the diagnosis of schistosomiasis is microscopic examination of eggs in feces (*S. mansoni*) or urine (*S. haematobium*). Eggs can be quantified by Kato-Katz as number of eggs per gram of feces (epg) (Katz et al., 1972), or by urine filtration

as number of eggs per 10ml of urine (ep10ml), respectively (WHO, 1991). Since there is considerable day-to-day variation in the number of excreted eggs, samples from consecutive days are usually examined (Jordan et al., 1993). Egg counts give a crude, indirect approximation of worm burden (Jordan et al., 1993), and are used as a measure of infection intensity (Table 1.3). In addition, immunological methods (antibody- or antigen-based assays) can be used (Polman, 2000). For *S. haematobium*, indirect methods are also available. Visual urine inspection for macrohematuria (red urine) can reveal heavy infections, while reagent strips can detect invisible hematuria, i.e. microhematuria, in lighter *S. haematobium* infections (Montresor et al., 1998).

Table 1.3. Classes of intensity of infection (WHO, 2002).

	S. mansoni (epg)	*S. haematobium* (ep10ml)
Light	1-99	1-49
Moderate	100-399	
Heavy	≥400	≥50

Schistosoma-induced morbidity can be visualized by a variety of techniques such as tissue biopsy, endoscopy, and radiography (Gryseels et al., 2006). Ultrasonography is one of the least invasive techniques. Standard protocols are available to classify *S. mansoni*-specific hepatic fibrosis and *S. haematobium*-specific urinary tract morbidity by ultrasound (Figure 1.4). While such protocols are very useful for epidemiological studies, they are less suitable for routine clinical use in the primary health care setting as they require specific expertise and experience (Richter et al., 1996).

Treatment

Praziquantel is the recommended treatment for *Schistosoma* infections. It has been available since the 1980s and is administered as a single oral dose of 40 mg/kg. The drug is relatively cheap and effectively kills adult worm stages of all *Schistosoma* species. Side-effects are related to the intensity of infection. They are generally mild and include nausea, vomiting, malaise, and abdominal pain (Gryseels et al., 2006). Early morbidity regresses faster upon treatment than late and advanced disease, and in some cases, late disease does not regress at all (Richter et al., 1996).

Control and elimination

Schistosomiasis belongs to the so-called NTDs. Together with other (mainly helminthic) infections such as soil-transmitted helminthiases (STH), lymphatic filariasis, and onchocerciasis, it is among the most prevalent NTDs. The distribution of these diseases clusters and overlaps in low-income populations, living in adverse environmental

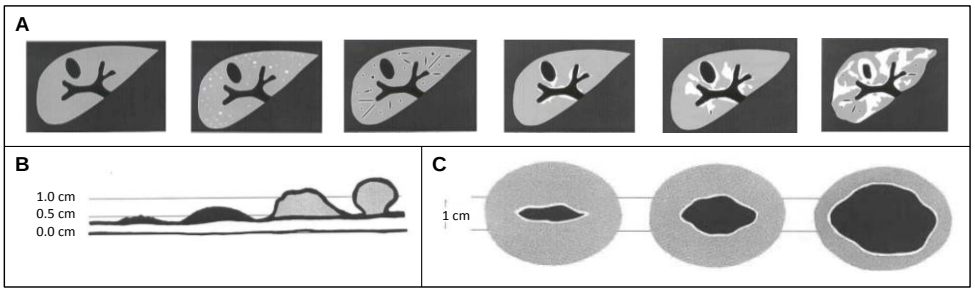


Figure 1.4. Diagnosis of *Schistosoma*-associated morbidity by ultrasound.

Adapted from Richter et al. (1996). **Panel A** shows the classification of *S. mansoni*-specific hepatic fibrosis: from unaffected liver parenchyma, 'image pattern A' (score=0), on the left, to advanced periportal fibrosis, 'pattern F' (score=8), on the right. **Panel B** shows the classification of *S. haematobium*-specific lesions of the bladder wall: from 'irregularity' (score=1) on the left, to 'pseudopolyp' (score=2) on the right. **Panel C** shows the measurement of upper urinary tract morbidity, i.e. congestive dilation of the renal pelvis, using a longitudinal scan on an empty bladder: from no dilation (score=0) on the left, to 'marked hydronephrosis' with compressed parenchyma (score=8) on the right.

conditions with poor sanitation and hygiene. As a result many people are affected by multiple NTDs.

For schistosomiasis, mass drug administration (MDA) with praziquantel is now the main component of most national control programs (WHO, 2002; WHO, 2013). Schistosomiasis MDA programs aim to reduce current infection and, by keeping infection intensity down, prevent the development of severe disease. Such MDA programs – often integrated in broader NTD control programs – mainly target school children. The rationale is that this age group typically has the highest infection intensities.

The WHO recently put forward an ambitious goal for the year 2020: to control schistosomiasis globally, and to initiate interventions towards local elimination. To sustain control achievements, multi-faceted, integrated control packages are needed, including not only anti-schistosomal treatment, but also the provision of clean water and improved sanitation, snail control, and behaviour change (Sturrock, 1989; King, 2009; Gray et al., 2010; Rollinson et al., 2013; Freeman et al., 2013).

Schistosomiasis in northern Senegal

The major part of the work described in this thesis (Chapters 2-5) was carried out on the Nouk Pomo peninsula in Lac de Guiers (Guiers Lake), in the north of Senegal. This lake is connected to the Senegal River, the longest river of West Africa. Following devastating droughts in the 1970s, the Diama dam was constructed near Saint-Louis at

the mouth of the river (Figure 1.5). This dam prevents upstream intrusion of saltwater and thereby protects freshwater sources. It allows large areas to be irrigated for agriculture. Furthermore, the dam maintains several lakes, including Lac de Guiers. People living in the Senegal river basin greatly benefited from these positive effects of the dam (Organisation pour la Mise en Valeur du Fleuve du Sénégal, 2003), and the local economy improved (Talla et al., 1990).

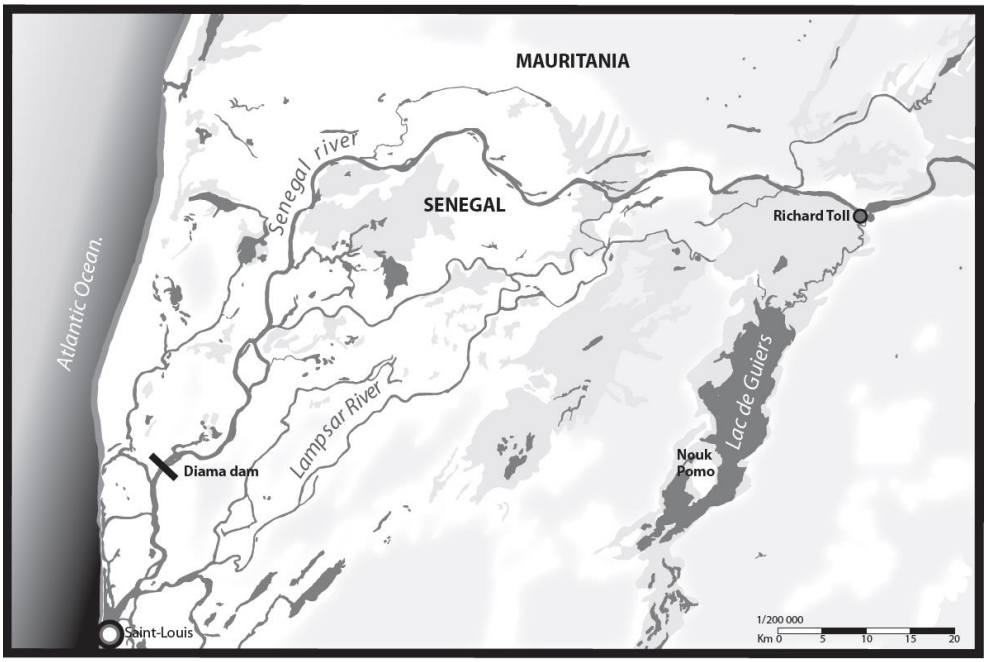


Figure 1.5. Lac de Guiers study area in northwest Senegal.

Unfortunately, the water development had a downside too. Rates of water-borne diseases such as diarrhea and malaria increased (Sow et al., 2002; Organisation pour la Mise en Valeur du Fleuve du Sénégal, 2003). *Schistosoma haematobium* was since long endemic in the north of Senegal (Becker and Collignon, 1994), but around 1989, just upon completion of the dam in 1986, its prevalence and distribution suddenly expanded. Moreover, the dam led to the introduction of *S. mansoni* into the area in 1988. The *S. mansoni* epidemic started in the community of Richard-Toll (Vercruysse et al., 1994). From this epicenter, *S. mansoni* rapidly spread throughout the region (Talla et al., 1990; Talla et al., 1992). In a few years, the prevalence in Richard-Toll rose from 0% to 75-100% in 1992, with highest intensities of infection ever described (Picquet et al., 1996). By 1994, virtually the whole Lac de Guiers area had become exposed to this species (Picquet et al., 1996). Subsequently, *S. haematobium* emerged in the Lac de

Guiers area between 2000 and 2005 (Southgate et al., 2000). As a result, many communities became co-endemic for *S. mansoni* and *S. haematobium* (De Clercq et al., 1999; Southgate et al., 2001; Ten Hove et al., 2008; Huyse et al., 2009).

Co-endemic *S. mansoni* and *S. haematobium*

There is a large overlap of *S. mansoni*- and *S. haematobium*-endemic areas in Africa (Figure 1.1). Thus, many areas are co-endemic for both species, implying that many people are at risk of co-infection. In spite of this, schistosomiasis studies generally investigate either *S. mansoni* or *S. haematobium*. Sometimes co-infected subjects are even purposely excluded from these investigations. So far, little is known about the epidemiology of *Schistosoma* infection and associated disease in co-endemic populations, and about their underlying mechanisms. Yet, the (potentially adverse) effects of co-infections on schistosomiasis transmission, morbidity and control can be significant (Robert et al., 1989; Ernould et al., 1999a; Koukounari et al., 2010; Webster et al., 2013; Gouvras et al., 2013). Table 1.4 gives an overview of the handful of studies on co-endemic *S. mansoni* and *S. haematobium* that have been described in literature so far. Most of these studies were performed in schoolchildren only. As yet, little is known about the effect of mixed infections on morbidity. Also, nothing is known about the immunological differences between single and mixed, or between *S. mansoni* and *S. haematobium* infections, and only one study investigated micro-geographical patterns in a co-endemic community (Farooq, 1966).

Aims

In co-endemic areas, *S. mansoni* and *S. haematobium* can interact, either directly, or indirectly via the host immune system. This may have important implications for infection and morbidity levels in such populations. In this thesis, we investigate the differences and interactions between these two *Schistosoma* species, as well as their effects on host morbidity. More specifically, the aim of this thesis is to get a detailed insight into the epidemiological patterns of *Schistosoma* infection and associated morbidity on a micro-geographical scale in *S. mansoni* and *S. haematobium* co-endemic areas. The main research questions of this thesis are:

- What are the differences between single and mixed infections in terms of:
  - Infection intensity? (Chapter 2)
  - *S. mansoni*- and *S. haematobium*-specific morbidity? (Chapter 3)
- How do **demographic** and **geographic** distribution patterns differ between
  - Single and mixed infection? (Chapter 2 and 4)
  - *S. mansoni* and *S. haematobium* infection? (Chapter 2 and 4)
  - *S. mansoni*- and *S. haematobium*-specific morbidity? (Chapter 3 and 4)

Table 1.4. Non-exhaustive list of literature on co-endemic *S. mansoni* and *S. haematobium*.

Location	Population	Prevalence (%)			Findings	Reference
		Sm	*Sh*	Mix		
Rhodesia	Communities	55	39	16	Ectopic *Sm* occurs in people with *Sh*	(Husting, 1965)
		4-70	11-84		Hypothesized that *Sh* infection does not protect against *Sm*, based on age-infection curves	(Clarke, 1966)
Egypt		29	30	17	*Sm* is acquired more slowly than *Sh* during childhood and more persistent in adulthood	(Farooq et al., 1966a)
		30	38	17	Single *Sh* causes more morbidity than single *Sm*; Mixed infections associated with higher risk of morbidity; Association between morbidity and *Sh* but not *Sm* depends on infection intensity	(Farooq et al., 1966b)
	4 to 5-year-olds	17	30	~12	*Sh* and *Sm* cluster in different sections of one community	(Farooq, 1966)
	Autopsies: people who died in hospital	?	?	?	*Sh* eggs were retained in tissues more than *Sm* eggs; Increased *Sh* infection intensities in mixed infections; *Sh* but not *Sm* worm loads decrease with age from teenage onwards; *Sh* may affect distribution of *Sm* eggs in the body	(Cheever et al., 1977)
Sudan, near Khartoum North	Infected schoolboys & employees				Infections with *Sh* or *Sm* eggs in urine were more sensitive to treatment than those with *Sm* eggs in feces	(Omer and Teesdale, 1978)
Egypt, Kafr El Sheikh	Communities	20	30	8	Decline of *Sh* after adolescence more steep than that of *Sm*	(DeWolfe Miller et al., 1981)
Liberia, Bong County		25	23	?		(Dennis et al., 1983)
	School children	29	32	14	Same levels of infection in single and mixed infections	
Egypt, Beni-Suef	Hamsters infected with local strain				Proposed *Sm* 'strain' with preferential oviposition in urine to be a *Sm* x *Sh* hybrid	(Soliman et al., 1986)
South Africa, Embokodweni	Informal settlement	55	53	36	*Sh* but not *Sm* infection correlates with exposure to water; *Sm* is more persistent in adulthood than *Sh*	(Kvalsvig and Schutte, 1986)
Zimbabwe	Snails	0-10	~2-19		Infected *Sm* and *Sh* snail hosts show geographical clustering within one river habitat; Human to snail transmission on scale of 40m	(Woolhouse and Chandiwana, 1989)
Cameroon	Fishing villages	15	26	7	2 to 3-fold higher levels of *Sh* but not *Sm* in mixed as compared with single infections	(Robert et al., 1989)
	School children	9	36	5	More ectopic *Sm* eggs under conditions of high *Sh* and low *Sm* transmission; High level of *Sm* infection not related to ectopic *Sm*	(Ratard et al., 1991)
Sudan, White Nile Province		15	26	5	Patterns of distribution varied between species; Same levels of infection in single and mixed infections	(Ahmed et al., 1996)

Table 1.4. Non-exhaustive list of literature on co-endemic *S. mansoni* and *S. haematobium* (continued).

Location	Population	Prevalence (%)			Findings	Reference
		Sm	*Sh*	Mix		
Zimbabwe	School children	85	71	62	Mixed as compared with single infections associated with hepatomegaly	(Friis et al., 1996)
Tanzania		~5?	~40	~3?	Same levels of infection in single and mixed infections upon correction for age; *Sm* and *Sh* infections do not cluster in same individuals	(Booth et al., 1998)
		11	57	5 ?	Inverse spatial relationship between *Sm* and *Sh* at school level; Trend with age for *Sh* but not *Sm*; Twofold higher levels of *Sm* in mixed as compared with single infections	(Lwambo et al., 1999)
Senegal (north)	Communities	8-76	1-64	?	Levels of *Sm* increased while *Sh* decreased 10 months after MDA	(Ernould et al., 1999a)
		91	28	21	Cure and intensity reduction rates of *Sm* lower than those of *Sh* upon MDA	(De Clercq et al., 1999)
Egypt		0-43	0-14		*Sm* is acquired more slowly than *Sh* during childhood and more persistent in adulthood	(El Khoby et al., 2000)
Cameroon, near Bessoum	School children	31	71	?	*Sh* may reduce hepato- and splenomegaly; Ectopic eggs associated with infection intensity of either species	(Cunin et al., 2003)
Mali		?	?	?	Mixed infections reduce the risk of *Sm* but increase the risk of *Sh* morbidity; Single *Sh* associated with *Sm* morbidity; MDA may favor reinfection with *Sm* rather than *Sh*	(Koukounari et al., 2010)
Kenya, Taveta		~11	~64	7	Mixed infections reduce the risk of *Sh* morbidity; Single *Sh* associated with *Sm* morbidity; *Sm* infection increases 12 months after MDA	(Gouvras et al., 2013)
Cameroon		49-87	75-96	44-84	Mixed infections disappeared after MDA; lower cure rates for *Sh* than for *Sm*; pattern of reinfection depends on transmission setting	(Tchuem Tchuente et al., 2013)
Senegal (north)		?	?	?	Identified *Sm* x *Sh* hybrids	(Huyse et al., 2013)
		79–100	81–97		*Sm* more persistent than *Sh* after MDA	(Webster et al., 2012)
Yemen	Children	9	24	4	More hepatomegaly in mixed versus single infections; Same levels of anemia in mixed and single infections	(Sady et al., 2013)
Kenya, south Nyanza	School children	0-21	0-22	0-9	Heterogeneous spatial distribution; *Sm* more prevalent in coastal and *Sh* more prevalent in inland schools; Same levels of infection in single and mixed infections	(Sang et al., 2014)

Sm: *S. mansoni* infections (including single and mixed); *Sh*: *S. haematobium* infections (including single and mixed); Mix: Mixed *S. mansoni* and *S. haematobium* infections.

- How do **adaptive cytokine responses** differ between *S. mansoni* and *S. haematobium* infections, in terms of
  - Adult worm antigens (AWA)? (Chapter 5)
  - Soluble *Schistosoma* egg antigens (SEA)? (Chapter 5)
- How do **TLR-induced cytokine responses** differ between *S. haematobium*-infected and -uninfected subjects? (Chapter 6)

A co-endemic focus, Nouk Pomo, a peninsula in Lac de Guiers in the north of Senegal (see Figure 1.5), was selected as the study site. The small scale of this study allowed a detailed, multidisciplinary analysis of complex (co-)infection and morbidity patterns. Moreover, it enabled us to take the well-known focality of schistosomiasis into account. Chapters 2 to 5 describe the research conducted in this population. Demographic patterns and associations between (co-)infection and morbidity are described in **Chapter 2** and **3**, and geographic patterns in **Chapter 4.** In **Chapter 5**, cytokine responses to adult worm antigens (AWA) were used as a proxy for host responses to living adult worms. These responses may thus be related to immunological resistance in some individuals or conversely, to susceptibility to infection in others. Secondly, cytokine responses to soluble *Schistosoma* egg antigens (SEA), were used as a proxy for the egg-induced responses, and may as such be related to the observed morbidity patterns. Subsequently, **Chapter 6** explores the innate immune responses that generally precede and shape these adaptive responses. To this extent, another population was studied, consisting of children from a *S. haematobium*-mono-endemic area in Lambaréné, Gabon.

Finally, **Chapter 7** provides a general discussion of the epidemiological and immuno-epidemiological findings described in Chapter 2 to 6. It points out the significance of our observations for our general understanding of the disease as well as the practical relevance for schistosomiasis control and elimination, and proposes directions for future research.

Chapter 2

Epidemiology of mixed *Schistosoma mansoni* and *Schistosoma haematobium* infections in northern Senegal

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International journal for parasitology 2012;42(3):305-11

Abstract

Due to the large overlap of *Schistosoma mansoni*- and *Schistosoma haematobium*-endemic regions in Africa, many people are at risk of co-infection, with potential adverse effects on schistosomiasis morbidity and control. Nonetheless, studies on the distribution and determinants of mixed *Schistosoma* infections have to date been rare. We conducted a cross-sectional survey in two communities in northern Senegal (n=857) to obtain further insight into the epidemiology of mixed infections and ectopic egg elimination. Overall prevalences of *S. mansoni* and *S. haematobium* infection were 61% and 50% respectively, in these communities. Among infected subjects, 53% had mixed infections and 8% demonstrated ectopic egg elimination. Risk factors for mixed infection - i.e. gender, community of residence and age - were not different from what is generally seen in *Schistosoma*-endemic areas. Similar to overall *S. mansoni* and *S. haematobium* infections, age-related patterns of mixed infections showed the characteristic convex-shaped curve for schistosomiasis, with a rapid increase in children, a peak in adolescents, and a decline in adults. Looking at the data in more detail however, the decline in overall *S. haematobium* infection prevalences and intensities appeared to be steeper than for *S. mansoni*, resulting in a decrease in mixed infections and a relative increase in single *S. mansoni* infection with age. Moreover, individuals with mixed infections had higher infection intensities of both *S. mansoni* and *S. haematobium* than those with single infections, especially those with ectopic egg elimination ($p<0.05$). High infection intensities in mixed infections, as well as age-related differences in infection patterns between *S. mansoni* and *S. haematobium*, may influence disease epidemiology and control considerably, and merit further studies into the underlying mechanisms of *Schistosoma* infections in co-endemic areas.

Introduction

Schistosomiasis is amongst the most common human parasitic diseases with an estimated 207 million people infected worldwide. More than 90% of them live in sub-Saharan Africa (Hotez and Kamath, 2009). The global distribution map of *Schistosoma* shows a large overlap of *Schistosoma mansoni*- and *Schistosoma haematobium*-endemic areas in Africa (Doumenge et al., 1987; Gryseels et al., 2006; Montgomery, 2011), indicating that many people are at risk of co-infection with both species. Nevertheless, little is known about the distribution and determinants of such mixed infections in human populations (Dennis et al., 1983; Robert et al., 1989; Ahmed et al., 1996; Booth et al., 1998; Lwambo et al., 1999). Animal models have described that *S. mansoni* and *S. haematobium* interact in the host. The two species have been shown to form heterologous male-female pairs with the male determining the oviposition site and the female producing eggs characteristic of her species (Khalil and Mansour, 1995; Southgate et al., 1998; Webster et al., 1999). This phenomenon probably contributes to the occurrence of ectopic egg elimination, i.e. *S. mansoni* eggs in urine or *S. haematobium* eggs in feces, in mixed foci (Ratard et al., 1991; Cunin et al., 2003).

Recently, differences have been observed between single and mixed infections regarding their association with bladder as well as liver pathology (Koukounari et al., 2010). Also, unforeseen increases in *S. mansoni* infection have been observed after praziquantel treatment in co-endemic areas (Ernould et al., 1999a; Koukounari et al., 2010). Moreover, mixed infections may lead to the hybridization of *Schistosoma* spp. or parthenogenesis (Jourdane et al., 1995; Khalil and Mansour, 1995), with as yet unclear consequences for disease and transmission (Wright and Ross, 1980; Webster and Southgate, 2003; Huyse et al., 2009). Understanding the epidemiology of mixed infections will help us to answer important standing questions on the underlying mechanisms towards morbidity and to develop effective strategies for the prevention and control of schistosomiasis in co-endemic areas.

During the past decades, many communities in northern Senegal have become co-endemic for *S. mansoni* and *S. haematobium* (De Clercq et al., 1999; Ernould et al., 1999a; Southgate et al., 2001; Van der Werf et al., 2002; Ten Hove et al., 2008; Huyse et al., 2009). *Schistosoma mansoni* was introduced in Richard-Toll in 1988 upon construction of the Diama dam and rapidly spread throughout the region (Talla et al., 1990; , 1992). By 1994, virtually the whole Lac de Guiers area had become exposed to this species (Picquet et al., 1996). Today, both *S. mansoni* and *S. haematobium* are widespread, resulting in a large number of people with mixed infections in the communities around the lake.

Here, we report the results of a cross-sectional study investigating the epidemiology of mixed *Schistosoma* infections in two communities on the banks of Lac de Guiers in

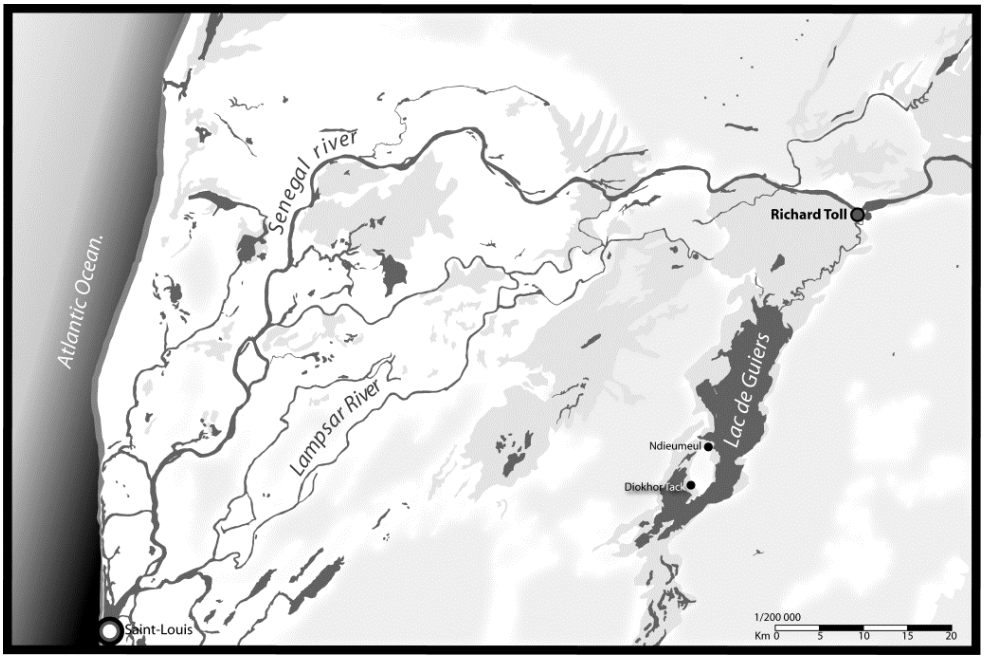

Figure 2.1. Map of northern Senegal indicating the communities participating in this study, Ndieumeul and Diokhor Tack.

northern Senegal. We studied the patterns of *S. mansoni* and *S. haematobium* infection in these mixed foci, and compared mixed with single infections. Possible underlying mechanisms and the impact of the reported findings are discussed.

Material and methods

Study area

Ndieumeul (also known as Thiekène; 16°13'12"N 15°51'36"W) and Diokhor Tack (16°11'24"N 15°52'48"W), are the largest communities on the Nouk Pomo peninsula in Lac de Guiers, Senegal and are situated 4 km apart (Figure 2.1). These Wolof communities have a total estimated population size of 1,300 people. Cultivation is the main means of subsistence and the farmlands are irrigated with water from the lake. Although the water from Lac de Guiers is piped to the capital city of Dakar, 250 km away (Berger et al., 2006), the people living nearby do not have access to safe water. To our knowledge, there have been no periodic anthelminthic treatment programs in these villages prior to our study.

The present study was conducted in 2009 as part of a larger investigation on the immuno-epidemiology of *Schistosoma* infection and morbidity (SCHISTOINIR:

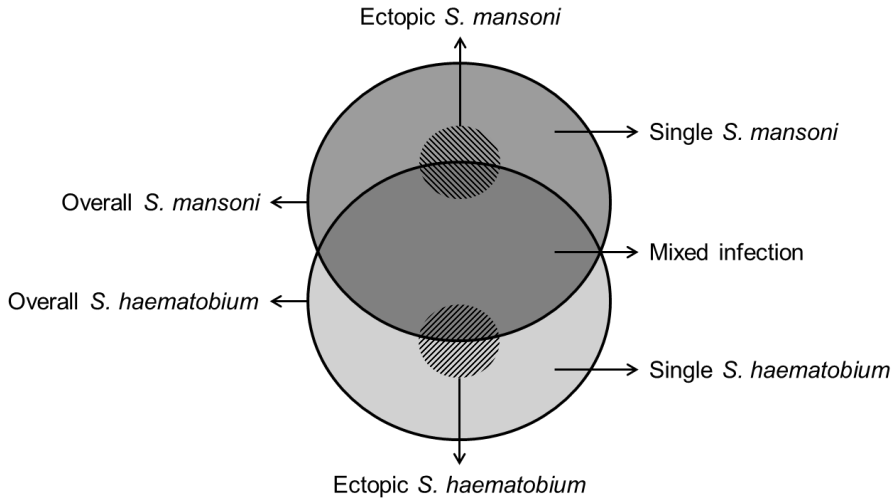

Figure 2.2. Schematic overview of *Schistosoma* infection groups.

www.york.ac.uk/res/schistoinir) for which approval was obtained by the review board of the Institute of Tropical Medicine in Belgium, the ethical committee of the Antwerp University Hospital in Belgium and 'Le Comité National d'Ethique de la Recherche en Santé' in Senegal.

Informed and written consent were obtained from all participants. After the study, praziquantel (40 mg/kg) and mebendazole (500 mg) treatment were offered to all community members to treat and prevent schistosomiasis and soil-transmitted helminthiasis, respectively, according to WHO guidelines (WHO, 2006).

Parasitology

Two feces and two urine samples were collected from each participant on consecutive days. For each feces sample, two Kato-Katz slides of 25 mg of fecal material each were prepared and microscopically examined for *Schistosoma* spp., *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm (Katz et al., 1972; WHO, 1991). *Schistosoma mansoni* infection intensity was expressed as the number of eggs detected per gram of feces (epg). Urine filtration was performed using a filter of 12µm pore-size (Isopore, USA) according to standard procedures (WHO, 1991). *Schistosoma haematobium* infection intensity was expressed as the number of eggs detected per 10 ml of urine (ep10ml). World Health Organization (WHO) standards were used to categorize schistosomal infection intensity into light (1-99 epg for *S. mansoni* and 1-49 ep10ml for *S. haematobium*), moderate (100-399 epg for *S. mansoni*) and heavy infections (≥400 epg for *S. mansoni* and ≥50 ep10ml for *S. haematobium*) (Montresor et al., 1998). Ectopic eggs were measured qualitatively (positive/negative).

In this paper, the following definitions are used (see also Figure 2.2): single infection is defined as passing eggs of only one species, and mixed infection as passing eggs of both *S. mansoni* and *S. haematobium.* Ectopic egg elimination refers to the elimination of schistosomal eggs via the unusual route – i.e. *S. haematobium* eggs in feces or *S. mansoni* eggs in urine. Overall *S. mansoni* infection refers to both mixed and single *S. mansoni* infections. Overall *S. haematobium* infection includes both mixed and single *S. haematobium* infections.

Statistical analyses

IBM SPSS 19.0 (SPSS, Inc.) was used for statistical analyses. Results were considered significant when the *p*-value was <0.05. Data were characterized by percentages, geometric means, and 95% confidence intervals (CIs). As egg output showed skewed distributions, data were normalized by 10log-transformation. Geometric means of egg counts (GM epg or ep10ml) were calculated for microscopically positive individuals to analyze the intensity of infection. The Mann-Whitney U test was used to determine age differences between the communities. The Pearson Chi-square test was used to determine the association between community and gender as well as between *S. mansoni* and *S. haematobium* infection status. The independent-samples T-test was used to compare GM infection intensities between single and mixed infections.

Multivariable logistic regression models were used to identify independent risk factors for overall *S. mansoni* and overall *S. haematobium* infection, respectively. In the first model, *S. mansoni*-positive subjects were compared with *S. mansoni*-negative subjects. In the second model, *S. haematobium*-positive subjects were compared with *S. haematobium*-negative subjects. Age, gender and community of residence were included as potential risk factors. Similarly, the association between mixed infection and these risk factors was assessed, with single *S. mansoni* and single *S. haematobium* infection as reference groups, respectively.

To assess the association between infection intensity (*S. mansoni* or *S. haematobium* infection) and mixed infection, multivariable linear regression was performed, using a dummy variable for mixed infection (1=mixed, 0=single), and age, gender and community of residence as other risk factors.

The association between ectopic egg elimination and intensity of infection in mixed infections was assessed by multivariable logistic regression, with non-ectopic mixed infections as a reference group. Infection intensity of *S. mansoni* and *S. haematobium*, age, gender, and community of residence were included as risk factors.

Due to the skewed trend of infection prevalences and intensities with age, the population was divided into seven age groups (0-4, 5-9, 10-14, 15-19, 20-29, 30-39 and ≥ 40 years) in all models. In addition, significant interaction terms ($p<0.05$) were added to the equations.

Results

Complete data (based on ≥1 feces sample and ≥10 ml of urine) were obtained from 857 individuals. This group consisted of 428 males and 429 females, 253 subjects from Ndieumeul and 604 from Diokhor Tack, with a median age of 16 (range 0-85) years. There were no significant differences regarding age or gender between the two communities (p>0.8).

Seventy-three per cent of the study population was infected with at least one *Schistosoma* spp. (Table 2.1). The overall prevalence of *S. mansoni* infection was 61% (520/857) and that of *S. haematobium* 50% (431/857), with 15% (129/857) and 9% (76/857) of heavy infections, respectively. Among infected subjects, 53% (328/623) had mixed infections: 8% (49/623) had mixed infections with ectopic egg elimination and 45% (279/623) without ectopic egg elimination.

Table 2.1. Schistosomal infection prevalences and intensities.

Schistosoma mansoni infection		*Schistosoma haematobium* infection		Prevalence n (%)		*S. mansoni* infection intensity		*S. haematobium* infection intensity	
feces	urine	feces	urine			GM epg (95%CI)		GM ep10ml (95%CI)	
Positive subjects				**623**	**(72.7)**				
Single infections				**295**	**(34.4)**				
+	-	-	-	191	(22.3)	76.6	(62.0-94.4)		
-	-	-	+	102	(11.9)			2.3	(1.8-3.0)
-	-	+	-	1	(0.1)				
-	+	-	-	1	(0.1)				
Mixed infections				**328**	**(38.3)**	**167.4**	**(142.0-197.2)**	**9.9**	**(8.0-12.1)**
without ectopic eggs				279	(32.6)				
+	-	-	+	279	(32.6)	148.9	(124.7-177.7)	7.6	(6.1-9.5)
with ectopic *S. haematobium* eggs				2	(0.2)				
+	-	+	+	1	(0.1)	420.0		25.0	
+	-	+	-	1	(0.1)	60.0			
with ectopic *S. mansoni* eggs				47	(5.5)				
+	+	-	+	39	(4.6)	383.1	(264.2-554.5)	58.3	(36.2-93.9)
-	+	-	+	8	(0.9)			9.5	(1.7-48.7)
Negative subjects				**234**	**(27.3)**				
-	-	-	-	234	(27.3)				
Overall *S. mansoni* infections				520	(60.7)	125.3	(109.7-143.1)		
Overall *S. haematobium* infections				431	(50.3)			7.1	(5.9-8.4)
Total				**857**	**(100%)**				

GM: geometric mean; 95% CI: 95% confidence interval; epg: number of *Schistosoma mansoni* eggs / gram feces; ep10ml: number of *S. haematobium* eggs / 10 ml of urine.

