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Tracking the spatial and longitudinal dynamics of mixed infections of urogenital and intestinal schistosomiasis, inclusive of *Schistosoma mattheei*, in two sentinel rural communities from southern Malawi

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The World Health Organization's 2030 neglected tropical disease roadmap aims to eliminate schistosomiasis as a public health problem with preventive chemotherapy (PC); however, mixed infections of *Schistosoma haematobium* with zoonotic species, inclusive of putative hybrids, present a potential challenge. We sought to address the importance of mixed species infections through a 2-year, longitudinal epidemiological investigation at two villages in southern Malawi (Samama and Mthawira). Participants (approx. 2000) were sampled at baseline (BL), a 12-month follow-up (FU1) and a 24-month follow-up (FU2). PC was provided annually (BL-FU1) and biannually (FU1–FU2). Urine samples underwent microscopical examination and CCA rapid-diagnostic testing, with egg-patent urine filters undergoing additional molecular screening for five non-*S. haematobium* species using real-time polymerase chain reaction (rtPCR). Prevalence of schistosomiasis by microscopy was statistically higher in

Samama than Mthawira (±0.0563, p-value = 1.3×10^{-11}), as was mixed infections with *Schistosoma mattheei*, by rtPCR (± 0.17, p-value = 3.84×10^{-10}). By FU2, PC reduced the prevalence of *S. haematobium* and *Schistosoma mansoni*, but that of *S. mattheei* remained relatively stable, rising by 0.98% at Samama (± 0.19, p-value = 0.41) and decreasing by 0.43% at Mthawira (± 0.39, p-value = 0.33). We conclude that treatment will not be sufficient for control of zoonotic *S. mattheei*, but additional interventions will be required.

This article is part of the Science+ Meeting Issue 'Parasite evolution and impact in action: assessing the importance of hybrid schistosomes in Africa'.

1. Background

Among the parasitic neglected tropical diseases (NTDs), schistosomiasis is currently the most significant [1], estimated to infect 240 million people globally, resulting in an annual mortality rate of approximately 13 000 [2,3]. Up to 779 million people are living at risk of the disease [4,5]. Although the current mortality rate is believed to be an underestimate [6], some analyses suggest it may be as high as 24 000 deaths per year [7]. The majority (91%) of active cases occur in sub-Saharan Africa [8,9] and present either as urogenital or intestinal schistosomiasis, and sometimes as mixed infections of both, with all forms of the disease causing significant morbidity.

Urogenital schistosomiasis is primarily caused by the parasitic blood fluke *Schistosoma haematobium* (*S. haematobium*), while intestinal schistosomiasis is primarily caused by *Schistosoma mansoni* (*S. mansoni*). Infection by each blood fluke occurs through exposure to contaminated water where infected intermediate snail hosts of the respective genera *Bulinus* or *Biomphalaria* are present locally. The World Health Organization (WHO) 2030 NTD Roadmap aims to eliminate schistosomiasis as a public health problem (EPHP), defined as <1% proportion of heavy intensity schistosomiasis infections, in all 78 endemic countries by 2030 [10]. The foundational strategy is by preventive chemotherapy (PC) through mass drug administration (MDA) with the anthelminthic praziquantel (PZQ). For *S. haematobium* infections, heavy intensity infections are defined as 50+ eggs per 10 ml of urine [11]. However, the WHO Roadmap also identifies the lack of understanding surrounding the emergence of mixed infections, inclusive of hybrid and zoonotic schistosomes, implicated in urogenital schistosomiasis and their potential to derail current control efforts.

Since the early 2000s, hybrids between *S. haematobium* and *Schistosoma bovis* have been widely reported from communities in West Africa [12–17]. They have since been reported from further afield in East Africa [18] and outside the typical transmission zone, such as in Corsica [19] and Spain [20]. Studies looking at the introgression of *S. haematobium* and the zoonotic species *S. bovis* have suggested that hybridization events were rare and largely ancestral [21,22]. In consideration of another veterinary schistosome species, *Schistosoma mattheei* (*S. mattheei*), another zoonotic species common in livestock, particularly cattle, hybridization events have different introgression dynamics and appear more contemporary as shown in the laboratory and nature [18,23,24]. More importantly, meticulous laboratory studies carried out by Wright & Ross [25] demonstrated that *S. haematobium* x *S. mattheei* hybrids exhibited heterosis in their ability to infect snails and experimental animals, as well as demonstrating increased growth rate and egg output [25].

It should also be noted that the historical environments (inclusive of aquatic and terrestrial habitats) and epidemiological conditions resulting in past hybridization events likely do not reflect today's zoonotic and anthroponotic transmission patterns, which are shaped by denser human and domestic animal populations following agricultural expansion.

The human population in sub-Saharan Africa has increased tenfold since 1900, from an estimated 140 million to 1.4 billion [26], and cattle numbers have also increased from <5 million to over 300 million across the same time scale [27]. The decrease in nomadism [28] coupled with the creation of both large- and small-scale water-management programmes [29] has likely increased the chances for non-target species infection for both zoonotic and anthroponotic schistosomes at these mixed species-transmission sites. In Malawi, the potential significance that mixed species, inclusive of hybrids or zoonotic infections, may have on the maintenance of urogenital schistosomiasis disease is unknown and requires investigation [30]. To address this knowledge gap, the Wellcome Trust funded the Hybridisation in UroGenital Schistosomiasis investigation, which started in 2021 with the aim of revealing the transmission biology, epidemiological impact and clinical importance of *S. mattheei* hybrids in Malawi.

Our study reported here was carried out over a 4-year period and involved veterinary and human-health studies, of which the latter consisted of a 2-year longitudinal study enrolling approximately 2000 participants from two sentinel study communities in southern Malawi, based at Samama (S 14.41876700, E 35.22098500) and Mthawira (S 16.84980200, E 35.29004100) villages. Here, at least three species of the *S. haematobium* group are known to be endemic: *S. haematobium, S. mattheei* and *S. bovis* [18] as well as *S. mansoni*, whose incidence is increasing in Southern Malawi. Prior studies noted *S. mattheei* (and *S. haematobium*) within locally sampled cattle [31] and in a clinical case report [32]. This raised key questions about the spread of mixed zoonotic and/or hybrid infections among the local populations, the variation in the prevalence of such schistosomes between sites and demographics and their infection dynamics after PC by MDA with PZQ [33].

Here, we describe the results of a 2-year longitudinal study investigating the prevalence of urogenital and intestinal schistosomiasis, inclusive of mixed infections with *S. mattheei* species markers, in two study populations located in Southern Malawi (figure 1). Our objectives were to determine the prevalence of zoonotic *Schistosoma* species at both study sites across the 2 years of the longitudinal study, to identify any association between demographics relating to age and gender with putative-zoonotic *Schistosoma* sp. infection and to assess the spatial distribution of positive cases at the household level and identify any overt heterogeneities.

