The blackfly vectors and transmission of *Onchocerca volvulus* in Mahenge, south eastern Tanzania

Adam Hendy[a,*], Andreas Krüger[b], Kenneth Pfarr[c,d], Jacobus De Witte[a], Addow Kibweja[e], Upendo Mwingira[f], Jean-Claude Dujardin[a], Rory Post[g,h], Robert Colebunders[i], Sarah O'Neill[j], Akili Kalinga[e]

[a] *Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium*
[b] *Department of Tropical Medicine, Bundeswehr Hospital, Hamburg, Germany*
[c] *Institute for Medical Microbiology, Immunology and Parasitology, University Hospital Bonn, Bonn, Germany*
[d] *German Center for Infection Research (DZIF), Bonn-Cologne Partner Site, Bonn, Germany*
[e] *National Institute for Medical Research, Tukuyu Research Centre, Tukuyu, Tanzania*
[f] *Neglected Tropical Diseases Control Programme of Tanzania, Dar es Salaam, Tanzania*
[g] *Department of Disease Control, London School of Hygiene and Tropical Medicine, London, United Kingdom*
[h] *School of Natural Sciences and Psychology, Liverpool John Moores University, Liverpool, United Kingdom*
[i] *Global Health Institute, University of Antwerp, Antwerp, Belgium*
[j] *Department of Public Health, Institute of Tropical Medicine, Antwerp, Belgium*

A R T I C L E   I N F O

A B S T R A C T

The Mahenge Mountains onchocerciasis focus in south eastern Tanzania was historically one of the most heavily infected areas in the country. The vectors of *Onchocerca volvulus* are mainly *Simulium damnosum* complex blackflies, but a species of the *Simulium neavei* group may also contribute to transmission in some areas. The only detailed studies of parasite transmission in Mahenge were conducted in the late 1960s. The taxonomy of the *S. damnosum* complex has since been revised and onchocerciasis control through annual community directed treatment with ivermectin (CDTI) commenced in 1997. This study aimed to provide a cytogenetic and molecular update of the *S. damnosum* complex cytoforms present in Mahenge, and to evaluate the current status of *O. volvulus* transmission by blackflies following 19 years of annual CDTI.

Rivers were surveyed to identify sites of *S. damnosum* s.l. breeding among the eastern slopes of the mountains, and human landing collections of adult female blackflies were made close to breeding sites. Identification of *S. damnosum* complex cytoforms was by cytotaxonomy of late-instar larvae and ITS1 amplicon size polymorphisms of larvae and adults. Adult blackflies were pool screened for *O. volvulus* infection using a triplex real-time PCR. The cytoforms 'Nkusi', *Simulium kilibanum* and 'Turiani' were found breeding in perennial rivers. 'Nkusi' and *S. kilibanum* were collected on human bait at 7/7 catch sites and possessed ITS1 profiles most closely resembling the molecular forms 'Nkusi J' and *S. kilibanum* 'T'. Whereas 'Turiani' was present in rivers, it was not collected on human bait and appears to be zoophilic. *Simulium nyasalandicum* was collected in low numbers on human bait at 3/7 catch sites. In total, 12,452 *S. damnosum* s.l. were pool screened and *O. volvulus* infection was detected in 97/104 pools of bodies and 51/104 pools of heads. The estimated percentage of *S. damnosum* s.l. carrying infective L3 stage parasites was 0.57% (95% CI 0.43%–0.74%).

Onchocerca volvulus transmission by *S. damnosum* s.l. is continuing in the Mahenge Mountains after 19 years of annual CDTI. Infection rates appear similar to those reported in the 1960s, but a more detailed study is required to fully understand the epidemiological significance of the ongoing transmission. These results provide further evidence that annual CDTI may be insufficient to eliminate the parasite in formerly hyperendemic foci.

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1. Introduction

1.1. Onchocerciasis in Tanzania

Human onchocerciasis, or river blindness, results from repeated bites of infected blackfly (Diptera: Simuliidae) vectors of the parasitic nematode *Onchocerca volvulus* (Nematoda: Filarioidea) (World Health Organization, 2017). In sub-Saharan Africa, the disease is endemic in 31 countries, although many are now working towards control and elimination (Tekle et al., 2016; World Health Organization, 2016, 2015). Onchocerciasis epidemiology is largely defined by the presence of suitable vector breeding sites (World Health Organization, 2016). These can be in fast-flowing rivers, or smaller riverine habitats of freshwater crab (*Potamonautes* spp.) carriers of certain phoretic blackfly species (Crosskey, 1990). In Tanzania, endemic foci are scattered and are closely associated with the Eastern Arc Mountains and southern highlands where an estimated 4 million people are at risk of the disease (Mweya et al., 2007; Post et al., 2007) .