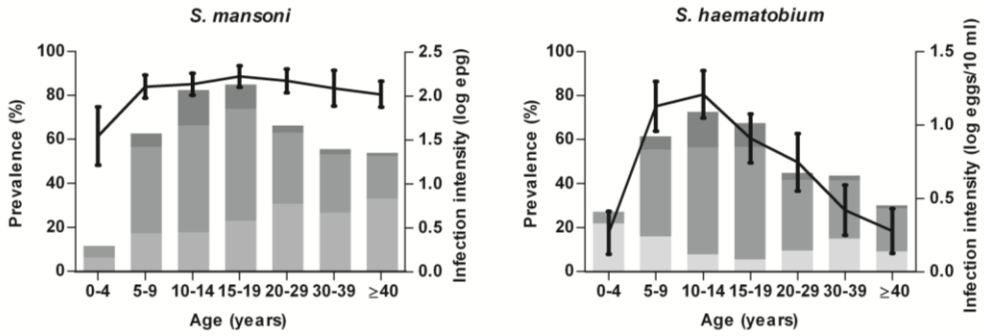

Figure 2.3. Age-prevalence and -intensity curves for schistosomal infection in the communities studied.

The bars indicate overall infection prevalences per age group. Dark grey stacks indicate infections with ectopic egg elimination. Grey stacks in the middle indicate mixed infections, and lighter grey stacks designate single *Schistosoma mansoni* and single *Schistosoma haematobium* infections (all without ectopic egg elimination). Lines indicate mean 10log-transformed infection intensities among positive subjects with 95% confidence intervals (whiskers). Epg: number of *S. mansoni* eggs detected per gram of feces; ep10 ml: number of *S. haematobium* eggs detected per 10 ml of urine.

Individuals who were positive for *S mansoni* were more likely to be infected with *S. haematobium* and vice versa ($p<0.001$). Among positive subjects, *S. mansoni* and *S. haematobium* infection intensities were significantly higher in mixed compared with single infections ($p<0.001$).

Furthermore, 2.5% (21/857) of the study population harbored one or more intestinal helminths. *Ascaris lumbricoides* was found in 17 and *T. trichiura* in six individuals. No hookworm infections were detected.

Mixed *Schistosoma* infections

Figure 2.3 indicates that overall *S. mansoni* infection prevalences were higher than those for *S. haematobium* in all age groups, except in children under five years. Accordingly, single *S. haematobium* infection was the most dominant infection in the youngest subjects (< 5 years). The prevalences and intensities of overall *S. mansoni* and *S. haematobium* infection, as well as mixed infections, increased up to the second decade of life, with a subsequent decrease in adults (Figure 2.3). The decline in prevalence and intensity was sharper and occurred at an earlier age for *S. haematobium* than for *S. mansoni*. As a result, single *S. mansoni* infection was the most dominant infection in the oldest age group (≥40 years). These patterns were similar in the two communities. Table 2.2 summarizes the risk factors for overall *S. mansoni* and *S. haematobium* infection, respectively. Age and community of residence were strongly

Table 2.2. Results from multivariable logistic models examining risk factors of overall *Schistosoma mansoni*, overall *Schistosoma haematobium*, as well as mixed infections.

Risk factors		Overall infection				Mixed infection			
		S. mansoni-positive versus *S. mansoni*-negative		*S. haematobium*-positive versus *S. haematobium*-negative		Mixed infection versus single *S. haematobium*		Mixed infection versus single *S. mansoni*	
		n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)
Age [a]	0-4 years	96	0.02 (0.01-0.1)***	96	0.1 (0.03-0.2)***	26	0.02 (0.004-0.1)***	11	0.1 (0.01-1.0)
	5-9 years	163	0.3 (0.2-0.6)**	163	0.6 (0.3-1.4)	100	0.2 (0.1-0.7)**	102	0.8 (0.3-1.9)
	10-14 years	142	0.9 (0.4-1.8)	142	0.8 (0.3-1.7)	103	0.7 (0.2-2.0)	116	0.7 (0.3-1.8)
	15-19 years	92	Ref.	92	Ref.	62	Ref.	78	Ref.
	20-29 years	127	0.3 (0.2-0.6)**	127	0.1 (0.1-0.3)***	57	0.2 (0.1-0.8)*	84	0.2 (0.1-0.5)***
	30-39 years	94	0.2 (0.1-0.5)***	94	0.1 (0.05-0.3)***	41	0.1 (0.04-0.4)**	52	0.1 (0.02-0.3)***
	≥40 years	143	0.2 (0.1-0.4)***	143	0.1 (0.05-0.3)***	42	0.2 (0.1-0.6)**	77	0.1 (0.04-0.4)***
Gender	Male	428	Ref.	428	Ref.	202	Ref.	256	Ref.
	Female	429	1.2 (0.9-1.7)	429	0.5 (0.2-1.2)	229	1.5 (0.9-2.4)	264	0.5 (0.2-1.4)
Community	Ndieumeul	253	Ref.	253	Ref.	168	Ref.	190	Ref.
	Diokhor	604	0.4 (0.2-0.5)***	604	0.3 (0.2-0.5)***	263	0.4 (0.3-0.7)**	330	0.5 (0.3-0.7)***
Interaction	Age*Gender		N/A	857	***		N/A	520	*

Ref.: reference category; OR: odds ratio; 95% CI: 95% confidence interval; N/A, not applicable. *: $p<0.05$; **: $p < 0.01$; ***: $p < 0.001$.

[a] Associations with age were significant at the level of $p<0.001$.

associated with both infections. For *S. haematobium* infection, gender differences were more pronounced in adults than in children (p=0.008 for the age*gender interaction term); age-stratified analysis indicated that adult women (≥20 years of age) were more at risk of being infected with *S. haematobium* compared with their male counterparts (odds ratio (OR) 2.5; 95% CI: 1.6-4.0). A similar trend was found for *S. mansoni* infection, although it was not significant. The risk factors for mixed infection are summarized in Table 2.2, and reflect those of overall *S. mansoni* and *S. haematobium* infection. Again, gender differences were more pronounced in adults than in children. Age-stratified analysis showed that adult women were more at risk of being infected with mixed infections than their male counterparts (OR=2.7; 95% CI: 1.5-4.9 for mixed infection versus single *S. mansoni*, and OR=1.6; 95% CI: 0.7-3.7 for mixed infection versus single *S. haematobium*).

Similar risk factors were identified for infection intensities as for infection prevalences (data not shown). In addition, *S. mansoni* infection intensity was associated with the presence of *S. haematobium* infection and vice versa (standardized β=0.21 and 0.22 for *S. mansoni* and *S. haematobium* infection intensity, respectively (p<0.001)). These associations were similar in both communities.

Ectopic *Schistosoma* egg elimination

Ectopic *S. mansoni* egg elimination was found in 48 subjects and ectopic *S. haematobium* egg elimination in three subjects. Within the first group, most individuals also had *S. mansoni* eggs in feces and *S. haematobium* eggs in urine (n = 39, Table 2.1). This combination was restricted to 5-29-year-old individuals. Within this age range, ectopic elimination of *S. mansoni* eggs was significantly associated with schistosomal infection intensities: the OR for ectopic *S. mansoni* elimination was 3.5 (95%CI: 2.0-6.2) for a 10-fold increase in *S. haematobium* and 2.2 (95%CI: 1.1-4.4) for a 10-fold increase in *S. mansoni* infection intensity. Similarly, the average *S. haematobium* infection intensity was four times lower in those with single *S. haematobium* infection (GM=2.3 eggs/10 ml) than in those with additional ectopic *S. mansoni* eggs (GM=9.5 eggs/10 ml), suggesting a positive association between ectopic *S. mansoni* egg elimination and *S. haematobium* infection intensity in the other groups as well (Table 2.1).

Discussion

Due to the large overlap of *S. mansoni*- and *S. haematobium*-endemic regions in Africa, many people are at risk of co-infection, with potential adverse effects on schistosomiasis morbidity and control (Cheever et al., 1977; Friis et al., 1996; Koukounari et al., 2010). Nonetheless, studies on the distribution and determinants of mixed infections have to date been rare. Here, we report on the epidemiology of mixed *S.*

mansoni and *S. haematobium* infections as well as ectopic egg elimination in two mixed foci in northern Senegal.

The age-related patterns of overall *S. mansoni* and *S. haematobium* infection corresponded to the characteristic convex-shaped curve for schistosomiasis, with a peak in adolescence (Woolhouse, 1998; Cook and Zumla, 2009). Mixed infections followed the same pattern. However, a more detailed analysis revealed that the decline in overall *S. haematobium* infection prevalences and intensities after adolescence appeared to be steeper than for *S. mansoni*. Previous studies in either *S. mansoni*- or *S. haematobium*-endemic foci (summarized by Agnew et al. (1993)), as well as in mixed foci (Hairston, 1965; Clarke, 1966; Farooq et al., 1966a; Dennis et al., 1983; Kvalsvig and Schutte, 1986; Robert et al., 1989; De Clercq et al., 1999; Lwambo et al., 1999; El Khoby et al., 2000) have shown similar trends, but only Agnew et al. (1993) explicitly mentioned this. In an early autopsy study, Cheever et al. (1977) reported a more pronounced reduction of *S. haematobium* than *S. mansoni* worm loads with age, which is also in line with our results. The underlying mechanism for this apparently rather common phenomenon is unknown and merits further investigation. Different mechanisms could play a role and are discussed below.

Cheever et al. (1977) showed that *S. haematobium* eggs have a higher tendency to accumulate at the oviposition site than *S. mansoni* eggs. Accumulating *S. haematobium* eggs lead to progressive bladder wall pathology which may subsequently obstruct egg passage into the lumen of the bladder and lead to a reduction of *S. haematobium* egg excretion. Furthermore, differences between *Schistosoma* spp. in age-dependent reduction of fecundity have been suggested, possibly mediated by the host's immune system (Agnew et al., 1996).

Differences in vulnerability to the host's immune response between the two species may also explain the more pronounced reduction of *S. haematobium* compared with *S. mansoni* worm load with age as observed by Cheever et al. One human study indicated that the two species induce different types of humoral immune responses (van Remoortere et al., 2001). The authors showed that *S. haematobium*-infected subjects produced IgM as well as IgG antibodies against specific carbohydrate epitopes, while IgM – which is thought to inhibit protective host immune responses (Butterworth et al., 1987) – dominated in *S. mansoni* infections. Also, animal studies reported vaccination with cercariae to convey a higher degree of protection against *S. haematobium* than against *S. mansoni* infection (Taylor et al., 1973; Webbe et al., 1976; Agnew et al., 1993; Dean et al., 1996). Similarly, animal models provided evidence for differences in 'concomitant immunity': adult *S. mansoni* worms appear to have a greater capacity than *S. haematobium* to elicit an immune response which prevents infection with new worms, while they themselves remain invulnerable to the host's immune defense (Agnew et al., 1993; Terry, 1994).

Omer and Teesdale (1978) suggested that the apparent increased vulnerability of *S. haematobium* compared with *S. mansoni* may be related to the location of the worms in the human blood circulation. The authors suggested that treatment-induced damage to, and dislodgement of, adult *S. haematobium* would result in displacement of the worms within the blood stream from the vesical plexus via the heart to the lungs. Trapped in the capillary beds of the lung alveoli, they would not be able to recover from this damage (i.e. so-called 'irreversible lung-shift'). Damaged adult *S. mansoni* worms, on the other hand, would be carried away from the mesenteric plexus via the portal vein to the liver where they can recover and then return to their oviposition site. Although speculative, similar mechanisms may play a role upon immune damage to adult worms.

The observed differences between age-related *S. mansoni* and *S. haematobium* patterns may also be related to differences in exposure to the two species; a lower cumulative exposure to *S. mansoni* compared with *S. haematobium* could have resulted in a lower protective immune response against the first species (Woolhouse, 1998; Mitchell et al., 2008). However, this scenario is unlikely in this specific area because *S. mansoni* was introduced before *S. haematobium* (Picquet et al., 1996), and current exposure to the first appeared more intense; peak and overall (heavy) infection prevalences were higher for *S. mansoni* at the time of this current study.

This suggests that differences in host-parasite interactions play a more important role than parasite exposure in the observed, more pronounced decline of *S. haematobium* than of *S. mansoni* infection with age. It should be noted however, that most evidence relies on animal models and/or old data. Caution should be used when extrapolating observations from animal models to the human host, particularly with regard to *S. haematobium* for which humans are assumed to be the only natural final host (Cook and Zumla, 2009). More recent human studies are needed to corroborate our age-related observations on mixed *Schistosoma* infections as well as on the proposed underlying mechanisms.

Not only age, but also other risk factors for mixed infection observed in this study were similar to those generally observed for schistosomiasis. Schistosomiasis is characterized by a focal epidemiology (Anderson and May, 1985a). It is therefore not surprising that also for mixed infections, community of residence was identified as an important risk factor. This could be due to differences in water contact behavior, snail distribution, genetic differences and other factors (Robert et al., 1989; Pinot de Moira et al., 2007). Furthermore, women were more at risk of infection than men. Gender differences have been previously observed (Dennis et al., 1983; Robert et al., 1989; El Khoby et al., 2000; Cunin et al., 2003), and have been attributed to hormonal differences (Remoue et al., 2002; Klein, 2004; Escobedo et al., 2005) and differences in

water contact between males and females (Fulford et al., 1996; Mahmoud, 2001; Scott et al., 2003; Sow et al., 2011).

Understanding the exact relation between mixed infection and infection intensity is crucial, as increased egg loads can have important repercussions on the development of morbidity (Gryseels et al., 2006). We found higher *S. mansoni* and *S. haematobium* infection intensities in mixed than in single infections and a positive association between *S. mansoni* and *S. haematobium* infections in these mixed foci. Robert et al. also found higher infection intensities in mixed infections (1989). However, other studies on a larger scale (at county, provincial or district level) reported inconsistent results (Dennis et al., 1983; Ahmed et al., 1996; Booth et al., 1998; Lwambo et al., 1999). Possibly, the relationship between mixed infection and infection intensity varies according to local differences in *S. mansoni* and *S. haematobium* transmission, which may be diluted on a larger scale. As such differences can even occur at a community level (Pinot de Moira et al., 2007), it is important to further investigate the relationship between mixed infection, infection intensity and morbidity in small-scale studies and different endemic settings.

Ectopic eggs were found in 15% of mixed infections, and most of those were *S. mansoni* eggs. Single infection with ectopic egg elimination was uncommon. Ectopic *S. mansoni* egg elimination was associated with *S. mansoni* and *S. haematobium* infection intensity. A 'spilling over' of *S. mansoni* worms and/or eggs towards the urinary bladder may have partly contributed to the elimination of *S. mansoni* eggs via the urine in heavily infected children (Husting, 1965). It has also been proposed that increased portal pressure – due to severe *S. mansoni*-associated hepatic fibrosis – might contribute to this phenomenon (Cook and Jordan, 1970; Cheever et al., 1977). Nevertheless, ectopic *S. mansoni* egg elimination was more strongly associated with *S. haematobium* than with *S. mansoni* infection intensity. Previous studies have consistently found this association (Blair, 1965; Husting, 1965; Ratard et al., 1991; Cunin et al., 2003) which has been attributed to sexual interactions between the two species (Webster et al., 1999). Experimental models have shown that *S. mansoni* and *S. haematobium* can form heterologous male-female pairs. This results in *S. haematobium* males carrying *S. mansoni* females to the vesical plexus. These females will then lay eggs with a *S. mansoni*-like morphology that are passed into the urine (Southgate et al., 1998). In addition, single males can remove homo- or heterologous females from other male worms (Tchuem Tchuente et al., 1995; Pica-Mattoccia et al., 2000; Steinauer, 2009). Male *S. haematobium* appear to be competitively stronger in taking heterologous females away from their male partner than *S. mansoni* (Webster et al., 1999; Cunin et al., 2003). This might explain why *S. mansoni* eggs are more commonly found to be eliminated via the unusual route than *S. haematobium* eggs (Ratard et al., 1991; Ernould et al., 1999a; Cunin et al., 2003);

prevalences of ectopic *S. mansoni* elimination of up to 31% have been reported (Ernould et al., 1999a).

In the present study, ectopically eliminated eggs were categorized as either *S. mansoni* or *S. haematobium.* However, we cannot exclude that some of these may have been genetic hybrids (Huyse et al., 2009), or parthenogenetic eggs, which could not be distinguished from regular *Schistosoma* eggs by microscopy. Experimental studies suggest that ectopic *S. mansoni*-like eggs are likely to be of parthenogenetic origin (Taylor, 1970; Basch and Basch, 1984; Imbert-Establet et al., 1994; Tchuem Tchuente et al., 1994; Jourdane et al., 1995; Khalil et al., 1995; Southgate et al., 1998). To date however, the possibility that *S. mansoni* x *S. haematobium* hybrids exist in nature has not been excluded. It is essential to determine the exact genetic nature and viability of ectopic *Schistosoma* eggs since hybrid species are assumed to be more infective and pathogenic than their parental species (Wright and Ross, 1980; Webster and Southgate, 2003; Huyse et al., 2009).

Initially, the distributions and risk factors for mixed infections did not appear to differ much from those of overall *S. mansoni* or *S. haematobium* infections in these mixed foci. Looking at the data in more detail, however, the decline in infection prevalences and intensities in adults was steeper for *S. haematobium* than for *S. mansoni*, resulting in a decrease in mixed infections and a relative increase in single *S. mansoni* infections over age. These observations are in line with previous studies in humans. Also, animal studies suggested *S. mansoni* to be less vulnerable to the host's age-dependent immune response than *S. haematobium*. Furthermore, a positive association was found between mixed infection, ectopic *S. mansoni* egg elimination and infection intensity of both species, with potentially important consequences for the development of morbidity in co-endemic areas. The significance of these findings should be confirmed by further epidemiological studies at a micro-geographical level, taking host- and parasite-related as well as environmental factors into account.

Acknowledgements

We gratefully thank the population of Ndieumeul and Diokhor Tack, Senegal, and the village chiefs, Daoure Mbaye and Daouda Pene, for their hospitality and participation in this study. This study would not have been possible without the field workers in Richard-Toll, Abdoulaye Yague, Mankeur Diop, Moussa Wade and Ngary Sy, who helped with the sample collection and did excellent microscopic analysis. We would also like to thank Claire Bourke and Adrian Mountford for sharing their experience on animal work, Tine Huyse, Frederik van den Broeck, Nele Boon and Kirezi Kanobana for critically reading the manuscript, and the medical staff of the Health Centre in Richard-Toll for their support. This work was funded by the European Union's sixth framework program (INCO-CT-2006-032405).

Chapter 3

Bladder morbidity and hepatic fibrosis in mixed *Schistosoma haematobium* and *Schistosoma mansoni* infections: a population-wide study in northern Senegal

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PLoS negled tropical diseases 2012;6(9):e1829

Abstract

Background: The global distribution map of schistosomiasis shows a large overlap of *Schistosoma haematobium*- and *S. mansoni*-endemic areas in Africa. Yet, little is known about the consequences of mixed *Schistosoma* infections for the human host. A recent study in two neighboring co-endemic communities in Senegal indicated that infection intensities of both species were higher in mixed than in single infections. Here, we investigated the relationship between mixed *Schistosoma* infections and morbidity in the same population. So far, this has only been studied in children.

Methods: *Schistosoma* infection was assessed by microscopy. *Schistosoma*-specific morbidity was assessed by ultrasound according to WHO guidelines. Multivariable logistic regression models were used to identify independent risk factors for morbidity.

Principal findings: Complete parasitological and morbidity data were obtained from 403 individuals. *Schistosoma haematobium*-specific bladder morbidity was observed in 83% and *S. mansoni*-specific hepatic fibrosis in 27% of the participants. Bladder morbidity was positively associated with *S. haematobium* infection intensity (OR=1.9 (95% CI 1.3-2.9) for a 10-fold increase in intensity). Moreover, people with mixed infections tended to have less bladder morbidity than those with single *S. haematobium* infections (OR=0.3 (95% CI 0.1-1.1)). This effect appeared to be related to ectopic *S. mansoni* egg elimination in urine. Hepatic fibrosis on the other hand was not related to *S. mansoni* infection intensity (OR=0.9 (95% CI 0.6-1.3)), nor to mixed infections (OR=1.0 (95% CI 0.7-1.7)).

Conclusions: This is the first population-wide study on the relationship between mixed *Schistosoma* infections and morbidity. Mixed infections did not increase the risk of *S. mansoni*-associated morbidity. They even tended to reduce the risk of *S. haematobium*-associated morbidity, suggesting a protective effect of *S. mansoni* infection on bladder morbidity. These unexpected results may have important consequences for schistosomiasis control in co-endemic areas and warrant further investigation.

Introduction

Worldwide more than 207 million people are infected with *Schistosoma,* 85% of whom live in Africa (WHO, 2010). Due to the large overlap in *Schistosoma haematobium*- and *S. mansoni*-endemic areas (Doumenge et al., 1987; Gryseels et al., 2006; Montgomery, 2011) many people are at risk of co-infection. Yet, little is known about mixed *Schistosoma* infections and their impact on host morbidity.

Clinical manifestations of schistosomiasis are associated with the species-specific oviposition sites (Gryseels et al., 2006). Both *S. haematobium* and *S. mansoni* mature in the portal vein of the human host and form male-female pairs. Subsequently, the *S. mansoni* male carries the female to the mesenteric plexus whereas the *S. haematobium* couple continues its way to the veins of the pelvis. In these respective sites, they lay eggs, which are eventually eliminated from the body via the urine (*S. haematobium*) or feces (*S. mansoni*). About half of the eggs, however, are carried away with the blood stream and/or trapped in the tissues. These retained eggs provoke inflammatory and granulomatous immune responses (Gryseels et al., 2006). For *S. haematobium*, this can lead to inflammation, ulceration and pseudopolyposis of bladder and ureteral walls, and in children this is often accompanied by hematuria. Chronic lesions may evolve to fibrotic and sandy patches with severe sequelae such as hydroureter, hydronephrosis, and squamous cell bladder cancer (Smith and Christie, 1986; King, 2002; Gryseels et al., 2006). For *S. mansoni*, egg deposition can lead to inflammatory hepatic schistosomiasis and hepatosplenomegaly in children and adolescents (Wilson et al., 2011). Trapped schistosomal eggs are gradually replaced by fibrotic deposits, and give rise to chronic hepatic schistosomiasis (Gryseels et al., 2006).

In mixed *S. mansoni* and *S. haematobium* infections, the above-described processes act in parallel, which may result in more severe or other abnormalities than in single infections. So far, only two studies have investigated the relationship between mixed infections and host morbidity. In Zimbabwe, a positive association between *S. mansoni* egg output and liver size was found in the presence but not in the absence of *S. haematobium*. Yet, this effect was very little (Friis et al., 1996). In a study in Mali, mixed infection was associated with reduced hepatic morbidity on one hand and increased bladder morbidity on the other (Koukounari et al., 2010). Both studies were performed in schoolchildren, in whom severe hepatic schistosomiasis is unlikely to have developed already (Richter et al., 1996; Gryseels et al., 2006; Wilson et al., 2011). Community-wide studies would therefore be more appropriate to investigate mixed infections and morbidity.

In northern Senegal, many communities have in the past decades become co-endemic for *S. mansoni* and *S. haematobium* (De Clercq et al., 1999; Ernould et al., 1999a; Southgate et al., 2001; Van der Werf et al., 2002; Ten Hove et al., 2008; Huyse et al., 2009). *Schistosoma mansoni* was introduced in Richard-Toll in 1988 upon construction

of the Diama dam and rapidly spread throughout the region (Talla et al., 1990; Talla et al., 1992). By 1994, virtually the entire Lac de Guiers area had become exposed to this species (Picquet et al., 1996). Today, both *S. mansoni* and *S. haematobium* are widely spread, resulting in a large number of people with mixed infections in the communities around the lake.

Recently, we reported on the distribution of and risk factors for mixed *Schistosoma* infections in two communities on the banks of Lac de Guiers. Individuals with mixed infections were found to have higher infection intensities than those with single infections (Chapter 2). In the present study, we set out to investigate the relationship between mixed *Schistosoma* infections and morbidity in the same communities. We studied the patterns of *S. haematobium*-specific bladder morbidity and *S. mansoni*-specific hepatic morbidity in this co-endemic focus, and compared morbidity in people with mixed *Schistosoma* infections to those with single infections.

Methods

Ethics statement

This study was part of a larger investigation on the epidemiology of schistosomiasis and innate immune responses (SCHISTOINIR: www.york.ac.uk/res/schistoinir) for which approval was obtained from the review board of the Institute of Tropical Medicine, the ethical committee of the Antwerp University Hospital and 'Le Comité National d'Ethique de la Recherche en Santé' in Dakar. Informed and written consent was obtained from all participants prior to inclusion into the study.

Study area

This study was conducted from July until November 2009 in Ndieumeul (also known as Thiekène) and Diokhor Tack, two neighboring communities on the Nouk Pomo peninsula in Lac de Guiers. Details on the study area have been described elsewhere (Chapter 2).

Parasitology and urine dipstick

Two urine and two feces samples were collected from each participant on consecutive days for microscopic analysis (Katz et al., 1972; WHO, 1991). Per feces sample, two Kato-Katz slides of 25 mg fecal material each were prepared and microscopically examined for *Schistosoma* species (Katz et al., 1972). *Schistosoma mansoni* infection intensity was expressed as the number of eggs detected per gram of feces (epg). Urine filtration was performed using a filter of 12µm pore-size (Isopore) according to standard procedures (WHO, 1991). *Schistosoma haematobium* infection intensity was expressed as the number of eggs detected per 10 ml of urine (ep10ml). Ectopic eggs were measured

qualitatively (positive/negative). Ectopic egg elimination refers to elimination of schistosomal eggs via the unusual route – i.e. *S. mansoni* eggs in urine or *S. haematobium* eggs in feces. Single infection was defined as passing eggs of only one species, and mixed infection as passing eggs of both *S. mansoni* and *S. haematobium*, regardless of the route of egg elimination (Chapter 2). Microhematuria was determined in a subsample using Combur 7 dipsticks (Roche) on the first urine sample. All community members were offered praziquantel (one dose of 40 mg/kg body weight) and mebendazole (one dose of 500 mg) treatment after the study according to WHO guidelines (WHO, 2006).

Ultrasound

Participants were examined using a portable ultrasonography device with convex transducer. Pathologic lesions associated with *S. haematobium* or *S. mansoni* infection were recorded according to the Niamey guidelines (Richter et al., 1996). All examinations were performed by the same clinician who was blind to the participant's infection status. Participants with severe pathology that needed further treatment were referred to the appropriate health authority. For *S. haematobium*-specific morbidity, the urinary bladder score was determined (Richter et al., 1996). A score of ≥1 was considered as *S. haematobium*-specific urinary bladder morbidity in accordance with previous studies (Hatz et al., 1990; Medhat et al., 1997; Leutscher et al., 2000; King et al., 2001; Kouriba et al., 2005; Koukounari et al., 2006; Shiff et al., 2006; Koukounari et al., 2010). Individuals with a score of 0 were categorized as controls. For *S. mansoni*-specific morbidity, the liver image pattern was determined (Richter et al., 1996). Additional measurement of periportal thickening was not included as this approach has been shown to be not reproducible (King et al., 2003). Liver image patterns of C to F were categorized as *S. mansoni*-specific hepatic morbidity (Boisier et al., 2001; King et al., 2003; Booth et al., 2004a; Booth et al., 2004b; Malenganisho et al., 2008). Individuals with liver image pattern A or B are not likely to have periportal fibrosis (Richter et al., 1996) and were therefore categorized as controls. Individuals with signs of hepatic morbidity that were not specific for *S. mansoni* (e.g. hepatitis, cirrhosis or fatty liver) were excluded (Richter et al., 1996) (Table 3.2).

Statistical analysis

IBM SPSS 19.0 (SPSS, Inc.) was used for statistical analysis. Results were considered significant when the *p*-value was <0.05. As egg outputs showed skewed distributions, data were normalized by log (base 10)-transformation after adding half of the detection limit to allow for zeroes.

Differences between groups were determined by the Pearson Chi-square test for community and gender, and by the Mann-Whitney U test for age. Furthermore, the

Pearson Chi-square test was used to determine the association between bladder morbidity and microhematuria, as well as between bladder and liver morbidity.

Because of the non-linear trend of morbidity over age, the population was divided into four age groups (0-9, 10-19, 20-39 and ≥40 years) for multivariable regression analysis. Multivariable logistic regression models were used to identify independent risk factors for *S. haematobium*-specific bladder morbidity and *S. mansoni*-specific hepatic fibrosis, respectively. Age, gender, community of residence, *S. haematobium* infection intensity and *S. mansoni* infection intensity were included as potential risk factors. Moreover, significant interaction terms with age ($p<0.05$) were added.

Similar models were used to assess the independent effect of mixed infection (as compared to single infection) on *S. haematobium*-specific bladder morbidity and *S. mansoni*-specific hepatic fibrosis, respectively. Among *S. haematobium*-positive subjects, the association between bladder morbidity and mixed infection was investigated using a dummy variable for mixed infection (1=mixed, 0=single), and age, gender, community of residence and *S. haematobium* infection intensity as other determinants. Likewise, the association between hepatic fibrosis and mixed infection was investigated in *S. mansoni*-positive subjects with a dummy variable for mixed infection and upon correction for age, gender, community of residence and *S. mansoni* infection intensity.

Results

Complete parasitological data were obtained from 857 individuals (Chapter 2). Ultrasound data were collected from a random subsample of 403 individuals. The latter group consisted of 207 males and 196 females with a median age of 16 (range 3-85) years. There were no significant dissimilarities between those who participated in the ultrasound examination and those who did not, except for a slightly lower percentage of individuals participating from the community of Ndieumeul as compared to Diokhor Tack ($p=0.005$), and from the youngest as compared to the older age groups ($p=0.030$). No significant differences in age or gender were observed between the two communities.

Morbidity prevalences

Schistosoma haematobium-specific bladder morbidity was observed in 83% of the study population (334/403; Table 3.1). Most common lesions concerned multifocal or diffuse bladder wall thickening (n=189), irregularities (n=94) or a single mass (n=19). Microhematuria was twice as prevalent among those with bladder morbidity as compared to those without (44 % *versus* 21%, $p=0.002$).

Table 3.1. *Schistosoma haematobium*-associated bladder morbidity, hematuria and *S. haematobium* infection in the two co-endemic communities studied.

Morbidity	Urinary bladder score	n	(%)	Microhematuria (%)	*S. haematobium* infection	
					%	GM (95%CI)
Negative	**0**	**69**	**(17)**	**21**	**33**	**3.9 (1.8-8.1) ep10ml**
Positive	**≥1**	**334**	**(83)**	**44**	**58**	**8.8 (6.7-11.5) ep10ml**
	1	15	(4)	62	73	8.5 (2.2-31.5) ep10ml
	2	301	(75)	42	56	8.5 (6.4-11.3) ep10ml
	≥3	18	(4)	71	78	13.1 (4.2-39.5) ep10ml
Total		**403**	**(100)**	**40**	**54**	**8.1 (6.2-10.4) ep10ml**

GM: Geometric Mean, calculated for microscopically *S. haematobium*-positive individuals only.

Table 3.2. *Schistosoma mansoni*-associated hepatic fibrosis and *S. mansoni* infection in the two co-endemic communities studied.

Morbidity	Liver image pattern	n	(%)	*S. mansoni* infection	
				%	GM (95%CI)
Negative	**A-B**	**282**	**(70)**	**72**	**123 (99-153) epg**
	A	142	(35)	75	131 (98-175) epg
	B	140	(35)	69	115 (83-160) epg
Positive	**C-F**	**109**	**(27)**	**61**	**113 (76-168) epg**
	C	89	(22)	63	125 (81-193) epg
	D	10	(2)	40	113 (29-400) epg
	E	9	(2)	67	45 (10-160) epg
	F	1	(0.2)	0	N/A
Excluded		12	(3)		
Total		**403**	**(100)**	**69**	**120 (99-145) epg**

GM: Geometric Mean, calculated for microscopically *S. mansoni*-positive individuals only; N/A: Not Applicable.

Table 3.3. Liver and bladder co-morbidity in the two co-endemic communities studied. [a]

Bladder morbidity	Hepatic fibrosis		
	Negative	**Positive**	**Total**
Negative	51	16	**67**
Positive	231	93	**324**
Total	**282**	**109**	**391**

[a] Number of cases.

S. mansoni-specific fibrosis was present in 27% (109/403) of the population (Table 3.2). Liver image patterns up to F were observed, but the large majority had pattern C (89/109).

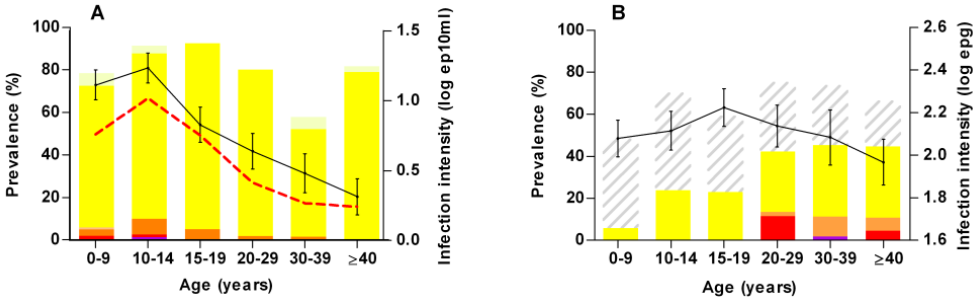

Figure 3.1. Age distribution of schistosomiasis morbidity in the two co-endemic communities studied.

Colored stacks indicate morbidity prevalences and continuous black lines indicate mean 10log-transformed infection intensities among positive subjects with the standard error of the mean (whiskers). **Panel A**: Different forms of *S. haematobium*-specific bladder morbidity are denoted by a color gradient: light yellow stacks designate a urinary bladder score of 1, bright yellow a score of 2 and orange (3 and 4), red (5) and violet (6) indicate higher morbidity scores. The dotted red line indicates hematuria prevalence in a subsample (n=317). **Panel B**: The severity of *S. mansoni*-specific fibrosis is denoted by a color gradient. Yellow stacks designate liver image pattern C, orange pattern D, red pattern E, and violet stacks indicate pattern F. Striped stacks designate those with borderline liver morbidity (pattern B, not classified as morbidity).

Morbidity of both liver and bladder was observed in 24% (93/391) of the study participants (Table 3.3). Those who had bladder morbidity tended to be more at risk for hepatic fibrosis and vice versa (odds ratio (OR)=1.3 (95% confidence interval (CI) 0.7-2.4)).