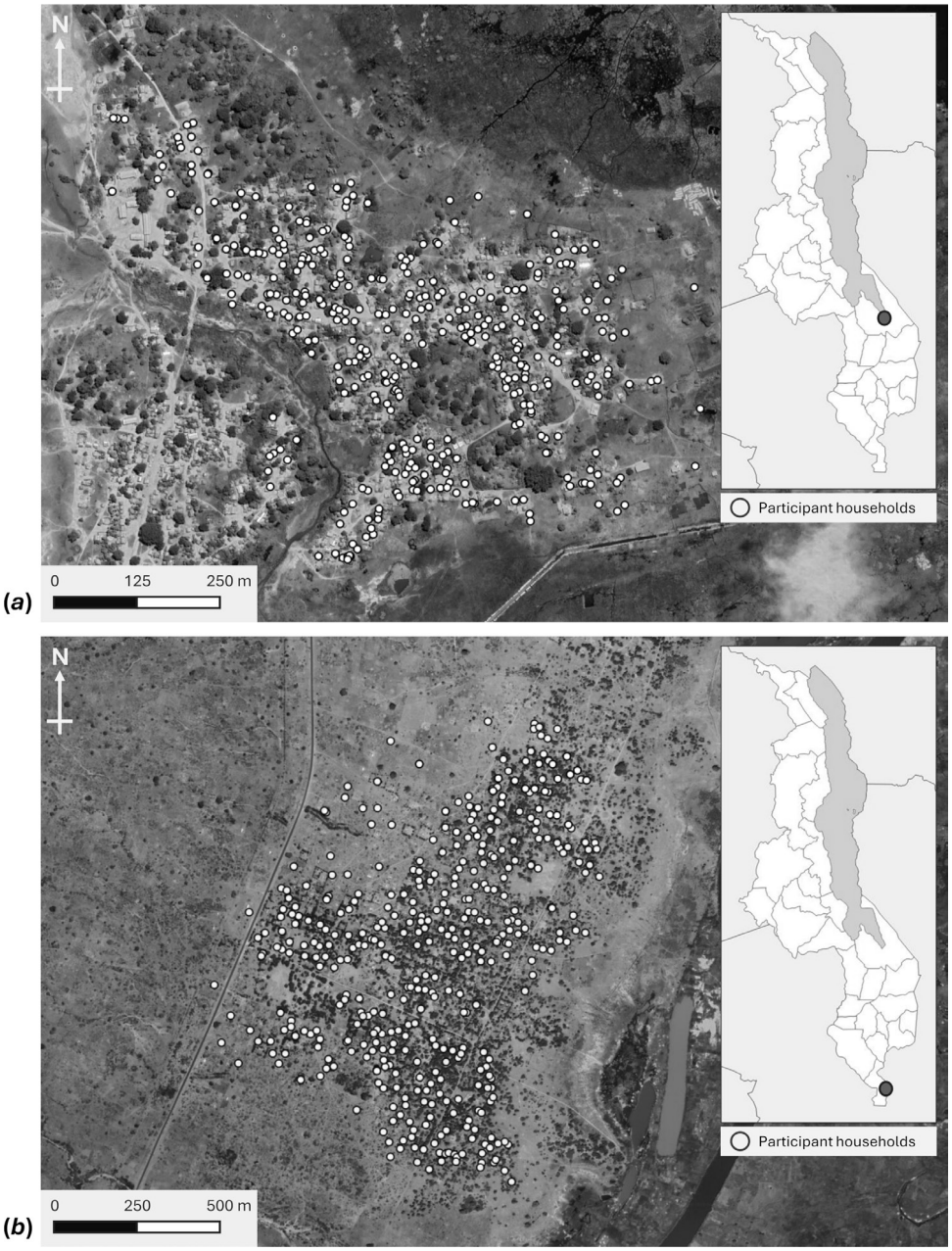

Figure 1. Mapping of the participant households (white circles) for both communities: (*a*) Samama (Mangochi) and (*b*) Mthawira (Nsanje).

2. Methods

(a) Study sites

Our longitudinal study extended across 2 years, comprising a baseline survey (BL, 2022) and two annual follow-ups (FU1, 2023, and FU2, 2024), and enrolled participants from two sentinel rural communities: Samama is located near Lake Malawi in the Mangochi District, and Mthawira is located near the Shire River in the Nsanje District (figure 1). Both districts are endemic for urogenital schistosomiasis and have previously reported suspected mixed zoonotic or hybrid cases of schistosomiasis in either local human or cattle populations [18,31]. At BL, the study enlisted approximately 2000 participants from 2 to 65 years of age, distributed across both study sites (Samama: $n = 1141$; Mthawira: $n = 959$) and with balanced sex representation (females = 1129, males = 971). Recruitment occurred at the household level, i.e. the entirety of a selected household, excluding pregnant women, would be recruited into the study and followed across all three time points (173 participants dropped out of the study by FU2). PC with MDA of PZQ was offered at 40 mg kg^{-1} of body weight to each member of the community, including pre-school age children (PSAC), annually between BL and FU1 on month two and biannually between FU1 and FU2 on months 13 and 18 (electronic supplementary material, figure S2). For each household, the GPS coordinates were recorded, and participants were assigned a unique identifier. Information on household, age, and sex, as well as additional general knowledge, attitude and practice associated with schistosomiasis, was gathered alongside urine samples allowing for comparative analysis [34].

(b) Microscopy urine filtration

Urine samples were collected from each participant, and an aliquot of 10 ml underwent urine filtration. Urine filters were then examined at ×20 magnification to determine schistosomiasis positivity status using compound microscopes. Each filter was assigned the following egg count grade: 1 = 0 egg, 2 = 1–10 eggs, 3 = 10–50 eggs and 4 = 50 + eggs. Following the WHO guidelines, 50+ eggs per 10 ml of urine is designated as a heavy infection [11]. Microscopy analysis also allowed for the identification of non-*S. haematobium* eggs by morphology (electronic supplementary material, figure S1). Only positive urine filters, with the exception of a small subset of microscopy negative samples (approx. 2%), were then stored in 100% EtOH for further downstream molecular analysis, with the goal of identifying individuals with non-*S. haematobium* urogenital infections. In addition to urine filtration, samples also underwent screening with the POC CCA (ICT International, Cape Town, Republic of South Africa), where trace results were considered positive as described by O'Ferrall *et al.* [23].

(c) Urine filter DNA extraction

Schistosoma haematobium microscopy-positive urine filters underwent DNA extraction as described in Cunningham *et al*. [35] to gain an insight into the species signature of each filter. Briefly, the ethanol was evaporated from the samples overnight in an incubation oven at approximately 60°C before DNA extraction using the Qiagen DNEasy blood and tissue kit (Qiagen, Manchester, UK) with two initial adaptations: 0.5 µl of Phocine herpesvirus-positive extraction control DNA and 0.5 g of 1.4 mm ceramic beads were added to the ATL buffer, and the samples were treated by bead-beating for 30 s at 3000 rpm using the MagnaLyser tissue lysis platform (Roche, Burgess Hill, UK). The rest of the DNA extraction followed the recommended protocol provided by the manufacturer.