The main vector of *O. volvulus* in Tanzania is *Simulium damnosum sensu lato* (s.l.). It is primarily responsible for transmission in the Uluguru and Mahenge Mountains, and the Kilosa, Kilombero, Ruvuma and Tukuyu foci (Raybould and White, 1979). Blackflies of the *Simulium neavei* group (*sensu* McMahon 1957), whose immature stages are associated with freshwater crabs, are responsible for transmission in the Usambara and Nguru Mountains (Raybould and White, 1979; Kalinga and Post, 2011; McMahon, 1957). Whereas species within the *S. neavei* group can be identified by adult morphology, *S. damnosum* s.l. is a complex of isomorphic sibling species, sometimes referred to as cytospecies, that are usually identified by fixed or sex-linked inversion differences in their larval polytene chromosomes (Post et al., 2007). The *S. damnosum* complex consists of approximately 60 named cytospecies and cytotypes (chromosomally distinct populations of unconfirmed taxonomic status), collectively known as cytoforms (Post et al., 2007; Adler and Crosskey, 2014). About 26 of these are known from East Africa (Krüger, 2006). Each cytoform differs in distribution, ecology, behaviour and ability to transmit parasites, and correct identification is therefore important in understanding disease epidemiology (Post et al., 2007; Adler et al., 2010). In East Africa, chromosomal identification can sometimes be supplemented with molecular identification based on PCR amplicon size polymorphisms of blackfly ITS1 rDNA (Krüger, 2006).

1.2. The Mahenge focus

The Mahenge Mountains onchocerciasis focus located in Ulanga district, south eastern Tanzania, was historically one of the most heavily infected areas in the country. Disease prevalence was as high as 87% in some communities, but the focus was generally thought to be mesoendemic (microfilarial prevalence 40–59%) (Pedersen and Kolstrup, 1986; Mwaiko et al., 1990; Häusermann, 1969; Geigy et al., 1965; Wegesa, 1970a; World Health Organization, 2001). More recent pre-control epidemiological surveys have since demonstrated that the area was hyperendemic (microfilarial prevalence ≥60%) (World Health Organization, 2001; National Onchocerciasis Control Programme of Tanzania (NOCP), 2000). Mahenge was also the location of the first described cases of nodding syndrome, a childhood seizure disorder which has been associated with *O. volvulus* infection (Dowell et al., 2013). Children with nodding syndrome have been diagnosed at Mahenge Epilepsy Clinic and those affected have come from villages throughout the area, but it is not clear whether all are within the extent of the focus (A Winkler pers. comm.).

The blackflies of Mahenge are known mainly from two studies conducted by Häusermann in the 1960s (Häusermann, 1969, 1966). Of the *S. damnosum* complex cytoforms present, 'Nkusi' was the most abundant and presumed anthropophilic species. 'Sanje' was considered to be zoophilic and the biting behaviour of 'Ketaketa' was unknown (Häusermann, 1969). The list was updated by Raybould and White and included 'Nkusi', 'Sebwe', 'Turiani', 'Hammerkopi' and 'Ketaketa' (Raybould and White, 1979). In addition, the authors stated that 'Turiani' was previously misidentified as 'Nyamagasani' (=*S. kilibanum*). Current taxonomic classifications place 'Nkusi', 'Sebwe' and 'Turiani' within the 'Sanje' subcomplex (Krüger, 2006), while 'Hammerkopi' and 'Ketaketa' have been synonymised with *Simulium plumbeum* which is classified within the 'Ketaketa' subcomplex (Krüger et al., 2006a). *Simulium nyasalandicum* or another undescribed species within the *S. neavei* group (originally thought to be *Simulium woodi*), has occasionally been collected on human bait in Mahenge (Häusermann, 1966; Lewis and Raybould, 1974). *Simulium adersi*, *Simulium bovis* (species-group) and *Simulium vorax* have also been collected during larval and pupal surveys (Häusermann, 1966). Whereas these latter species are occasionally anthropophilic in Tanzania and elsewhere in Africa, they are not known vectors of *O. volvulus* (Raybould, 1966; Wegesa, 1970b; Crosskey, 1957; Wahl and Renz, 1991; Hendy et al., 2017).

Häusermann dissected 12,416 *S. damnosum* s.l. collected on human bait in the Mzelezi Valley between 1966 and 1967 (Häusermann, 1969). He showed that 6.9% (856) of all flies had developing *O. volvulus* infections and 0.68% (85) contained infective L3 stage parasites in the head (L3H) (Häusermann, 1969). At this time, the prevalence of human onchocerciasis in nearby communities was as high as 65.1% (Häusermann, 1969). No *S. nyasalandicum* were collected in the Mzelezi Valley, although it was previously shown that they could ingest microfilariae when fed on an *O. volvulus* infected individual, and that these developed to 'sausage forms' of first stage (L1) larvae which were found in the thoracic flight muscles (Häusermann, 1966). However, there was no evidence that they developed to infective stages.

1.3. Onchocerciasis control and evaluation

Attempts to control onchocerciasis in Mahenge started in 1994 through a vertical programme of mass drug administration (MDA) with ivermectin (National Onchocerciasis Control Programme of Tanzania (NOCP), 2000). In 1997, the control strategy changed to a more effective community-based treatment approach, before annual community directed treatment with ivermectin (CDTI) was implemented by the African Programme for Onchocerciasis Control as an appropriate and cost-effective means of large-scale and sustainable drug distribution (National Onchocerciasis Control Programme of Tanzania (NOCP), 2000). There have been no attempts at vector control in the area since the late 1960s (Häusermann, 1969).