Age-related patterns

Figure 3.1 shows that bladder morbidity was mainly observed in children (<20 years), with a peak in 10-to-19-year-olds. The age-related distribution of bladder morbidity coincided with that of *S. haematobium* infection intensity and microhematuria, although the peak was slightly later in adolescence, and the subsequent decline in adults less pronounced.

The prevalence of hepatic fibrosis increased from 6% in 0-to-9-year-olds to 44% in those ≥20 years old. The more severe forms of hepatic fibrosis (liver image patterns D, E and F) only became apparent in adults (≥20 years old). The peak in the age-related distribution of hepatic fibrosis occurred more than 10 years later in life than the *S. mansoni* infection intensity peak (Figure 3.1).

Risk factors

Multivariable analysis showed age to be a significant risk factor for bladder morbidity as well as hepatic fibrosis (Table 3.4). However, age-related patterns of hepatic fibrosis differed between the two communities (p=0.033): while the ORs for hepatic fibrosis increased with age in Diokhor Tack (p<0.001), they did not vary with age in Ndieumeul (data not shown). Individuals from Diokhor Tack were significantly more at risk for hepatic fibrosis but tended to be less at risk for bladder morbidity than their counterparts from Ndieumeul (Table 3.4). Furthermore, females were less at risk for both forms of morbidity than males. Neither *S. mansoni* intensity nor *S. haematobium* intensity was identified as an independent risk factor for hepatic fibrosis. On the other hand, *S. haematobium* (but not *S. mansoni*) infection intensity was a strong risk factor for bladder morbidity.

Effect of mixed infection

After including mixed infection to the multivariable model, the above-described trends remained the same (Table 3.5). The risk of hepatic fibrosis did not differ between subjects with single *S. mansoni* and those with mixed infections. Interestingly however, mixed infection tended to be negatively associated with *S. haematobium*-specific bladder morbidity, suggesting a protective effect of current *S. mansoni* infection (p=0.068).

Ectopic egg elimination and bladder morbidity

Ectopic *S. haematobium* eggs were found in one (0.6%) and ectopic *S. mansoni* eggs in 23 (13%) out of 176 individuals with mixed infections. Table 3.6 illustrates the importance of the route of *S. mansoni* egg elimination in the development of bladder morbidity. Those who eliminated *S. mansoni* via both urine and feces (n=17) had highest *S. haematobium* infection intensities and prevalences of bladder morbidity. Lowest prevalences of morbidity were observed in those who exclusively eliminated *S. mansoni* eggs via the urine (and not via the feces; n=6), despite relatively high *S. haematobium* infection intensities.

Discussion

The global distribution map of schistosomiasis shows a large overlap of *Schistosoma mansoni*- and *S. haematobium*-endemic areas in Africa (Doumenge et al., 1987; Gryseels et al., 2006; Montgomery, 2011). Yet, little is known about the consequences of mixed *Schistosoma* infections for the human host. Here, we report on the relationship of mixed *Schistosoma* infections with *S. haematobium*-specific bladder morbidity and *S. mansoni*-specific hepatic morbidity in two co-endemic communities.

Table 3.4. Risk factors for schistosomiasis morbidity in the total study population.

Risk factors		*S. haematobium*-specific bladder morbidity (n=403)			*S. mansoni*-specific hepatic fibrosis (n=391)		
		n	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	n	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Age[a]	<10y	102	0.3 (0.1-0.7)**	0.3 (0.1-0.8)*	102	0.1 (0.03-0.2)***	0.1 (0.01-1.3)
	10-19y	121	Ref.	Ref.	119	0.4 (0.2-0.7)**	1.4 (0.4-5.1)
	20-39y	109	0.3 (0.1-0.7)**	0.6 (0.3-1.3)	105	Ref.	Ref.
	≥40y	71	0.4 (0.2-0.97)*	0.9 (0.3-2.3)	65	1.0 (0.6-1.9)	0.7 (0.1-3.8)
Gender	Male	207	Ref.	Ref.	200	Ref.	Ref.
	Female	196	0.4 (0.2-0.7)***	0.3 (0.2-0.6)***	191	0.8 (0.5-1.3)	0.5 (0.3-0.9)*
Community	Ndieumeul	100	Ref.	Ref.	99	Ref.	Ref.
	Diokhor Tack	303	0.4 (0.2-0.8)*	0.6 (0.3-1.3)	292	1.9 (1.1-3.3)*	5.1 (1.3-20.8)*
S. haematobium	Infection intensity [b]	403	2.0 (1.4-2.9)***	1.9 (1.3-2.9)**	391	0.6 (0.5-0.8)***	0.9 (0.7-1.3)
S. mansoni	Infection intensity [b]	403	1.5 (1.1-2.1)*	1.1 (0.8-1.6)	391	0.8 (0.6-1.0)	0.9 (0.6-1.3)
Interaction	Age x community	N/A	N/A	N/A	391	N/A	p=0.033 [b]

OR: Odds Ratio; 95%CI: 95% Confidence Interval; Ref.: Reference category; N/A: Not Applicable.*: p<0.05; **: p < 0.01; ***: p < 0.001.

[a] For *S. haematobium*-specific bladder morbidity, the trend with age was significant at the level of p=0.025 in the uni- and p=0.043 in the multivariable analysis. For *S. mansoni*-specific hepatic fibrosis, the trend with age was significant in the crude analysis (p<0.001). In the adjusted analysis the ORs for hepatic fibrosis increased with age in Diokhor Tack (p<0.001) but they did not vary with age in Ndieumeul.

[b] OR for a 10-fold increase in infection intensity.

Table 3.5. The effect of mixed *Schistosoma* infection on bladder morbidity and on hepatic fibrosis.

Risk factors		Bladder morbidity in *S. haematobium*-infected subjects (n=216)			Hepatic fibrosis in *S. mansoni*-infected subjects (n=270)		
		n	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	n	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Age [a]	<10y	63	0.3 (0.1-1.1)	0.2 (0.1-0.9)*	65	0.1 (0.03-0.3)***	0.1 (0.02-0.2)***
	10-19y	88	Ref.	Ref.	102	0.4 (0.2-0.8)**	0.3 (0.2-0.7)**
	20-39y	44	0.2 (0.1-0.8)*	0.3 (0.1-1.2)	66	Ref.	Ref.
	≥40y	21	0.3 (0.1-1.4)	0.5 (0.1-3.0)	37	0.8 (0.3-1.8)	0.8 (0.4-1.9)
Gender	Male	109	Ref.	Ref.	140	Ref.	Ref.
	Female	107	0.3 (0.1-0.8)*	0.3 (0.1-0.9)*	130	1.0 (0.6-1.8)	0.6 (0.3-1.2)
Community	Ndieumeul	70	Ref.	Ref.	87	Ref.	Ref.
	Diokhor Tack	146	0.2 (0.04-0.8)*	0.2 (0.04-0.7)*	183	1.5 (0.8-2.8)	1.6 (0.8-3.2)
S. haematobium	Infection intensity [b]	216	1.8 (0.96-3.3)	1.8 (0.9-3.7)		N/A	N/A
S. mansoni	Infection intensity [b]		N/A	N/A	270	0.9 (0.6-1.4)	1.0 (0.7-1.7)
Mixed infection	No	40	Ref. [c]	Ref. [c]	97	Ref. [d]	Ref. [d]
	Yes	176	0.6 (0.2-2.2)	0.3 (0.1-1.1)	173	0.8 (0.6-1.1)	1.1 (0.8-1.5)

OR: Odds Ratio; 95%CI: 95% Confidence Interval; Ref.: Reference category; N/A: Not Applicable. *: $p<0.05$; **: $p < 0.01$; ***: $p < 0.001$.

[a] The trends with age were not significant for *S. haematobium*-specific bladder morbidity, but for S. *mansoni*-specific hepatic fibrosis, they were at the level of $p<0.001$ in both analyses.

[b] OR for a 10-fold increase in infection intensity.

[c] Mixed infections as compared to single *S. haematobium* infections.

[d] Mixed infections as compared to single *S. mansoni* infections.

Table 3.6. *S. mansoni* egg elimination, infection intensity and bladder morbidity in subjects passing *S. haematobium* eggs in urine.

Route of *S. mansoni* egg elimination		n	*S. haematobium* infection intensity (GM (95% CI))	Bladder morbidity (%)
No elimination	(single *S. haematobium* infection)	39	3.1 (2.0-4.7) ep10ml	92
Exclusively via feces	(mixed infection)	153	8.0 (6.0-10.8) ep10ml	89
Via feces & urine	(mixed infection)	17	60.1 (27.2-132.3) ep10ml	100
Exclusively via urine	(mixed infection)	6	12.0 (1.5-86.5) ep10ml	50
Total		215	**8.1 (6.2-10.4) ep10ml**	89

GM: Geometric Mean; 95%CI: 95% Confidence Interval.

Risk factors for schistosomal morbidity in this co-endemic area – i.e. age, gender, community of residence – were similar to those generally observed in *Schistosoma*-endemic areas. In line with other studies in mono-endemic areas, we observed that children are more at risk of bladder morbidity (King et al., 2004; Garba et al., 2010), while adults are more at risk for hepatic fibrosis (Gryseels et al., 2006; Berhe et al., 2007). Participants from one community tended to be less at risk for bladder morbidity but were significantly more at risk for hepatic fibrosis than participants from the other. Micro-geographical differences (i.e. between neighboring communities) have been reported before (Butterworth et al., 1994; Boisier et al., 2001; Booth et al., 2004b; Malenganisho et al., 2008), and are probably due to geographical heterogeneities in *Schistosoma* exposure (history) (Anderson and May, 1985a; Robert et al., 1989; Pinot de Moira et al., 2007, and Chapter 2 of this thesis), although other factors (e.g. genetic or environmental factors) may also be involved (Butterworth et al., 1994). Males were more at risk for bladder morbidity and hepatic fibrosis than females. Previous studies in either *S. mansoni*- or *S. haematobium*-endemic areas have found similar associations for both forms of morbidity (Heurtier et al., 1986; Hatz et al., 1990; Serieye et al., 1996; Medhat et al., 1997; Traore et al., 1998; Mohamed-Ali et al., 1999; Wagatsuma et al., 1999; El Khoby et al., 2000; Boisier et al., 2001; King et al., 2003; Brouwer et al., 2003; Booth et al., 2004b; Berhe et al., 2007; Malenganisho et al., 2008; Garba et al., 2010). Possibly, males are more prone to *Schistosoma*-associated pathology because of hormonal and immunological differences. It has been proposed that hepatic fibrosis might be more pronounced in men because androgens can reduce interferon (IFN)-γ levels while estrogens have the opposite effect on this antifibrogenic cytokine (Mohamed-Ali et al., 1999). Similarly, a study in Uganda showed that the risk of hepatic fibrosis was associated with different cytokine profiles in men and women (Booth et al., 2004a).

Recently, we found infection intensities of both *S. mansoni* and *S. haematobium* to be elevated in mixed as compared to single infections (Chapter 2). In the present study, *S. haematobium* infection intensity was identified as an independent risk factor for bladder morbidity, in accordance with previous studies from mono-endemic areas (Heurtier et al., 1986; Serieye et al., 1996; Medhat et al., 1997; Traore et al., 1998; El Khoby et al., 2000; Leutscher et al., 2000; Brouwer et al., 2003; Brouwer et al., 2004; Garba et al., 2010). However, subjects with mixed infections tended to have less bladder morbidity than those with single *S. haematobium* infections, independently of infection intensity. This would suggest a protective effect of *S. mansoni* on bladder morbidity.

The observed reduction in bladder morbidity in mixed infections appeared to be related to ectopic *S. mansoni* egg elimination. Lowest prevalences of bladder morbidity were observed in *S. haematobium*-positive individuals who also eliminated *S. mansoni* eggs in urine (but not in feces), despite the relatively high *S. haematobium* infection intensities in this group. Experimental models have shown that *S. mansoni* and *S. haematobium* can form heterologous male-female pairs. As the *S. haematobium* male is assumed to be competitively stronger than *S. mansoni* (Webster et al., 1999; Cunin et al., 2003), this would result in more heterologous pairs in the vesical than in the mesenteric plexus and thus in more ectopic *S. mansoni* than ectopic *S. haematobium* egg elimination in mixed foci (Ratard et al., 1991; Khalil and Mansour, 1995; Southgate et al., 1998; Webster et al., 1999; Ernould et al., 1999a; Cunin et al., 2003; and Chapter 2 of this thesis). Nothing is known yet about the pathogenicity of eggs from heterologous pairs (Jourdane et al., 1995; Khalil and Mansour, 1995). It could be speculated however, that these heterologous pairs would produce less (pathogenic) eggs than homologous *S. haematobium* worm pairs, or that their eggs would deviate to other sites (Mansour et al., 1984; Soliman et al., 1986). Competition between *S. mansoni* and *S. haematobium* females for *S. haematobium* males would then lead to a reduction of bladder morbidity in mixed as opposed to single infections. Other factors may also underlie the potential protective effect of *S. mansoni* infection. As our study population has been exposed to *S. mansoni* for a longer period of time than to *S. haematobium* (Picquet et al., 1996), one could argue that *S. mansoni*-induced cross-resistance to *S. haematobium* might have played a role as well (Webbe et al., 1979). Obviously, more research is needed to confirm the observed relation between mixed *Schistosoma* infection and bladder morbidity, and to understand the underlying mechanisms.

Only one epidemiological study has looked into mixed infections and bladder morbidity before. In contrast to our study, the authors reported a positive association between mixed infection and bladder morbidity in Malian subjects (Koukounari et al., 2010). However, they studied only schoolchildren (7-14 years) and did not take ectopic egg elimination into account. Restricting our analysis to children between 5 and 14 years

(the limited sample size did not allow us to perform the analysis on a smaller age range), the association between mixed infection and bladder morbidity still tended to be negative (OR=0.5 (95%CI 0.1-3.4)). Disregarding ectopic eggs as well, the association became positive (OR=1.7 (95%CI 0.4-7.6)), which is in line with the Malian study (data not shown). These discrepancies clearly demonstrate the importance of considering ectopic egg elimination in mixed *Schistosoma* infections and morbidity.

In contrast to our findings for bladder morbidity, hepatic fibrosis was neither associated with current *S. mansoni* infection intensity nor with mixed infections. Hepatic fibrosis only develops after 5-15 years of exposure to *S. mansoni* (Gryseels et al., 2006). This was illustrated by the >10 years' time lag between the *S. mansoni* infection and *S. mansoni*-associated hepatic fibrosis peaks in the respective age-related curves (Figure 3.1). While inflammatory hepatic morbidity is generally positively associated with current *S. mansoni* infection (Guyatt et al., 1995; Kardorff et al., 1996; Gryseels et al., 2006), the progression of morbidity into chronic schistosomiasis is driven by cumulative exposure to *S. mansoni* eggs rather than current infection (Chan et al., 1996; Mahmoud, 2001; Wilson et al., 2011).

So far, only two other studies have investigated the relationship between mixed infections and liver morbidity, with contrasting results. A Zimbabwean study found a slight positive association between mixed infections and liver size (Friis et al., 1996). The afore-mentioned Malian study observed a negative association between mixed infections and hepatic fibrosis (Koukounari et al., 2010). However, the latter classified liver image pattern B as abnormal (Koukounari et al., 2006), which is not according to the Niamey guidelines (Richter et al., 1996; Boisier et al., 2001; King et al., 2003; Booth et al., 2004a; Booth et al., 2004b; Malenganisho et al., 2008), and hampers an adequate comparison with our findings. Adopting the criteria of the Malian study, we still found a positive association between mixed infections and liver fibrosis in children (7-14 years), although not significant (data not shown). The divergences between the Malian study and our study – for bladder as well as liver morbidity – could furthermore be due to differences in the distribution of *S. haematobium* and *S. mansoni* infection status and intensity between the three Malian study areas, which were not accounted for (Koukounari et al., 2010). Also other differences in e.g. transmission dynamics (Butterworth et al., 1994), between the Malian and present study cannot be excluded.

Conclusion

Up to now, the relationship between mixed *Schistosoma* infection and morbidity has only been studied in schoolchildren. This population-wide study is the first to include adults. Mixed infections were not associated with an increased risk of *S. mansoni*-associated morbidity and even tended to reduce the risk of bladder morbidity. These unexpected results warrant further investigation of a possible protective effect of *S.*

mansoni on bladder morbidity. Especially the role of interspecies interactions and ectopic *S. mansoni* egg elimination should be studied in more detail, as these phenomena may have important consequences for schistosomiasis morbidity and control in co-endemic areas.

Acknowledgements

We gratefully thank the population of Ndieumeul and Diokhor Tack and the village chiefs, Daoure Mbaye and Daouda Pene, for their hospitality and participation in this study. This study would not have been possible without the field workers in Richard-Toll, Abdoulaye Yague, Mankeur Diop, Moussa Wade and Ngary Sy, who assisted in the sample collection and microscopic analysis. We would also like to thank Boubacar Fofana and Ahmed Dieng for their help with the ultrasound examinations, the medical staff of the Health Centre in Richard-Toll for their support, and Tine Huyse for her helpful suggestions.

Chapter 4

Micro-geographical heterogeneity in *Schistosoma mansoni* and *S. haematobium* infection and morbidity in a co-endemic community in northern Senegal

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PLoS neglected tropical diseases 2013 Dec 26;7(12):e2608

Abstract

Background: *Schistosoma mansoni* and *S. haematobium* are co-endemic in many areas in Africa. Yet, little is known about the micro-geographical distribution of these two infections or associated disease within such foci. Such knowledge could give important insights into the drivers of infection and disease and as such better tailor schistosomiasis control and elimination efforts.

Methodology: In a co-endemic farming community in northern Senegal (346 children (0-19y) and 253 adults (20-85y); n=599 in total), we studied the spatial distribution of *S. mansoni* and *S. haematobium* single and mixed infections (by microscopy), *S. mansoni*-specific hepatic fibrosis, *S. haematobium*-specific urinary tract morbidity (by ultrasound) and water contact behavior (by questionnaire). The Kulldorff's scan statistic was used to detect spatial clusters of infection and morbidity, adjusted for the spatial distribution of gender and age.

Principal findings: *Schistosoma mansoni* and *S. haematobium* infection densities clustered in different sections of the community (p=0.002 and p=0.023, respectively), possibly related to heterogeneities in the use of different water contact sites. While the distribution of urinary tract morbidity was homogeneous, a strong geospatial cluster was found for severe hepatic fibrosis (p=0.001). Particularly those people living adjacent to the most frequently used water contact site were more at risk for more advanced morbidity (RR=6.3; p=0.043).

Conclusions: *Schistosoma* infection and associated disease showed important micro-geographical heterogeneities with divergent patterns for *S. mansoni* and *S. haematobium* in this Senegalese community. Further in depth investigations are needed to confirm and explain our observations. The present study indicates that local geospatial patterns should be taken into account in both research and control of schistosomiasis. The observed extreme focality of schistosomiasis even at community level, suggests that current strategies may not suffice to move from morbidity control to elimination of schistosomiasis, and calls for less uniform measures at a finer scale.

Introduction

Schistosomiasis is amongst the most common human parasitic diseases with over 230 million people affected worldwide (WHO, 2010). More than 90% of them live in sub-Saharan Africa (Hotez and Kamath, 2009). The two major species are *Schistosoma mansoni* and *S. haematobium*, which are co-endemic in many regions (Gryseels et al., 2006). However, little is known about the geographical distribution of both species within such co-endemic regions. Knowledge on micro-geographical variations of single and mixed *Schistosoma* infections and associated disease could provide important insights into the drivers of infection and disease and as such better tailor schistosomiasis control and elimination efforts.

Recent progress in geographic information systems (GIS) has facilitated a better understanding of geospatial dimensions of schistosomiasis on the large scale. On continental and national scales, climatic (e.g. temperature and rainfall) and physical factors (e.g. vegetation, large water bodies, altitude) have been identified as major determinants of the heterogeneous geographical distribution of *Schistosoma* infection (either *S. mansoni* or *S. haematobium* (Dennis et al., 1983; Kloos et al., 1988; Ahmed et al., 1996; Tsang et al., 1997; Brooker et al., 2001; Handzel et al., 2003; Raso et al., 2005; Simoonga et al., 2008; Brooker and Clements, 2009; Clements et al., 2009)). On subnational levels, distance to water contact sites, land use and the distribution of infected snails have been reported to contribute to these heterogeneities (Gryseels and Nkulikyinka, 1988; Chandiwana et al., 1988; Gryseels and Nkulikyinka, 1990; Coutinho et al., 1997; Gazzinelli, 2006; Kapito-Tembo et al., 2009; Odiere et al., 2011).

Few studies have however exploited these techniques to address the geospatial dimensions of schistosomiasis on the micro-scale, i.e. within communities or among households (Utzinger et al., 2003; Clennon et al., 2004; Clennon et al., 2006; Brooker et al., 2006; Matthys et al., 2007; Pullan et al., 2008; Yiannakoulias et al., 2010; Mutuku et al., 2011; Stothard et al., 2011). Most of these considered spatial patterns of only one *Schistosoma* species even though *S. mansoni* and *S. haematobium* often occur together (Gryseels et al., 2006). Moreover, micro-geographical clustering of *Schistosoma* infection has never been studied in relation to *Schistosoma*-specific morbidity.

In the present study, we set out to investigate the spatial patterns of *S. mansoni* and *S. haematobium* infection and morbidity in a co-endemic community on the bank of Lac de Guiers in the north of Senegal (Chapter 2 and 3). During the past decades, many communities around Lac de Guiers in the north of Senegal have become co-endemic for *S. mansoni* and *S. haematobium* (De Clercq et al., 1999; Southgate et al., 2001; Ten Hove et al., 2008; Huyse et al., 2009). *Schistosoma mansoni* was introduced in Richard-Toll in 1988 upon construction of the Diama dam and rapidly spread throughout the region (Talla et al., 1990; , 1992). By 1994, virtually the whole Lac de Guiers area had become exposed to this species (Picquet et al., 1996). Today, both *S. mansoni* and *S.*

haematobium are wide-spread in the communities around the lake, and the situation is still dynamic.

Methods

Ethics statement

This study was part of a larger investigation on the epidemiology of schistosomiasis and innate immune responses (SCHISTOINIR: www.york.ac.uk/res/schistoinir) for which approval was obtained from the review board of the Institute of Tropical Medicine, the ethical committee of the Antwerp University Hospital and 'Le Comité National d'Ethique de la Recherche en Santé' in Dakar. Informed and written consent was obtained from all participants prior to inclusion into the study. For minors, informed and written consent was obtained from the legal guardian.

Participants with severe pathology that needed further treatment were referred to the appropriate health authority. After the study, all community members were offered praziquantel (40 mg/kg) and mebendazole (500 mg) to treat and prevent schistosomiasis and soil-transmitted helminthiasis, respectively (Chapter 2), according to WHO guidelines (WHO, 2006).

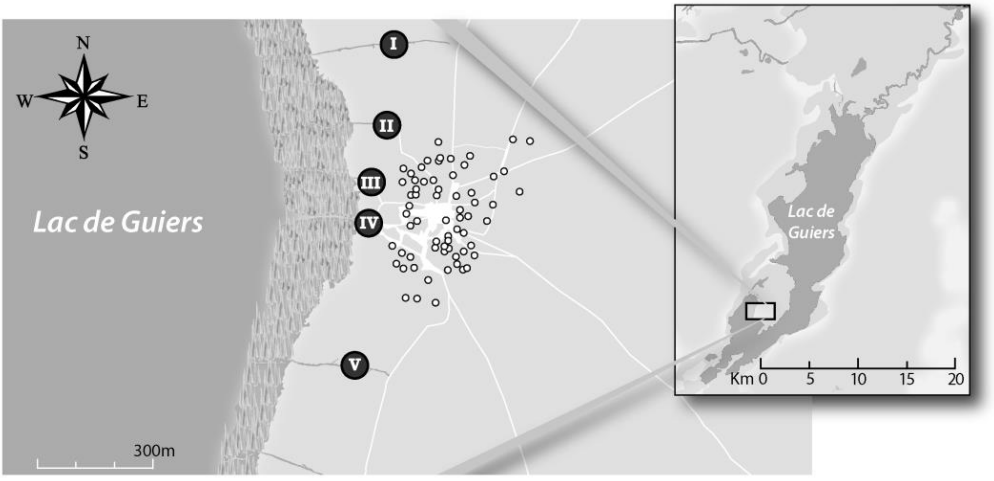

Figure 4.1. General map of the study village.

The inset shows that Diokhor Tack is located in the south-west of the Nouk Pomo peninsula in Lac de Guiers. The main figure indicates the locations of the 68 households that participated in the study (white dots). Black dots with Roman numerals refer to the sites where people come into direct contact with water from the lake. Water contact site III and IV are open spaces enclosed by dense vegetation. Site IV is the largest water contact site. The more remote water contact sites (I, II, and V) are located along irrigation canals in which vegetation was sparser at the time of study.

Study area

This cross-sectional study was conducted from July until November 2009 in Diokhor Tack (16°11'24"N 15°52'48"W), the largest community on the Nouk Pomo peninsula in Lac de Guiers. Details on the study area have been described elsewhere (Chapter 2). In short, it is an isolated, compact and homogeneous Wolof community of Muslim faith with a surface of ~0.25 km². Cultivation is the main means of subsistence and the farmlands that surround the village are irrigated with water from the lake. Although the water from Lac de Guiers is piped to the capital city of Dakar, 250 km away (Berger et al., 2006), the people living nearby do not have access to safe water. Water contact takes place in the lake or in specific sites in canals that are connected to the lake in the west (Figure 4.1). There were no periodic anthelminthic treatment programs prior to our study and the community does not have a health facility. The nearest 'health post' is ~12 km away. All community members that gave informed consent (or their legal guardians) were included in the study. Participants were registered and recruited from door to door for the parasitological and ultrasound surveys. The community consisted of 71 households, 68 of which participated in this study (Figure 4.1). This corresponded to a total study population of 599 individuals.

Data collection and definitions

For microscopic diagnosis of *Schistosoma* infection, two feces and two urine samples were collected from each participant on consecutive days. For each feces sample, two Kato-Katz slides of 25 mg fecal material each were prepared, and urine samples were filtered and processed according to standard procedures, as previously described (Chapter 2 and 3). In analogy with earlier micro-geographic studies (Clennon et al., 2004; Clennon et al., 2006; Mutuku et al., 2011), *S. mansoni* and *S. haematobium* infection densities were expressed as the number of eggs detected per gram of feces (epg) or per 10 ml of urine (ep10ml), respectively, including both negative (0 epg or 0 ep10ml) and positive individuals (Kitron and Higashi, 1985). Single infection was defined as passing eggs of only one species, and mixed infection as passing eggs of both *S. mansoni* and *S. haematobium*, irrespective of the route of egg elimination (Chapter 2). *Schistosoma*-specific morbidity was determined by ultrasound, as previously described (Chapter 3). Pathologic lesions associated with *S. haematobium* or *S. mansoni* infection were recorded according to the Niamey guidelines (Richter et al., 1996). Individuals with signs of hepatic morbidity that were not specific to *S. mansoni* (e.g. hepatitis, cirrhosis or fatty liver) were excluded (Richter et al., 1996). To assess the presence or absence of *S. mansoni*-specific hepatic fibrosis, the liver image pattern was determined (Richter et al., 1996). Liver image patterns of C ("periportal fibrosis possible") to F ("very advanced periportal fibrosis") were categorized as *S. mansoni*-specific hepatic fibrosis (Chapter 3). Individuals with liver image pattern A ("no sign of periportal fibrosis") or B ("incipient periportal fibrosis not excluded") were categorized as

controls (Richter et al., 1996). To assess the presence or absence of *S. haematobium*-specific urinary tract morbidity, the urinary bladder score was determined (Richter et al., 1996). A score of ≥1 was considered as *S. haematobium*-specific bladder morbidity in accordance with previous studies (Medhat et al., 1997; Leutscher et al., 2000; and Chapter 3 of this thesis). The severity of morbidity was represented by the liver image pattern score for *S. mansoni*- and by the upper urinary tract score for *S. haematobium*-specific morbidity (Richter et al., 1996). Finally, individual questionnaires were used to explore water contact behavior in a random subsample of people older than 5 years of age.

Mapping and geospatial processing

Water contact sites as well as the center of each household were located using a hand-held differential global positioning system with an accuracy of 3m (Garmin Etrex H). Household locations in latitude and longitude were then linked to the collected individual infection, morbidity and questionnaire data (multiple observations per location). These data were imported into SaTScan 9.1.1 (Software for the spatial and space-time scan statistics, developed by M. Kulldorff, Harvard Medical School, Boston and Information Management Services Inc., Silver Spring, Maryland, USA. Available at www.satscan.org) according to the software's user guide (Kulldorff, 2010). ArcMap 9.3 (ESRI, Redlands, California, USA) was used to project the geographic coordinates and statistically significant clusters (see below) on to the Universal Transverse Mercator zone 28N (1984 datum).

Spatial statistics

The widely used Kulldorff's scan statistic in SaTScan™ tests whether events such as disease cases are distributed randomly in space and, if not, identifies the approximate location of significant geospatial clusters (Kulldorff, 1997). The test uses a moving circular window that varies up to a predefined size. Each window is a potential cluster. For each window, a likelihood ratio test is applied based on the observed and expected number of cases inside and outside the window to test the null hypothesis of absolute spatial randomness against the alternative hypothesis that there is an elevated risk within the window as compared to outside. The window with the maximum likelihood is the 'most likely cluster'. The *p*-value of the maximum likelihood ratio test statistic was obtained after 999 Monte Carlo replications. A maximum window size of 50% of the study population was chosen upon sensitivity analysis using maximum sizes from 10 to 50%. Only statistically significant ($p<0.05$) most likely clusters were reported, and standard settings (i.e. non-overlapping secondary clusters) were used throughout all analyses. In case the most likely (significant) cluster contained only one household, an additional check was performed to increase the robustness of cluster detection. The standard analysis was repeated while allowing for overlapping secondary clusters

(using the "criteria for reporting secondary clusters" option "no restriction = most likely cluster for each grid point" (Kulldorff, 2010)), and the secondary cluster (including the first household) was reported, if it remained significant. Additionally, clusters with $p<0.06$ were displayed to indicate households that tended to have increased risks.

Infection densities of *S. mansoni* and *S. haematobium* showed skewed distributions, and were therefore normalized by log (base 10)-transformation after adding half of the detection limit to allow for zeroes. The detection limit for *S. mansoni* infection was 10 epg and that for *S. haematobium* infection 0.5 ep10ml. The spatial distribution of log-transformed infection densities was assessed using normal models (Kulldorff et al., 2009). Geometric mean (GM) infection densities in- and outside spatial clusters were computed to quantify significant spatial heterogeneities. Subsequently, Bernoulli models (Kulldorff, 1997) were run to investigate the distribution of single *S. mansoni*, single *S. haematobium* and mixed infections, comparing spatial distributions of people with:

- single *S. mansoni* (1) *versus* those without single *S. mansoni* infections (0);
- single *S. haematobium* (1) *versus* those without single *S. haematobium* infections (0);
- mixed (1) *versus* those without mixed infections (0).

The spatial distribution of the prevalence of hepatic fibrosis and urinary tract morbidity was tested using binary variables in separate Bernoulli models. Ordinal models were used to assess the distribution of the severity of *S. mansoni*- and *S. haematobium*-specific morbidity (Jung et al., 2007). Relative risks (RR) comparing people in- and outside clusters, as well as prevalences in- and outside clusters were calculated to quantify significant spatial heterogeneities based on Bernoulli and ordinal models.

Gender and age are important risk factors for both *Schistosoma* infection (Chapter 2), and morbidity (Chapter 3). To investigate whether these demographic factors 1) caused clustering of infection and morbidity, and/or 2) impacted on the size and exact locations of statistically significant clusters, the abovementioned analyses were adjusted using multiple datasets (Kulldorff et al., 2007). Six datasets were prepared, containing either males or females from 0 to 9, 10 to 19, or ≥20 years old (Table 4.1). SaTScan™ incorporated all datasets into a single log likelihood function. This function is defined as the sum of the individual log likelihoods for those data sets for which the observed case count is more than the expected. Since this adjustment was only possible for the Bernoulli and normal models, separate Bernoulli models were run for the ordinal morbidity model showing significant spatial heterogeneities in the unadjusted analysis.

Finally, Bernoulli models were used to compare the geospatial distribution of people reporting to frequent a particular water contact site versus the distribution of people who did not report to frequent that site.

Table 4.1. Characteristics of the 6 datasets used for the gender- and age-adjusted spatial analyses.

Gender	Age (years)	Infection[a]				Morbidity[a]		
		n_{total}	Single *S. mansoni*	Single *S. haematobium*	Mixed infections	n_{total}	Hepatic fibrosis	Urinary tract morbidity
Male	0 – 9	109	15	17	27	49	3	41
Female	0 – 9	79	9	16	17	28	2	18
Male	10 – 19	90	21	10	49	49	12	48
Female	10 – 19	68	11	6	42	34	6	26
Male	≥ 20	103	39	3	15	49	33	44
Female	≥ 20	150	44	20	39	82	34	56
Total		**599**	**139**	**72**	**189**	**291**	**90**	**233**

[a] Total study population in the first columns and numbers of cases in subsequent columns.

Results

Characteristics of the study population

Complete parasitological data were obtained from a total of 599 individuals from 68 households. The median household size was 8 people (range 1-20). The total study population consisted of 302 males and 297 females with a median age of 15 (range 0-85) years. Ultrasound and questionnaire data were obtained from random subsamples of 291 individuals (64 households), and 277 individuals (63 households), respectively. The prevalence of overall *S. mansoni* infection was 55% (328/599) and that of *S. haematobium* 44% (261/599). Mixed infections were observed in 32% of the population (189/599). The prevalence of *S. mansoni*-specific hepatic fibrosis was 31% (90/291). Most cases had liver image pattern C (71/90), 9/90 had pattern D, while advanced periportal fibrosis was observed in 10/90 cases (nine with liver image pattern E and one with F). The prevalence of *S. haematobium*-specific urinary tract morbidity was 80% (233/291). Positive upper urinary tract scores (range 3-12) were observed in 6% of the study population (18/291). Distributions of single and mixed *Schistosoma* infections, *S. mansoni*-associated hepatic fibrosis and *S. haematobium*-associated bladder morbidity according to gender and age are summarized in Table 4.1.