(d) Five-plex mitochondrial DNA real-time polymerase chain reaction

The mitochondrial DNA (mtDNA) HRM assay described by Cunningham *et al*. [35] was used to identify the following five non-*S. haematobium* species: *S. mattheei, S. curassoni, S. bovis, S. mansoni* and *S. margrebowiei*. The mtDNA HRM reaction consisted of 400 nM of each of the species-specific primers and 6 µl of Type-IT HRM supermix (Qiagen, Manchester, UK), with the addition of nuclease-free water making up the remaining volume to 12 µl. The cycling conditions were as follows: 95°C for 5 min followed by 35 cycles of 95°C for 10 s, 58°C for 30 s and 72°C for 10 s, with a final melt step from 65°C to 92 °C with a ramp rate of 0.1°C per second.

(e) Data analyses

Statistical analyses were performed using R v. 4.4.2 within RStudio. The egg count categories used to classify the results from microscopy were carried over into the analysis as four ordinal levels: absent (0), low (1–10), medium (11–50) and high (>50 eggs). For both *S. mansoni* and *S. mattheei*, infection status was treated as a binary outcome: individuals were classified as positive [1] or negative (0) for DNA signatures. Co-infection was defined as being positive for both species simultaneously (1 and 1). When mentioned, participants were grouped in four age groups: PSAC (PSAC: age 0–5), primary school-aged children (primary SAC: age 6–13), secondary school-aged children (secondary SAC: age 14–17) and adults (age 18 and above). Results of the POC CCA (a proxy for intestinal schistosomiasis) tests were fitted against polymerase chain reaction (PCR) species identification in a generalized linear model corrected for binomial distribution for a comparison of the diagnostic tools [36].

To visualize infection patterns, prevalence and/or abundance were plotted using local polynomial regression fitting (LOESS) against variables of interest such as age and sampling year, stratified by host sex or parasite species [37]. For clarity, the *y*-axes in age-related plots were truncated at 45 years. For regression-based analysis, we fitted generalized additive models (GAMs) using the 'mgcv' package [38]. Binary prevalence outcomes were modelled with a binomial distribution, while egg count data were modelled using a Tweedie distribution to accommodate zero inflation and overdispersion. Age and age-by-sex interactions were modelled using thin plate regression splines, while location, sampling year and sex were included as categorical predictors. GAMs were fitted using restricted maximum likelihood, and model selection was based on the Akaike information criterion. Post hoc comparisons of sampling years were performed using estimated marginal means (least-squares means) via the 'emmeans' package [39].

Precise positive and negative household placement was carried out using QGIS software with the addition of kernel density heatmaps depicting frequency of infection at the household level (distance set at 150 m) across all time points (Styled Heatmap (kernel density estimation) application from the Density Analysis plugin tool [40]).

(f) Research ethics

Ethical approval for this study was obtained from both the Kamuzu University of Health Sciences (KUHeS) Research Ethics Committee (KUREC; approval # P.08/21/3381) and the Liverpool School of Tropical Medicine (LSTM) Research Ethics Committee prior to the start of the study. Written informed consent for participation in the study was secured from all participants either as a signature or a thumbprint. For children participating, individuals under the age of 18, written assent was obtained from their parents or guardians on their behalf. All participation documents were provided in local languages (Chichewa, Chiyao and Chisena) with copies of information leaflets and signed consent forms provided to each participant [34].

3. Results

(a) Participant recruitment and retention

A total of 3316 samples with sufficient demographic data to identify age and sex were collected from Samama across BL (n = 1 141), FU1 (n = 1 083) and FU2 (n = 1 092), and 2602 samples were collected from Mthawira at BL (n = 959), FU1 (n = 808) and FU2 (n = 835). The ratio of female and male participants recruited from Samama (1.26 : 1) and from Mthawira (1.13 : 1) remained stable across the different sampling rounds, thanks to the high percentage of participant retention (94.8%).

(b) Microscopy

The microscopy analysis of the urine filters identified an overall egg-patent difference between sites with higher schistosomiasis prevalence in Samama than Mthawira (E = −0.38, se = 0.06, p-value = 1.3×10^{-11}). Patterns of temporal variation in prevalence were different between both sites. In Samama (Mangochi), schistosomiasis prevalence decreased from 51.6% (± 1.48, n = 1141) at BL to 42.7% (± 1.50, n = 1083) at FU1, but increased from 34.7% (± 1.54, n = 959) to 45.2% (± 1.75, n = 808) in Mthawira (Nsanje) in the same timeframe. From FU1 to FU2, it decreased in both locations to 31.7% (± 1.41, n = 1092) and 21.0% (± 1.41, n = 835) at Samama and Mthawira, respectively.

The same pattern was observed in egg counts. Generalized additive models, fitted independently for both sites, showed a significant reduction of egg counts over time in Samama in comparison to the BL (FU1: E = −0.25, se = 0.05, p-value = 2.24×10^{-7}; FU2: E = −0.52, se = 0.05, p-value = $<2 \times 10^{-16}$). This reduction was influenced by an important nonlinear effect of age (edf = 5.65, F = 6.84, p-value = 2.07×10^{-8}) and interaction with sex (age × female: edf =<0.01, $F = 4.394 \times 10^{7}$, p-value =$<2 \times 10^{-16}$; age × male: edf = 3.53, F = 12.76, p-value = $<2 \times 10^{-16}$), but not by sex alone (E = −0.10, se = 0.05, p-value = 0.06). In Mthawira, the temporal dynamic is different, with significantly increased egg counts at FU1 (E = 0.29, se = 0.06, p-value = 3.36×10^{-7}) followed by a significant decrease at FU2 (E = −0.45, se = 0.07, p-value = 1.14×10^{-10}) in comparison to the BL. This variation was again impacted by a strong nonlinear effect of age (edf = 5.91, F = 6.70, p-value = 6.16×10^{-7}) and not by sex (E = −0.08, se = 0.05, p-value = 0.159). Detailed schistosomiasis prevalence by age group is reported in table 1, showing an overall higher burden in school-age children in both sites. Details of temporal dynamics are graphically represented by sex and age, with the separation of egg counts into prevalence and intensity in figure 2.

The POC CCA tests produced a positive rate of 28.1% (± 1.33, n = 1139) in Samama and 0.8% (± 0.29, n = 958) in Mthawira at BL. This increased at both sites to 49.2% (± 1.52, n = 1083) and 4.0% (± 0.83, n = 569) at FU1, respectively. However, by FU2, the positivity rate of urines tested with the POC CCA decreased at both sites, with Samama reporting a positivity rate of 15.1% (± 1.08, n = 1092) and Mthawira reporting 1.2% (± 0.38, n = 835). The odds ratios calculated from generalized linear model coefficients indicate that *S. mansoni*-positive samples were 3.18 (p-value = 1.07×10^{-15}) times more likely to get a positive POC CCA test, while the likelihood was lower for *S. haematobium* (1.89, p-value $<2 \times 10^{-16}$) and *S. mattheei* (1.51, p-value = 0.0076) positive samples. These results indicate a weak correlation with the *Schistosoma* species identification from urine and the POC CCA tests.