The most recent estimates of onchocerciasis prevalence in Mahenge were ascertained following skin snip evaluations carried out in 10 villages in 2009 (Tekle et al., 2016). At this time, there had been seven annual CDTI rounds with > 60% therapeutic coverage (defined as the proportion of the total population receiving treatment). The mean village microfilarial prevalence of 8.3% (max. 21.9%) was much lower than ONCHOSIM modelled estimates of 43.8%, suggesting that the focus was progressing towards elimination faster than expected (Tekle et al., 2016). According to World Health Organization guidelines, the anticipated duration of treatment phases of MDA programmes should typically last between 12 and 15 years, and should continue with a minimum 80% annual therapeutic coverage until *O. volvulus* transmission is interrupted (World Health Organization, 2016). Pool screen analysis of blackflies should then be used to demonstrate interruption of transmission before a focus enters a phase of post-treatment surveillance. This requires testing a minimum 6000 blackflies from across the focus and demonstrating that the upper bound of the 95% confidence interval of those carrying infective L3H parasites is < 0.05% (< 1/2000 in all flies assuming a parity rate of 50%) (World Health Organization, 2016).

1.4. Objectives

In the 50 years since Häusermann published his work in Mahenge, the taxonomy of the *S. damnosum* complex has been revised and onchocerciasis has been targeted for elimination. This study aimed to provide a cytogenetic and molecular update of the *S. damnosum* complex cytoforms present in Mahenge, and to evaluate the current status of *O. volvulus* transmission by blackflies following 19 years of annual CDTI.

2. Materials and methods

2.1. Study area

The Mahenge Mountains rise to approximately 1500 m at their highest point and are situated between 8°24′ and 9°00′ S, and 36°00′ and 37°00′ E in Ulanga district, south eastern Tanzania (Häusermann, 1969). Annual rainfall is between 1000 mm and 1500 mm, and occurs mainly between November and May (Häusermann, 1966; Lovett and Pocs, 1993). Perennial rivers that provide suitable habitats for *S. damnosum* s.l. breeding include the Luli in the north, the Mbalu and Lukande rivers in the east, and the Mzelezi, Ruaha and Msingizi rivers in the south (Fig. 1, Section 3.1) (Häusermann, 1969). Whereas *S. damnosum* s.l. breeding and biting takes place throughout the focus, *S. nyasalandicum* appears to be restricted to higher altitudes and biting has only been recorded near Sali and Mahenge (Häusermann, 1966). Detailed descriptions of seasonal changes in blackfly breeding and biting are provided elsewhere (Häusermann, 1969, 1966). The 2012
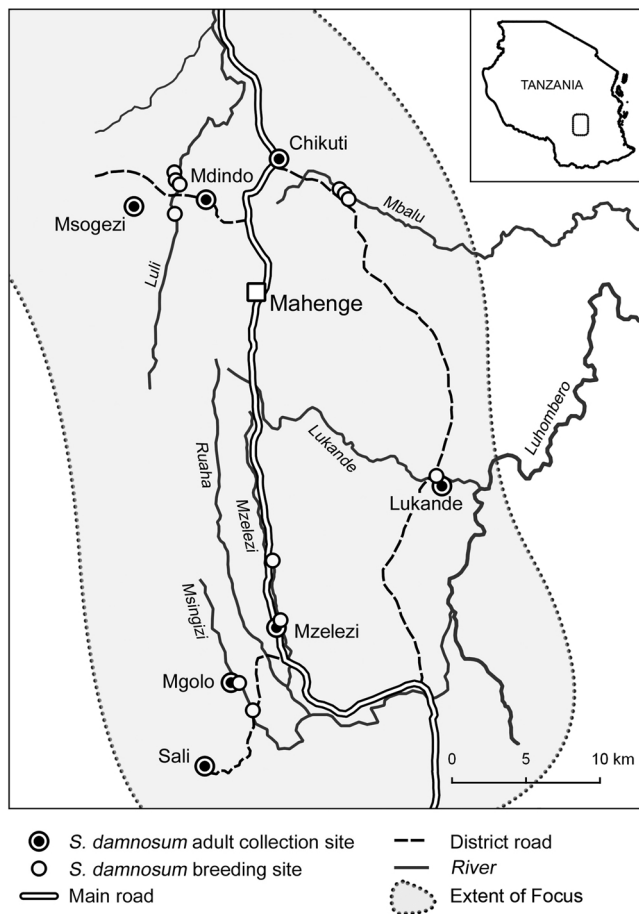

Fig. 1. Map of *S. damnosum* s.l. breeding and adult collection sites. The shaded area represents the approximate extent of the onchocerciasis focus on the eastern slopes of the Mahenge Mountains as defined by Häusermann (Häusermann, 1966). Inset shows the location of the study area in south eastern Tanzania.

population census reported 265,203 people living in Ulanga district (National Bureau of Statistics, 2013), where the majority ethnic group are the Pogoro (Häusermann, 1969; Winkler et al., 2008). Animals kept include chickens, goats and occasionally pigs. Cattle are rare in Mahenge and in the past were only kept by the missions, although some migrant populations have brought small numbers to the area in recent years (J Irani pers. comm.) (Häusermann, 1969).