Spatial distribution of *Schistosoma* infection

Figure 4.2A depicts the heterogeneous geospatial distribution of *S. mansoni* and *S. haematobium* infection densities (p=0.001 for both unadjusted analyses). While the size of the *S. haematobium* infection density cluster increased upon correction for the spatial distribution of gender and age, both *S. mansoni* and *S. haematobium* clusters remained statistically significant (Figure 4.2B; p=0.002 and p=0.023, respectively).

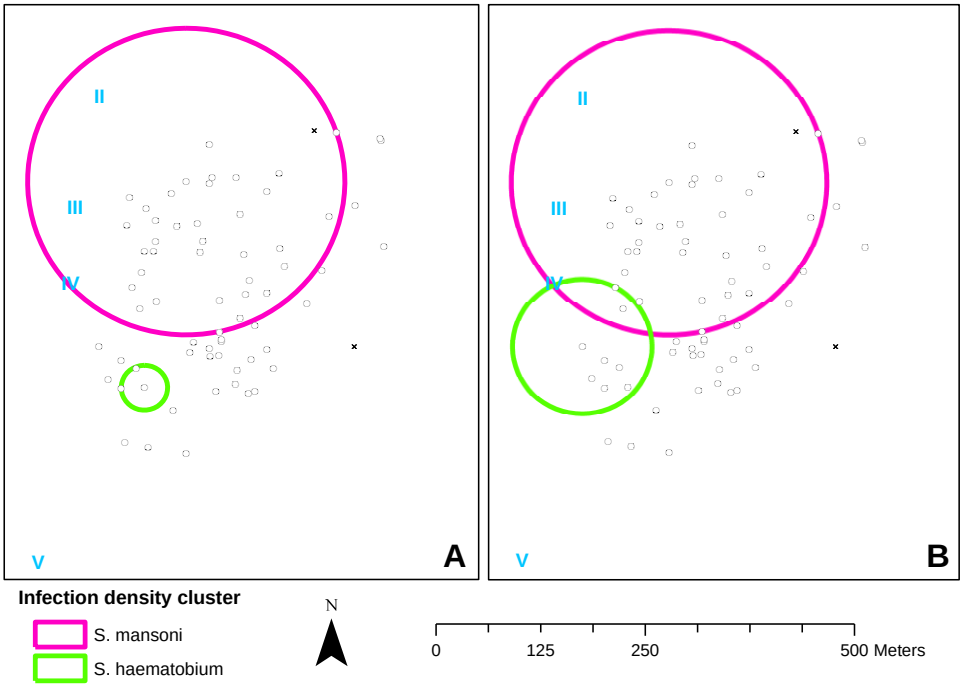

Figure 4.2. Spatial distribution of *S. mansoni* and *S. haematobium* infection densities.

Black circles and crosses indicate households that were included in and excluded from the analysis, respectively. Roman numerals indicate water contact sites. **Panel A** depicts the unadjusted clusters (p=0.001 for both *S. mansoni* and *S. haematobium*). The geometric mean (GM) *S. mansoni* infection density was 33 epg for those living in the northern *S. mansoni* cluster (n=285) compared to 12 epg in the rest of the community (n=314). The GM *S. haematobium* infection density was 4.7 ep10ml inside the southern *S. haematobium* cluster (n=34) and 0.7 ep10ml outside (n=565). **Panel B** depicts the gender- and age-adjusted clusters (p=0.002 for *S. mansoni* (north), and p=0.023 for *S. haematobium* (south).

Participants with mixed and those with single *S. haematobium* infections were randomly distributed (p=0.16 and p=0.080, respectively), while those with single *S. mansoni* infections tended to cluster geographically (Figure 4.3A; RR=1.7; p=0.053). Figure 4.3B indicates that the clustering of single *S. mansoni* was independent of the spatial distribution of gender and age ($p \leq 0.050$), although the cluster size and exact location were slightly altered upon adjustment.

Spatial distribution of *Schistosoma*-specific morbidity

Figure 4.4A indicates that people with hepatic fibrosis (RR=1.9; p=0.054) and urinary tract morbidity (RR=1.2; p=0.053) tended to cluster in the same area. Adjusted analysis however indicated that these heterogeneous patterns were dependent on the

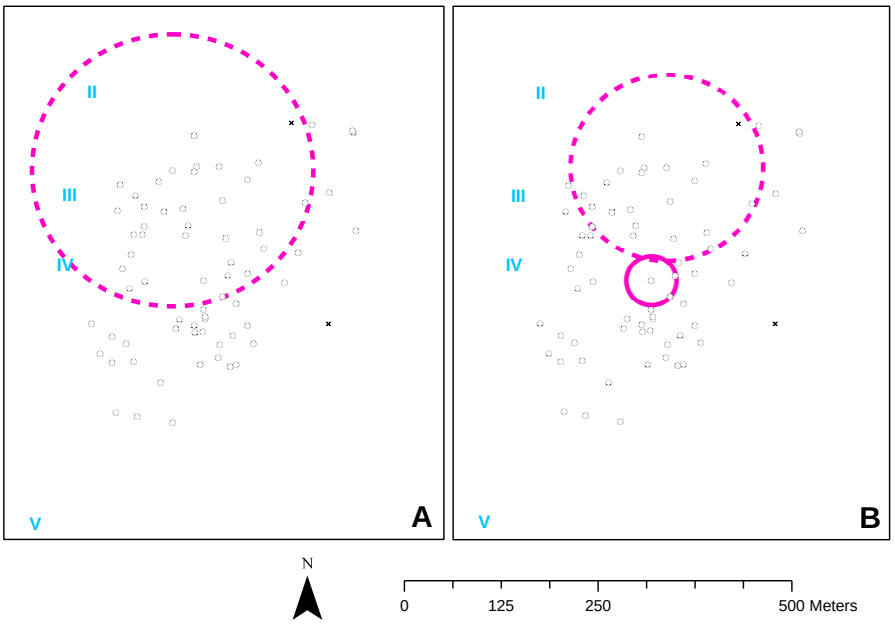

Figure 4.3. Spatial distribution of single *S. mansoni* infections.

Black circles and crosses indicate households that were included in and excluded from the analysis, respectively. Blue Roman numerals indicate water contact sites. Continuous pink circles are statistically significant clusters ($p<0.05$) and dotted circles are borderline significant ($p<0.06$). **Panel A** depicts the unadjusted cluster (RR=1.7; $p=0.053$). The prevalence of single *S. mansoni* infection was 30% (83/278) inside and 17% (56/321) outside the cluster. **Panel B** shows the gender- and age-adjusted clusters ($p=0.045$ for the most likely cluster (center) and $p=0.050$ for the secondary cluster (north).

distribution of gender and age ($p=0.087$ for hepatic fibrosis and $p=0.071$ for urinary tract morbidity in the adjusted analysis). In order to assess the distribution of morbidity by severity, ordinal analyses were performed for liver image pattern and upper urinary tract scores. Figure 4.4B shows that this resulted in one significant cluster ($p=0.001$) in which the RR increased with the severity of hepatic fibrosis: the RR for a healthy liver image pattern A was 0.3, that for B 1.3, for C 1.4, for D 2.7 and for E 4.3. Moreover, the only person with pattern F in the community lived in this cluster. Bernoulli models were used to investigate whether this cluster of severe hepatic fibrosis was independent of the distribution of gender and age. Since more severe hepatic fibrosis was only observed in adults, these analyses were restricted to ≥20-year-olds. Unadjusted Bernoulli models revealed that image patterns D-F (as opposed to A-C) clustered in the households within the hepatic fibrosis cluster that were closest to water contact site IV

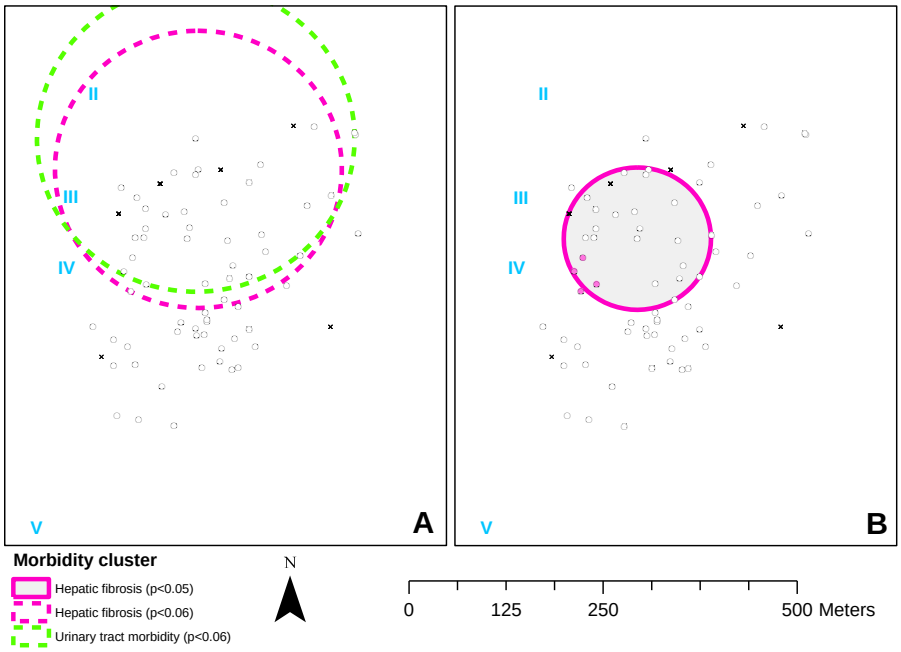

Figure 4.4. Spatial distribution of *S. mansoni*- and *S. haematobium*-specific morbidity.

Black circles and crosses indicate households that were included and excluded from the analysis, respectively. Continuous colored circles are statistically significant clusters ($p<0.05$) and dotted circles are borderline significant ($p<0.06$). Roman numerals indicate water contact sites. **Panel A** depicts the unadjusted clusters for the prevalence of morbidity. Both *S. mansoni*-specific hepatic fibrosis (pink dotted circle; RR=1.9) and *S. haematobium*-specific urinary tract morbidity clusters (green dotted circle; RR=1.2) were borderline significant ($p=0.054$ and $p=0.053$, respectively). The prevalence of hepatic fibrosis was 41% (59/145) in- and 21% (31/146) outside, and that of urinary tract morbidity was 89% (117/131) in- and 73% (116/160) outside the cluster. Gender- and age-adjusted analysis revealed no (borderline) significant clusters for the prevalence of morbidity. **Panel B** depicts the clusters of morbidity by severity. The risk of severe hepatic fibrosis was elevated in the circle with a RR of 0.3 for liver image pattern A, 1.3 for B, 1.4 for C, 2.7 for D and 4.3 for E, and the only person with pattern F lived here ($p=0.001$; unadjusted ordinal model). The gender- and age-adjusted cluster for patterns D-F (as opposed to A-C) in adults constituted of the households indicated in pink ($p=0.031$; Bernoulli model).

(RR=6.3; $p=0.043$; data not shown). The combined distribution of patterns E and F (as opposed to A-D) was homogeneous ($p=0.20$). In the adjusted model, the cluster of pattern D-F remained statistically significant (Figure 4.4B; $p=0.031$).

The risk of severe urinary tract morbidity was homogeneously distributed ($p=0.38$ in the ordinal analysis).

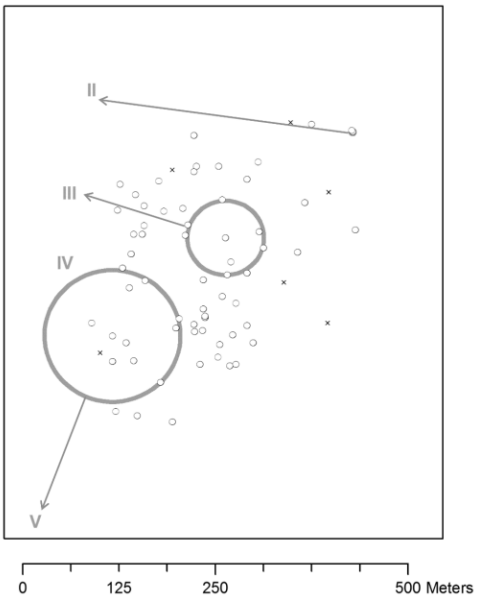

Figure 4.5. Spatial distribution of self-reported use of the different water contact sites.

Black circles and crosses indicate households that were included and excluded from the analysis, respectively. Roman numerals indicate water contact sites. Gray circles indicate clusters of people that reported to frequent a given water contact site indicated by an arrow. Participants in the northeastern cluster were more likely to frequent site II (3/4 *versus* 5/273; p=0.005), participants in the middle cluster to frequent site III (4/17 *versus* 2/260; p=0.022), and those from the southwestern cluster to frequent site V (8/53 *versus* 1/224; p=0.001) than those living outside the respective clusters.

Water contact behavior

Water contact activities were concentrated at site IV with 62% of the interviewees (172/277) reporting to frequent this site. Numbers of observations in the other sites were limited. Nonetheless, spatial analysis of the questionnaire data revealed significant heterogeneities in the self-reported use of the different water contact sites (Figure 4.5). People from two adjacent households in the northeast were more likely to frequent site II than those from the rest of the community (3/4 in- *versus* 5/273 outside the cluster; p=0.005). People living in the center of the community were more likely to frequent site III than others (4/17 *versus* 2/260; p=0.022). Those from the southwest were more likely to frequent site V (8/53 *versus* 1/224; p=0.001). Use of site I and IV did not appear to be linked to a particular group of households (p=0.31 and p=0.16 respectively).

Discussion

The present micro-geographical study revealed significant clusters of *S. mansoni* and *S. haematobium* infection density in different sections of one community in a co-endemic area, possibly related to heterogeneities in the use of different water contact sites. While the distribution of urinary tract morbidity was homogeneous, a strong geospatial cluster was found for severe hepatic fibrosis. Particularly those people living adjacent to the most frequently used water contact site were more at risk for advanced morbidity than those living farther away.

These findings confirm the well-known focality of schistosomiasis (Cook and Zumla, 2009). Even within one community, one cannot assume the risk of schistosomiasis to be homogenous. More remarkably even, the two *Schistosoma* species clustered in different sections of the community; *Schistosoma mansoni* infections clustered in the north while *S. haematobium* clustered in the south. A series of recent GIS studies showed significant micro-geographical heterogeneities in *S. haematobium* infection within a mono-endemic Kenyan community (Clennon et al., 2004; Clennon et al., 2006). Those in *S. mansoni* mono-endemic communities showed conflicting results with heterogeneous spatial patterns in some studies and homogeneous patterns in others (Utzinger et al., 2003; Brooker et al., 2006; Matthys et al., 2007; Pullan et al., 2008; Yiannakoulias et al., 2010; Stothard et al., 2011). To our knowledge, only Farooq et al. have so far investigated the spatial distribution of both infections in a co-endemic community in Egypt in the 1960s. They reported higher infection levels of *S. mansoni* in small children in one section of the community and higher levels of *S. haematobium* in another section (Farooq, 1966). This is in agreement with the divergent distributions of *S. mansoni* and *S. haematobium* infection densities observed in the present study. Several interrelated factors may underlie these observations, and are discussed below.

First of all, the micro-geographic distributions of the intermediate snail host of *S. mansoni* and *S. haematobium*, which belong to the genus *Biomphalaria* and *Bulinus*, respectively, may be divergent as well. Unfortunately, it was logistically impossible to collect snails in the present study. Yet, it is known that these snail species prefer different niches and that their distribution is influenced by chemical, physical, and biological factors (Polderman et al., 1985; Woolhouse and Chandiwana, 1989; Amankwa et al., 1994; Ernould et al., 1999b; Johnson et al., 2009; Al-Sheikh and Dagal, 2011). Indeed, Woolhouse and Chandiwana demonstrated that the snail hosts of *S. mansoni* and *S. haematobium* occupy different locations in one single habitat in a co-endemic focus (Woolhouse and Chandiwana, 1989). Ecological factors may thus have favored *S. mansoni* transmission in the north and *S. haematobium* transmission in the south.

At the human host level, behavioral factors may have played a role in the observed spatial pattern of infection. Although based on a small number of observations, our questionnaire data indeed indicated that people from the north and center were more likely to frequent the northern sites than other community members, whereas those from the southwest were more likely to use the southernmost site. It thus seems that the first group maintained *S. mansoni* transmission in the north and the second *S. haematobium* transmission in the south. This corresponds to the study of Woolhouse and Chandiwana reporting 1) a similar geospatial segregation of *S. mansoni* and *S. haematobium* infection in the snail host population between transmission sites, and 2) a very focal man-to-snail transmission, within a distance of 40m. Interestingly, they proposed that these divergent patterns most likely reflected differences in the

distribution of defecation from that of urination, favoring *S. mansoni* and *S. haematobium* transmission, respectively (Woolhouse and Chandiwana, 1989). In contrast to water contact behavior, age and gender of the human host were shown to have a negligible impact on the divergent pattern of *S. mansoni* and *S. haematobium* infection. Spatial clustering in the different sections of the community remained significant upon correction for age and gender. Other factors that may have contributed to the spatial pattern include genetic differences in susceptibility to infection (Bethony et al., 2002; Grant et al., 2011; Grant et al., 2012). Indeed, extended families tended to live together in this community (L. Meurs, personal observation). Also, people from the same section/extended family are more likely to have similar behavioral patterns (Farooq, 1966; Farooq et al., 1966a; Lima e Costa MF et al., 1987; Kloos et al., 1998).

In contrast to the spatial distribution of *S. haematobium* infection, the distribution of *S. haematobium*-associated urinary tract morbidity was homogeneous. This was unexpected as *S. haematobium* infection has consistently been reported as an independent risk factor for urinary tract morbidity (Heurtier et al., 1986; King et al., 1988; Serieye et al., 1996; Medhat et al., 1997; Traore et al., 1998; El Khoby et al., 2000; Leutscher et al., 2000; Brouwer et al., 2003; Brouwer et al., 2004; Garba et al., 2010). The fact that *S. haematobium* was only introduced in this region approximately 6 years prior to this study (Southgate et al., 2000), may explain the relatively low severity of urinary tract morbidity in this community and the consequent absence of a spatial pattern. The severity of urinary tract morbidity is expected to progress over time with cumulative exposure to *S. haematobium* eggs (King et al., 1992).

On the other hand, a strong geospatial cluster was found for severe *S. mansoni*-specific hepatic fibrosis which overlapped with that of *S. mansoni* infection density. At first sight, this seems to be in contrast with previous studies by this group showing that current *S. mansoni* infection is not associated with hepatic fibrosis (Chapter 3), which usually develops after 5-15 years of exposure (Gryseels et al., 2006). However, a closer look at the overlapping clusters showed that teenagers had the highest infection densities and contributed most to the *S. mansoni* infection density cluster (data not shown). Adults on the other hand had more advanced morbidity, and contributed most to the severe hepatic fibrosis cluster. This suggests that these adults were in fact the teenagers with the highest *S. mansoni* infection densities earlier in life.

Moreover, the clustering of severe hepatic fibrosis in adults seemed to be associated with the distance to the water. Those living within ~100 m of the major water contact site (Figure 4.4B) were at least six times more likely to develop advanced hepatic fibrosis (liver image pattern D-F) than those living farther away. However, other factors cannot be excluded such as genetic predisposition (Bethony and Quinnell, 2008), diet or nutritional status (Gong et al., 2012), or co-infections, which may have put those

living in close vicinity of the water at a higher risk of developing hepatic morbidity than the rest of the community.

To our knowledge, only Booth et al. have so far investigated micro-geographical variations in *Schistosoma*-associated morbidity. They found an association between splenomegaly and the combined exposure to *S. mansoni* and *Plasmodium falciparum* but did not explicitly investigate spatial clustering (Booth et al., 2004c).

To our knowledge this is the first study to quantify micro-geographical infection patterns of *S. mansoni* and *S. haematobium* in a co-endemic community, and the first to relate these to patterns of *Schistosoma*-specific morbidity. Apart from the strengths, it is also important to address some limitations of our study. First, the study was cross-sectional and the results were merely descriptive in nature. The present study was a first attempt to describe patterns of schistosome infection and morbidity on a micro-scale, and it was not designed to explain the underlying mechanisms of potential micro-geographical clustering. Based on the limited data that were available, we generated a number of hypotheses, but other risk factors, including environmental, malacological, genetic, immunological and socio-economic factors, should be included in future studies. In addition, more spatial as well as spatio-temporal studies (Clennon et al., 2004; Clennon et al., 2006; Mutuku et al., 2011) are necessary to confirm our observations in other geographical areas and to explain them. Another limitation was that there is as yet no standard technique available to investigate spatial patterns. The emergence of various statistical methods has greatly boosted geospatial studies on schistosomiasis and increased our understanding of this disease. On the other hand, the large variety of methods has also hampered the comparison between the different micro-geographical studies that have been conducted so far and standardization is recommended.

Current WHO schistosomiasis control strategies aim to prevent morbidity in later life through regular mass drug administration (MDA) to at risk populations in so-called homogeneous ecological zones (WHO, 2011; WHO, 2013). However, the strong micro-geographical clustering of infection and morbidity observed in the present study suggests that less uniform strategies should be developed to better tailor control efforts at the local level. A more targeted approach will be even more relevant in view of resolution WHA65.21 on the elimination of schistosomiasis, recently adopted by the WHO (WHO, 2013). It is expected that MDA alone cannot break the *Schistosoma* life cycle and that complementary interventions will have to be put in place (Sturrock, 1989). Micro-geographical studies will help to get much needed insights into local transmission dynamics of *S. mansoni* and *S. haematobium* and hence to develop sustainable control and elimination strategies (Peng et al., 2010).

Acknowledgements

We gratefully thank the community of Diokhor Tack and the village chief Daouda Pene, for their hospitality and participation in this study. This study would not have been possible without the field workers in Richard-Toll, Abdoulaye Yague, Mankeur Diop, Moussa Wade and Ngary Sy, who assisted in the sample collection and microscopic analysis. We would like to thank Boubacar Fofana and Ahmed Dieng for their help with the ultrasound examinations, and the medical staff of the Health Centre in Richard-Toll for their support. Furthermore, we greatly appreciated discussions with Guy Hendrickx and Els Ducheyne from Avia-GIS (Zoersel, Belgium), and with Sophie Vanwambeke from Université Catholique de Louvain (Louvain-La-Neuve, Belgium), as well as advice from Martin Kulldorff, Harvard Medical School and Harvard Pilgrim Health Care (Boston, Massachusetts, USA).

Chapter 5

Cytokine responses to *Schistosoma mansoni* and *Schistosoma haematobium* in relation to infection in a co-endemic focus in northern Senegal

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PLoS neglected tropical diseases 2014 Aug 7;8(8):e3080

Abstract

Background: In Africa, many areas are co-endemic for the two major *Schistosoma* species, *S. mansoni* and *S. haematobium*. Epidemiological studies have suggested that host immunological factors may play an important role in co-endemic areas. As yet, little is known about differences in host immune responses and possible immunological interactions between *S. mansoni* and *S. haematobium* in humans. The aim of this study was to analyze host cytokine responses to antigens from either species in a population from a co-endemic focus, and relate these to *S. mansoni* and *S. haematobium* infection.

Methodology: Whole blood cytokine responses were investigated in a population in the north of Senegal (n=200). Blood was stimulated for 72 h with schistosomal egg and adult worm antigens of either *Schistosoma* species. IL-10, IL-5, IFN-γ, TNF-α, and IL-2 production was determined in culture supernatants. A multivariate (i.e. multi-response) approach was used to allow a joint analysis of all cytokines in relation to *Schistosoma* infection.

Principal findings: *Schistosoma haematobium* egg and worm antigens induced higher cytokine production, suggesting that *S. haematobium* may be more immunogenic than *S. mansoni*. However, both infections were strongly associated with similar, modified Th2 cytokine profiles.

Conclusions: This study is the first to compare *S. mansoni* and *S. haematobium* cytokine responses in one population residing in a co-endemic area. These findings are in line with previous epidemiological studies that also suggested *S. haematobium* egg and worm stages to be more immunogenic than those of *S. mansoni*.

Introduction

Schistosomiasis is a parasitic disease of major public health importance. *Schistosoma mansoni* and *S. haematobium* are the main human species. Both species are endemic in Africa, where their distributions show a great overlap (Gryseels et al., 2006). Schistosomes are known to down-regulate host immune responses and to induce so-called modified Th2 responses. The exact phenotype of the induced response depends on a complex immunological 'dialogue' that involves cytokines and immune cells of Th2, but also Th1, Th17 and regulatory components of the immune system (Allen and Maizels, 2011).

So far, little is known about differences in host immune responses to schistosomes and possible immunological interactions between *S. mansoni* and *S. haematobium* in humans. Yet, epidemiological studies have suggested that host immunological factors may play an important role in co-endemic areas. Interspecies differences in immunogenicity for example, may explain why infection-age curves and morbidity patterns differ between *S. mansoni* and *S. haematobium*. Also, immunological interspecies differences and/or immunological interactions between *S. mansoni* and *S. haematobium* may explain differences in morbidity levels between single and mixed *Schistosoma* infections. Cheever et al. reported a more pronounced reduction of *S. haematobium* than *S. mansoni* worm loads with age (1977). Similarly, in a mixed focus in northern Senegal, we found the age-infection curve of *S. haematobium* to decline more steeply after adolescence than that of *S. mansoni* (Chapter 2), indicating that protective immunity against *S. haematobium* may develop more rapidly. In addition, we found that mixed *S. mansoni* and *S. haematobium* infection as compared with single *S. haematobium* infection tended to decrease the risk of *S. haematobium*-specific urinary tract pathology (Chapter 3). This appeared mainly due to ectopically excreted, possible hybrid eggs (Huyse et al., 2013). Others also found *S. mansoni* to affect *S. haematobium*-specific morbidity and vice versa (Koukounari et al., 2010; Gouvras et al., 2013), indicating that the two infections may have different effects on the egg-induced immune responses that provoke morbidity.

The present study set out to compare *Schistosoma*-specific cytokine responses induced by *S. mansoni* and *S. haematobium* antigens, and to relate these to *Schistosoma* infection in a *S. mansoni* and *S. haematobium* co-endemic area. *Schistosoma* infection status (single and mixed) and infection intensities as well as *Schistosoma*-specific cytokine responses were determined in residents from a co-endemic focus in northern Senegal. A multivariate (i.e. multi-response) approach was used to allow a joint analysis of multiple cytokine responses (interleukin (IL)-10, IL-5, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-2) (Bourke et al., 2013).

Materials and methods

Ethics statement

This study was part of a larger investigation on the epidemiology of schistosomiasis and innate immune responses (SCHISTOINIR) for which approval was obtained from the review board of the Institute of Tropical Medicine, the ethical committee of the Antwerp University Hospital and 'Le Comité National d'Ethique de la Recherche en Santé' in Dakar. Informed and written consent was obtained from all participants prior to inclusion into the study. For minors, informed and written consent was obtained from their legal guardians.

All community members were offered praziquantel (40 mg/kg) and mebendazole (500 mg) treatment after the study according to WHO guidelines (WHO, 2006).

Study area

This study was conducted in Ndieumeul and Diokhor Tack, two neighboring communities on the Nouk Pomo peninsula in Lake Guiers. Details on the study area have been described elsewhere (Chapter 2 and 3). Between July 2009 and March 2010, parasitological data were collected from 857 individuals (Chapter 2). A random subsample of 200 subjects was followed up immunologically. These subjects were between 5 and 53 years of age. Individuals who had lived in an urban area in the 5 years preceding the study (n=7), had taken praziquantel within the last year (n=2), or had clinical signs of malaria (recruited upon recovery), and pregnant women (n=18) were excluded from the immunological study.

Parasitology

Two feces and two urine samples were collected from each participant on consecutive days. Infection with *Schistosoma* spp. was determined quantitatively (by Kato-Katz and urine filtration), and infection with soil-transmitted helminths (STHs) *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm, was assessed qualitatively (by Kato-Katz), as described elsewhere (Chapter 2). Aliquots of the first fecal samples were preserved in ethanol to confirm microscopy results by multiplex PCR (*A. lumbricoides*, hookworm and *Strongyloides stercoralis*) (n=198) (Wiria et al., 2010). Infection with *Plasmodium* was determined by Giemsa-stained thick blood smears.

Whole blood culture

Five hours after venipuncture, heparinized blood was diluted 1:4 in RPMI 1640 (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM pyruvate and 2 mM glutamate (all from Sigma). This mixture (200 µl sample volume) was incubated in 96-well round bottom plates (Nunc) at 37 °C under 5% CO_2 atmosphere

for 72 h, together with one of four schistosomal water-soluble antigen preparations at a final concentration of 10 µg protein/ml:

- *Schistosoma* egg antigen (SEA) derived from *S. mansoni* (SEAm);
- SEA from *S. haematobium* (SEAh);
- Adult worm antigen (AWA) from *S. mansoni* (AWAm); or
- AWA from *S. haematobium* (AWAh).

Medium (see above) without stimulus was used as a negative control. After harvesting, supernatants were stored at -80 °C. *Schistosoma* eggs and adult worms were isolated from either *S. mansoni*- or *S. haematobium*-infected golden hamsters. SEAm, SEAh, AWAm and AWAh were prepared from this material using identical procedures. In brief, eggs or worms were freeze-dried and then homogenized in phosphate-buffered saline (PBS) with 10% n-octyl-β-D-glucopyranoside. Subsequently, this mixture was sonicated, frozen, thawed and washed with PBS. The resulting pellet was dialyzed and filter-sterilized. While AWAm and AWAh batches were lipopolysaccharide (LPS)-free, SEAm and SEAh antigens contained equivalent amounts of LPS (final concentrations of 1-5 ng/ml).

Cytokine measurement

IL-10, IL-5, IFN-γ, TNF-α, and IL-2 in culture supernatants were analyzed simultaneously using custom Luminex cytokine kits (Invitrogen) according to the manufacturer's instructions. Samples with concentrations below the detection limit were assigned values corresponding to half of the lowest value detected. Lowest values detected were 0.063 pg/ml for IL-10, 0.044 pg/ml for IL-5, 0.090 pg/ml for IFN-γ, 0.051 pg/ml for TNF-α, and 0.063 pg/ml for IL-2.

Statistical analysis

Results were considered significant when the *p*-value was <0.05. The Pearson Chi-square test was used to determine the association between infection status on the one hand, and age and gender on the other. Nonparametric techniques were chosen because cytokine concentrations were not normally distributed. Univariate statistics were used to compare single antigen-induced responses within individuals (IBM SPSS 21.0). McNemar's tests were used to compare cytokine response frequencies between *S. mansoni* and *S. haematobium* antigen-induced responses within individuals (e.g. SEAm- versus SEAh-induced responses). Similarly, Wilcoxon Signed Rank tests were used to compare cytokine response levels between *S. mansoni* and *S. haematobium* antigen-induced responses within individuals. Multivariate (i.e. multi-response) statistics were used to collectively analyze multiple cytokine responses – i.e. cytokine profiles - in the study population, and to investigate interrelationships between these responses (Bourke et al., 2013). We chose the nonparametric technique nonmetric

multidimensional scaling (nMDS; in R with the 'Vegan' package (R Core Team, 2012; Oksanen et al., 2013)). This is a variant of the parametric principal component analysis (PCA), but with fewer assumptions about the nature of the data and the interrelationship of the variables (Clarke and Warwick, 2001). This is important because cytokine response levels were not normally distributed, even after log-transformation. Also, levels of different cytokines typically correlate with one another. Upon computation of the cytokine profiles, associations between these cytokine profiles and *Schistosoma* infection were assessed. The approach is illustrated in the Supporting information, page 84. Before nMDS, cytokine concentrations in the negative control were subtracted from those in antigen-stimulated samples to obtain net cytokine responses. Negative values were set to zero. Net cytokine responses were normalized by log(base 10)-transformation after adding 1 pg/ml to allow for zeroes. *Schistosoma* infection intensities were normalized after adding half of the detection limit (i.e. 5 eggs per gram of feces and 0.25 eggs per 10ml of urine for *S. mansoni* and *S. haematobium*, respectively). One nMDS was performed for each of the four *Schistosoma*-specific whole blood stimulations (either SEAm, SEAh, AWAm or AWAh) using the 'metaMDS' function (Oksanen et al., 2013). Each nMDS was repeated several times to assess the robustness of the resulting pattern (Clarke and Warwick, 2001). The Euclidean dissimilarity index was used (Oksanen et al., 2013), and cytokine profiles - i.e. the matrix of IL-10, IL-5, IFN-γ, TNF-α, and IL-2 - were plotted in three dimensions (3D) to adequately represent the variation in the data (Clarke and Warwick, 2001). Afterwards, gradients of the separate cytokine responses, on which the nMDS was based, were fitted using the 'envfit' function (Oksanen et al., 2013). The same function was used to fit infection intensities onto each 3D nMDS, and to statistically test associations of antigen-induced cytokine profiles with *Schistosoma* infection intensity or infection status, i.e. uninfected, single *S. mansoni*, single *S. haematobium*, versus mixed *S. mansoni* and *S. haematobium* infection. The 'ordiellipse' function was used to fit average group scores - with their 95% confidence intervals (CIs) - for different infection statuses (Oksanen et al., 2013). In contrast to individual *S. mansoni*- and *S. haematobium*-induced cytokine responses which can be compared quantitatively within individuals as described above (univariate statistics), qualitative differences between *S. mansoni*- and *S. haematobium*-induced cytokine profiles could only be assessed visually by nMDS, not by formal statistical testing.

Results

Characteristics of the study population

The study population consisted of 88 males and 112 females with a median age of 16 (range 5-53) years. Malaria and STHs *T. trichiura* and hookworm were absent in this population, and *A. lumbricoides* and *S. stercoralis* rare (n=3 and 2, respectively, with

Table 5.1. *Schistosoma* infections in the study population.

Subjects	*S. mansoni* infection		*S. haematobium* infection		Prevalence (n)	Code for Infection Status in Figure 5.2
	Feces	Urine[a]	Feces	Urine		
Positive					**158**	
Single infections					63	
	+	-	-	-	42	M (dark blue)
	-	-	-	+	21	H (light blue)
Mixed infections					95	MH
	+	-	-	+	81	MH (pink)
	+	+	-	+	13	MH (yellow)
	-	+	-	+	1	MH (red)
Negative	-	-	-	-	**42**	N (green)
Total	**136**	**14**	**0**	**116**	**200**	

[a] *Schistosoma mansoni* eggs that were ectopically excreted in the urine had a *S. mansoni*-like morphology but may have had a genetically hybrid constitution (Chapter 2; Huyse et al., 2013).