4. Mitochondrial DNA real-time polymerase chain reaction analysis

(a) Modelling infection rates by year and species

Analysis of urine filters targeting non-*S. haematobium* schistosome DNA markers only definitively identified two non-*S. haematobium* species present, namely *S. mattheei* and *S. mansoni*. Across all time points, a total of 168 *S. mattheei*-positive urine filters were detected from Samama, with a 4.7% (± 0.60), 4.3% (± 0.60) and 5.7% (± 0.69) prevalence at BL, FU1 and FU2, respectively. A similar analysis of Mthawira samples identified 47 *S. mattheei*-positive urines with a prevalence of 1.7% (± 0.40), 2.2% (± 0.51) and 1.3% (± 0.39) at BL, FU1 and FU2. Alongside *S. mattheei*, the mtDNA real-time PCR identified *S. mansoni* in urine filters in differing prevalences across the two sites, with 222 *S. mansoni*-positive urine filters identified from Samama samples across all three time points (BL: 10.4% (± 0.87), FU1: 4.6% (± 0.63), FU2: 3.9% (± 0.58)) and only two (FU1 and FU2) *S. mansoni*-positive urine filters from Mthawira.

Similarly to the prevalence of *S. haematobium*, *S. mansoni* prevalence also dropped over time in Samama (figure 3*a*). The greatest drop occurred between BL and FU1 by 5.2% (p-value = 1.60×10^{-8}), whereas the decrease from FU1 to FU2 was only 0.5% (p-value = 0.776). In contrast to both the gross schistosomiasis and *S. mansoni* prevalence, the prevalence of *S. mattheei* stayed stable with a slight non-significant increase (0.98%) from BL to FU2 (E = 0.16, se = 0.19, p-value = 0.411). Similarly, *S. mattheei* prevalence in Mthawira remained stable, but following the same trend as the gross schistosomiasis with a slight increase at FU1 (E = 0.17, se = 0.34, p-value = 0.620) and decrease at FU2 (E = −0.38, se = 0.39, p-value = 0.325), although these variations were not significant (figure 3*a*).

Here, we interpret PCR Cq values as a proxy for infection load (electronic supplementary material, figure S3). In both locations, *S. mattheei* load follows a similar pattern to prevalence, with an increase in the Cq values over time in Samama. This increase in Cq values indicates a decrease in *S. mattheei* genetic markers and therefore an assumed decrease in intensity. In Mthawira, Cq values dip at FU1 indicating an increase in *S. mattheei* genetic markers in samples, suggesting an increase in

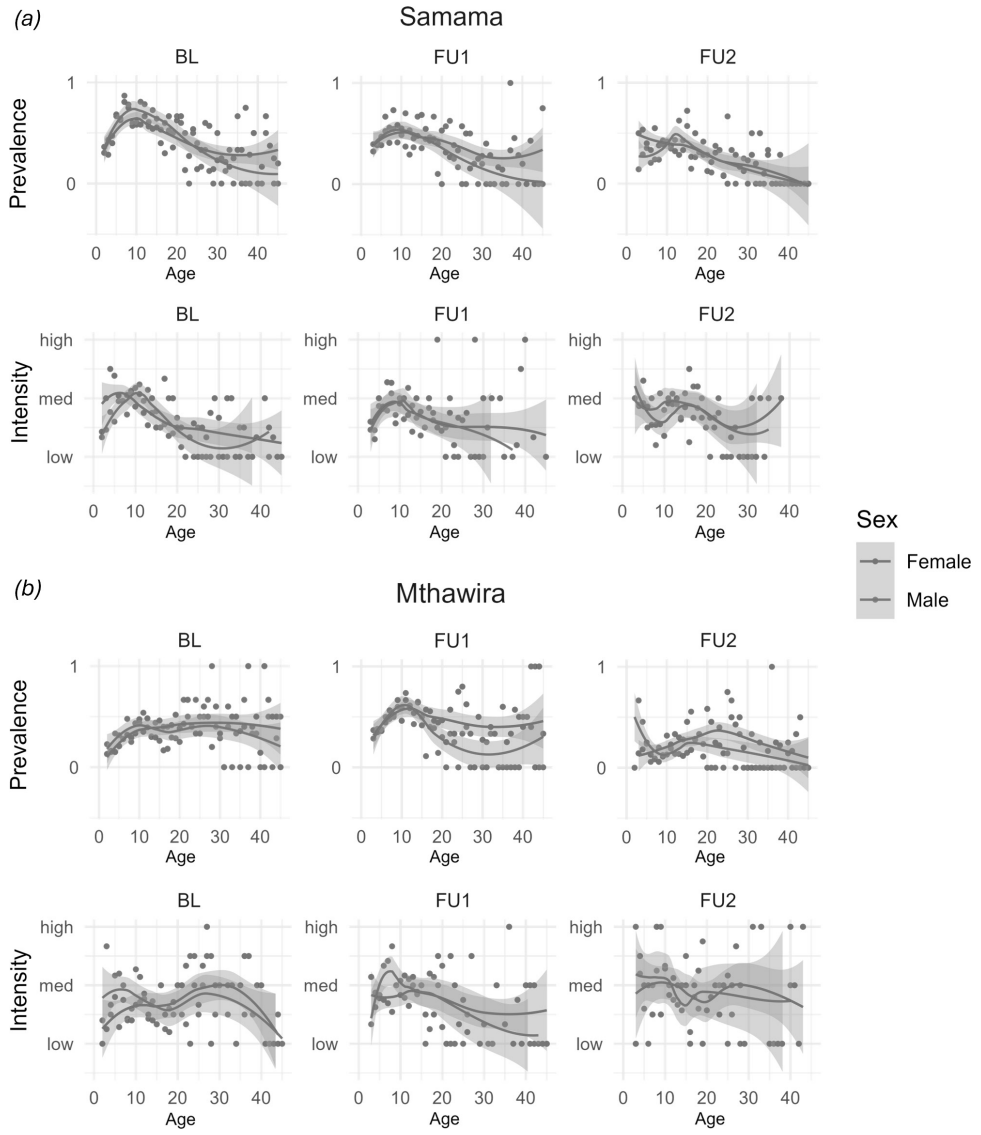

Figure 2. Prevalence and intensity of urine egg-patent infection over time by age and gender. Prevalence considers infection as a binary outcome, and intensity of infection excludes uninfected individuals (egg count: low = 1–10, medium = 11–50, high >50). The data are separated by communities (Samama and Mthawira) and sampling years: baseline (BL), follow-ups 1 (FU1) and 2 (FU2). Dots represent the mean at each age for both genders (red = female, blue = male) and locations, while lines project the LOESS with the 95% CI in grey. The *y*-axes are truncated at age 45 for better clarity.