2.2. Collection and preservation of blackflies

Communities in villages that were historically meso- or hyperendemic for onchocerciasis were identified in consultation with the Programme Manager for Neglected Tropical Diseases (Dr A Kilimba) at Mahenge Hospital. Larvae were collected from rocks and vegetation in rivers near these villages in January 2015 and June 2016, and were fixed in three changes of Carnoy's fixative ($\approx$ 3: 1 ethanol: glacial acetic acid) for cytotaxonomic study. Pupae were collected and preserved in the same way, but were subsequently transferred to absolute ethanol in the laboratory.

Adult blackfly collections were timed to coincide with periods of peak biting activity and *O. volvulus* transmission at the end of the rainy season in June and July 2016. This was approximately five months after the previous ivermectin treatment round, which took place between 30th December 2015 and 30th January 2016 (A Kilimba, pers. comm.). Two people (vector collectors) from each of the villages surveyed for blackfly breeding were trained in standard 'human landing collection' methods for the collection of adult blackflies (Walsh et al., 1978). Trial catches were conducted for a single day between 07:00 and 18:00 to identify sites of highest blackfly activity, before routine collections were carried out at the most productive sites. Collections were not fully supervised, although spot-checks were conducted and regular mobile phone communication was maintained with the collectors. Adult blackflies were preserved in absolute ethanol and delivered weekly to the field station in Mahenge town.

All immature and adult specimens were kept in the dark at ambient temperatures for the duration of the field work, before being stored at −20 °C upon returning to the laboratory.

2.3. Identification of blackflies

Simulium damnosum complex larvae were identified morphologically by the presence of dorsal abdominal tubercles and scales on the prothoracic proleg (Crosskey, 1990). Late-instar larvae, pupae and adult blackflies were otherwise identified where possible using morphological keys in Freeman and de Meillon (1953). Adults of the *S. neavei* group were identified using keys in Lewis and Raybould (1974) and were compared with reference specimens at the Natural History Museum (London, UK), including those collected by Häusermann (1969, 1966).

Prior to cytotaxonomic study, heads and thoraces of late-instar *S. damnosum* s.l. larvae were removed from specimens in the laboratory and were stored individually in absolute ethanol for ITS1 analysis. Salivary glands were then dissected from abdominal cavities of associated specimens and chromosomes were prepared for cytotaxonomy following a Feulgen-staining method outlined by Adler et al. (2004). Larvae were identified with reference to published chromosome maps (Boakye, 1993; Procunier and Muro, 1993; Krüger, 2006).

ITS1 amplicon size polymorphisms of *S. damnosum* complex larvae and adults were interpreted with reference to Krüger (2006). DNA was extracted using QIAGEN DNeasy Blood & Tissue Kits (Qiagen, N.V.) and amplified using ITS1 Fw and ITS1 Rev primers (Table 1) and a modified protocol based on methods outlined by Tang et al. (1996). Reactions were carried out in 25 μL total volumes containing 10 pmol of each primer, 5 μL template DNA and GoTaq® G2 Hot Start Colorless Master Mix (Promega Benelux B.V.). Cycling conditions involved Taq polymerase activation at 95 °C for 2 mins, followed by 35 cycles at 90 °C for

Table 1

Primers and hybridisation probe sequences and concentrations used in the ITS1 and triplex PCRs. Ov = *Onchocerca volvulus*, Oo = *Onchocerca ochengi*.

PCR	Primers	Sequence and Modification	Concentration (nM)
ITS1	ITS1 Fw	TTACTATCTTATTTCCACAA	400
ITS1	ITS1 Rev	CCCCTGTCTAGATGTTAT	400
Triplex real-time	OvOo ND5 forward	GCTATTGGTAGGGGTTTGCAT	300
Triplex real-time	OvOo ND5 reverse	CCACGATAATCCTGTTGACCA	300
Triplex real-time	Ov probe	Fam-TAAGAGGTTATTGTTTATGCAGATGG-BHQ1	100
Triplex real-time	Oo probe	Hex- TAAGAGATTGTTGTTTATGCAGATAGG-BHQ1	100
Triplex real-time	16S rDNA forward	AATTACTCCGGAGTTAACAGG	500
Triplex real-time	16S rDNA reverse	TCTGTCTCACGACGAACTAAAC	500
Triplex real-time	16S rDNA probe	Cy5-TACAACATCGATGTAGCGCAGC-BBQ-650	150

60 s, 45 °C for 60 s, and 72 °C for 60 s, before a final extension at 72 °C for 5 mins. Amplicons were run on 2% (w/v) agarose gels, stained with ethidium bromide and visualised under UV light.