100% concordance between microscopy and PCR). In contrast, 137 (69%) subjects were infected with *S. mansoni*, and 116 (58%) with *S. haematobium*. Sixty percent (95/158) of all *Schistosoma* infections were mixed *S. mansoni* and *S. haematobium* infections (Table 5.1). The distributions of *S. mansoni* and *S. haematobium* infections in the study population according to age and gender are shown in Table 5.2. Both *Schistosoma* infections peaked in adolescents (10 to 19 year-olds), but gender differences were not statistically significant. Epidemiological patterns of infection have been described in more detail elsewhere (Chapter 2).

General cytokine profiles

Insight into the different antigen-induced cytokine responses relative to one another was obtained by nMDS. Figure 5.1 and 5.2 show the variation in multivariate cytokine responses in the study population, with dots representing individuals. Distances between dots approximate inter-individual dissimilarities in cytokine responses with stress values (i.e. discrepancies) of 0.051 for SEAm, 0.041 for SEAh, 0.058 for AWAm, and 0.061 for AWAh. Red arrows indicate increasing gradients of IL-10, IL-5, IFN-γ, TNF-α and IL-2 responses, respectively. The level of a cytokine response increases in the direction of the corresponding arrow (see also Supporting information, page 84). The length of a cytokine arrow indicates the goodness of fit of that arrow (or cytokine gradient).

The nMDS outcomes for the first axis (nMDS1) show that for each of the four antigen stimulations, all cytokine responses point to the left. Individuals plotted on the left produced consistently higher levels of all cytokines measured than those on the right.

Table 5.2. Distribution of *Schistosoma* infection in the study population.

		n	*S. mansoni* infection		*S. haematobium* infection	
			Percentage of positives (%)	*p*-value	Percentage of positives (%)	*p*-value
Age (in years)				**0.001**		**0.001**
	5-9	51	58.8		66.7	
	10-19	59	88.1		72.9	
	20-39	55	58.2		49.1	
	≥40	35	65.7		34.3	
Gender				0.32		0.20
	Male	88	72.7		63.6	
	Female	112	65.2		53.6	

In other words, nMDS1 indicates a gradient of high (left) to low (right) cytokine responses. In analogy, the second axis (nMDS2), indicates a gradient of Th1-like (IFN-γ and TNF-α, top) to Th2-like (IL-5, bottom) phenotypes for each of the antigen stimulations. In contrast to SEA-induced IL-5, AWA-induced IL-5 was not accompanied by production of IL-10. IL-2 levels increased with Th1 cytokines, except for SEAm. The third axis (nMDS3) indicates a gradient of TNF-α and IL-2 (left) to IFN-γ and IL-10 (right).

In contrast to antigen-induced cytokines, spontaneously induced levels of cytokines in the control (medium only), did not show significant gradients, except for IL-5 on the third nMDS axis (stress=0.11, data not shown).

Comparison between *S. mansoni*- and *S. haematobium*-induced cytokine responses and cytokine profiles

Figure 5.1 and 5.2 indicate that *S. mansoni* and *S. haematobium* antigens induced very similar cytokine profiles; cytokine profiles differed more between adult (AWA) and egg (SEA) life stages of the parasite than between the two *Schistosoma* species. Within individuals, *S. haematobium*-induced cytokine response levels were higher than those induced by *S. mansoni* (Table 5.3). This was statistically significant for all SEA- and AWA-induced cytokine responses that were measured, except for SEA-induced IFN-γ and IL-10.

Relation between cytokine profiles and *Schistosoma* infection intensity

Subsequently, we related the above-described variation in cytokine responses in the study population (i.e. plotted cytokine profiles) to infection intensity. Table 5.4 shows that all associations between *Schistosoma* antigen-induced cytokine profiles and infection intensity were statistically significant. In Figure 5.1, the direction of the black arrows represents the increasing gradients of *S. mansoni* and *S. haematobium* infection

Table 5.3. Levels of *Schistosoma*-induced cytokine responses in 72h whole blood cultures (n=200).

Antigen	Species	Cytokine	Response (%)	Median Concentration in pg/ml (IQR) [a]		*p*-value [b]
SEA [c]	***S. mansoni***					
		IL-10	92.0	12.7	(5.2 - 32.4)	0.874
		IL-5	78.5	3.7	(1.0 - 19.0)	**<0.001**
		IFN-γ	67.5	3.4	(0.05 - 7.8)	0.729
		TNF-α	64.5	0.7	(0.03 - 2.2)	**0.046**
		IL-2	80.0	6.3	(2.0 - 18.8)	**<0.001**
	S. haematobium					
		IL-10	90.5	13.1	(4.7 - 32.2)	
		IL-5	77.0	5.2	(0.9 - 47.4)	
		IFN-γ	63.0	4.2	(0.05 - 7.8)	
		TNF-α	67.5	1.0	(0.03 - 4.3)	
		IL-2	80.5	8.2	(2.1 - 54.7)	
AWA [d]	***S. mansoni***					
		IL-10	98.5	25.7	(13.2 - 48.2)	**0.008**
		IL-5	94.5	69.3	(11.8 - 201.2)	**<0.001**
		IFN-γ	74.5	5.4	(0.05 - 9.4)	**0.002**
		TNF-α	90.5	4.6	(1.2 - 10.9)	**<0.001**
		IL-2	98.0	60.3	(22.4 - 152.1)	**<0.001**
	S. haematobium					
		IL-10	99.0	30.0	(17.0 - 50.4)	
		IL-5	96.0	108.6	(25.9 - 237.9)	
		IFN-γ	78.5	6.3	(1.7 - 12.1)	
		TNF-α	96.5	6.0	(2.7 - 15.1)	
		IL-2	98.0	99.5	(42.4- 224.5)	
None						
		IL-10	59.5	1.7	(0.03 - 4.9)	
		IL-5	57.0	0.9	(0.02 - 2.6)	
		IFN-γ	58.0	2.2	(0.05 - 5.8)	
		TNF-α	63.5	0.4	(0.03 - 1.5)	
		IL-2	45.5	0.03	(0.03 - 2.9)	

Blood samples from one individual were divided into five and stimulated with *Schistosoma* antigens (SEAm, SEAh, AWAm, or AWAh), and with medium only (negative control; see Materials and methods).

[a] Crude cytokine levels are reported. IQR: Interquartile range (Tukey's hinges)

[b] Wilcoxon Signed Rank test comparing *S. mansoni*- and *S. haematobium*-induced cytokine levels within individuals (either for SEA or AWA)

[c] *Schistosoma* egg antigen

[d] Adult worm antigen

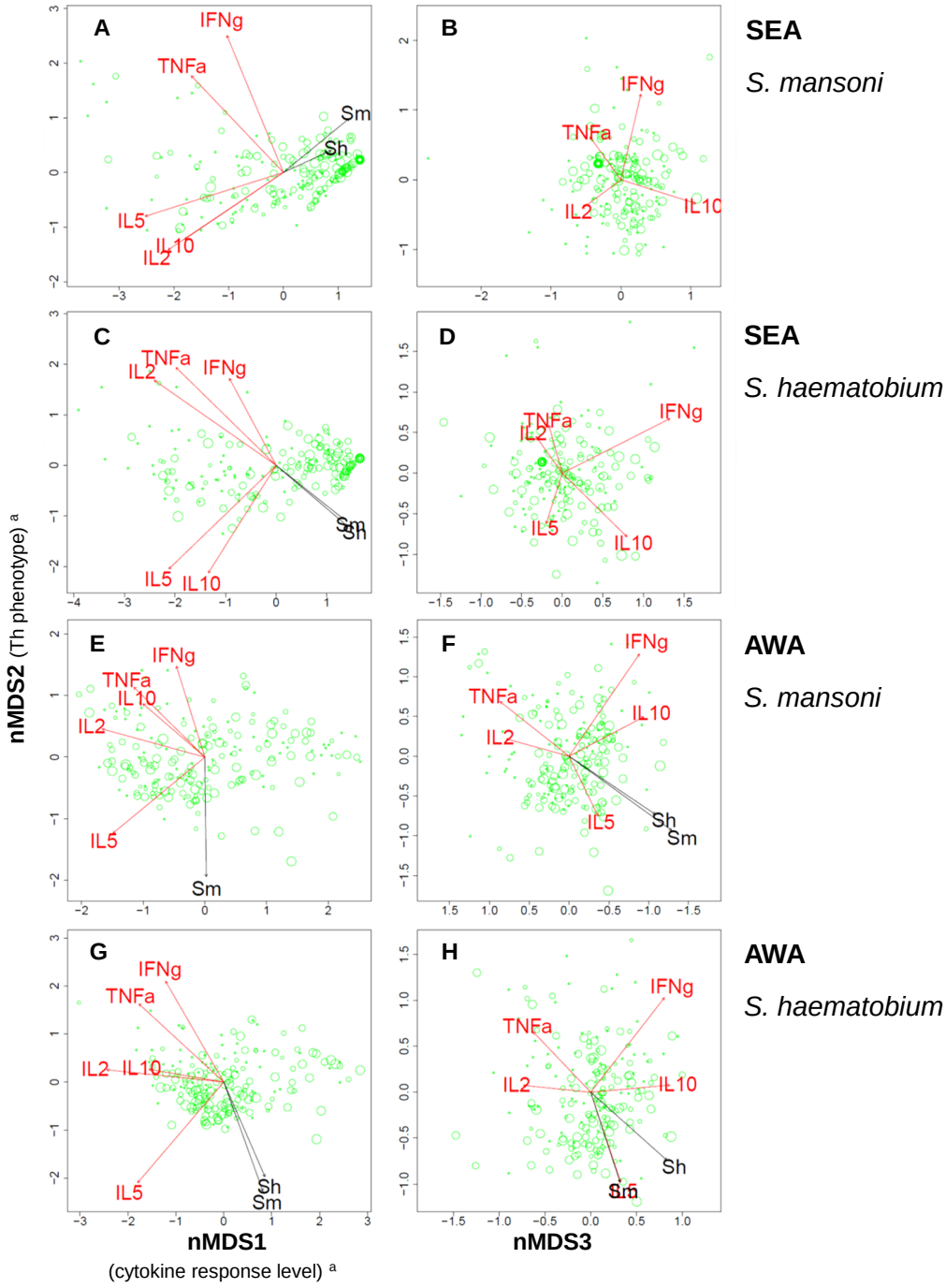


Figure 5.1. Variation in *Schistosoma* antigen-induced cytokine responses in relation to *Schistosoma* infection intensity.

Each three-dimensional (3D) nMDS ordination is represented in two 2D planes (Supporting information, page 84). Left and right panels represent the 1st and 2nd, and 2nd and 3rd dimensions, respectively. **Panels A** and **B** show the *S. mansoni* egg antigen (SEAm)-induced cytokine profile, **Panels C** and **D** that of *S. haematobium* SEA(h), **Panels E** and **F** that of *S. mansoni* adult worm antigens (AWAm), and **Panels G** and **H** show *S. haematobium* AWA(h)-induced cytokine profiles. Green dots represent individuals. Distances between dots approximate the rank order of dissimilarities in cytokine profiles between the respective individuals with stress values (i.e. discrepancies) of 0.051 for SEAm, 0.041 for SEAh, 0.058 for AWAm, and 0.061 for AWAh. Red arrows indicate linear gradients of normalized net cytokine responses on which the nMDS is based. Green dot sizes are proportional to individual values of normalized infection intensity of *S. mansoni* (for simplicity dots were only labelled with *S. mansoni* (not *S. haematobium*) infection intensity). Black arrows indicate linear gradients of post hoc fitted normalized infection intensity of *S. mansoni* ('Sm') and *S. haematobium* ('Sh'). The length of the arrows is proportional to the goodness of fit onto the cytokine profile within one 2D plane, but lengths cannot be compared between cytokine and infection intensity arrows. Arrows are only depicted if their fit was significant at the level of p=0.05 in 3D ordinations (see Table 5.4), as well as in the respective 2D planes. In Panel H, the arrows of IL-5 response and *S. mansoni* infection intensity are overlapping and their labels are therefore illegible.

[a] The biological a posteriori interpretation of nMDS1 (left x-axis) and nMDS2 (y-axis) were added between brackets on the axis labels, but nMDS3 (right x-axis) could not be interpreted.

Table 5.4. Association between *Schistosoma* infection and *Schistosoma* antigen-induced cytokine profiles.

Infection		Antigen-induced cytokine profile			
		SEAm	SEAh	AWAm	AWAh
***S. mansoni* infection intensity**					
	R^2	0.14	0.17	0.10	0.13
	p-value	**0.001**	**0.001**	**0.001**	**0.001**
***S. haematobium* infection intensity**					
	R^2	0.05	0.18	0.07	0.15
	p-value	**0.02**	**0.001**	**0.003**	**0.001**
Infection status					
	R^2	0.09	0.18	0.02	0.04
	p-value	**0.001**	**0.001**	0.2	**0.01**

Figure 5.1 shows the fit of infection intensity and Figure 5.2 that of infection status (uninfected, single *S. mansoni*, single *S. haematobium*, versus mixed infections) onto each of the four *Schistosoma* antigen-induced cytokine profiles (either SEAm, SEAh, AWAm or AWAh), obtained by the 'metaMDS' and 'envfit' functions (see also Supporting information, page 84) (R Core Team, 2012; Oksanen et al., 2013). Here, the goodness of these fits, i.e. squared correlation coefficients (R^2), is shown. The statistical significance was assessed using permutation tests (n=999), and presented *p*-values are approximations.

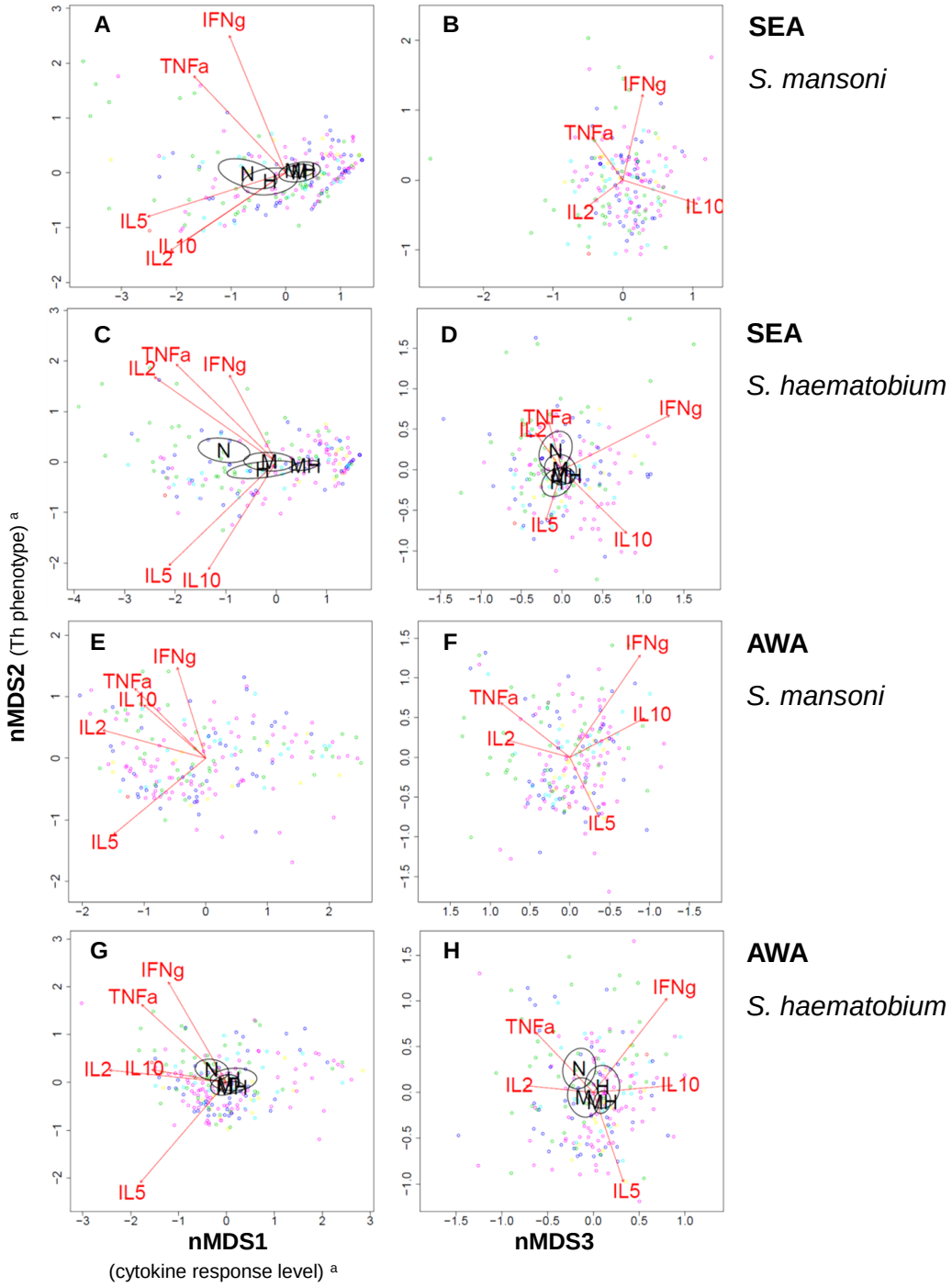


Figure 5.2. Variation in *Schistosoma* antigen-induced cytokine responses in relation to *Schistosoma* infection status.

Each three-dimensional (3D) nMDS ordination is represented in two 2D planes (Supporting information, page 84) as in Figure 5.1: Left and right panels represent the 1st and 2nd, and 2nd and 3rd dimensions, respectively. **Panels A** and **B** show the *S. mansoni* egg antigen (SEAm)-induced cytokine profile, **Panels C** and **D** that of *S. haematobium* SEA(h), **Panels E** and **F** that of *S. mansoni* adult worm antigens (AWAm), and **Panels G** and **H** show *S. haematobium* AWA(h)-induced cytokine profiles. Dots represent individuals and distances between dots approximate the rank order of dissimilarities in cytokine profiles between the respective individuals with stress values (i.e. discrepancies) of 0.051 for SEAm, 0.041 for SEAh, 0.058 for AWAm, and 0.061 for AWAh. Red arrows indicate linear gradients of normalized net cytokine responses on which the nMDS is based. The length of the arrows is proportional to the goodness of fit onto the cytokine profile within one 2D plane, and arrows are only depicted if their fit was significant at the level of p=0.05 in 3D ordinations (see Table 5.4), as well as in the respective 2D planes. Green dots represent uninfected individuals, dark blue those with single *S. mansoni* infections, light blue single *S. haematobium*, and the other colors indicate people with mixed infections: pink indicates mixed infections without ectopic egg elimination, yellow mixed infections with *S. mansoni* in feces as well as in urine and *S. haematobium* in urine, and red dots represent one individual with both *S. mansoni* and *S. haematobium* eggs in urine (possibly a hybrid species (Huyse et al., 2013; Chapter 2 and 3); see also Table 5.1). Ellipsoids represent 95% confidence intervals for average group scores, for different infection statuses: uninfected ('N'), single *S. mansoni* ('M'), single *S. haematobium* ('H'), versus mixed infection ('MH'). Ellipsoids are drawn using the function 'ordiellipse', and only depicted if the fit of infection status onto the cytokine profile was significant at the level of p=0.05 in 3D ordinations (see Table 5.4), as well as in the respective 2D planes. In Panel A and G, the labels for single *S. mansoni* ('M') and mixed infection ('MH') are overlapping.

[a] The biological a posteriori interpretation of nMDS1 (left x-axis) and nMDS2 (y-axis) were added between brackets on the axis labels, but nMDS3 (right x-axis) could not be interpreted.

intensity, respectively (see also Supporting information, page 84). On the first axis, which indicates cytokine response levels (see above), these arrows generally point into the opposite direction of cytokine responses. This indicates that people with elevated *Schistosoma* infection intensities are more likely to have lower cytokine responses, and vice versa. On the second axis, which indicates the Th1 versus Th2 response phenotype (see above), infection intensity generally increases with IL-5 and decreases with Th1 cytokines TNF-α, IFN-γ, and IL-2 (except for SEAm-induced IL-5 which decreases with increasing infection intensity). Briefly, as infection intensity increased, cytokine response levels decreased and the Th2 phenotype became more pronounced. The association between infection intensity and reduced cytokine responsiveness was more pronounced for SEA than for AWA stimulation. *Schistosoma* infection intensity increased with AWA-induced IL-5, but decreased with SEA-induced IL-5 levels, indicating that people with higher infection intensities produced more of a Th2-like response against AWA and more of a suppressive response (i.e. with low cytokine response levels) against SEA than people with lower infection intensities, and vice versa.

We did not observe differences in induced cytokine profiles between the two *Schistosoma* infections. Associations between cytokine profiles and infection intensity were comparable for *S. mansoni* and *S. haematobium* infections (Figure 5.1). Table 5.4 shows significant correlations between cytokine profiles and *Schistosoma* infection intensity for homologous combinations (i.e. infection intensity and antigen stimulation of the same species) as well as for heterologous combinations (i.e. infection intensity of one and antigen stimulation of the other species).

Relation between cytokine profiles and infection status (mixed versus single infections)

Schistosoma antigen-induced cytokine profiles were significantly associated with *Schistosoma* infection status, except upon stimulation with AWAm (Table 5.4). Figure 5.2 shows how antigen-induced cytokine profiles differed according to infection status (except for AWAm, which was not significantly associated with infection status), with 95% CI ellipsoids indicating the average nMDS scores per infection group: uninfected ('N'), single *S. mansoni* ('M'), single *S. haematobium* ('H'), versus mixed ('MH') *Schistosoma* infection group. In analogy with Figure 5.1, uninfected individuals had higher cytokine responses than *Schistosoma*-infected subjects, and their cytokine profiles were skewed more towards the Th1 phenotype. On the whole, there was a gradient in cytokine profiles from uninfected individuals, to people with single and then mixed *Schistosoma* infections (Figure 5.2) and these profiles were in the same direction as the gradient of infection intensity (Figure 5.1). In other words, people with low cytokine responses of the Th2 phenotype tended to have both mixed and heavier infections, people with strong Th1 responses tended to be uninfected, and those with an intermediate cytokine profile tended to have both single and lighter *Schistosoma* infections.

For the SEAm-induced cytokine profile, there was a clear difference (i.e. separation between ellipsoids) between *S. mansoni*-infected individuals (with either single or mixed *S. mansoni*), and those without *S. mansoni* (no *Schistosoma* infection, or single *S. haematobium* infection; Figure 5.2A). There were no significant differences in this cytokine profile between single and mixed *S. mansoni* infections, or between uninfected individuals and those with single *S. haematobium* infections. This indicates that, in contrast to *S. mansoni*, *S. haematobium* infection status was not associated with SEAm-induced cytokine profiles. *Schistosoma haematobium*-induced cytokine profiles on the other hand, showed similar relationships with *S. mansoni* as well as with *S. haematobium* infection status. Cytokine profiles of people with single and mixed infections differed significantly from those of uninfected people, and cytokine profiles did not appear to differ between single *S. mansoni* and single *S. haematobium* infections.

Discussion

The objective of this study was to compare cytokine responses induced by *S. mansoni* and *S. haematobium* antigens, and to relate these to *Schistosoma* infection in a *S. mansoni* and *S. haematobium* co-endemic area. We showed that *Schistosoma* infection intensity was significantly associated with *Schistosoma* antigen-induced cytokine profiles and that it may explain up to 18% of the variation in cytokine responses observed in this population. As *Schistosoma* infection intensity increased, cytokine responses decreased and the Th2 phenotype became more pronounced. This was exemplified by relatively higher IL-5 (and IL-10) and relatively lower IFN-γ, TNF-α and IL-2 levels. Lightly infected and uninfected subjects on the other hand, had elevated cytokine responses, with a Th1 phenotype. These patterns are consistent with the modified Th2 response characteristic for schistosomiasis (Allen and Maizels, 2011). nMDS also indicated that the association between infection and the Th2 phenotype was more pronounced for AWA, while that between infection and (reduced) cytokine responsiveness was more pronounced for SEA. These observations fit with a previous study by Joseph et al. describing similar immunological differences between *Schistosoma* adult worm and egg life stages in a population from a *S. mansoni* mono-endemic area, using more conventional analyses (Joseph et al., 2004).

Secondly, we demonstrated that increased *Schistosoma* infection intensity and mixed (as compared to single) infections were associated with similar, modified Th2, cytokine profiles. This is probably due to the fact that subjects with mixed infections were more likely to have higher infection intensities than those with single infections (Chapter 2). Also, similar, modified Th2, cytokine profiles were observed for both *S. mansoni* and *S. haematobium* infection intensity, whether blood was stimulated with antigens from the homo- or heterologous species. This may be indicative of immunological cross-reactivity between species. For *S. mansoni*-induced cytokine profiles however, this was unlikely, because profiles did not differ between single and mixed *S. mansoni* infection groups. While *S. haematobium*-induced cytokine profiles did differ between single and mixed *S. haematobium* infection groups, we could not determine whether these differences were due to mixed infection per se, or to higher *S. haematobium* infection intensity in mixed as compared to single infections. Other potentially confounding factors such as age may have been involved as well (Chapter 2), and future studies should be performed to assess their respective roles in determining cytokine responses. To obtain more evidence on the existence of cross-reactivity between the two major human *Schistosoma* species, it is important to compare immune responses between different co- and mono-endemic areas, using different immunological parameters (e.g. cytokine, humoral and cytological data). To our knowledge, only one human study reported on functional *S. mansoni* – *S. haematobium* cross-reactivity. This study from 1974 reported lethal in vitro activity of sera from subjects infected with one

species against schistosomula of the same but not of the other species (Smith and Webbe, 1974). Indeed, *S. mansoni* and *S. haematobium* may share few if any epitopes that are involved in protective immunity because they belong to genetically distinct groups. Potential cross-reactivity or the lack thereof merits further investigation as this may have important implications for our understanding of the epidemiology of schistosomiasis as well as for the development of an effective schistosomiasis vaccine.

The present study demonstrated that nMDS can be used successfully to analyze host cytokine responses collectively. In this way, it was possible to analyze cytokine responses in relation to one another, and in relation to *Schistosoma* infection. nMDS is a nonparametric, multivariate and visual method. It is a robust and powerful tool because it avoids problems of multiple statistical tests and violations of data assumptions (Clarke and Warwick, 2001). Moreover, nMDS makes it easier to interpret complex data than traditional one-by-one graphs, tables, and tests. Here, we used this approach to study multivariate cytokine responses, but it can be used equally well to increase our understanding of other complex, multidimensional data, such as cytological and/or serological data (Durnez et al., unpublished data), as well as infection data on multiple co-endemic parasite species.

Additional analyses showed that, within individuals, *S. haematobium* antigens induced higher cytokine responses in 72h whole blood cultures than those of *S. mansoni*. A very similar pattern was observed in parallel investigations in Ghana, in a population which was - in contrast to the Senegalese study population - first exposed to *S. haematobium* and then to both *S. mansoni* and *S. haematobium*, and with lower prevalences of *S. mansoni* and higher prevalences of *S. haematobium* (Amoah et al., unpublished data; and Chapter 2 of this thesis). This suggests that this finding does not depend on the level of transmission or on exposure history, and that the two *Schistosoma* species may differ in their immunogenicity. This hypothesis is in line with observations from Van Remoortere et al. (2001) who found *S. mansoni* to induce mainly IgM antibodies – which are thought to inhibit protective host immune responses (Butterworth et al., 1987) – while *S. haematobium* induced both IgM and IgG antibodies against shared carbohydrate epitopes. It is therefore tempting to speculate that lower cytokine response levels may prevent Ig class switching from IgM to IgG for these epitopes in *S. mansoni* infection, while stronger cytokine responses may promote class switching in *S. haematobium* infection. Alternatively, differences in their biochemical composition may underlie interspecies differences in both immunogenicity and humoral immune responses. These two immunological interspecies differences may also have contributed to earlier epidemiological findings. Several studies observed a steeper decline of the age-infection curve of *S. haematobium* as compared to *S. mansoni* after adolescence, indicating that protective immunity against *S. haematobium* might develop more rapidly (Cheever et al., 1977; Chapter 2). Secondly, higher levels of *S.*

haematobium- as compared to *S. mansoni*-specific morbidity have been observed in co-endemic populations (Koukounari et al., 2010; Gouvras et al., 2013; Chapter 3), suggesting that the immune responses provoked by *S. haematobium* eggs might be more pathogenic. It should be noted however, that other factors may also explain these two epidemiological observations. For example, *S. mansoni* and *S. haematobium* eggs accumulate in different organs, i.e. the liver and the urinary tract, respectively, and these differences in anatomical context may also explain the differences in the extent of morbidity between the two species. More research is necessary to investigate the abovementioned immunological interspecies differences and their implications for epidemiological patterns of infection and morbidity in more detail.

Conclusion

In conclusion, this is the first study to comprehensively investigate *S. mansoni*- and *S. haematobium*-induced cytokine responses in a *S. mansoni* and *S. haematobium* co-endemic area, and to relate these cytokine responses to *Schistosoma* infection. The present study demonstrates that nMDS can be used successfully as a tool for the joint analysis of multiple cytokine responses in relation to *Schistosoma* infection. We showed strong associations between *Schistosoma* infection and *Schistosoma*-induced cytokine profiles, and provided a first insight into potential differences and interactions between human *S. mansoni* and *S. haematobium* infections. This knowledge will contribute to an improved understanding of the mechanisms underlying *Schistosoma* infection and morbidity in co-endemic populations.

Acknowledgements

We gratefully thank the population of Ndieumeul and Diokhor Tack and the village chiefs, Daoure Mbaye and Daouda Pene, for their hospitality and participation in this study. This study would not have been possible without the field workers in Richard-Toll, Abdoulaye Yague, Mankeur Diop, Moussa Wade and Ngary Sy, who assisted in the sample collection and microscopic analysis. We would also like to thank the medical and technical staff of the Health Centre in Richard-Toll for their support, Yvonne Kruize for providing the immunologic stimuli and preparatory work, Rogier Achterberg, Mareen Datema and Churnalisa Doran for the cytokine measurements in Leiden, Pierre Legendre from the University of Montreal and Vincent Sluydts from Antwerp for their useful advice on multivariate analyses, as well as Lies Durnez from Antwerp for critically reviewing the statistical methods used. In addition, we would like to thank one of the Reviewers who made important contributions to our manuscript in two Review rounds.

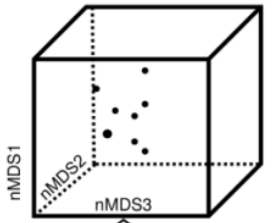
Supporting information

Schematic representation of nonmetric multidimensional scaling

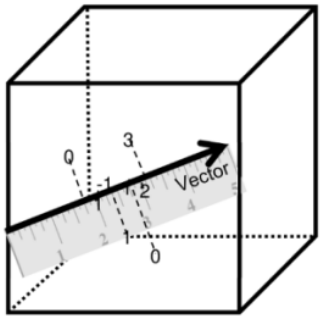
The nonparametric data reduction technique nonmetric multidimensional scaling (nMDS) aims to plot the individuals (dots) in such a way that the inter-point distance in the plot approximates the same rank order as corresponding dissimilarities in multivariate cytokine profiles (i.e. matrix of net IL-10, IL-5, IFN-γ, TNF-α, and IL-2 production) (Clarke and Warwick, 2001; R Core Team, 2012; Oksanen et al., 2013; Bourke et al., 2013). In other words: it aims to minimize the stress, i.e. discrepancy between inter-point distances and true dissimilarities. This is achieved in an iterative procedure. Subsequently, the plot (i.e. a 3D cloud of dots) is rotated in such a way that the first axis represents the largest variance, followed by gradually less variance in the consecutive axes. This method is analogous to principal component analysis which is, in fact, the parametric equivalent of nMDS. In order to obtain acceptable stress levels and robust patterns, it was necessary to plot individuals in three dimensions (3D) (Clarke and Warwick, 2001). One 3D nMDS (cube) was performed for each of the four *Schistosoma*-specific whole blood stimulations (either SEAm, SEAh, AWAm or AWAh) using the 'metaMDS' function (in R using the 'Vegan' package (R Core Team, 2012; Oksanen et al., 2013)). Afterwards, the 'envfit' function was used to test whether a gradient of infection intensity is present in the 3D cloud of dots. This is done by searching the vector (direction represented by an arrow) that shows a maximal correlation with the individual values of log-transformed infection intensity (Oksanen et al., 2013). *Schistosoma mansoni* as well as *S. haematobium* infection intensity were fitted onto each 3D cytokine profile. Table 5.4 presents the goodness of fit (R^2) and statistical significance (p-value) for each of these combinations. Two 2D planes (squares) were produced from each 3D nMDS: A) nMDS axis 1 by 2; and B) axis 3 by 2. Significant 3D correlations were tested and fitted (pointers) in the two 2D planes (Figure 5.1). Similarly, 'envfit' was used to test whether people with different infection status (uninfected, single *S. mansoni*, single *S. haematobium*, versus mixed infections) differed in their cytokine profiles (not shown). The 'ordiellipse' function was used to visualize how cytokine responses varied with infection status (Figure 5.2).

Nonmetric Multidimensional Scaling (nMDS)
Cytokine Profile

3D nMDS

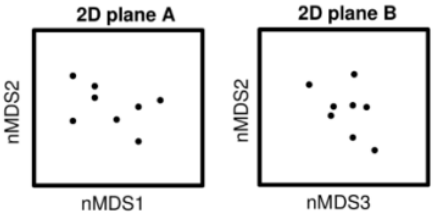


'envfit'



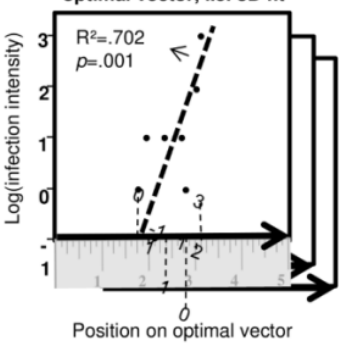
Dots replaced by individual values of log(infection intensity) for either *S. mansoni* or *S. haematobium*

Corresponding 2D plots



Correlations between these values and their projections onto different vectors (i.e. directions) is calculated.

Correlation plot for optimal vector, i.e. 3D fit

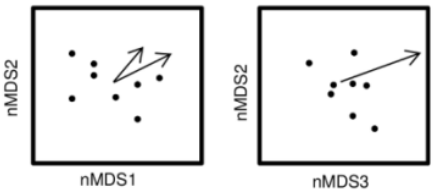


The vector with maximal correlation is selected, i.e. the 3D fit

Statistical Testing
Infection Intensity

***S. mansoni*:**
If 3D fit is significant → **2D fit A** + **2D fit B**

***S. haematobium*:**
If 3D fit is significant → **2D fit A** + **2D fit B**



Chapter 6

Enhanced pro-inflammatory cytokine responses following Toll-like-receptor ligation in *Schistosoma haematobium*-infected schoolchildren from rural Gabon

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PLoS one 2011;6(9):e24393

Abstract

Background: *Schistosoma* infection is thought to lead to down-regulation of the host's immune response. This has been shown for adaptive immune responses, but the effect on innate immunity, that initiates and shapes the adaptive response, has not been extensively studied. In a first study to characterize these responses, we investigated the effect of *Schistosoma haematobium* infection on cytokine responses of Gabonese schoolchildren to a number of Toll-like receptor (TLR) ligands.