Table 1. Urine egg-patent schistosomiasis prevalence for age groups across time points in Samama and Mthawira.

		percentage positive of different age groups across time points (± se)			
site	sampling round	PSAC	primary SAC	secondary SAC	adult 18+
Samama	BL	43.1 (± 3.48)	67.6 (± 2.29)	56.9 (± 4.14)	36.4 (± 2.49)
	FU1	39.5 (± 3.85)	51.1 (± 2.44)	49.7 (± 4.17)	31.3 (± 2.46)
	FU2	37.8 (± 4.32)	37.9 (± 2.37)	38.8 (± 3.80)	19.7 (± 2.04)
Mthawira	BL	20.0 (± 3.12)	37.2 (± 2.57)	34.2 (± 4.35)	39.8 (± 2.75)
	FU1	35.2 (± 4.29)	50.5 (± 5.16)	50.5 (± 5.16)	35.3 (± 2.92)
	FU2	27.3 (± 5.52)	17.1 (± 2.04)	25.2 (± 3.81)	22.1 (± 2.41)

intensity of infection. The results for *S. mansoni* Cq values remain stable over time with a slight increase in Samama, indicating no significant change in intensity of infection for those testing positive, even though prevalence has reduced.

Dynamics of *S. mansoni* and *S. mattheei* prevalence can be further analysed in Samama (prevalence in Mthawira is too low for accurate analysis, $n = 2$; table 2). *Schistosoma mansoni* prevalence tended to be non-significantly lower in males ($E = -0.60$, se = 0.53, p-value = 0.261) and peaked in school-age children, with the highest prevalence in secondary SAC (important nonlinear effect of age: edf = 4.61, $\chi^2 = 41.06$, p-value = 1.83×10^{-6}). Inversely, *S. mattheei* prevalence tended to be non-significantly higher in males ($E = 0.37$, se = 0.20, p-value = 0.0617), peaking in primary school-age boys (important nonlinear interaction of age and male: edf = 3.05, $\chi^2 = 15.68$, p-value = 0.0056).

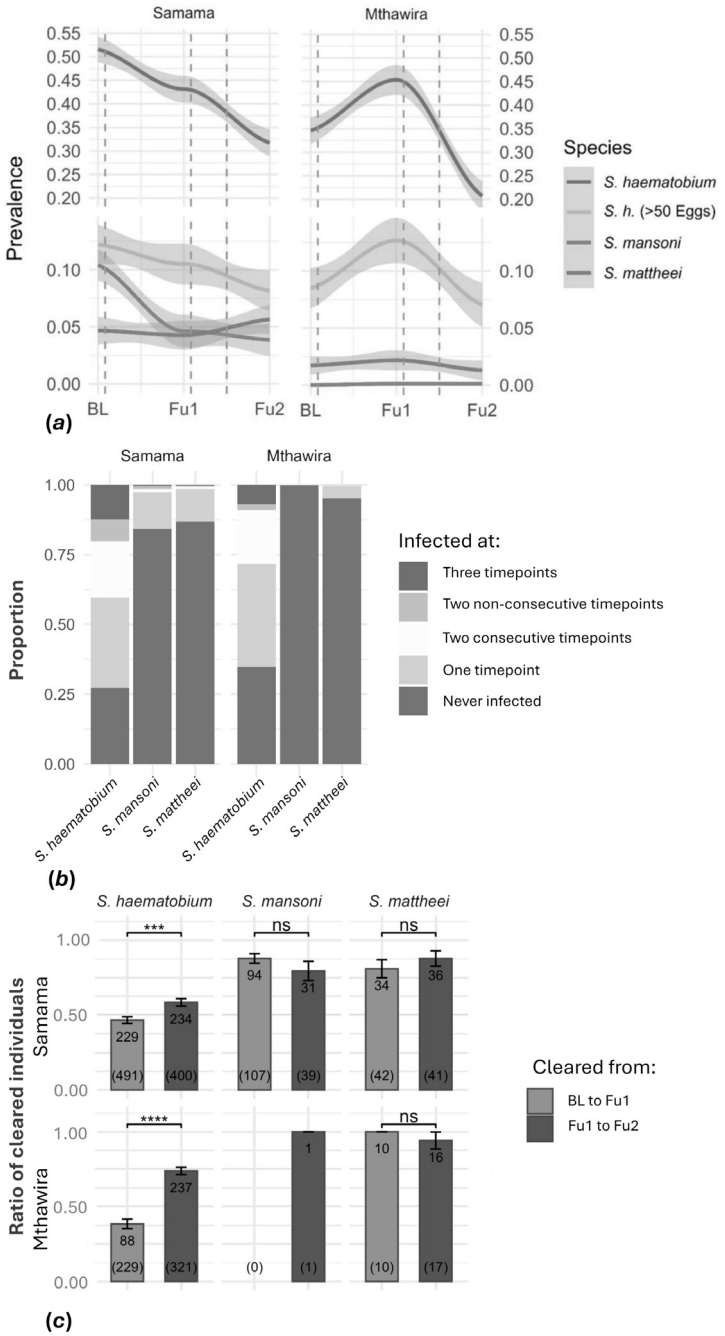

Figure 3. (*a*) Infection rate of gross-urogenital schistosomiasis diagnosed with microscopy (purple) compared to high-egg count cases (lilac), species-specific 5-plex real-time PCR-positive results for *S. mansoni* (green) and *S. mattheei* (orange), across the three sampling time points for both sites (Samama and Mthawira). Treatment regimens (at month 1, 13 and 18 post-BL sampling) are indicated by vertical dashed grey. (*b*) Proportion of single and multiple infections to the different schistosomiasis species at both locations. Only individuals sampled at all time points are included (Samama: $n = 950$, Mthawira: $n = 678$). (*c*) Proportion of *Schistosoma* sp.-positive participants who tested negative in the year following annual treatment (from BL to FU1 in yellow) and biannual treatment (from FU1 to FU2 in red). Sample sizes are indicated in brackets, while the number of cleared individuals is indicated at the top of each bar. Error bars represent standard error. Statistical significance was assessed using GLMs corrected for binomial distribution (*p*-value <0.0001 = ****, <0.001 = ***, <0.01= **, <0.05 = *, >0.05 = ns).

(b) Persistence of zoonotic schistosomiasis

Positive individuals were tracked across all three sampling rounds to determine persistence of positivity over time across BL, FU1 and FU2 for microscopy-positive *S. haematobium* samples and samples positive for real-time PCR *S. mattheei* and *S. mansoni* markers. For the purpose of analysis, only those individuals who had participated at each time point were included. Of the microscopy-positive individuals from Samama, 691 participants sampled across all three time points were positive, of which 44.4% were positive once, 38.5% were positive across two timepoints and 17.1% were positive across all three timepoints. Similarly, of the 168 *S. mattheei*-positive individuals, 125 were sampled at all three time points, of which 88.8% tested positive at one time point, 8.8% tested positive at two time points and 1.6% were found to be persistently positive at each of the three time points. The *S. mansoni*-positive individuals at Samama that were sampled at each time point numbered 150; of these, 82.7% were positive once, 14.6% were positive at two points and 2.7% tested positive across all time points (figure 3*b*).