2.4. Pool screening

Adult *S. damnosum* s.l. were prepared in pools of heads and corresponding bodies according to collection site. Flies with visibly distended abdomens, which may have blood fed on vector collectors, were discarded prior to pooling. Heads were then separated from bodies of the remaining flies in glass petri dishes using No.3 entomological pins and a dissecting microscope. Petri dishes were washed with 0.5% sodium hypochlorite (NaClO) and pins were sterilised using a FIREBOY safety Bunsen burner (INTEGRA Biosciences, Switzerland) after each use to reduce the risk of contamination. Pooled samples were placed in 2 mL microcentrifuge tubes and incubated overnight to allow excess ethanol to evaporate. Samples were disrupted using a FastPrep-24™ (MP Biomedicals, LLC) homogeniser before DNA was extracted using QIAGEN DNeasy Blood & Tissue Kits (Qiagen, N.V.).

Prior to pool screening, extracts were tested for PCR inhibiting factors as described previously (Colebunders et al., 2016). If detected, samples were diluted 1:10 or until no PCR inhibition remained (usually 1:100 or 1:1000). Pooled samples were then analysed using a triplex real-time PCR that differentiates *O. volvulus* from *Onchocerca ochengi* (a bovine parasite also transmitted by *S. damnosum* s.l.) based on differences in respective ND5 genes (GenBank: AY462885.1 and FM206483.1). The PCR also includes genus-specific primers and hybridisation probes for 16S rDNA genes. Reactions were carried out using a Rotor Gene 6000 cycler (Qiagen, Hilden, Germany) in 20 μL total volumes containing 2 μL template DNA, 1X HotStar *Taq* Buffer (Qiagen, N.V.), 4.5 mM MgCl$_2$, 40 mM dNTP, 2.5 units HotStar *Taq*, and primers and hybridisation probes listed in Table 1. Cycling conditions involved Taq polymerase activation at 95 °C for 15 min, followed by 45 cycles at 95 °C for 10 s and 61 °C for 30 s with fluorescence acquisition on the Fam, Hex and Cy5 channels at the end. Plasmids containing the respective sequences were used as PCR positive controls in every run (Colebunders et al., 2016).

A positive pool of bodies was interpreted as being infected with microfilariae or developing *O. volvulus* larvae, whereas a positive pool of heads was interpreted as containing infective L3H parasites. Poolscreen v2.0 (Katholi, 2010) was used to estimate *O. volvulus* infection rates in pools of unequal size, with 95% confidence intervals. Transmission potentials were not calculated due to the short duration of the study, which would produce artificially high estimates.

2.5. Ethics statement

Blackfly collections involving human participants were subject to review and approval by the Institutional Review Board at the Institute of Tropical Medicine, Antwerp, Belgium (1089/16) and the Medical Research Coordinating Committee at the National Institute for Medical Research, Dar es Salaam, Tanzania (NIMR/HQ/R.8a/Vol.IX/2212).

Vector collectors were from local communities and were already participating in the CDTI programme as 'Community Drug Distributors'. They were receiving annual ivermectin treatment in accordance with the National Onchocerciasis Control Programme and were also given ivermectin before participating in blackfly collections in order to reduce their skin microfilarial loads. All participants were adults over the age of 18 years who provided written informed consent.

3. Results

3.1. Identification of blackflies

Twenty one riverine sites surveyed in January 2015 and June 2016 were positive for blackfly larvae or pupae, while twelve of these were positive for *S. damnosum* s.l. (Table 2). The *S. damnosum* complex was collected from rivers close to adult blackfly collection sites, with the exception of Sali, a relatively isolated mountain community situated above 850 m in the south of the focus (Fig. 1, Table 3). Blackflies of other species were abundant in the Mbezi River at Sali, and included *S. vorax*, which was identified by the morphology of respiratory organs dissected from three mature larvae, and those present on a single pupa (Table 2). *Simulium vorax* was also present in the Luli and Mbalu rivers, while *S. adersi* was collected from the Mzelezi and Lukande rivers. No blackflies of the *S. bovis* species-group were found.

The cytoforms 'Nkusi', *S. kilibanum* and 'Turiani' were identified based on analysis of larval chromosomes and ITS1 rDNA. Inversions were only present in chromosome arms 2L and 3S of the larvae studied. The remaining chromosome arms in all specimens corresponded to standard sequences found in *S. kilibanum*, which is the chromosomal standard for the complex (Table 3). All 74 specimens from four sites along the Luli River possessed inversion 2L-5 which is fixed in 'Nkusi', but polymorphic in *S. kilibanum* and 'Turiani' (Fig. 2). A further 18/20 specimens from the Mbalu River also possessed 2L-5, whereas the remaining two were chromosomally standard. In five specimens from rivers south of Mahenge, four were standard and one was heterozygous for inversion 2L-5. Two male specimens, one from the Luli River and one from the Msingizi River, showed inversion 3S/1, which is sex-linked and diagnostic for 'Turiani' cytoform (Fig. 2).