Methodology: Peripheral blood mononuclear cells (PBMCs) were collected from *S. haematobium*-infected and uninfected schoolchildren from the rural area of Zilé in Gabon. PBMCs were incubated for 24h and 72h with various TLR ligands, as well as schistosomal egg antigen (SEA) and adult worm antigen (AWA). Pro-inflammatory TNF-α and anti-inflammatory/regulatory IL-10 cytokine concentrations were determined in culture supernatants.

Principal findings: Infected children produced higher adaptive IL-10 responses than uninfected children against schistosomal antigens (72h incubation). On the other hand, infected children had higher TNF-α responses than uninfected children and significantly higher TNF-α to IL-10 ratios in response to FSL-1 and Pam3, ligands of TLR2/6 and TLR2/1 respectively. A similar trend was observed for the TLR4 ligand LPS while Poly(I:C) (Mda5/TLR3 ligand) did not induce substantial cytokine responses (24h incubation).

Conclusions: This pilot study shows that *Schistosoma*-infected children develop a more pro-inflammatory TLR2-mediated response in the face of a more anti-inflammatory adaptive immune response. This suggests that *S. haematobium* infection does not suppress the host's innate immune system in the context of single TLR ligation.

Introduction

Schistosomiasis is a parasitic disease of major public health importance and is largely chronic in nature. The WHO estimates that more than 207 million people are infected worldwide (Chitsulo et al., 2000; WHO, 2010). Chronic helminth infections are generally assumed to cause down-regulation of immune responses allowing long-term survival of the parasite on the one hand and minimizing immunopathology on the other (Burke et al., 2009) with important consequences to the host's health (Maizels and Yazdanbakhsh, 2003; Gryseels et al., 2006; Diaz and Allen, 2007; King and Dangerfield-Cha, 2008). Effects of schistosomal infection on adaptive immunity are widely studied in this context and it has been shown that chronic schistosomiasis inhibits *in vitro* proliferation of human lymphocytes in response to schistosomal antigens (Rocklin et al., 1980; Grogan et al., 1998). The anti-inflammatory cytokine interleukin (IL)-10 plays an important role in this down-regulation (King et al., 1996; Mwatha et al., 1998; Montenegro et al., 1999; Wamachi et al., 2004; Booth et al., 2004a). Van den Biggelaar and colleagues (2000) have demonstrated that children chronically infected with schistosomes produce more IL-10 together with IL-5 and IL-13 in response to adult worm antigen (AWA), than those free of *Schistosoma* infection. Helminth-induced immunomodulation may also have an impact on other infections and vaccine effectiveness. For example, in filarial infections, IL-10 down-regulates responses to unrelated antigens such as tetanus toxoid (Cooper et al., 1998; Nookala et al., 2004). Similar mechanisms might explain the impaired reaction to tetanus toxoid observed in *Schistosoma mansoni*-infected individuals (Sabin et al., 1996). Another feature of IL-10 is that it might suppress atopy in *Schistosoma haematobium*-infected children (van den Biggelaar et al., 2000; Smits et al., 2007).

Although it is becoming clearer how chronic schistosomiasis impacts on adaptive immune responses in terms of immunoregulation, the effects of schistosomal infection on innate immunity is a less studied area. In general, the innate immune system detects invading pathogens through a set of 'pattern-recognition receptors' (PRRs) which recognize 'pathogen-associated molecular patterns' (PAMPs) (Iwasaki and Medzhitov, 2004; Lee and Kim, 2007). Engagement of PRRs, such as Toll-like receptors (TLRs) initiates early immune events prior to the full activation of adaptive immunity. TLRs act as homo- (e.g. TLR3 or TLR4) or heterodimers (e.g. TLR2/1 or TLR2/6), or in combination with other PRRs such as C-type lectins. Most TLRs induce IL-12p70 which in turn promotes differentiation of Th1 cells and inflammatory responses, characterized by production of tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ). On the other hand, C-type lectins can be in part responsible for the induction of anti-inflammatory responses (Geijtenbeek and Gringhuis, 2009). The interplay between innate receptors is thought to form the signals that will determine the development of adaptive immune responses (Lee and Kim, 2007; Chang, 2010).

The present study is part of the SCHISTOINIR project that aims to explore innate immune responses in schistosomiasis (for further information: www.york.ac.uk/res/schistoinir). This pilot study was set out to investigate how chronic schistosomiasis affects the peripheral blood mononuclear cell (PBMC) response to various TLR ligands. Here, we report the results from children living in a *S. haematobium*-endemic rural area near Lambaréné, Gabon.

Methods

Ethics statement

The study was approved by the "Comité d'Ethique Regional Independent de Lambaréné" (CERIL). Written informed consent was obtained from parents or legal guardians of all children participating prior to inclusion into the study.

Study site and population

This pilot study was performed in Zilé, a rural area situated 16 km from Lambaréné, Gabon (van der Kleij et al., 2004; Retra et al., 2008). This area is endemic for *S. haematobium*, but with important focal differences. Within Zilé there are two smaller communities, one with high (PK15) and one with low transmission (PK17), 2 km apart from each other. In June and July 2008, we recruited 17 *S. haematobium*-infected schoolchildren from PK15 and 13 uninfected from PK17, and compared their immune profiles. Study participants had no history of anti-helminthic treatment within the last 3 months prior to this study.

Parasitological examination

S. haematobium infection was determined a maximum of seven days prior to blood collection by examining the residue of 10 ml of urine passed through a filter of 12-µm pore-size (Millipore). Children were classified as *S. haematobium*-infected if at least one *S. haematobium* egg was detected in the urine, or uninfected if three consecutive urine samples were negative. Geometric mean infection intensity was calculated for infected children using the first urine reading. Infections with intestinal helminths *Ascaris lumbricoides* and *Trichuris trichiura* were determined by analyzing one fresh stool sample using the Kato-Katz method 30-45 minutes after preparation (Katz et al., 1972). *Necator americanus* larvae were detected in a 7-day coproculture of the same stool sample. To this end the classical charcoal culture procedure was used (Polderman et al., 1991; Krepel et al., 1992; Reiss et al., 2007). Infection with *Plasmodium falciparum* and microfilaria was determined by Giemsa-stained thick blood smears (Planche et al., 2001).

After collection of blood samples, all *S. haematobium*-infected children were treated with a single dose of praziquantel (40 mg/kg), and those with intestinal helminths were given a single dose of albendazole (400 mg). Children with other infections or clinical complaints were provided with appropriate medical care.

Hematology

Hematological parameters were analyzed using the ADVIA® 120 Hematology System (Bayer Health Care) and erythrocyte sedimentation rate was determined manually.

PBMC cultures

PBMCs were isolated from heparinized venous blood by density centrifugation on Ficoll (AZL pharmacy, The Netherlands) and cultured in 200µl of IMDM supplemented with 5% fetal bovine serum (Greiner Bio-One), 100 U/ml penicillin (Astellas), 10 µg/ml streptomycin, 1 mM pyruvate and 2mM L-glutamine (Sigma) in 96-well round bottom plates (Nunc). PBMCs were seeded at 1 x 10^6 cells/well for stimulation with schistosomal egg antigen (SEA) or adult worm antigen (AWA), and at 5 x 10^5 cells/well for stimulation with specific TLR ligands and phyto-hemagglutinin (PHA; positive control). IMDM medium was used as a negative control. Supernatants were collected after 24h and 72h of incubation at 37°C in a 5% CO_2 atmosphere and stored at -80°C until cytokine analysis.

Innate and adaptive stimuli

A panel of four TLR ligands was used:

- TLR2/1 ligand, Pam3CSK4 (Pam3), a synthetic bacterial triacylated lipopeptide (final concentration of 100 ng/ml; EMC microcollections GmbH),
- TLR2/6 ligand, FSL-1, a synthetic diacylated lipoprotein mimicking the N-terminal part of LP44 from *Mycoplasma salivarium* (50 ng/ml; InvivoGen),
- TLR3 ligand polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of viral double-stranded RNA (50µg/ml; InvivoGen),
- TLR4 ligand, ultrapure lipopolysaccharide (LPS) derived from *Escherichia coli* (100 ng/ml; InvivoGen).

In addition to the classical innate ligands, we used the schistosomal antigen-containing products SEA and AWA from *S. haematobium* at a final concentration of 10 µg protein/ml. Purified PHA (Remel) was used as a positive control at a final concentration of 2 µg/ml.

Cytokine analysis

Two cytokines were measured in this pilot study; one key pro-inflammatory cytokine, TNF-α (Martich et al., 1993; Feldmann et al., 1996; Abraham et al., 1998), and the anti-

inflammatory/regulatory cytokine IL-10 (Spits and De Waal, 1992; De Vries, 1995; Papadakis and Targan, 2000; Booth et al., 2004a; Grant et al., 2011). Cytokine concentrations were measured in supernatants from single PBMC cultures by ELISA according to the manufacturer's instructions, using half of the reaction volume (PeliKine Compact™, Human TNF-α and IL-10 ELISA kit, Sanquin).

A pro-inflammatory index was computed as the TNF-α : IL-10 ratio upon stimulation relative to the spontaneously produced TNF-α : IL-10 ratio (i.e. in medium):

$$\frac{[\text{TNF-}\alpha]_{\text{stimulusA}} / [\text{IL-10}]_{\text{stimulusA}}}{[\text{TNF-}\alpha]_{\text{medium}} / [\text{IL-10}]_{\text{medium}}}$$

Table 6.1. General characteristics of the study population.

	Uninfected	Infected
n	13	17
Age (mean (range))	10.2 y (7-16)	11.4 y (7-16)
Sex (boys / girls)	8 / 5	9 / 8
BMI (median (interquartile range))	15.5 kg/m² (2.83)	15.7 kg/m² (1.64)
***S. haematobium* infection intensity (geometric mean (range))**	Not applicable	9.8 eggs/10ml (1-1002)
Intestinal helminth infection (n/total)	5/12	5/11
***N. americanus* infection(n/total)**	1[a]/12	2/12
***A. lumbricoides* infection (n/total)**	2[b]/12	2[b]/11
***T. trichiura* infection (n/total)**	4[c]/12	2[b]/11
Malaria (n/total)	1/12	0/17
Microfilariasis (n/total)	0/12	1/17
Erythrocyte sedimentation rate (median (range))	15 mm/h (6-50)	15 mm/h (10-88)
Level of white blood cells (median (range))	8.70·10³/µl (3.80-10.30)	7.20·10³/µl (5.00-12.40)
Level of lymphocytes (median (range))	3.22·10³/µl (1.68-5.00)	3.33·10³/µl (1.26-4.60)
Level of monocytes (median (range))	0.68·10³/µl (0.36-1.00)	0.64·10³/µl (0.49-1.04)
Level of neutrophils (median (range))	2.99·10³/µl (1.06-6.08)	2.14·10³/µl (1.05-5.79)
Level of eosinophils (median (range))	0.79·10³/µl (0.20-1.93)	1.53·10³/µl (0.50-2.49)
Level of basophils (median (range))	0.09·10³/µl (0.04-0.27)	0.07·10³/µl (0.04-0.12)
Level of hemoglobin (median (range))	12.2 g/dl (11.4-13.2)	11.3 g/dl (9.1-13.6)

Thick smears (n=1) for the diagnosis of blood parasites as well as stool samples (n=6 for Kato-Katz and n=5 for coproculture) for the diagnosis of intestinal helminths were missing at random.

[a] Including one child with a *T. trichiura - N. americanus* co-infection.

[b] Including one child with a *T. trichiura - A. lumbricoides* co-infection.

[c] Including one child with a *T. trichiura - N. americanus* co-infection and another one with a *T. trichiura - A. lumbricoides* co-infection.

Statistical analysis

Differences between schistosome-infected and uninfected groups were determined by the Fisher's exact test for sex and intestinal helminth infections and by the Mann-Whitney U test for helminth infection intensity, body mass index (BMI) and hematological parameters. Age was normally distributed and differences between infection groups were tested using the independent student's T test. As cytokine concentrations were not normally distributed, nonparametric tests were used. Differences in an individual's cytokine response to different stimuli (e.g. medium *versus* stimulus-induced cytokine concentration, or ratio) were tested by the Wilcoxon matched-pair signed-rank test.

Differences between infection groups in cytokine concentrations and pro-inflammatory indices were tested by the Mann-Whitney U test. Cytokine concentrations were corrected for spontaneous cytokine production. This was done by subtracting the spontaneously induced cytokine concentration (i.e. in medium) from the stimulus-induced cytokine concentration. For the pro-inflammatory index, this was done by dividing the stimulus-induced ratio by the spontaneously induced ratio (see formula above). Similarly, Spearman's ρ was calculated to estimate the correlation between cytokine responses and infection intensity.

SPSS 18.0 (SPSS Inc.) and GraphPad Prism 5 (GraphPad Software, Inc.) were used for statistical analysis. Results were considered significant when the *p*-value was <0.05.

Results

Characteristics of the study population

Table 6.1 shows that the demographic, parasitological and hematological characteristics of the infected and uninfected groups of schoolchildren were comparable. Although *S. haematobium*-infected children tended to be more anemic and to have higher eosinophil levels, there were no significant differences between the groups for any of the parameters. In addition, the prevalence and infection intensity of *A. lumbricoides*, *T. trichiura* or *N. americanus* did not significantly differ between *S. haematobium*-positive and -negative children (data not shown for infection intensity). *Schistosoma haematobium* infection intensities were low (<50 ep10ml) in 14/17 infected children (Montresor et al., 1998). Malaria and filariasis are endemic in this area (Wildling et al., 1995). However, only one study participant was infected with *P. falciparum* and one with microfilariae.

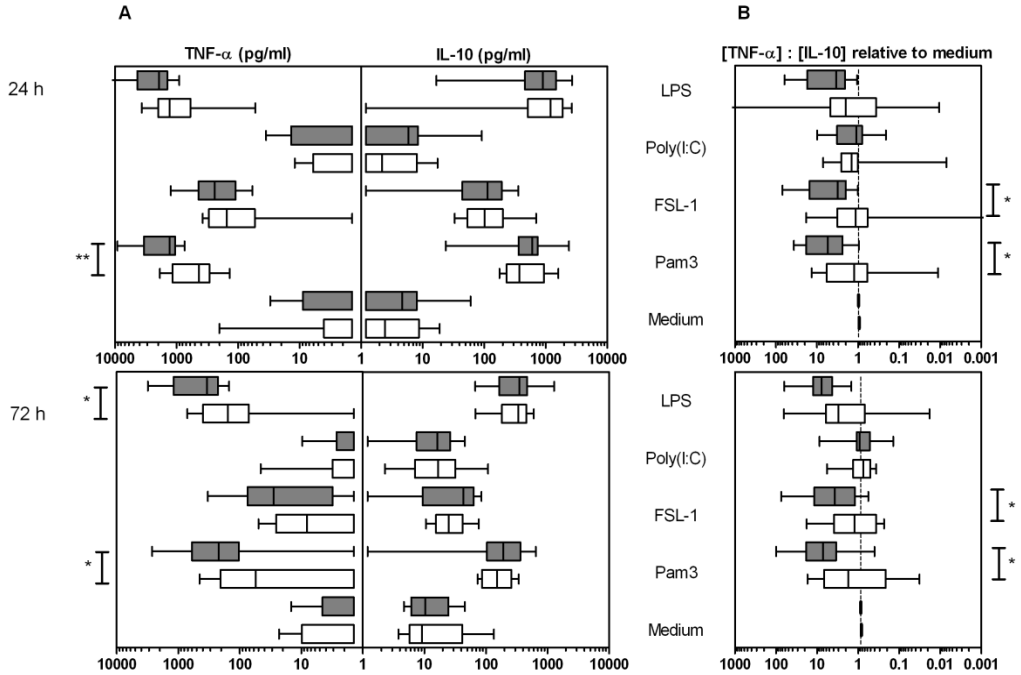


Figure 6.1. Cytokine production in response to TLR ligands in PBMC cultures.

White and grey boxes correspond to *S. haematobium*-free and infected children respectively with the whiskers indicating minimal and maximal concentrations. ***** $p<0.05$; ****** $p<0.01$. **Panel A**: At 24h, the 'innate' time-point, infected children produced significantly more TNF-α in response to the TLR2/1 ligand Pam3CSK4 (Pam3) compared with uninfected children. TLR-mediated IL-10 production did not significantly deviate between infection groups. When innate cytokine responses faded at 72h, similar trends were observed. These plots were not adjusted for spontaneous cytokine production. **Panel B**: At both time points, pro-inflammatory indices (i.e. cytokine ratio induced by one of the stimuli relative to the spontaneously produced ratio) induced by the TLR2 ligands, Pam3 and FSL-1, were significantly higher in infected versus uninfected children.

Cytokine responses to TLR-ligands

TLR-induced cytokine production by PBMC cultures from infected and uninfected groups was measured at 24h (Figure 6.1A, upper panel). While Pam3, FSL-1 and LPS led to substantial and significant cytokine production in both groups compared to the medium control, the TLR3 ligand poly(I:C) did not stimulate significant levels of TNF-α or IL-10. Pam3, FSL-1 and LPS tended to induce the production of greater quantities of the pro-inflammatory cytokine TNF-α in infected compared to uninfected children (Pam3; $p<0.01$), while the production of IL-10 was comparable between the two groups.

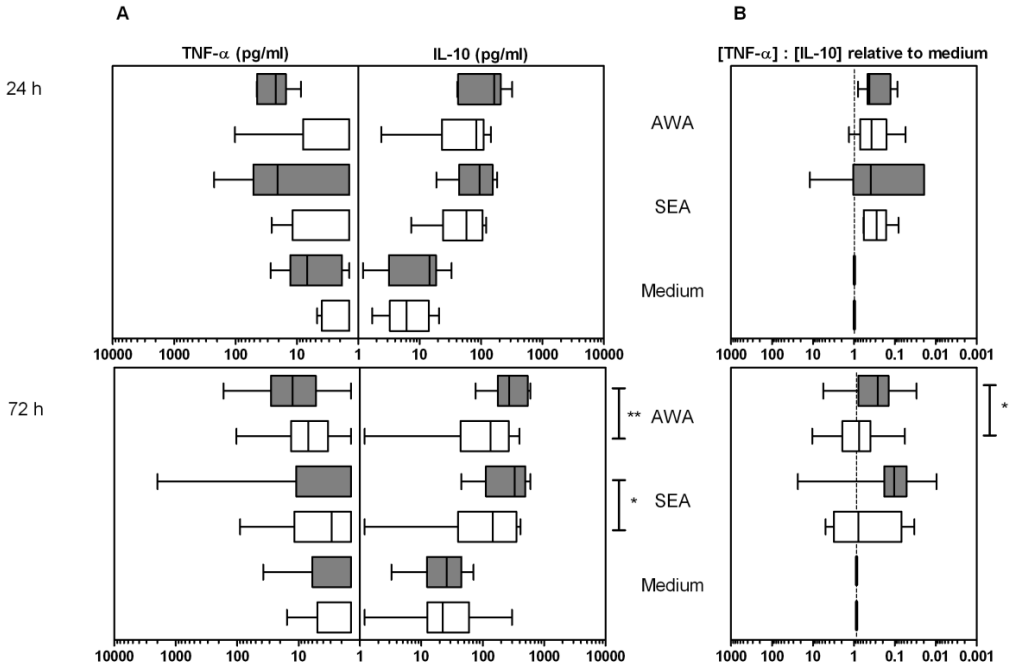


Figure 6.2. Cytokine production in response to schistosomal products in PBMC cultures.

PBMCs were stimulated with schistosomal egg antigen (SEA) or adult worm antigen (AWA). White and grey boxes correspond to *S. haematobium*-free and infected children respectively with the whiskers indicating minimal and maximal concentrations. * $p < 0.05$; ** $p < 0.01$. **Panel A**: Cytokine production did not differ between groups at 24h (mainly innate response) but the adaptive IL-10 response after 72h of incubation to both schistosomal products was significantly higher in infected children than in *S. haematobium*-free children. Furthermore, only infected children produced a significant innate TNF-α response to schistosomal products at 24h. These plots are not adjusted for spontaneous cytokine production. **Panel B**: After 24h (predominantly innate), most cytokine ratios induced by schistosomal products were more anti-inflammatory than the ratios induced by medium alone. When the adaptive response had developed after 72h however, only infected children produced significant anti-inflammatory cytokine balances while pro-inflammatory indices were lower in infected children than in uninfected children (AWA; $p < 0.05$).

There was a stronger pro-inflammatory index for PBMC responses of infected children (Figure 6.1B, upper panel) upon TLR-stimulation (except poly(I:C)) than those from uninfected children. These differences were significant for both Pam3 and FSL-1 ($p<0.05$) which are ligands for TLR2.

Furthermore, *S. haematobium* infection intensity was positively associated with TLR2- and TLR4-mediated TNF-α but not with IL-10 responses. This was significant for Pam3

(ρ=0.80) and FSL-1 (ρ=0.66). Also, pro-inflammatory indices tended to be positively associated with infection intensity (significant for Pam3 with ρ=0.60).

In order to get an idea of the dynamics of the innate response, TLR responses were also measured after 72h. Cytokine responses to TLR ligands decreased over time, being less pronounced after 72h. Nevertheless, the difference between infected and uninfected subjects was similar to that observed in 24h cultures with a largely pro-inflammatory TLR response in infected subjects (Figure 6.1, lower panels).

Cytokine responses to schistosomal products

We examined adaptive immune responses by analyzing cytokine production by PBMCs after 72h of incubation with schistosomal antigens (Figure 6.2, lower panels). Since these products not only contain antigenic components but also innate ligands (Van der Kleij et al., 2002; Thomas, 2003; Van Die, 2003; Van Vliet, 2005; Aksoy et al., 2005; Van Liempt et al., 2007; Caldas et al., 2008; Ritter et al., 2010), cytokine production was assessed at both 24h (innate) and at 72h (adaptive response).

SEA- and AWA-induced IL-10 was detected in PBMC culture supernatants after 24h and increased further in 72h cultures. IL-10 production in infected children was consistently higher than in uninfected children at 24h but this difference was only significant at 72h: both SEA- and AWA-induced IL-10 responses were significantly higher in infected children at 72h (Figure 6.2A).

After 24h, AWA-stimulated cultures from infected children secreted significant levels of TNF-α ($p<0.05$ *cf.* medium), while in uninfected children, antigen-induced TNF-α was only detected after 72h.

After 24h, the cytokine ratios following stimulation with schistosomal products seemed more anti-inflammatory than pro-inflammatory (i.e. increased IL-10 : TNF-α; Figure 6.2B, upper panel). After 72h, the pro-inflammatory index tended to increase in uninfected children, and was significantly higher than in infected children upon stimulation with AWA (Figure 6.2B, lower panel).

Discussion

In this pilot study we investigated the effects of chronic *Schistosoma* infection on TLR-mediated cytokine production. We showed that innate TNF-α responses and TNF-α : IL-10 ratios upon TLR2 stimulation of PBMCs were significantly higher in *S. haematobium*-infected children compared with those without infection, in the face of enhanced regulatory adaptive responses to schistosomal antigens. This suggests that schistosomal infection is associated with elevated pro-inflammatory TLR2 responses.

While cytokine responses upon TLR stimulation tended to decrease over time, the reverse was seen after stimulation with schistosomal products. Although at 24h, TNF-α

levels tended to be more pronounced in infected than in uninfected children, analogous with the observed TLR-mediated cytokine responses, after 72h of stimulation, PMBCs from *S. haematobium*-infected children produced significantly more IL-10 than uninfected children, as has been described elsewhere (van den Biggelaar et al., 2000). Schistosomal products are more complex than single TLR stimuli. SEA and AWA stimulate the innate immune system through TLRs, C-type lectins and other innate receptors (Van der Kleij et al., 2002; Thomas, 2003; Van Die, 2003; Van Vliet, 2005; Aksoy et al., 2005; Van Liempt et al., 2007; Caldas et al., 2008; Ritter et al., 2010) but in addition, they contain antigens which can be processed and presented to the T cell receptor forming the basis of acquired immune responses. Schistosomal products are thus able to activate both innate and adaptive pathways. This may explain the observed trend of an initial pro-inflammatory-like immune response. Concurrent with a fading innate cytokine response, a clear anti-inflammatory adaptive response comprising elevated IL-10 was detected in infected children at 72h.

To our knowledge there are only two other human studies on TLR-mediated cytokine profiles in schistosomiasis. Van der Kleij et al. reported reduced TLR responses in Gabonese *S. haematobium*-infected as compared to uninfected children (Van der Kleij et al., 2004). These results seem to contradict the current findings, but can be explained by differences in the selection of the uninfected control groups. While in the present study both infected and uninfected children were from the same *S. haematobium*-endemic rural area (Zilé), Van der Kleij et al. recruited the uninfected control group from a non-endemic neighboring semi-urban area to ensure that the negative subjects were truly negative with no history of exposure. However, when we examined the dataset of the Van der Kleij study to compare the infected (n=5) and uninfected (n=10) groups from the same rural area of Zilé, infected children tended to produce higher TNF-α levels as well as higher pro-inflammatory indices in response to LPS than their uninfected counterparts, which is in line with our results.

The second study of TLR-mediated cytokine profiles was carried out in Brazil and also corresponds with our results. Although they did not highlight it, Montenegro et al. showed that 48h whole blood cultures from *S. mansoni*-infected adults produced more TNF-α than those of uninfected adults in response to LPS (Montenegro et al., 2002).

In addition, a mouse model of infection pointed towards a similar association between TLR function and schistosomal infection and suggests that schistosomal infection induces elevated pro-inflammatory TLR responses (Joshi et al., 2008).

Few field studies on TLR responses in other helminth infections than schistosomiasis have been published and show varying results. In contrast to *S. haematobium* infection, a negative association has been found between infection with the filarial nematode *Wuchereria bancrofti* and intracellular pro-inflammatory cytokine expression upon TLR stimulation in lymphocytes and monocytes in a population in South India (Babu et al.,

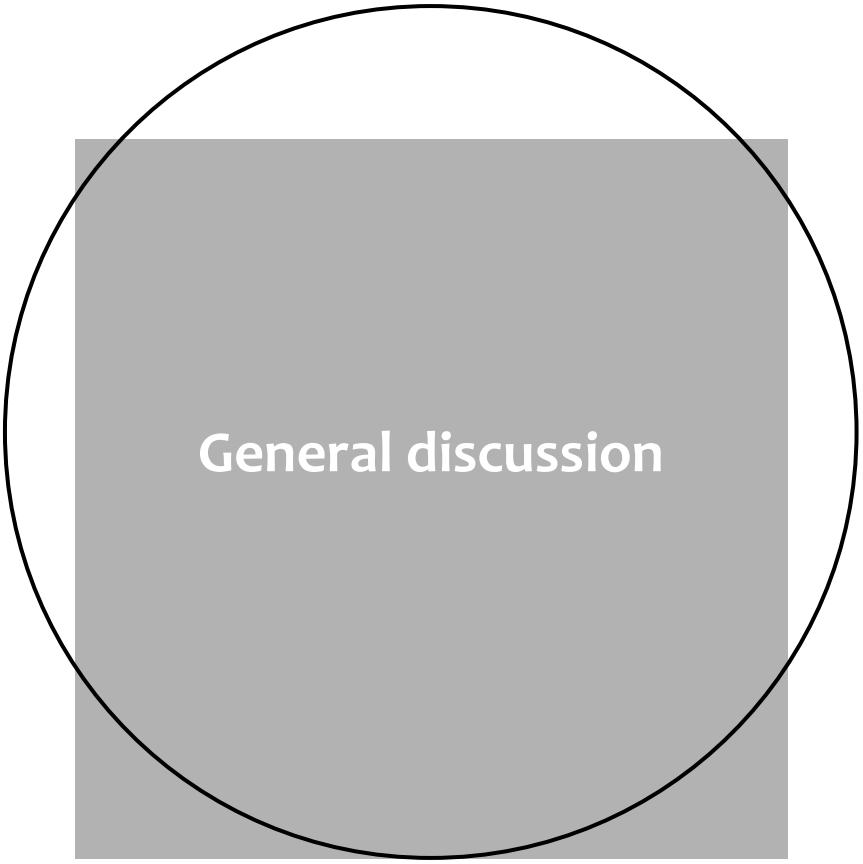
2005; Babu et al., 2006). In addition, *W. bancrofti* was associated with reduced pro-inflammatory cytokine responses upon TLR stimulation of PBMCs in a population from the same area with latent tuberculosis (Babu et al., 2009). On the other hand, Jackson et al. showed infections with intestinal nematodes, i.e. hookworm and *Trichuris trichiura*, to be associated with pro-inflammatory cytokine production, but not with regulatory cytokine production, upon TLR2 and TLR4 stimulation of monocytes from children from Pemba Island (Jackson et al., 2006). Apparently, the effect of helminth infections on the innate immune responses is species specific; some helminth species may be associated with more pro-inflammatory TLR responses while other helminths are associated with more anti-inflammatory TLR responses. In the present study, *S. haematobium*-infected and uninfected children did not significantly differ with respect to the number and species of other helminth infections. However, information on soil-transmitted helminth infections was not available for some of the participants and we cannot entirely rule out that these infections may have altered the observed innate cytokine responses. In addition, other parasites such as malaria may influence immune balances (Hartgers and Yazdanbakhsh, 2006). The duration as well as the intensity of infection might further influence innate immune responses. It is therefore important to confirm our findings in a larger study population and in varying epidemiological settings.

In conclusion, this pilot study on innate immune responses in schistosomiasis shows a more pro-inflammatory response to single TLR2 ligands in the face of an anti-inflammatory adaptive immune response in *S. haematobium*-infected children. Whilst the precise biological mechanisms for these observations remain to be ascertained, it seems that the commonly accepted view that schistosomal infection suppresses the host's immune system does not hold for ligation of single TLRs. Many receptors – TLRs as well as non-TLRs such as C-type lectins – are involved in innate signaling and their interactions as well as down-stream pathways are at the basis of the immune response to invading pathogens (Lee and Kim, 2007; Carvalho et al., 2009). Given the fact that a *Schistosoma* worm is a complex mix of ligands stimulating the innate immune system, further research into this innate cross-talk is necessary.

Acknowledgments

We thank all school children and the Zilé community for their cooperation. Furthermore, we thank Yvonne Kruize for the training she has provided, Sunny Sapthu and Alwin van der Ham for performing most of the ELISAs, Anne Marie Nkoma, Teddy Mabamba, Olivier Kouma and Paule Valerie Mbadinga from the Medical Research Unit of the Albert Schweitzer Hospital in Lambaréné who collected and analyzed stool and urine samples. We would also like to thank Joris Menten from the Institute of Tropical Medicine in Antwerp and Linda May from LUMC for their help on the statistical analysis.

Chapter 7



Thesis outline

Schistosomiasis is one of the most common human parasitic diseases with over 230 million people affected worldwide. The two major species are *Schistosoma mansoni* and *S. haematobium*, which occur together in many African regions (Gryseels et al., 2006). In such co-endemic areas, the two species can interact, either directly or indirectly via the host immune system. This may have important implications for infection and morbidity levels in the communities involved. However, few studies have investigated this. This thesis is one of the first investigations into differences and interactions between the two major human *Schistosoma* species, and their effects on host morbidity.

In the previous chapters, patterns of infection and morbidity were investigated in a detailed way by zooming in into one co-endemic focus in the north of Senegal, Nouk Pomo, and by adopting a multidisciplinary approach including epidemiological, parasitological, clinical, geographical, and immunological investigations. We compared demographic determinants of *S. mansoni* and *S. haematobium,* single and mixed infections, *S. mansoni* and *S. haematobium* morbidity, as well as the effect of mixed and single infections on morbidity (Chapter 2 and 3). The micro-geographical distribution of *S. mansoni* and *S. haematobium* infection and morbidity, and that of single and mixed infections were studied (Chapter 4), and immunological patterns of *S. mansoni* and *S. haematobium* infection were compared in order to gain more insight into the mechanisms that may underlie epidemiological observations (Chapter 5 and 6).

In this chapter, we will integrate the findings of the different chapters and take them one step further. We present a comprehensive analysis of factors related to mixed infections and morbidity, and provide an in-depth analysis of age-related infection patterns and immune responses in (co-)endemic situations. The relevance of the findings in this thesis for schistosomiasis control and elimination will be highlighted, and we will conclude with directions for further research.

Mixed infections and morbidity

In Africa, many people are at risk of infection with both *S. mansoni* and *S. haematobium* (Gryseels et al., 2006). It is therefore important to understand, whether and how such mixed *Schistosoma* infections may alter the risk of developing chronic morbidity. This is a challenging research question because chronic morbidity due to schistosome infection generally develops over the course of many years, and it is difficult to follow a population for such a long period of time, if only for ethical reasons. The transversal, multidisciplinary studies in this thesis nonetheless provide some important insights into the possible determinants of morbidity in populations co-endemic for *S. mansoni* and *S. haematobium*. Here, we will further discuss and link the possible effects of

heterologous worm pairing, infection intensity, and egg-induced immune responses on host morbidity in mixed infections.

Heterologous worm pairing

We found that people with the heaviest infections often had ectopic *S. mansoni* eggs in urine. A 'spilling over' of the surplus of *S. mansoni* worms and/or eggs towards the urinary bladder may have contributed to this phenomenon. However, ectopic *S. mansoni* eggs were also observed in a minority of people without *S. mansoni* eggs in feces, and in whom 'spilling over' was therefore less likely. In Chapter 3, this specific subgroup of people with both *S. haematobium* and ectopic *S. mansoni* eggs in urine, but without *S. mansoni* eggs in feces (n=6), tended to be less at risk of *S. haematobium*-specific urinary tract morbidity than those with other *S. haematobium* infections. It was argued that this may have been due to direct interactions between the two *Schistosoma* species. *Schistosoma haematobium* males probably outnumber and/or outcompete *S. mansoni* males, and are able to pair with heterologous *S. mansoni* females. In such heterologous male-female pairs, the *S. haematobium* male determines the oviposition site (region of the urinary tract) and the *S. mansoni* female the egg morphology (lateral spine). Perhaps, less (pathogenic) eggs are produced upon heterologous pairing, or these eggs may be more likely to deviate to other sites. While this thesis was in preparation, a study in Kenyan school children observed very similar patterns, which may also be explained by heterologous worm pairing. Although that study did not take ectopic egg elimination into account, children with mixed infections were less at risk for urinary tract morbidity than those with single *S. haematobium* infections (Gouvras et al., 2013). Moreover, Huyse et al. (2013) discovered *S. mansoni* x *S. haematobium* hybrid eggs in children living in the same area as our study population. Like ectopic eggs, these hybrids were observed more frequently in urine than in feces. It is therefore tempting to speculate that the ectopic *S. mansoni* eggs observed in this thesis may have included genetic hybrids.