Table 2. Schistosomiasis species by HRM-rtPCR prevalence for age groups by gender in Samama.

		percentage positive of different age by sex (± se)			
species	sex	PSAC	primary SAC	secondary SAC	adult 18+
S. mattheei	females	5.1% (± 1.51)	5.7% (± 0.91)	4.2% (± 1.36)	1.8% (± 0.48)
	males	2.9% (± 1.00)	9.2% (± 1.17)	6.7% (± 1.63)	2.9% (± 0.91)
S. mansoni	females	2.8% (± 1.13)	8.3% (± 1.09)	10.6% (± 2.10)	2.3% (± 0.54)
	males	1.4% (± 0.72)	10.5% (± 1.24)	12.2% (± 2.12)	2.3% (± 0.82)

At Mthawira, the microscopy-positive individuals who were sampled at all three time points numbered 442, of which 56.6% were positive once, 32.8% were positive across two time points and 10.6% were positive across all three time points. The real-time PCR assay identified 33 *S. mattheei*-positive individuals who participated at each time point; of these, 93.9% were positive at one time point, and 6.1% were positive at two time points, with zero participants testing positive across all three time points (figure 3*b*).

(c) Performance of preventive chemotherapy by mass drug administration

As previously described, the prevalence of *S. haematobium* and *S. mansoni* decreased following treatment from BL to FU2; however, for *S. mattheei,* no significant change in prevalence was observed. Analysis of positive individuals who received treatment between sampling rounds BL-FU1 or FU1-FU2 demonstrated that treatment was effective between sampling rounds by a reduction in *S. haematobium* prevalence. Moreover, a significantly higher proportion of individuals were cleared following the biannual treatment between FU1-FU2 compared to the annual treatment between BL-FU1 at both sites. In contrast, no significant difference in the proportion of cleared individuals between BL-FU1 and FU1-FU2 was observed among the participants positive for either *S. mansoni* or *S. mattheei* (figure 3*c*).

(d) Household *Schistosoma mattheei* and *Schistosoma mansoni* mapping

Mapping of households with individuals positive for *S. mattheei* across the two sites demonstrated two distribution patterns of *S. mattheei*-positive households. In Samama, while the distribution of positive households appeared homogenous across the community when factoring frequency of cases per household, the results of the spatial kernel density show a distinct cluster of cases towards the S.W. of the community, bordered by an intermittent stream running N-S (figure 4*a*). The distribution of *S. mansoni* households in Samama was again homogenous throughout the community, although spatial kernel density analysis showed a greater frequency of cases per household was found towards the North of the community (figure 4*b*).

In Mthawira, the distribution of *S. mattheei*-positive households is far more heterogeneous, with several distinct small clusters observable towards the South of the community. This is made even more evident in the kernel frequency heatmaps, which show several small clusters of several households with multiple cases of *S. mattheei*-positive urine samples across the study period (figure 4*c*).

5. Discussion

(a) Urogenital schistosomiasis and relationship between age and gender

The overall prevalence of urine egg-patent urogenital schistosomiasis was significantly different between the two sites of Mthawira (Nsanje) and Samama (Mangochi), with Samama having a significantly higher prevalence (p-value = 1.3×10^{-11}). The prevalence of the infection decreased from BL and FU2 at both sites; however, at Mthawira, a significant increase in prevalence was observed at FU1 (p-value = 3.36×10^{-7}), whereas at Samama, a significant sequential decrease from BL was observed at each time point (FU1 p-value = 2.24×10^{-7}; FU2 p-value = $<2 \times 10^{-16}$; figure 2). The increase in disease incidence at Mthawira between BL and FU1 coincided with flooding caused by the tropical cyclone Freddy, which made landfall in 2023 and was the longest-lasting tropical system on record, causing extensive flooding in Southern Malawi, particularly around Nsanje [41]. The flooding may well have resulted in an increase in transmission due to the floodwater distributing infected snails across the floodplains, creating a larger ecological zone for transmission of schistosomiasis within the Mthawira community, resulting in the spike of cases observed. Additionally, outside of PC MDA, no other forms of intervention targeting the halting or reduction of transmission were implemented, resulting in no protection as people go about their daily lives.

An important nonlinear effect of age and interaction with sex was observed, with young women from Mthawira exhibiting markedly higher levels of schistosomiasis at FU1 compared to males (figure 2). This pattern was not observed at BL, suggesting it may be attributable to treatment failure or increased levels of re-infection rates among females, potentially linked to sex-specific water contact behaviours following the flooding caused by Tropical Cyclone Freddy [34]. Such differences in infection rates between the sexes have previously been reported from other locations [42–44]. Intensity did not appear to change over the course of the study, with high-intensity case numbers (50+ eggs per 10 ml of urine) remaining stable throughout the study