Molecular identification of larvae collected in 2015 showed that 6/14 analysed from the Luli, Mbalu and Mzelezi rivers produced 310 (+450) bp ITS1 amplicons, while 8/14 produced 310 + 380 (+450) bp amplicons (Fig. 3). Larvae with both of these ITS1 profiles were also found sympatrically in the Mzelezi and Msingizi rivers in 2016, although specimens producing 310 (+450 bp) amplicons were more common in the Mzelezi. Whereas many specimens exhibited 450 bp ITS1 amplicons that have not been previously reported, the cytological and molecular profiles most closely resemble *Simulium kilibanum* 'T', which produces 310 (+340) bp amplicons, and 'Nkusi J' which produces 310 + 380 bp amplicons (Krüger, 2006; Krüger et al., 2006b). The Mahenge specimens may represent genetic variants of these cytoforms. ITS1 amplicon sizes of the two male 'Turiani' larvae (270 bp) were consistent with previous findings (Fig. 3) (Krüger, 2006). A

Table 2

Sites of blackfly breeding, indicating presence/absence of *S. damnosum* s.l. and other species identified based on morphology of the pupal respiratory organ.

Collection Dates	Nearest Village	River	Latitude	Longitude	Altitude	S. damnosum	Other Larvae	S. adersi	S. hargreavesi	S. hirsutum	S. mcmahoni	S. rotundum	S. vorax
10, 16, 17/ 01/15	Mdindo/ Msogezi	Luli	−8.609717	36.665633	513m	✓	270				5		
10/01/2015		Luli	−8.614200	36.667600	527m	✓	89				14		
10/01/2015		Luli	−8.616817	36.670017	530m	✓	153						1
16/01/2015		Luli	−8.622133	36.664733	538m	✓	13						
17/01/2015	Mdindo	Luli	−8.634883	36.667050	569m	✓	145				2		
11/01/2015	Chikuti	Mbalu	−8.623517	36.771450	423m	✓	4						
11/01/2015		Mbalu	−8.626433	36.770833	431m	✓	16						
11/01/2015		Mbalu	−8.628900	36.768183	415m	✓	7						
18/01/2015	Mahenge	?	−8.657883	36.723300	806m		35		8		3		
12/01/2015	Mzelezi	Mzelezi	−8.840200	36.725400	526m		9						
12/01/2015		Mzelezi	−8.848683	36.725350	480m	✓	1						
12/01/2015		Mzelezi	−8.866733	36.728500	432m		60	2			6		
13/01/2015		Mzelezi	−8.868617	36.728317	437m		38						
13/01/2015		Mzelezi	−8.810650	36.720567	543m		3						
14/01/2015	Sali	Mbezi	−8.977783	36.679367	859m		21						
14/01/2015		Mbezi	−8.960833	36.685800	924m		233			2		1	4
14/01/2015	Isyaga	Msingizi	−8.940300	36.717533	446m	✓	78				7		
15/01/2015	Lukande	Lukande	−8.790600	36.835250	342m		47	1			11		
15/01/2015		Lukande	−8.790833	36.828333	346m	✓	8						
17, 20/06/ 2016	Mdindo/ Msogezi	Luli	−8.609717	36.665633	513m	✓	51						1
22/06/2016	Chikuti	Mbalu	−8.628900	36.768183	415m	✓	111		2				7
27/06/2016	Mzelezi	Mzelezi	−8.886917	36.732083	333m	✓	3		1				
8/06/2016	Mgolo	Msingizi	−8.920950	36.709450	465m	✓	23						
27/06/2016	Isyaga	Msingizi	−8.940300	36.717533	446m	✓	7						

further three female larvae and one of undetermined sex, from the Mzelezi and Msingizi rivers, exhibited 270 bp amplicons and probably represent the same cytoform (Table 4). 'Sanje' cannot be excluded as it also produces a 270 bp amplicon (Krüger, 2006), however, given the known presence of 'Turiani' and the lack of chromosomal evidence for Sanje, 'Turiani' is the most likely designation.

Of the adult *S. damnosum* s.l. collected on human bait in 2016, 16/57 produced 310 (+450) bp, and 38/57 produced 310 + 380 (+450) bp amplicons (Table 4). Specimens with these ITS1 profiles were collected at all adult catch sites. Four specimens collected from or near the Mzelezi and Msingizi rivers had additional ITS1 profiles (Table 4). One larva and one adult from the Mzelezi River each exhibited ≈ 380 + 450 bp ITS1 amplicons, while two adults caught at Mgolo each had ≈ 340 + 380 bp amplicons. No (0/57) adult blackflies collected on human bait produced 270 bp amplicons, and 'Turiani' therefore appears to be zoophilic.