Heterologous worm pairing in the intestinal tract on the other hand, is less likely to occur as *S. haematobium* males have been shown to be competitively stronger than *S. mansoni* males (Webster et al., 1999; Cunin et al., 2003). Consistent with this, ectopic *S. haematobium* egg elimination in feces was rare in the present study (Chapter 2).

Infection intensity

In Chapter 2, we found higher infection intensities of *S. mansoni* as well as *S. haematobium* in mixed as opposed to single infections. As far as we are aware, this is the only study that has been performed on such a small geographical scale. Studies that were performed on a larger geographical scale reported inconsistent results, which could have been due to local variations in transmission dynamics of the two

species. Robert et al. (1989) for example, reported comparable infection intensities in mixed and single *S. mansoni* infections but elevated intensities in mixed as compared with single *S. haematobium* infections in one population living in different communities on the banks of the Lagdo Lake in northern Cameroon. Preliminary results from our group in another village - Pakh - in the Lac de Guiers area, indicated significantly elevated levels of *S. haematobium* but reduced levels of *S. mansoni* in mixed as compared with single infections. As it is unknown which factors underlie these differences, it remains unclear to which extent the association between mixed infection and infection intensity observed in this thesis can be extrapolated to other populations.

The association between *S. haematobium* infection intensity and *S. haematobium*-specific urinary tract morbidity appeared stronger than that between *S. mansoni* infection intensity and *S. mansoni*-specific hepatic fibrosis. In Chapter 3, a direct association was found between active infection and urinary tract morbidity, but not hepatic fibrosis. Similar patterns were found in a *S. mansoni* and *S. haematobium* co-endemic focus in Egypt (Farooq et al., 1966b). The micro-geographical patterns in Chapter 4 strongly suggested an association between the level of *S. mansoni* infection intensity earlier in life and the severity of hepatic fibrosis later on in adult life. The difference in the delay between infection and the onset of morbidity may be due to interspecies differences in egg pathogenicity (see 'Egg-induced immune responses' below), or to differences between the anatomical context of the liver and urinary tract.

The same micro-geographical analyses showed that severe hepatic fibrosis clustered close to the central water contact site, in the same area where children showed the heaviest infections of the community. Preliminary analyses did not indicate any evidence of spatial heterogeneity in egg-induced cytokine responses, nor in any of the other adaptive or innate cytokine responses measured (data not shown). This strongly suggests that, on the community level, (cumulative) exposure to *Schistosoma* eggs may be more important in determining the risk of developing morbidity than individual host immune responses. Yet, it remains to be investigated whether there may be more subtle effects of cytokine responses on host morbidity, or whether immune parameters other than the cytokine responses measured here may provide a better indicator for who is likely to develop morbidity and who not, and how this relates to mixed infections.

Egg-induced immune responses

As explained above, people who excreted both *S. haematobium* and ectopic *S. mansoni* eggs in urine, but no *S. mansoni* eggs in feces tended to be less at risk of bladder morbidity. Due to an insufficient number of people with this type of infection in the immunological study, we could not draw any conclusions on the immune responses in

these subjects. Hence, it remains to be determined whether any protective effect of heterologous worm pairing might be immune-mediated. In general, people with mixed infections produced lower levels of cytokines than those with single infections, but with the approach used it was not possible to determine whether this was due to co-infection per se or due to elevated infection intensity, or to other potential confounders. The similarity of *S. mansoni*- and *S. haematobium*-induced cytokine profiles rather suggested that co-infection with *S. mansoni* is unlikely to have skewed immune response against *S. haematobium* eggs towards a potentially more or less pathogenic phenotype, and vice versa.

Nonetheless, it was shown that the magnitude of these responses were higher upon stimulation with *S. haematobium* as compared to *S. mansoni* (egg) antigens, suggesting that *S. haematobium* might induce stronger and therefore potentially more pathogenic immune responses than *S. mansoni*. Indeed, *S. haematobium*-specific morbidity was more common than *S. mansoni*-specific morbidity (Chapter 3), and age distributions indicated that the delay of onset of morbidity may be shorter for *S. haematobium* than for *S. mansoni* (Figure 3.1). Moreover, people without putative hybrid *S. mansoni* x *S. haematobium* eggs were more at risk for *S. haematobium*-specific morbidity than those with these ectopic *S. mansoni*(-like) eggs (Table 3.6).

As discussed in Chapter 5, this hypothesis is in line with findings of Van Remoortere et al. (2001). These authors showed that *S. mansoni* induces mainly IgM – which is thought to inhibit protective host immune responses (Butterworth et al., 1987) – while *S. haematobium* induces both IgM and IgG antibodies against shared carbohydrate

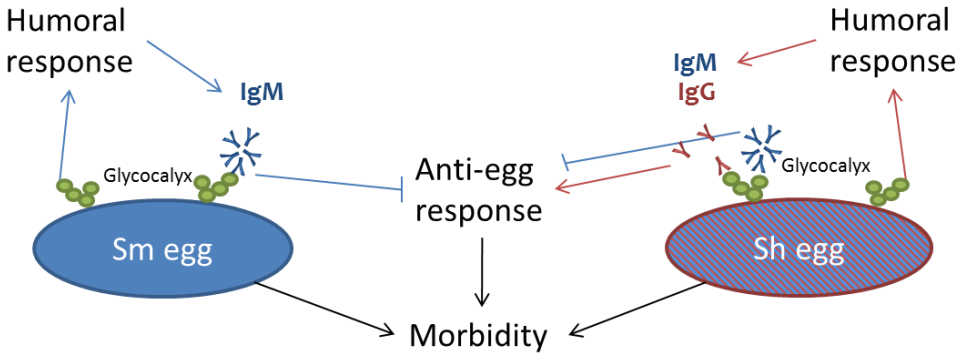


Figure 7.1. Hypothesized effects of *S. mansoni* and *S. haematobium* infection on host morbidity.

Van Remoortere et al. (2001) demonstrated that the antibodies to specific glycan epitopes from *S. mansoni* life stages induce more IgM and less IgG than those from *S. haematobium* life stages. The inhibitory effect of IgM might reduce anti-egg responses of the host immune system and thereby the development of host morbidity in *S. mansoni* co-infections, while increased IgG levels in *S. haematobium* co-infections may have the opposite effect.

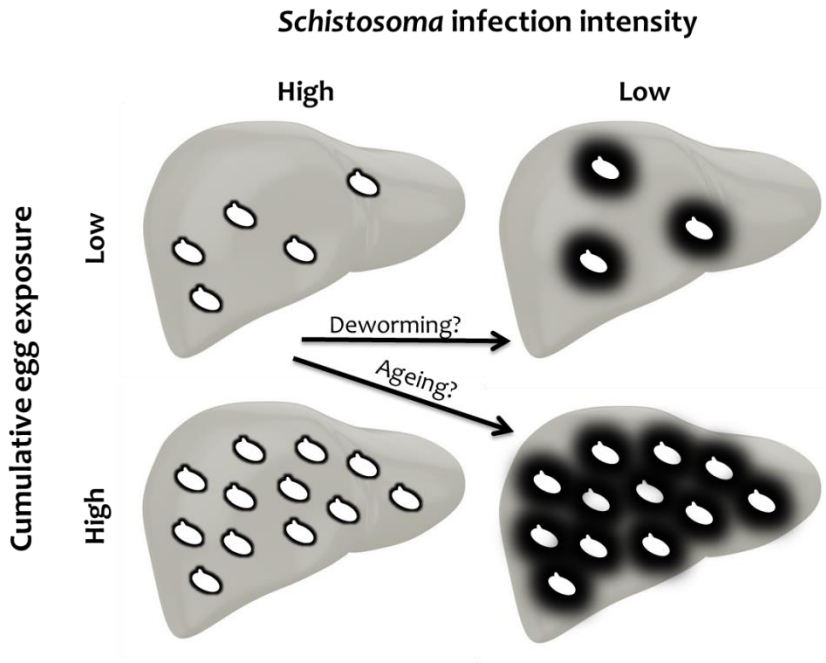


Figure 7.2. *Schistosoma* infection intensity, cumulative egg exposure, egg-induced immune responses, and morbidity in schistosomiasis.

Chronic hepatic schistosomiasis is used here as an example to demonstrate the relationship between *Schistosoma* infection intensity, cumulative exposure to *Schistosoma* eggs, and local immune responses to tissue-trapped eggs which may altogether lead to morbidity. The number of eggs in the liver indicates the number of trapped eggs or lesions with immuno-reactive remnants. Sizes of the black areas surrounding the eggs indicate the relative strength of the immune reactions in individual tissue lesions. We hypothesize that in the endemic setting, as people age, they will move from low to high cumulative egg exposure and from high to low infection intensities, and thus have enhanced immune responses against individual eggs (bottom right). After deworming, *Schistosoma* infection intensities decrease and immune responses against tissue-trapped eggs may become more pathogenic.

epitopes. IgM responses against trapped eggs might protect *S. mansoni*-infected individuals from developing morbidity, while increased IgG levels might predispose *S. haematobium*-infected individuals to more severe morbidity. Interestingly, IgG levels against the same carbohydrate epitopes were shown to be even higher in *S. japonicum* infections (Van Remoortere et al., 2001), which are associated with a more rapid onset of chronic morbidity and with more severe morbidity than *S. haematobium* (Jordan et al., 1993). We therefore cautiously hypothesize that *S. mansoni*-induced glycan-specific IgM may inhibit anti-egg responses and contribute to reduced levels of urinary tract morbidity in mixed as compared to single *S. haematobium* infections. Conversely, *S.*

haematobium-induced IgG might be hypothesized to enhance anti-egg responses and lead to increased levels of hepatic morbidity in mixed as compared to single *S. mansoni* infection, as illustrated in Figure 7.1.

It should be taken into account that the relationship between active infection and morbidity may not be as straightforward as is generally assumed. Chapter 5 suggested that heavily infected individuals have low immune responses to the eggs trapped in host tissues, while those with lighter infections have stronger immune responses to individual eggs. People with light infections are generally older, and are likely to have accumulated more *Schistosoma* eggs than younger individuals with heavy infections. This may imply that the pathogenicity of individual, trapped eggs might increase as the host ages. In other words, high cumulative exposure and reduced levels of infection might have synergistic effects on the development of chronic morbidity in schistosomiasis in older age. As explained in Figure 7.2, this may also imply that (temporary) worm elimination upon praziquantel treatment may lead to exacerbation of egg-induced morbidity.

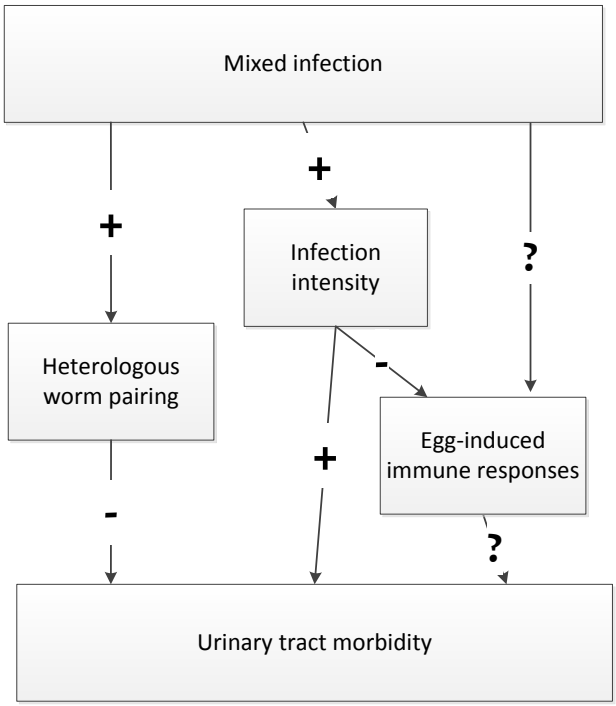


Figure 7.3. Overview of the factors that were studied and the relationships that were found with mixed infections and *S. haematobium*-specific urinary tract morbidity.

Net effect on morbidity

Figure 7.3 summarizes how heterologous worm pairing, infection intensity, and egg-induced immune responses may together (partly) determine the net effect of mixed infection on host morbidity, with *S. haematobium*-specific urinary tract morbidity as an example. Briefly, heterologous worm pairing might lead to less (pathogenic) *Schistosoma* eggs in the urinary tract, than pure *S. haematobium* infections. On the other hand, infection intensities were found to be higher in mixed *Schistosoma* infections, but it remains to be investigated whether a causal relationship exists between co-infection and infection intensity. Increased infection intensities lead to higher cumulative exposure of host tissues to *Schistosoma* eggs and thus to higher morbidity levels. We could not identify any interspecies differences in the induction of systemic cytokine profiles - i.e. regulatory, Th1, or Th2 phenotypes – but other immune parameters that were not measured (e.g. humoral immune responses) may still explain the observed differences in morbidity between single and mixed *Schistosoma* infections.

Age, *Schistosoma* infection and Th1 immunity

In Chapter 5, multiple cytokine responses to each of four *Schistosoma* antigens tested were visualized using nonmetric multidimensional scaling (nMDS). This resulted in four cytokine profiles. Associations between these cytokine profiles and *Schistosoma* infection were subsequently investigated. We found that both *S. mansoni* and *S. haematobium* infection were positively associated with antigen-induced Th2 and negatively with inflammatory/Th1 cytokines. This corresponds with results from earlier studies in either *S. mansoni* or *S. haematobium* mono-endemic areas (e.g. that of Joseph et al., 2004). These immuno-epidemiological patterns suggest that Th1 rather than Th2 responses may protect against infection, and confirms previous observations in mouse models (Wilson and Coulson, 2009). Moreover, worm antigen-induced Th1 responses consistently showed a linear increase with age (data not shown), suggesting that such protective immunity may be acquired with age - building up over time through cumulative exposure to schistosomes.

Besides *Schistosoma* antigen-induced cytokine profiles, we also studied cytokine profiles that are not specific for schistosomes, i.e. those induced by the T-cell mitogen phyto-hemagglutinin (PHA). Age as well as infection trends were very similar to those induced by *Schistosoma* worm antigen-induced cytokine profiles. nMDS furthermore showed that all PHA-induced cytokine responses were positively associated with *Schistosoma* worm antigen-induced Th1 cytokines ($p<0.01$; data not shown).

To investigate whether age may have confounded the association between cytokine profiles and infection intensity and/or vice versa, we used redundancy analysis (RDA) (Borcard et al., 2011). This is a statistical technique that is commonly used in

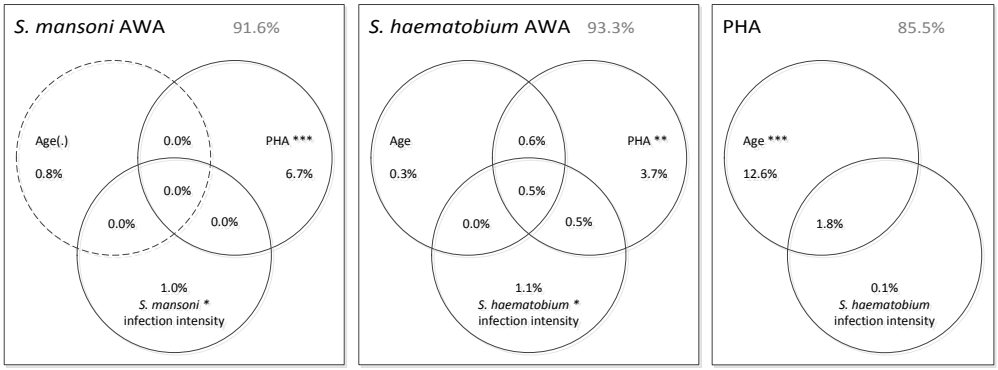


Figure 7.4. Variation partitioning by multivariable redundancy analysis: worm and mitogen-induced cytokine profiles, age and infection.

Venn diagrams indicate the percentages variance in antigen-induced cytokine profiles that could be explained by different explanatory variables (represented by circles) in multivariable RDA models and the percentages of variance that remained unexplained (outside circles) (n=199). Prior to RDA ('rda' function of Vegan package in R), net cytokine responses were log-transformed and standardized as for nMDS (see Chapter 5). Explanatory variables (age, *S. mansoni* and *S. haematobium* infection intensity, and for *Schistosoma* adult worm antigen (AWA)-induced cytokine profiles: PHA-induced cytokine profiles) were included in RDA models if they were significantly associated with respective 3D nMDS cytokine profiles. Except for the association between age and *S. mansoni* AWA-induced profiles (dashed circle), all associations were confirmed in simple RDA (continuous circles). The diagrams show that the association between infection intensity and AWA-induced cytokine responses remained in multivariable RDA. Also, the association between PHA- and AWA-induced cytokine profiles remained statistically significant in multivariable RDA. On the other hand, PHA responses were only associated with age - not with *Schistosoma* infection - and the significant effect of age on AWA-induced responses, was lost when PHA-responses were taken into account in the multivariable model. Asterisks indicate significance levels of the independent effects in full RDA models: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

ecology – but to our knowledge, never in biomedical sciences – and that combines linear regression with principal component analysis. Also, RDA was used to investigate the effect of unspecific, PHA-induced cytokine response profiles on *Schistosoma* worm-specific responses.

RDA showed a very specific and independent immunoregulatory/Th2-skewing effect of infection on *Schistosoma* worm-induced cytokine profiles. In addition, RDA indicated an infection-independent effect of age on general (i.e. nonspecific) adaptive immune responses as measured by PHA-induced cytokine profiles (Figure 7.4). Indeed, ageing itself is associated with dramatic changes in T cell function (Gardner and Murasko,

2002), and a Ghanaian study observed a similar age-associated increase in innate Th1 responses (May et al., 2009). The fact that, under adverse living conditions, subjects with a pro-inflammatory genetic predisposition are more likely to survive up to old age than those without might contribute to these observations (Van Bodegom et al., 2007; Kuningas et al., 2009). However, age-related increases in inflammation, termed 'inflamm-ageing', have also been described in more affluent populations (e.g. Franceschi et al., 2000).

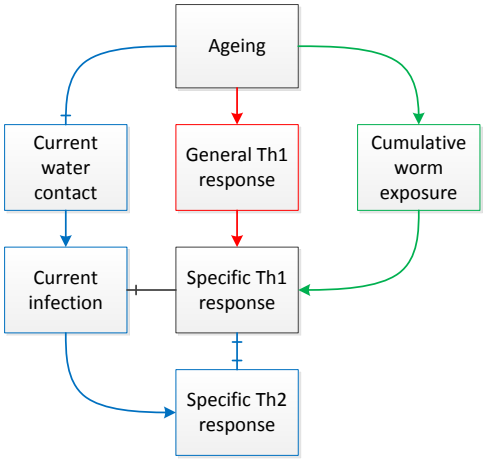


Figure 7.5. Conceptual framework of the hypothesized triangular relationship between age, Th1 responses, and *Schistosoma* infection.

Age-related infection and immunological patterns, as well as observations in Chapter 5 suggested that worm-specific Th1 responses might play a central role in immunological protection against infection. Arrowheads indicate positive and cross lines negative associations.

Disentangling the triangle

As illustrated in Figure 7.5, we hypothesize three different effects of ageing on *Schistosoma* infection and *Schistosoma*–specific Th1 immune responses. These may underlie *Schistosoma* infection-age infection curves in endemic areas:

- Due to differences in water contact behavior, adults are assumed to be less exposed to *Schistosoma* infection than children. This, or other factors that were not measured (e.g. age-related hormonal, metabolic, or dermatological changes) would lead to reduced infection levels across age groups, and the absence of worms would give rise to a relative decrease in Th2 and increase in Th1 responses (blue chain of events).
- In an endemic setting, exposure to *Schistosoma* antigens builds up as people age. This may give rise to a gradual increase in protective immunity and our observations indicate that such adaptive responses are most likely of the Th1 phenotype. These responses would in turn lead to a reduction of infection with age (green chain).

- Factors inherent to ageing itself may lead to a progressive increase in general pro-inflammatory/Th1 responses, or there may be selective survival of people with a genetic predisposition to produce pro-inflammatory/Th1 responses. Consequently, worm-specific immune responses would be skewed towards the Th1 phenotype in older individuals (red chain). If such Th1-type responses are indeed protective, this may lead to reduction of infection levels over age.

Relevance for schistosomiasis control strategies

A thorough knowledge of transmission of infection and disease etiology is key for successful schistosomiasis control. This thesis described the epidemiological patterns of *S. mansoni* and *S. haematobium* (co-)infection and disease at the smallest scale, i.e. at the micro-geographical level where disease transmission actually occurs. Strong spatial clustering at this level once more confirmed that such a fine geospatial resolution is essential to understand this complex disease and hence to develop sustainable control and elimination strategies. Based on our micro-epidemiological approach, we identified some opportunities and recommendations that should be taken into account to improve current strategies.

Mass drug administration

Currently, control strategies are based on periodic mass drug administration (MDA) with praziquantel. MDA aims to reduce infection levels early in life with the aim of reducing the development of chronic morbidity later in life. As explained above, our cross-sectional findings indeed suggest that cumulative exposure to infection is the main driver of severe morbidity (see 'Mixed infections and morbidity: Infection intensity'), and as such, support this basis.

The WHO recommends national control programs for schistosomiasis to target (mainly) school-age children and other high-risk groups (Table 7.1). Observations from this thesis suggest that optimizing current treatment allocation strategies may improve the effectiveness of MDA. Recently, the WHO has recognized that preschool-age children may be at a similar risk of schistosome infection and morbidity as their school-age siblings, and that they should be targeted as well (WHO, 2013). However, our results indicate that not only preschool-age but also children older than 14 years and young adults continue to be infected. For *S. mansoni*, infection prevalences and intensities only peaked after school-age, i.e. in 15-19-year olds (Chapter 2). These older age groups are thus likely to contaminate the environment, and to develop morbidity over time. Instead of school-(age-)based MDA, we thus recommend community-wide MDA, not only in high-risk areas, but also in areas that are at moderate and low risk (at least for *S. mansoni*).

Table 7.1. Treatment strategy for schistosomiasis recommended by the WHO (2013).

Category	Baseline prevalence among school-age children	Action to be taken	
High-risk community	≥50% by parasitological methods [a] (*S. mansoni* and *S. haematobium*) or ≥30% by questionnaire for history of hematuria	Treat all school-age children (enrolled and not enrolled) once a year	Also treat adults considered to be at risk [b] (from special groups to entire communities living in endemic areas)
Moderate-risk community	10-50% by parasitological methods (*S. mansoni* and *S. haematobium*) or <30% by questionnaire for history of hematuria	Treat all school-age children (enrolled and not enrolled) once every 2 years	Also treat adults considered to be at risk [b] (special groups only)
Low-risk community	<10% by parasitological methods (*S. mansoni* and *S. haematobium*)	Treat all school-age children (enrolled and not enrolled) twice during their primary schooling age (e.g. once on entry and once on exit)	Praziquantel should be available in dispensaries and clinics for treatment of suspected cases

[a] For *S. haematobium*, detection of hematuria by dipstick tests gives results equivalent to those determined by urine filtration.

[b] From special groups (pregnant and lactating women, groups with occupation involving contact with infested water such as fishermen, farmers, irrigation workers or women in their domestic tasks) to entire communities living in endemic areas

Current MDA allocation strategies do not reflect the spatial epidemiology of schistosomiasis infection and morbidity. In Chapter 4, we observed a highly focal distribution of both infection and associated disease on the micro-geographical level. We found that people living within 100m of the major water contact site had a more than six-fold higher risk of developing severe hepatic fibrosis than those who lived farther away. Nevertheless, the WHO recommends collecting baseline infection data based on parasitological surveys in sentinel sites that are assumed to be representative for a large so-called 'homogeneous ecological zone' (WHO, 2011). The recommended treatment strategy for this entire zone, i.e. 200,000-300,000 targeted children subsequently depends on the observed prevalence in 50 children from one randomly selected sentinel school. Obviously, such a uniform approach is prone to fail in reaching (all) those individuals who are most in need of treatment, and will result in unnecessary treatment of uninfected people. A substantial increase in the number of sentinel sites may help to overcome this problem, but would be incompatible with the logistical and economic advantages underlying current MDA-based control strategies (Molyneux et al., 2005). Novel ways are therefore urgently needed to enable a more targeted allocation of control interventions in *Schistosoma*-endemic areas. Based on the results of this thesis, we propose to use environmental factors such as the presence of infested water bodies and the geographical distance to these *Schistosoma* 'epicenters'

for treatment allocation, rather than parasitological data from randomly selected schoolchildren.

Other findings in this thesis suggested that removal of the causative agent might not simply lead to a proportional reduction of morbidity, especially in co-endemic areas. An unexpected effect of mixed *S. mansoni* and *S. haematobium* infections on *S. haematobium*-specific urinary tract morbidity was observed. The presence of *S. mansoni* appeared to protect against this form of morbidity in some cases. Also, immune responses in individuals with low levels of infection appeared more pathogenic than in individuals with higher infection levels. In addition, other studies in *S. mansoni* and *S. haematobium* co-endemic areas have shown an increase rather than a decrease of *S. mansoni* infection after deworming (Omer and Teesdale, 1978; De Clercq et al., 1999; Ernould et al., 1999a; Koukounari et al., 2010; Webster et al., 2013; Gouvras et al., 2013). Hence, periodic MDA and subsequent changes in immunological and infection equilibria may (in some cases) exacerbate the development of chronic morbidity instead of reducing it (Daffalla and Fenwick, 1982).

Elimination

Gaining and sustaining control of schistosomiasis and possibly achieving local elimination are the year 2020 targets set by the WHO (Knopp et al., 2013). When moving from control to elimination, active surveillance and detection of increasingly low-transmission areas is needed, but proven very difficult. Today, it is increasingly being recognized that malaria transmission becomes more and more focal as malaria prevalences are reduced (Cotter et al., 2013). As similar phenomena may occur in a post-control setting for schistosomiasis, strategies need to be developed with a sufficiently high geospatial resolution to detect even the smallest schistosomiasis hotspots. Because it will not be cost-effective to routinely collect parasitological data from human populations in a post-control setting, new ways of detecting schistosomiasis should be sought, e.g. through environmental sampling (see also 'Mass drug administration'). In addition, it is expected that MDA alone cannot break the *Schistosoma* life cycle and that complementary interventions will have to be put in place for schistosomiasis elimination, such as the provision of clean water and improved sanitation, snail control, and behaviour change (Sturrock, 1989; King, 2009; Gray et al., 2010; Rollinson et al., 2013; Freeman et al., 2013). Multidisciplinary studies on the micro-geographical level such as the ones described in this thesis will help to get much needed insights into local transmission dynamics of *S. mansoni* and *S. haematobium* and hence to move from control towards elimination.

Conclusions and directions for further research

More studies are needed to investigate to which extent the observations in this thesis can be extrapolated over time and to other communities. Future studies may build on the findings of this thesis, on the hypotheses that were generated, and on the novel methodologies used. Here, we will discuss some main directions for further schistosomiasis research.

Health impact of mixed *Schistosoma* infections

Helminth co-infections and mixed *Schistosoma* infections are the rule rather than the exception. Nonetheless, co-infection and mixed infection remain an understudied element of human medicine. This thesis provides a significant addition to the limited number of studies on mixed *Schistosoma* infections in humans. We demonstrated that on the small scale, mixed *Schistosoma* infections are significantly associated with increased infection intensities. However, different relationships between mixed infection and infection intensity have been found in other study populations. It is important to understand which context-specific factors impact on the association between mixed infection and infection intensity, and which causal mechanisms underlie this association. For example, it remains to be investigated whether previous and/or concomitant infection with *S. mansoni* might alter host resistance to infection with *S. haematobium*, or whether it may alter *S. haematobium* egg production (or vice versa). Secondly, we found that mixed infections affect the host's health differently than single infections (regardless of infection intensity). We hypothesized that this may be due to immunological differences between *S. mansoni* and *S. haematobium*, and also to heterologous worm pairing. Further studies are necessary to determine whether *S. haematobium* eggs are more immunogenic than those of *S. mansoni*, and to investigate the different features of anti-egg immune responses into more detail. Also, parasitological factors such as infection intensity, ectopic egg elimination, heterologous worm pairing, and hybridization between species should be studied in more detail to answer questions such as: What is the genetic nature of ectopically eliminated *Schistosoma* eggs? What are the morphological and immunological characteristics of *S. mansoni* x *S. haematobium* hybrid eggs? At the same time, confounding factors such as age and micro-geographical processes need to be taken into account. Only such a multidisciplinary approach can unravel the very complex mechanisms that determine the eventual effect of *Schistosoma* co-endemicity and mixed infections on the host's health.

Praziquantel treatment

Mixed *Schistosoma* infections may not only impact on the host's health, but also on the effect of praziquantel treatment. Unfortunately however, little attention is currently

paid to the possibility that concomitant infections in co-endemic populations may reduce drug effectiveness and increase the risk of side effects (Buck et al., 1978). In addition, chemotherapeutical removal of one or more pathogens may clear the way for invasion of other pathogens that may lead to worse health outcomes (Daffalla and Fenwick, 1982). One major question that was not addressed in this thesis was how praziquantel treatment impacts on *Schistosoma* co-infection and morbidity levels. Nonetheless, some of our observations suggested that it might indeed have adverse health effects (e.g. *S. mansoni* infections appeared to protect against *S. haematobium*-specific bladder morbidity, so elimination of *S. mansoni* might enhance this bladder morbidity). Given that praziquantel treatment may have unwanted effects in polyparasitism, and the recent scale-up of MDA programs, closer monitoring and investigations into both beneficial and adverse health effects of MDA with praziquantel are urgently warranted, especially in co-endemic areas (Humphries et al., 2012). Also, more evidence is needed from comprehensive, longitudinal studies to assess the effectiveness of MDA in terms of morbidity reduction, on the short as well as on the long term, and to improve WHO guidelines.

Focal geographic distribution

This thesis contains one of the few studies on the micro-geographical distribution of *Schistosoma* infection and the first on the micro-geographical distribution of associated morbidity. Significant spatial clustering of *Schistosoma* infection was observed even within one community, and *S. mansoni* and *S. haematobium* infection hotspots were found in different sections of the community. Future studies should investigate the drivers of this spatial clustering. It would be important to investigate whether similar divergent distributions of *S. mansoni* and *S. haematobium* also occur in other communities, and if so: what causes this divergence?

In Chapter 2, we found that *S. mansoni* and *S. haematobium* clustered together in the same individuals, while in Chapter 4, geospatial analyses revealed that the two infections clustered in separate parts of the community. These contrasting patterns on the individual and micro-geographical level seem to point to human factors (e.g. individual water contact behavior and/or immunological factors) as an explanation for the co-occurrence of *S. mansoni* and *S. haematobium* on the individual level, rather than micro-geographical factors such as co-exposure to both species in the same water contact site.

Not only *Schistosoma* infection but also associated chronic morbidity showed a very focal geographical distribution on the micro-geographical level, and people living within the close vicinity of the major water contact site of the community were disproportionally affected. This suggests that cumulative exposure to schistosomes may be the main driver of chronic morbidity on the community level, and warrants

further research into the role of this factor in the etiology of chronic *Schistosoma*-specific disease. In order to estimate the effect of cumulative exposure relative to other etiologic factors such as co-infection, host immunology and host genetics, a multidisciplinary approach will be key in unravelling the complexities of disease etiology.

Parasite immunology and vaccine development

Immunological mechanisms undoubtedly underlie epidemiological patterns of infection and disease. Yet, it remains to be investigated to which extent they explain the notoriously heterogenic distribution of schistosomiasis. Further studies should also assess the role of innate immunity in schistosomiasis. Also, the role of host immunological processes in polyparasitism is still unclear. Moreover, longstanding questions remain: To which extent do we develop protective immunity against schistosomes? What is the immunological phenotype of a protective response? Do acquired immune responses drive the typically convex age-infection curve? Further studies are needed to confirm the age-related inflammatory/Th1 shift observed in this thesis, to elucidate the exact biological mechanisms that may drive this shift, and to assess whether it may confer immunological protection against schistosomes in older age. The eventual success of the candidate schistosomiasis vaccines that are currently being tested (Kupferschmidt, 2013), critically depends on the true balance between the different possible underlying mechanisms of the age-infection curve, and whether or not adaptive immune responses are able to reduce levels of infection. Hence, such studies will give crucial insights for vaccine development.

Value of multidisciplinary research

Adding a geospatial dimension to classical immuno-epidemiological investigations proved particularly valuable to increase our understanding of schistosomiasis on the small scale. We demonstrated for example, how spatial analyses may be used as a 'workaround' to study longitudinal relationships, in a cross-sectional study (Chapter 4). This cannot be achieved through classical epidemiological methods. Also, the application of ecological techniques has proven very useful in studying the complexity of immunological responses (Chapter 5). In this way, this thesis provides a novel, more holistic approach to understanding schistosomiasis.

Taking into account the occurrence of co-infections, and a wider adoption of multidisciplinary research is likely to lead to more novel insights and speed up the snail pace at which our knowledge of schistosomiasis is advancing today. Such knowledge is key to rationalizing and optimizing current schistosomiasis control (and possibly future elimination) strategies, particularly in co-endemic areas.

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Short summary

The aim of this thesis was to get a detailed insight into the epidemiological patterns of *Schistosoma* infection and associated morbidity on a micro-geographical scale in *Schistosoma mansoni* and *S. haematobium* co-endemic areas. A *S. mansoni and S. haematobium* co-endemic focus in the north of Senegal was selected as the primary study site. The small scale of this study allowed a detailed, multidisciplinary analysis of complex infection and morbidity patterns. Moreover, it enabled us to take the well-known focality of schistosomiasis into account (Chapter 1).

We observed prevalences of 61% and 50% for *S. mansoni* and *S. haematobium* infection, respectively. Most of these infections were mixed *S. mansoni* and *S. haematobium* infections (53%). In comparison to single infections, mixed infections were of higher intensity. Eight percent of the infected people also showed ectopic excretion of *Schistosoma* eggs (mainly *S. mansoni* in urine; Chapter 2).