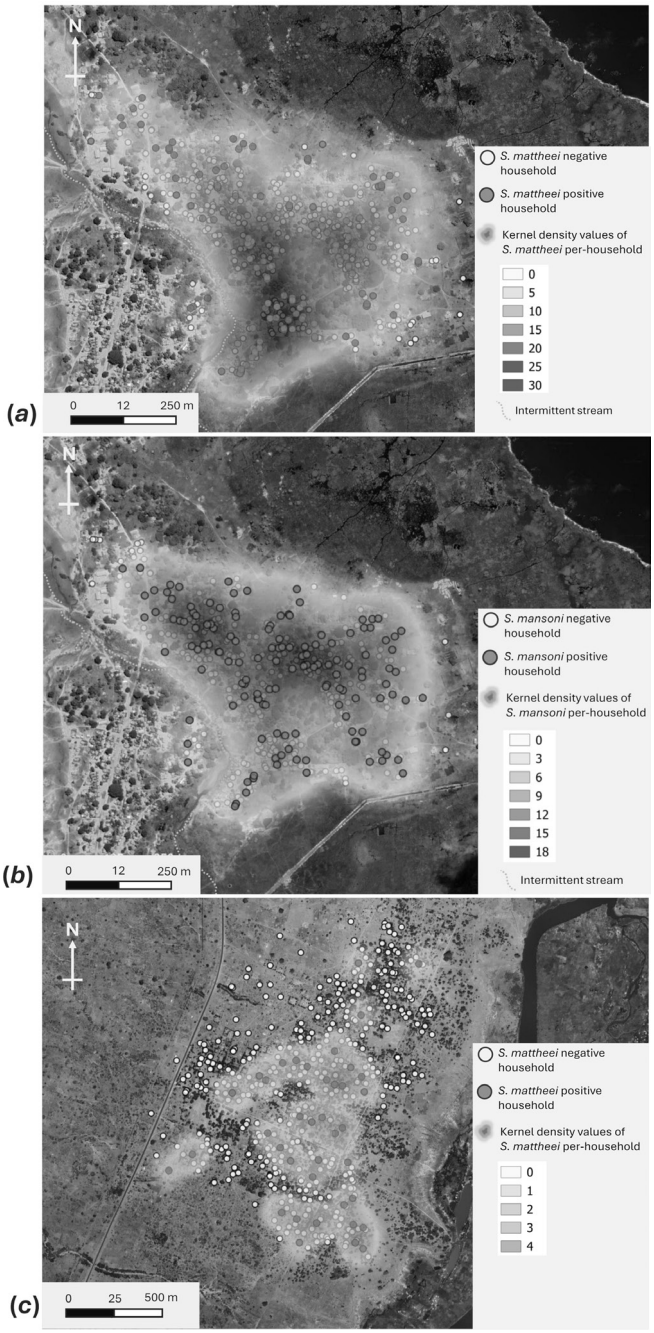

Figure 4. Distribution of (*a*) *S. mattheei* and (*b*) *S. mansoni* at Samama and (*c*) *S. mattheei* at Mthawira at the household level (solid red dots) plus heatmaps showing kernel density estimation for positive cases per household across the entirety of the study period.

at both sites. This was despite modifications to the standard protocol of MDA treatment, by introducing biannual treatment between FU1 and FU2, to try and increase the impact of treatment. This raises the question of whether the current MDA treatment alone is sufficient to meet the WHO 2030 goals for the EPHP.

(b) Mixed infections, hybridization and One Health

Although urine filters testing positive for genetic markers of *S. mattheei* were found at both sites, Samama was found to have a significantly higher prevalence (4.8%) of *S. mattheei* cases compared to Mthawira (1.8%). Across the course of the study, the prevalence of *S. mattheei* did not significantly decrease at either Samama (p-value = 0.4113) or Mthawira (p-value = 0.325–0.620) despite significant declines in both *S. haematobium* and *S. mansoni* by FU2 (figure 3*a*). This demonstrates that, despite the difference in prevalence between the two sites, the control of schistosomiasis using PZQ as a PC MDA reduced the incidence of *S. haematobium* and *S. mansoni* markers but did not overtly reduce the prevalence of *S. mattheei*. The persistence of the *S. mattheei* signal in both communities raises questions as to the nature of these signals and points to a degree of transmission durability, likely resulting from the permeability between the classic models of anthroponotic and zoonotic *Schistosoma* sp. cycles.

The importance of taking a One Health approach is highlighted by results from previously published studies that show local domestic Bovidae can have high prevalence levels of *S. mattheei* (49.1–87.7%), suggesting that there is a substantial amount of miracidial release into local water bodies around our study communities resulting in an increase in the prevalence of disease

in the snail populations [31,45]. Overlapping human/animal water contact points, with a high proportion of *S. mattheei*-infected snails, would result in a local environment with transmission of both anthroponotic and zoonotic schistosome species. This increases the chance of encounters between humans and zoonotic schistosome cercariae, which in turn increases the chance of successful infection of human community members with mixed infections, inclusive of zoonotic *Schistosoma* species.

In our study, we were able to identify that the majority (approx. 80–94%) of individuals who were positive for *S. mattheei* markers were only positive at one time point, during our study, suggesting that re-infection was rare (figure 3*b*). Despite this, persistent cases were observed, with two individuals from Samama testing positive at all three time points, one of which (patient X) has previously been reported on in a separate publication and had a rate of 93.3% *S. mattheei* x *S. haematobium* hybrids from stool-hatched miracidia [23]. These 'persistent' cases would imply either treatment failure or re-infection following treatment between each time point. The latter is perhaps more likely due to the high percentage of single-time point cases coupled with the success of treatment between BL-FU1 and FU1–FU2 (figure 3*c*).

Our manuscript reports the incidence of non-*S. haematobium* markers from urine filters in two communities from Southern Malawi. We took this approach so as to rapidly process a larger number of samples, with a high degree of sensitivity. However, such an approach has limitations, notably that the urine filter sample is a pool of multiple eggs. Consequently, it is not possible to say what the nature of the non-*S. haematobium* markers is with regard to being 'pure' *S. mansoni* or *S. mattheei* eggs or hybrids, with even further lack of clarity as to whether or not these are recent hybridization events or historic. The literature mainly concerns itself with investigating *S. bovis* x *S. haematobium* hybrids, where the evidence strongly suggests that hybridization is rare and most likely historic, with little evidence for recent cases of hybridization [19,21,22]. Studies show that the sex chromosomes are enriched with genes resistant to introgression between the two species, suggesting that hybridization between *S. bovis* and *S. haematobium* may be even rarer than previously thought. However, the relationship between *S. haematobium* and *S. mattheei* is less well understood, so the dynamics of introgression may be different between these two species, with the work of Wright and Ross showing that offspring of *S. mattheei* and *S. haematobium* may well be subject to hybrid vigour and so be more likely to occur and play a greater role in disease transmission. However, as discussed earlier, due to the nature of our sample being pooled eggs, it is not possible to determine if we are observing pure eggs or hybrids, and if hybrids are present, we are unable to reliably determine if these are ancestral events or if we are detecting markers of a more recent hybridization, but the parallel work of O'Ferrall *et al.* does demonstrate more conclusively that we are likely observing hybrid schistosomes as well as potentially pure forms. Their work took a similar investigative approach but proceeded to analyse individual miracidia, demonstrating both 'pure' forms of all three schistosome species mentioned in this paper, as well as *S. mattheei* x *S. haematobium* and *S. mattheei* x *S. mansoni* hybrids in the stool, but only pure *S. haematobium* or *S. haematobium* x *S. mattheei* hybrids in the urine. There was no detection of *S. haematobium* x *S. mansoni* hybrids, suggesting this may be the least likely pairing in an environment with all three species present, possibly due to *S. mattheei* and *S. mansoni* both concentrating in the mesenteric veins of the intestines, whereas *S. haematobium* is found in the veins surrounding the bladder, resulting in *S. mattheei* and *S. mansoni* being more likely to encounter each other than *S. haematobium*. Although we cannot rule out the possibility of the *S. mansoni* signals being the result of hybridization, as it has been reported to occur naturally [46,47].

Further circumstantial evidence that supports the theory that we are observing cases of hybrid infections is the impact of treatment. If the community at Samama is infected with a sub-population of *S. haematobium* with introgressed *S. mattheei* markers, then all being equal, this sub-population, being exposed to the same selection pressure from the MDA campaign, should see a proportional decrease in their numbers, reflecting the overall decrease in cases of urogenital schistosomiasis. However, what we observe is that the prevalence of the *S. mattheei* marker is remaining relatively stable, with it even increasing slightly. This suggests a difference in transmission, likely resulting from a stable reservoir, most likely the large number of untreated and infected cattle and goats.