3.2. Adult collections and pool screening

Routine adult blackfly collections were made for a maximum 17 days at the seven sites with highest biting activity between 13 June and 1 July (Fig. 1), yielding 16,911 *S. damnosum* s.l. and 32 *S. nyasalandicum* (Table 5). Whereas *S. damnosum* s.l. was collected on human bait at all sites, *S. nyasalandicum* was only identified from collections at Sali, Mgolo and Msogezi and represented 0.19% of the total catch. The morphology of *S. nyasalandicum* agreed with previous descriptions of specimens collected from Mahenge, lacking a band of copper scales on the fourth abdominal tergite and having minute or absent teeth on the tarsal claws (Lewis and Raybould, 1974).

Of the total adult catch, 12,452 *S. damnosum* s.l. were prepared in 104 pools of heads and bodies respectively, with pool-sizes ranging from 56 to 185 (mean 120). Results of the triplex real-time PCR showed that 97/104 pools of bodies and 51/104 pools of heads were infected

Table 3

Sites of *S. damnosum* s.l. breeding in January 2015 and June 2016, and inversions present on chromosome arms 2L and 3S. Only material collected in January 2015 was adequately preserved for cytotaxonomy. 'No.' = number of chromosome preparations made from larvae at each site.

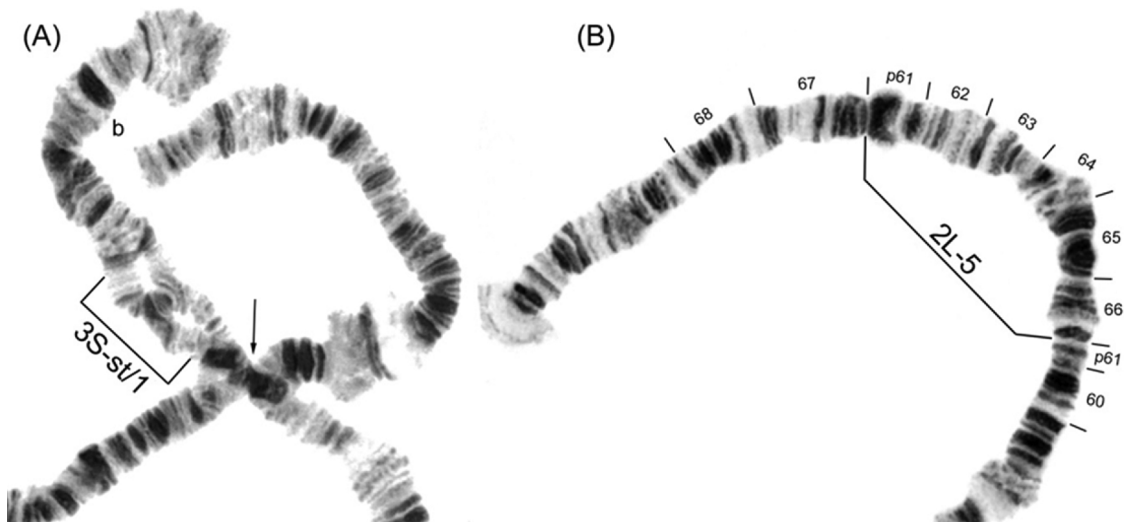| Year	Month	Nearest Village	River	Latitude	Longitude	Alt.	Larvae	Pupae	Cytotaxonomy					
									No.	2L-st	2L-5	2L-5/st	3S-st	3S-1/st
2015	January	Chikuti	Mbalu	−8.623517	36.771450	423m	3		3		3		3	
2015	January		Mbalu	−8.626433	36.770833	431m	15	2	7	1	6		7	
2015	January		Mbalu	−8.628900	36.768183	415m	25	8	10	1	9		10	
2015	January	Mdindo/Msogezi	Luli	−8.609717	36.665633	513m	174	18	44		44		43	1
2015	January		Luli	−8.614200	36.667600	527m	22	1	10		10		10	
2015	January		Luli	−8.616817	36.670017	530m	32	12	19		19		19	
2015	January	Mdindo	Luli	−8.634883	36.667050	569m	2		1		1		1	
2015	January	Lukande	Lukande	−8.790833	36.828333	346m		1						
2015	January	Mzelezi	Mzelezi	−8.848683	36.725350	480m	5	1	2	1		1	2	
2015	January	Isyaga	Msingizi	−8.940300	36.717533	446m	4		3	3			2	1
2016	June	Chikuti	Mbalu	−8.628900	36.768183	415m	7							
2016	June	Mdindo/Msogezi	Luli	−8.609717	36.665633	513m	79	1						
2016	June	Mzelezi	Mzelezi	−8.886917	36.732083	333m	21	6						
2016	June	Mgolo	Msingizi	−8.920950	36.709450	465m	177							
2016	June	Isyaga	Msingizi	−8.940300	36.717533	446m	35							
							Total	601	50	99	6	92	1	97	2