While elevated *S. haematobium* infection intensities predisposed for *S. haematobium*-specific urinary tract morbidity, the presence of *S. mansoni* in co-infections tended to protect against this. The latter effect was most pronounced in people with ectopic *S. mansoni* egg excretion. On the other hand, no association was found between *S. mansoni*-specific hepatic fibrosis and *Schistosoma* infection. This is likely due to a slower disease onset in *S. mansoni* as compared to *S. haematobium* infection. Indeed, the delay between the age peak for *S. mansoni* infection and morbidity was >10 years, while such a delay could not be observed for *S. haematobium* (Chapter 3).

We were the first to look into the micro-geographical distribution of chronic *Schistosoma* morbidity. In contrast to classical epidemiological analyses (see Chapter 3), spatial analysis did reveal a clear association between *S. mansoni* infection and morbidity. It was shown that adults living adjacent to the main *Schistosoma* transmission site were more than six times more likely to develop severe *S. mansoni*-specific hepatic fibrosis than their counterparts living farther away. Children living in the same spot were also more at risk of heavy *S. mansoni* infection, indicating that cumulative exposure to *Schistosoma* eggs is likely to be the main driver of chronic morbidity in schistosomiasis (Chapter 4).

We explored immune responses in the two *Schistosoma* infections in this population by means of whole blood cytokine responses. Cytokine responses to adult worm antigens were used as a proxy for host responses to living adult worms, and thus infection. Cytokine responses to soluble *Schistosoma* egg antigens were used as a proxy for the egg-induced responses that may have contributed to the observed morbidity patterns.

It was found that *S. haematobium* antigens induce higher cytokine levels than those of *S. mansoni*. This supports the idea that the first species may be more immunogenic. This hypothesis corresponds with our epidemiological findings: 1) in line with previous studies, we found the *S. haematobium* age curve to decline more steeply after adolescence than that of *S. mansoni*; and 2) we observed more *S. haematobium*- than *S. mansoni*-specific morbidity (83% versus 27%). Our investigations did not reveal any interspecies differences in the immunological phenotypes that were induced. Both *Schistosoma* species were associated with a modified Th2 response in a dose-dependent manner. Such a response is characteristic for *Schistosoma* infection (Chapter 5).

Subsequently, the innate immune responses that generally precede and shape these adaptive cytokine responses were investigated. This study was performed in children from a *S. haematobium* mono-endemic area in Lambaréné, Gabon. While children infected with *S. haematobium* produced more of the anti-inflammatory cytokine IL-10 than uninfected children in response to adaptive stimuli (egg and worm antigens), their innate immune response to TLR ligands was more pro-inflammatory (TNF-α) than that of uninfected children. Hence, schistosomal infection suppresses the host's adaptive immune system but does not appear to affect innate stimulation with single TLR ligands. This indicates that schistosomiasis does not induce a general suppression of the host immune system (Chapter 6).

This thesis provides a novel, holistic approach to understanding schistosomiasis. It has led to important new insights into demographic and geographic patterns of *S. mansoni* and *S. haematobium* on a micro scale, and provides new leads for further research on disease etiology and underlying mechanisms. Further studies are necessary to investigate to which extent these results can be extrapolated over time and to other populations. Such knowledge is key to rationalizing and optimizing current schistosomiasis control strategies, particularly in co-endemic areas. An improved understanding of this complex disease would furthermore greatly aid in developing successful elimination strategies in the future (Chapter 7).

Samenvatting

(Dutch summary)

Hoofdstuk één biedt achtergrondinformatie over schistosomiasis. Dit is een chronische parasitaire worminfectie die wereldwijd bij meer dan tweehonderd miljoen mensen voorkomt, vooral in Afrika ten zuiden van de Sahara. Volwassen mannetjes- en vrouwtjeswormen kunnen jarenlang in innige omhelzing in de menselijke bloedcirculatie voortleven. Hier produceren zij ontelbare *Schistosoma*-eitjes die door de gastheer worden uitgescheiden. Als deze eitjes in zoetwater terechtkomen, komen ze uit en kunnen de larven een bepaalde slakkensoort infecteren. Via deze tussengastheer vermenigvuldigt de parasiet zich, komt hij weer in het water terecht, en kan hij vervolgens de mensen via de huid penetreren en infecteren als zij in contact komen met het besmette water. In de menselijke bloedstroom begint de parasitaire levenscyclus dan weer van voor af aan. In endemische gebieden leidt een *Schistosoma*-infectie niet altijd tot direct zichtbare ziektesymptomen. Afhankelijk van factoren zoals het aantal herinfecties en de immuunstatus van de gastheer, kunnen er soms pas maanden tot jaren na een eerste infectie klinische symptomen optreden. Deze zijn het gevolg van de ophoping van (een deel van) de door de worm geproduceerde eitjes in de organen van de gastheer. De immuunreactie van de gastheer tegen deze eieren leidt op haar beurt tot een geleidelijke fibrotisering van het orgaanweefsel rondom de eitjes. In endemische gebieden ontstaat er zo sluipenderwijs het typische ziektebeeld van chronische schistosomiasis. Schistosomiasis kan door verschillende *Schistosoma*-soorten veroorzaakt worden. *Schistosoma mansoni* en *Schistosoma haematobium* zijn de meest voorkomende. Volwassen *S. mansoni*-wormen leven in de venen van het mesenterium waar hun eitjes deels via de stoelgang worden uitgescheiden en zich deels in de lever ophopen. Volwassen *S. haematobium*-wormen daarentegen, leven voornamelijk in de venen van de urinewegen, waar hun eitjes via de urine worden uitgescheiden, of zich ophopen in de aangrenzende organen, bijvoorbeeld in de wand van de urineblaas. In die delen van Afrika waar mensen van het besmette water afhankelijk zijn omdat er geen alternatieve waterbronnen zijn, en onder slechte hygiënische omstandigheden leven, komt schistosomiasis vaak voor. Door de geografische overlap van *S. mansoni* en *S. haematobium* is de kans groot dat mensen beide soorten dragen (coinfectie) in plaats van één van de twee (monoinfectie). Paradoxaal genoeg weten we maar heel weinig over dit soort coinfecties, en dit is een groot hiaat in onze kennis van schistosomiasis. Het voornaamste doel van dit proefschrift was dan ook om meer over deze *Schistosoma*-coinfecties te weten te

komen. Hiertoe voerden we een uitgebreide transversale studie uit in twee aangrenzende Wolofdorpen op een schiereiland in Lac de Guiers. Dit gebied ligt in het noorden van Senegal en is co-endemisch voor *S. mansoni* en *S. haematobium.*

Hoofdstuk twee beschrijft de epidemiologische infectiepatronen van de studiepopulatie in deze beide dorpen (n=857). *Schistosoma mansoni*- en *S. haematobium* infecties werden bepaald door middel van microscopische detectie van parasitaire eieren in de stoelgang en urine. Deze kwamen respectievelijk in 61% en 50% van de mensen voor. De meeste infecties waren coinfecties (53%). Deze infecties waren zwaarder (hogere eitellingen) dan de respectievelijke monoinfecties. Acht procent van de geïnfecteerde mensen had een afwijkend patroon van eiuitscheiding. Meestal werd bij hen *S. mansoni* in de urine teruggevonden in plaats van in de stoelgang, terwijl *S. haematobium* via de normale weg werd uitgescheiden. Dit kan deels verklaard worden door het feit dat vrouwelijke *S. mansoni*- en mannelijke *S. haematobium* wormen onderling kunnen paren ('heterologe paring'). Dit komt vaker voor dan andersom (*S. haematobium* vrouwtjes met *S. mansoni* mannetjes). In zulke gevallen bepaalt het vrouwtje het eifenotype en het mannetje de locatie van de eiproductie. Globaal gezien vertoonden *S. mansoni* en *S. haematobium* infecties eenzelfde demografische verdeling (volgens leeftijd, geslacht en dorp). Uit een meer gedetailleerde analyse bleek wel dat de leeftijdscurve voor *S. haematobium* infectie scherper daalde na de puberteit dan die van *S. mansoni*. Dit zou er misschien op kunnen wijzen dat *S. haematobium* een sterkere immuunrespons uitlokt dan *S. mansoni*.

Hoofdstuk drie gaat in op de epidemiologische patronen van *S. mansoni*-specifieke leverfibrose en *S. haematobium*-specifieke afwijkingen aan de urineblaas. Een deel van de studiepopulatie (n=403) werd onderzocht door middel van echografisch onderzoek met behulp van een gestandaardiseerd protocol. We observeerden meer blaas- dan leverafwijkingen (83% versus 27%). Waarschijnlijk heeft dit ermee te maken dat het langer duurt voordat *S. mansoni*-specifieke morbiditeit tot uiting komt. Dit zagen we ook terug in de infectie- en morbiditeitspatronen over de leeftijd. Deze toonden een verschil van >10 jaar tussen de leeftijd waarop *S. mansoni*-infectie en -morbiditeit pieken in deze populatie. Een dergelijk verschil kon voor *S. haematobium* niet waargenomen worden. Het zou er ook op kunnen wijzen dat *S. haematobium* immunogener is dan *S. mansoni*, zoals we al eerder suggereerden. Er kon geen statistische associatie aangetoond worden tussen *Schistosoma*-infectie en *S. mansoni*-specifieke leverfibrose op individueel niveau. Voor *S. haematobium*-specifieke morbiditeit werd een dergelijke associatie wel gevonden. Mensen met zwaardere *S. haematobium* infecties hadden een significant verhoogd risico op deze afwijkingen dan mensen met lichtere infecties. Daarbij leek de aanwezigheid van *S. mansoni* in coinfecties juist te beschermen tegen *S. haematobium*-specifieke blaasafwijkingen. Dit effect was het meest uitgesproken bij mensen met *S. mansoni*-eitjes in de urine in plaats van in de stoelgang, en dus mogelijk

het gevolg van de hierboven beschreven directe interacties tussen *S. mansoni* en *S. haematobium* in mensen met coinfecties in de vorm van heterologe wormparing.

In **hoofdstuk vier** brengen we infectie- en ziektepatronen nog beter in kaart door de bovenstaande klassieke epidemiologische analyses met spatiële clusterdetectie te combineren, in één van de twee bovengenoemde dorpen (n=599). We stelden vast dat *S. mansoni*- en *S. haematobium*-infecties in verschillende delen van het dorp clusterden. Dit kwam overeen met de watercontactpatronen die de mensen rapporteerden (door middel van vragenlijsten): mensen uit de *S. mansoni*-cluster hadden watercontact op andere plaatsen dan mensen uit de *S. haematobium*-cluster en vice versa. *Schistosoma haematobium*-specifieke blaasafwijkingen waren geografisch homogeen verdeeld over het dorp. Daar staat tegenover dat mensen die aangrenzend aan de drukstbezochte watercontactplaats woonden (<100 m), statistisch gezien zes keer meer risico hadden op een ernstiger vorm van *S. mansoni*-specifieke leverfibrose dan mensen die verder weg woonden. Dit suggereert dat de cumulatieve blootstelling aan *Schistosoma*-eitjes in de loop van het leven misschien wel de belangrijkste etiologische factor is in de ontwikkeling van chronische schistosomiasis.

Hoofdstuk vijf schetst vervolgens de immuunrespons van de menselijke gastheer tegen volwassen *S. mansoni*- en *S. haematobium*-wormen en tegen hun eieren, in relatie tot *S. mansoni*- en *S. haematobium*-infectie (n=200). Om de verworven immuunrespons in beeld te brengen, kwantificeerden we verschillende cytokine responsen (IL-10, IL-5, IFN-γ, TNF-α en IL-2) in bloedculturen 72 uur na stimulatie met worm- en eiantigenen. *Schistosoma haematobium*-antigenen bleken significant hogere cytokineresponsen op te wekken dan *S. mansoni*-antigenen. Dit suggereert dat de eerste soort meer immunogeen zou zijn, en is in overeenstemming met de bovengenoemde epidemiologische observaties: 1) dat de leeftijdscurve voor *S. haematobium*-infectie na de puberteit scherper daalde dan die van *S. mansoni*, en 2) dat *S. haematobium*-specifieke blaasafwijkingen vaker voorkwamen dan *S. mansoni*-specifieke leverafwijkingen. Er konden geen andere immunologische verschillen tussen *S. mansoni* en *S. haematobium* aangetoond worden. Antigenen van beide soorten induceerden eenzelfde soort immuunrespons, onafhankelijk van het feit of de mensen van wie de bloedmonsters afkomstig waren met *S. mansoni* of *S. haematobium* geïnfecteerd waren. Het is welbekend dat *Schistosoma*-infecties de verworven immuunrespons onderdrukken en in de richting van een Th2-respons sturen. Dit wordt een gemodificeerde Th2-respons genoemd, en dit type respons werd hier voor beide *Schistosoma*-soorten aangetoond. Hoewel deze studie nog geen afdoende bewijs geeft, leken er op het eerste gezicht dus geen aanwijzingen te zijn voor immunologische interacties tussen de twee *Schistosoma*-soorten, bijvoorbeeld in de vorm van immunologische kruisreacties.

Hoofdstuk zes gaat dieper in op de responsen van het aangeboren immuunsysteem die ten grondslag liggen aan de bovengenoemde *Schistosoma*-specifieke, verworven immuunresponsen. Hiertoe bestudeerden we een andere populatie, namelijk een groep van Gabonese schoolkinderen (n=30) uit een gebied waar alleen *S. haematobium* voorkomt. Omdat het onderzoek naar het aangeboren immuunsysteem nog erg pril is, richtten wij ons op de tot nu toe best beschreven responsen, die van de zogenaamde 'Toll-like' receptoren (TLR's). Tegen de verwachting in vonden we dat de TLR-responsen (TLR2 en TLR4) van de geïnfecteerde kinderen juist meer pro-inflammatoir waren dan die van de niet-geïnfecteerde kinderen, terwijl hun verworven responsen wel de kenmerkende immunosuppressie lieten zien. Dit toont aan dat er in ieder geval geen algemene onderdrukking van het immuunsysteem optreedt in schistosomiasis. Tegelijkertijd roept het de vraag op welk mechanisme dan wel bepalend is voor de gemodificeerde Th2-respons die zo kenmerkend is voor schistosomiasis.

Hoofdstuk zeven sluit af met een algemene discussie en een kritische evaluatie van alle bevindingen. We concluderen dat de multidisciplinaire aanpak in dit proefschrift tot nieuwe inzichten in schistosomiasis heeft geleid. Dit was met enkel conventionele methoden niet mogelijk geweest. Ons onderzoek bracht een aantal nieuwe aanknopingspunten voor verder onderzoek naar de etiologie van *Schistosoma*-specifieke morbiditeit in co-endemische gebieden aan het licht. Er zouden meer van dergelijke studies gedaan moeten worden om te bepalen in hoeverre de hier beschreven resultaten geëxtrapoleerd kunnen worden naar andere populaties. De huidige strategie om schistosomiasis te bestrijden is gebaseerd op massabehandeling met het medicijn praziquantel. Deze staat in schril contrast met de zeer focale verspreiding van schistosomiasis. Zelfs binnen één dorpje zijn er grote verschillen in infectie en ziekte waar te nemen. Verder geven onze onderzoeksresultaten aanleiding om te veronderstellen dat een dergelijke behandeling in co-endemische gebieden bepaalde infectie- en immunologische evenwichten kan verstoren, en daardoor negatieve gevolgen zou kunnen hebben op de volksgezondheid. Voor een effectievere ziektebestrijding zouden er nieuwe strategieën ontwikkeld moeten worden die wel rekening houden met de geografisch zeer focale ziektepatronen. Ook is meer inzicht nodig in de onderliggende mechanismen van coinfecties met verschillende *Schistosoma*-soorten, teneinde succesvolle strategieën te ontwikkelen om de ziekte op termijn uit te kunnen bannen.

Résumé

(French summary)

Le **premier chapitre** présente des informations générales sur la schistosomiase : Il s'agit d'une infection chronique causée par un ver parasite, qui atteint plus de deux-cent millions de personnes dans le monde, notamment en Afrique au sud du Sahara. Les vers adultes mâles et femelles peuvent vivre pendant des années en hôte de la circulation sanguine humaine. Au sein de cette dernière, les schistosomes produisent d'innombrables œufs excrétés par la suite par l'hôte. Quand ces œufs entrent dans l'eau douce, ils éclosent et les larves vont infecter une espèce particulière d'escargot. Dans cet hôte intermédiaire, les parasites se multiplient, ils reviennent dans l'eau, et peuvent alors infecter les humains à travers la peau quand ils entrent dans l'eau contaminée. Dans le sang humain, le cycle de vie du parasite commence alors à nouveau depuis le début. Dans les zones endémiques, les personnes sont en contact avec l'eau en permanence, et se réexposent donc à de nouvelles infections à *Schistosoma*, mais ces infections ne donnent habituellement pas de signes visibles immédiats de la maladie. Toutefois, une partie des œufs produits s'accumule avec le temps dans les organes de l'hôte. La réponse immunitaire de l'hôte contre ces œufs conduit à une fibrotisation progressive du tissu entourant les œufs. Dans les zones endémiques se crée ainsi insidieusement le tableau clinique typique de la schistosomiase chronique.

La schistosomiase peut être causée par différentes espèces de *Schistosoma*. *Schistosoma mansoni* et *S. haematobium* sont mondialement prépondérants. *Schistosoma mansoni* à l'état adulte, vit dans les veines du mésentère et ses œufs sont excrétés via les selles d'une part, et s'accumulent d'autre part dans le foie. Quant au *S. haematobium* adulte, il vit principalement dans les veines des voies urinaires, et ses œufs sont excrétés via l'urine ou s'accumulent dans les organes voisins, par exemple, dans la paroi de la vessie. Dans certaines régions d'Afrique - où les populations en contact avec l'eau contaminée, à défaut d'autres sources d'eau, vivent dans des conditions sanitaires déplorables - la schistosomiase est un fait divers. Aussi, en raison du chevauchement géographique de *S. mansoni* et *S. haematobium,* il arrive souvent qu'une personne soit infectée par les deux espèces de ver (coïnfection), à l'instar du cas classique de mono-infection. Paradoxalement, nous en savons très peu sur ce type de coïnfection, et c'est donc un grand vide dans notre connaissance de la schistosomiase. L'objectif principal de cette thèse était dès lors d'en savoir plus à ce sujet. À cette fin, nous avons mené une étude transversale étendue auprès de deux

villages Ouolofs voisins, sur une péninsule aux rives du Lac de Guiers. Le chevauchement de *S. mansoni* et de *S. haematobium* rend cette zone du nord du Sénégal co-endémique.

Le **chapitre deux** décrit les tendances épidémiologiques de l'infection auprès de la population de l'étude, notamment, dans les deux villages précités (n=857). Les infections par *S. mansoni* et *S. haematobium* ont été déterminées au moyen d'une détection au microscope des œufs du parasite dans les selles et les urines. Les taux d'infection s'élevaient respectivement à 61 et 50%. La plupart des infections étaient des coïnfections (53%). Le degré d'infection (nombre d'œufs) dans les cas de coïnfection s'avérait plus sévère que celui des mono-infections. De même, huit pourcent des personnes infectées présentaient un modèle atypique d'excrétion des œufs. En effet, le *S. mansoni* se retrouvait le plus souvent dans les urines plutôt que dans les selles, tandis que le *S. haematobium* transitait par la route normale, les urines. Ceci peut en partie s'expliquer par le fait que la femelle *S. mansoni* et le male *S. haematobium* parviennent à s'accoupler ('accouplement hétérologue'). Les cas d'accouplement inverses (*S. haematobium* femelle et *S. mansoni* mâle) sont moins fréquents. Dans de tels cas, c'est la femelle qui détermine le phénotype de l'œuf et le mâle l'emplacement d'œufs. Les infections *S. mansoni* et *S. haematobium* présentaient de prime abord une distribution démographique similaire (selon l'âge, sexe, et village). Cependant, vu de plus près, les cas d'infection à *S. haematobium* diminuaient de façon plus prononcée après la puberté que les cas d'infection à *S. mansoni*. Ce constat nous porte à croire que *S. haematobium* pourrait provoquer une réponse immunitaire plus forte que *S. mansoni*.

Le **chapitre trois** examine d'une part, les caractéristiques épidémiologiques spécifiques de la fibrose hépatique causée par *S. mansoni*, et d'autre part, les anomalies spécifiques de la vessie causée par *S. haematobium*. Une partie de la population de l'étude (n=403) a été examinée au moyen d'une échographie selon un protocole standardisé. Nous avons observé plus d'anomalies de la vessie que du foie (83% contre 27%). Ceci est probablement lié au fait que la morbidité propre à *S. mansoni* met plus de temps à se manifester. Les patrons d'infection et de morbidité en fonction de l'âge le suggéraient aussi: il se trouvait plus de 10 ans d'intervalle entre les pics d'infection et de morbidité de *S. mansoni*. Une telle différence n'a pas été observée pour *S. haematobium*. Ceci pourrait aussi signifier que *S. haematobium* est plus immunogène que *S. mansoni*, comme nous l'avons suggéré plus tôt. En effet, Il n'y avait pas d'association statistique entre l'infection à *Schistosoma* et la fibrose hépatique spécifique à *S. mansoni* au niveau individuel. Par contre, cette association a pu être établie dans la morbidité spécifique à *S. haematobium*. Le risque de ces anomalies était significativement élevé chez les personnes sévèrement infectées par *S. haematobium* par rapport à celles légèrement infectées. Ainsi, semblait la présence de *S. mansoni* dans la coïnfection, jouer un rôle dans la protection contre les anomalies de la vessie liées à *S. haematobium*. C'est chez

les personnes porteuses d'œufs de *S. mansoni* dans les urines plutôt que dans les selles que ce phénomène était le plus prononcé; ceci serait probablement une résultante de l'interaction directe décrite antérieurement entre *S. mansoni* et *S. haematobium* chez les personnes souffrant d'une coïnfection sous la forme d'un accouplement hétérologue de vers.

Dans le **chapitre quatre**, nous arrivons à mieux cartographier les patrons d'infection et de morbidité en combinant les analyses épidémiologiques classiques précitées avec la détection spatiale de clusters ou grappes, dans l'un des deux villages en question (n=599). Nous avons constaté que les infections à *S. mansoni* et *S. haematobium* se regroupaient dans différentes parties du village. Cela concordait avec les modèles de contact à l'eau rapportés par le biais de questionnaires: les personnes de la grappe *S. mansoni* ont été en contact avec l'eau dans des endroits autres que ceux de la grappe *S. haematobium* et vice versa. Géographiquement, les anomalies de la vessie spécifiques à *S. haematobium* étaient réparties de façon homogène dans le village. Cependant, les riverains du plan d'eau le plus fréquenté, couraient statistiquement six fois plus de risque d'avoir une forme plus grave de fibrose du foie spécifique à *S. mansoni* que ceux qui vivaient plus loin (>100m). Ceci suggère que l'exposition cumulative aux œufs de *Schistosoma* au cours de la vie, pourrait être le facteur étiologique le plus important dans le développement de la schistosomiase chronique.

Le **chapitre cinq** poursuit en décrivant la réponse immunitaire de l'hôte humain contre les vers *S. mansoni* et *S. haematobium* adultes et contre leurs œufs (n=200). Pour avoir une idée de la réponse immunitaire acquise, nous avons quantifié de multiples réponses de cytokine (IL-10, IL-5, IFN-γ, TNF-α et IL-2) dans des cultures de sang 72 heures après stimulation avec des antigènes de vers et d'œufs. Nous avons observé une plus forte réponse en cytokine aux antigènes *S. haematobium* qu'aux antigènes de *S. mansoni*. Cela suggère que la première espèce serait plus immunogène et confirme les observations épidémiologiques susmentionnés, à savoir: 1) Diminution de l'infection à *S. haematobium* après la puberté plus prononcée que pour *S. mansoni*, 2) Cas d'anomalies de la vessie spécifique à *S. haematobium* plus fréquent que les anomalies hépatiques spécifiques à *S. mansoni*. Il n'a pas été relevé d'autres différences immunologiques entre *S. mansoni* et *S. haematobium*. Les antigènes des deux espèces induisent le même type de réponse immunitaire, indépendamment du fait que les personnes dont les échantillons de sang ont été tirés aient été infectées par *S. mansoni* ou *S. haematobium*. Il est bien connu que les infections à *Schistosoma* influencent la réponse immunitaire acquise, et la dirige dans la direction de la réponse Th2 : C'est la réponse Th2 modifiée, et ce type de réaction a été montré ici pour les deux espèces de *Schistosoma*. Bien que cette étude ne fournisse pas de preuves concluantes, il semblait à première vue, ne pas y avoir d'indication d'interactions immunologiques entre les

deux espèces de schistosomes, par exemple sous la forme de réactions immunologiques croisées.

Le **chapitre six** se concentre sur les réponses du système immunitaire inné qui sous-tendent les réponses immunitaires acquises spécifiques de *Schistosoma* susmentionnées. Pour ce faire, nous avons étudié une population différente, à savoir un groupe d'écoliers gabonais (n=30) issu d'une zone où seul survient *S. haematobium*. Etant donné que la recherche dans le système immunitaire inné en est encore à ses prémisses, nous nous sommes concentrés sur les réponses les mieux décrites jusqu'à présent, à savoir celles des récepteurs Toll-like (TLRs). Nous avons constaté que les réponses TLR (TLR2 et TLR4) des enfants infectés étaient, contre toute attente, plus pro-inflammatoire que celles des enfants non infectés, alors que leur système immunitaire acquis laissait voir la suppression caractéristique. Cela montre qu'il ne se produit, en tout cas, pas de suppression générale du système immunitaire dans la schistosomiase. Dans le même temps, il se pose la question du mécanisme déterminant pour la réponse Th2 modifiée qui est si caractéristique de la schistosomiase.

Le **chapitre sept** conclut par une discussion générale et l'évaluation critique de tous les résultats. Nous en concluons que l'approche multidisciplinaire a donné de nouveaux éclairages sur la schistosomiase. En effet, cela n'aurait pas été possible avec les méthodes classiques uniquement. Notre recherche a fourni de nouveaux indices pour poursuivre les recherches sur l'étiologie de la morbidité spécifique à *Schistosoma* dans les zones co-endémiques. Il faudrait que d'autres études similaires soient menées afin de déterminer la mesure dans laquelle les résultats décrits ici pourraient faire l'objet d'une extrapolation à d'autres populations.

La stratégie actuelle de lutte contre la schistosomiase est basée sur le traitement de masse au praziquantel. Ceci est en contraste frappant avec la distribution très focale de la schistosomiase. Ainsi, dans un même village, de grandes différences dans l'infection et dans la maladie peuvent être observées. En outre, les résultats de notre recherche laissent supposer qu'un tel traitement dans les zones co-endémiques, pourrait perturber certains équilibres infectieux et immunologiques et donc avoir des conséquences négatives sur la santé publique.

Pour une lutte plus efficace contre la maladie, il faudrait développer de nouvelles stratégies qui prennent en compte les caractéristiques géographiques de la maladie. Il faudrait aussi plus d'éclaircissements sur les mécanismes sous-jacents de la schistosomiase et de la coïnfection avec différentes espèces de schistosomes, pour pouvoir développer des stratégies adéquates d'éradication de la maladie.

Acknowledgements

This thesis is the result of a whole army of people's hard work, but most of all, I would like to thank the people who were studied 'inside out', the schoolchildren from Zilé (Gabon), and especially, the people from Nouk Pomo (Senegal). It was a great pleasure to work with you, and you are unforgettable. I also thank the field team in Richard-Toll (Senegal) – Ngary, Yague, Moussa, Mankeur, Assane and Boly. You actually did all the (dirty) work and you did it wonderfully. I thank all my present and former colleagues from the District de Santé de Richard-Toll, la Région Médicale de Saint-Louis, the Laboratory of Bacteriology and Virology of Le Dantec, the Department of Parasitology at the LUMC, the Infectious Diseases Section of the VU, the former Parasitology Department and the present Eco-Epidemiology Research Pole of the ITM, and from various other places, for all your help, support, advice, input, encouragements, and lessons learnt. Thank you to each and every one of you for the nice time we spent together while drinking coffee or beer, while dancing, and while enjoying local food in Dakar, the Walo, Antwerp, Leiden, Lambaréné, and elsewhere. I would like to give a special thanks to my fellow SCHISTOINIR PhD students, Abena, Moustapha, and Łucja. I think we were a wonderful team together. You are great colleagues and friends, and I hope to have the honor to continue working with you for a long time to come. Moustapha, I think it was only possible to get through all those months of days and nights of field work in Senegal because you are such a nice person, and because you work so incredibly hard. As a duo, we managed to bring all the fieldwork to a good end. Kim, thank you for showing me the way in the ITM as well as in Richard-Toll, for all your support, and for the nice time. A big thanks to Kirezi, Liliane, Tine, Fré, and Nele too. Thank you to my 'scientific mothers' Maria and Katja for giving me the opportunity to work with you, and for being such inspirational role models. Katja, thank you for all the time and effort you invested in me, and for your endless patience.

Then, there are all those people at home to acknowledge. The time I spent away from home or behind my computer made me realize how precious you all are to me and the little time I spent with you gave me plenty of energy to continue my work. Thank you to Jan and my parents, especially my mother who travelled so often back and forth and even came to check on me in Senegal. Thank you to the Meurs and Wevers family, Twan, Willie, Wan Ying, Lino, Gijs, Chris, Freya, Ineke, Ronnie, and Maarten for being there. Thank you to the Diatta and Lopis family, including Vieux Sow, for spoiling me so much during my short breaks so that I always went back to Richard-Toll in great shape. Thank you to Mineke too, for bringing all this joy to my life in the last two years of my thesis, and Pépé, thank you for your continuous support.

Curriculum Vitae

Lynn Meurs was born in Bemmel (The Netherlands) on the 2nd of May in 1983. After completing secondary school (Stedelijk Gymnasium Nijmegen, 1995-2001), she studied Biomedical Sciences at the Radboud University in Nijmegen. Lynn's first internship was at the Nijmegen Centre for Molecular Life Sciences on the expression of a malaria transmission blocking vaccine candidate (Prof.dr. H. Stunnenberg & Dr. N. Outchkourov). After obtaining her cum laude Bachelors' degree in 2004, she performed an internship on the standardization of the direct membrane feeding assay - a test to study malaria transmission from man to mosquito - at the 'Institut de Recherche pour le Développement' (IRD) in Dakar, Senegal (Dr. C. Boudin & Dr. J.P. Verhave) for her Minor subject in International Health. For her Major Pathobiology, Lynn studied helminth glycans in immunomodulation during an internship at the VU Medical Centre in Amsterdam (Prof.dr. Y. Van Kooyk & Dr. I.M. Van Die). After obtaining her MSc degree in Biomedical Sciences in 2006 at the Radboud University, she moved back to Dakar. In 2007, she was appointed as a PhD candidate at the ITM in Antwerp and worked in the SCHISTOINIR project, under the wings of Prof.dr. Katja Polman and Prof.dr. Maria Yazdanbakhsh. Lynn was involved in several field studies in Senegal and one in Gabon, and in the laboratory work that was carried out at LUMC. Together with her fellow PhD student Moustapha Mbow, she carried out the fieldwork in Senegal and collected the parasitological, ultrasound, clinical, questionnaire, GPS, and immunological data described in this thesis. Lynn prepared, coordinated and documented this study and developed a keen interest in multidisciplinary research, (immuno- & eco-)epidemiology, multivariate analyses, polyparasitism and GIS. She also coached several Master students, and gave oral and poster presentations at international conferences. While finalizing her PhD project, Lynn worked part-time with Prof.dr. Lisette van Lieshout (LUMC) to compare different diagnostic methods for the detection of *Schistosoma* and STH infections. Lynn became a mother in 2012, and was awarded the Zoetis travel grant by the Belgian Society of Parasitology and Protistology in 2013. In 2014, she obtained three years of funding from the ITM for her postdoc project entitled 'An eco-epidemiological approach to understanding helminth co-infections and related morbidity patterns in humans'. Through this project, Lynn will build on the research performed in this thesis and take it one step further.

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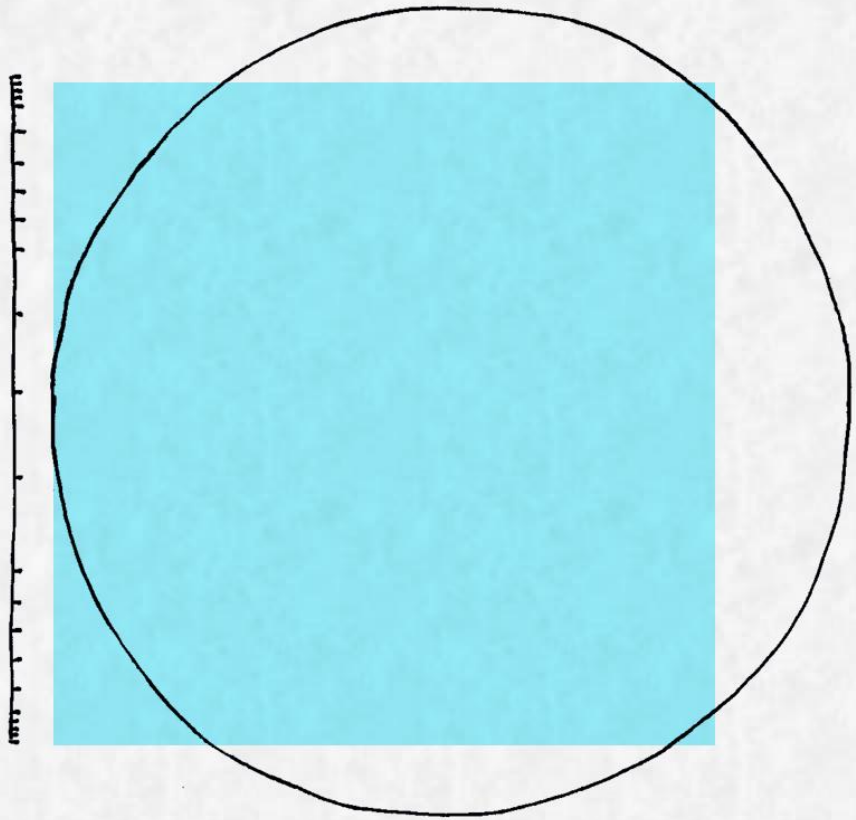

Lynn Meurs

Schistosoma mansoni **and** ***Schistosoma haematobium*** **infection and morbidity in a co-endemic focus**