It should also be noted that in our study, we only collected and screened urine filters and did not perform any analysis on faecal material regarding the screening for zoonotic markers. By concentrating only on urine samples and not including faecal samples, we may potentially underestimate the true extent of *S. mattheei*-positive individuals, as the previously mentioned paper by O'Ferrall *et al.* demonstrated that in cases of *S. mattheei*-infected individuals, a greater abundance of species diversity is observed in the faecal material compared to urine filters [23]. However, O'Ferrall *et al.* do demonstrate that the non-*S. haematobium* markers detected in the urine filters track with examples of 'hybrid' miracidia, with discordant species parentage, hatched from both the urine and faecal material.

(c) Age and gender and *Schistosoma mattheei*-positive participants

At Mthawira, no significant difference in the prevalence of *S. mattheei* between the sexes and age groups was observed. The situation at Samama was different, with a non-significantly higher prevalence of *S. mattheei* cases among males at FU1 and FU2. While *S. mattheei* prevalence tended to be non-significantly higher in males, when controlling for age, it was observed to peak significantly in primary SAC boys (p-value = 0.0056), suggesting behaviours specific to this demographic increase the likelihood of infection with *S. mattheei*. It should also be noted that *S. mattheei* was detected in PSAC from both sites. This is significant, as this age group typically has little water contact behaviour. The discovery of *S. mattheei* across all age groups raises the question of what behavioural risk factors are associated with infection from this species.

(d) Geospatial characteristics of *Schistosoma mattheei*-positive households

The distribution of *S. mattheei*-positive households varied between the two sites, with Samama having a rather homogenous distribution with positive households found across the community (figure 4*a*), whereas in Mthawira the distribution of *S. mattheei* was more heterogeneous and concentrated towards the centre and south of the community (figure 4*c*). When factoring the frequency of cases per household, a hotspot towards the S.W. of Samama can be observed. This concentration of cases tracks closely with the intermittent stream that runs North-South to the West of the village, implicating this temporary waterbody as a potential transmission zone for *S. mattheei;* of note, both *S. mattheei-* and *S. haematobium*-infected snails were observed here in February 2025 (unpublished). The kernel density analysis of *S. mattheei* cases per household shows a scattered distribution of clusters to the south of Mthawira at a significantly lower density (max 4) compared to the localization of cases seen in the kernel density distribution in Samama (max 30). Both sites indicate that there are likely localized transmission sites of *S. mattheei*, although the exposure of the wider community differs, with the individuals in Samama from across the community more likely to become infected than in Mthawira, where cases are localized to the South.

(e) A note on the dynamics of *Schistosoma mansoni*-positive urine samples

Alongside the detection of *S. mattheei*, a high number of urine filter samples also tested positive for *S. mansoni*, with a significant difference in prevalence between Mthawira (2/2 601) and Samama (198/3 329; figure 3*a*). This was reflected in the CCA results, although analysis showed only a weak correlation between *Schistosoma* species ID from urine and the CCA results. This poor correlation is likely due to the positive *S. mansoni* faecal samples, but urine negative. As our study did not conduct any faecal surveillance, further analysis cannot be performed.

No significant difference in prevalence was detected between males and females; however, secondary school children were found to have significantly higher levels of *S. mansoni* (p-value = 1.83×10^{-6}) compared to the other age groups. Spatial analysis shows widespread distribution of positives at the household level across Samama, but kernel density analysis of cases at the household level showed a strong clustering effect along the Northern portion of the community.

6. Conclusion

This study provides a seminal detailed analysis of mixed infections for urogenital and intestinal non-*S. haematobium* epidemiology in two communities from Southern Malawi. We demonstrate that there were raised numbers of individuals who were excreting mixed species infections of non-*S. haematobium* eggs in their urine, alongside the expected *S. haematobium* eggs. School-aged children were the most likely to test positive for genetic markers of both *S. mattheei* and *S. mansoni* in their urine, with a significantly higher proportion of both species found at Samama compared to Mthawira. The distribution of *S. mattheei* and *S. mansoni* appears focal at both study sites, most likely related to transmission sites associated with specific household water contact points and behaviours. In the future, to concretely identify the presence of hybrid eggs in communities, extensive screening of individual eggs using, at the very least, barcode sequences of the nuclear and mitochondrial genomes would be required. However, this has limitations with regard to determining the history of any hybridization detected. In order to answer the question of when these hybridization events occurred, more advanced methods would be required, similar to those used by Platt *et al*. However, such an approach is difficult to scale up to the number of participants involved in studies such as ours.

Finally, and most importantly, although analysis of our data shows a decrease in overall urogenital schistosomiasis from BL to FU2, the prevalence of *S. mattheei* did not decrease, despite annual and biannual MDA between BL-FU1 and FU1–FU2, respectively. This may prove to be a significant hurdle to the WHO's EPHP ambitions. Our study suggests that complementary control methods are needed, beyond MDA, incorporating One Health interventions to tackle infections both in snails and livestock reservoirs.

Ethics. Ethical approval for this study was obtained from both the Kamuzu University of Health Sciences (KUHeS), Research Ethics Committee (KUREC; approval # P.08/21/3381) and the Liverpool School of Tropical Medicine (LSTM). Research Ethics Committee prior to the start of the study. Written informed consent for participation in the study was secured from all participants either as a signature or a thumbprint. For children participating, individuals under the age of 18, written assent was obtained from their parents or guardians on their behalf. All participation documents were provided in local languages (Chichewa, Chiyao and Chisena) with copies of information leaflets and signed consent forms provided to each participant.

Data accessibility. The datasets generated and analysed for this study can be made available by contacting the corresponding author.

Supplementary material is available online.

Declaration of AI use. AI was used in the generation of some parts of the R stat analysis scripts.

Authors' contributions. L.C.: data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing; C.N.: data curation, investigation, writing—review and editing; M.R.: data curation, formal analysis, investigation, methodology, visualization, writing—original draft; P.M.: data curation, investigation, writing—review and editing; J.A.: investigation; G.N.: investigation, writing—review and editing; P.C.: investigation, writing—review and editing; D.K.: investigation, writing—review and editing; D.L.: investigation, writing—review and editing; B.P.N.: investigation, writing—review and editing; R.C.: investigation, writing—review and editing; A.M.O.: investigation, writing—review and editing; S.J.: investigation, writing—review and editing; S.R.: investigation, writing—review and editing; A.J.: investigation, writing—review and editing; B.M.: investigation, writing—review and editing; J.C.: investigation, writing—review and editing; L.J.: investigation; J.LaC.: investigation, writing—review and editing; S.K.: investigation, methodology, writing—review and editing; J.M.: conceptualization, funding acquisition, investigation, methodology, project administration, writing—review and editing; R.S.: funding acquisition, investigation, methodology, project administration, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interests. We declare we have no competing interests.

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