Fig. 2. *S. damnosum* s.l. chromosomes showing A) male sex-linked heterozygous inversion 3S-st/1, diagnostic for 'Turiani' cytoform, and also ectopic pairing of centromeres 2 and 3 (arrow); B) homozygous inversion 2L-5, which is fixed in 'Nkusi' and polymorphic in *S. kilibanum* and 'Turiani' cytoforms. 'b' = blister.
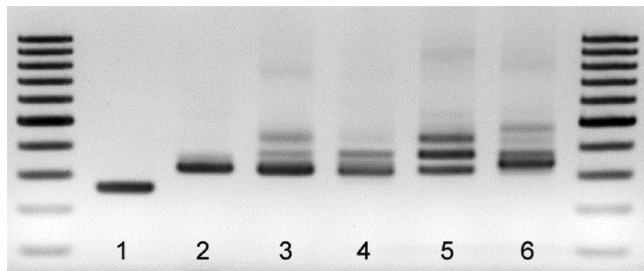

Fig. 3. Representative ITS1 banding patterns visualised alongside 100 bp DNA ladders (Thermo Scientific, Lithuania), showing variation among specimens. Interpreted as 1) 'Turiani' 270 bp, 2) *S. kilibanum* 310 bp, 3) *S. kilibanum* 310 + 450 bp, 4) 'Nkusi J' 310 + 380 bp, 5) 'Nkusi J' 310 + 380 + 450 bp, 6) Unknown ≈ 340 + 380 bp. Banding pattern 380 + 450 bp not shown.

with *O. volvulus*, and that positive pools of bodies and heads were detected at all seven catch sites. An estimated 0.57% (95% CI 0.43%–0.74%) of *S. damnosum* s.l. screened from the Mahenge Mountains contained infective L3H parasites (Table 5). A single pool of

S. nyasalandicum bodies collected at Sali was also positive for *O. volvulus* infection. No pools of either species were infected with *O. ochengi*.

4. Discussion

4.1. Simulium damnosum complex

Members of the *S. damnosum* complex previously reported from Mahenge include 'Nkusi', 'Sebwe', 'Turiani' and *S. plumbeum* (= 'Hammerkopi' and 'Ketaketa' cytoforms), and it was thought that 'Turiani' had been misidentified as *S. kilibanum* (Raybould and White, 1979; Krüger et al., 2006a; Procunier and Muro, 1993). Of these, 'Nkusi' was identified as the dominant cytoform and likely man-biting species by Häusermann (Raybould and White, 1979; Häusermann, 1969).

The molecular form, 'Nkusi J', is the assumed vector in the Uluguru Mountains, approximately 200 km north east of Mahenge, and anthropophilic 'Nkusi' *sensu* Dunbar (1969) is also known from nearby Kilosa focus where its vectorial status is unconfirmed (Raybould and White, 1979; Procunier and Muro, 1993; Krüger et al., 2006b; Dunbar, 1978). The 'Nkusi' collected during the current study possessed the fixed

Table 4
ITS1 amplicon sizes of *S. damnosum* s.l. larvae (L) and adults (A) collected in rivers and nearby villages in January 2015 and June 2016.

Year	Month	Nearest Village	River	ITS1 (bp)							
				310 (+450)		310 + 380 (+450)		270		Other	
				L	A	L	A	L	A	L	A
2015	January	Chikuti	Mbalu	3		5					
2015	January	Mdindo/Msogezi[a]	Luli	2		2		1			
2015	January	Mzelezi	Mzelezi	1		1		1			
2015	January	Isyaga	Msingizi					3			
2016	June	Idunda[b]	Luli				3				
2016	June	Chikuti	Mbalu		1		7				
2016	June	Mdindo/Msogezi[a]	Luli		6		10				
2016	June	Lukande	Lukande		1		2				
2016	June	Mzelezi	Mzelezi	11	3	1	4			1[c]	1[c]
2016	June	Mgolo	Msingizi	7	2	8	4	1			2[d]
2016	June	Ruaha[b]	Ruaha				3				
2016	June	Sali	Mbezi		3		5				
			Total	24	16	17	38	6	0	1	3

[a] Collections combined as the Luli River flows between the villages and adult catch sites were in close proximity.

[b] Trial catch sites not included in routine collections.

[c] ITS1 bands approx. 380 + 450 bp.

[d] ITS1 bands approx. 340 + 380 bp.

Table 5

Results of adult blackfly collections and pool screen analysis at each of the seven routine collection sites. Ov PCR +ve = number of pools positive for O. volvulus infection by real-time PCR, L3H = percentage of blackflies with infective L3 stage O. volvulus parasites in their heads.

										Ov PCR +ve		Infection Rate (Heads)[†]		
Collection Dates	Location	Latitude	Longitude	Alt.	No. Days	Total Catch	Mean Daily Catch	No. Pooled	No. Pools[*]	Bodies	Heads	L3H	95% CI -/+	
Simulium damnosum s.l.														
13 Jun–01 Jul	Msogezi	−8.630350	36.641916	603m	17	4273	251.4	2056	16	11	6	0.37%	0.13%	0.83%
13 Jun–01 Jul	Mdindo	−8.626194	36.686272	548m	17	4157	244.5	3210	25	25	15	0.72%	0.38%	1.26%
13 Jun–01 Jul	Chikuti	−8.602917	36.734533	459m	17	3001	176.5	2681	27	27	8	0.36%	0.14%	0.72%
13 Jun–01 Jul	Mgolo	−8.920950	36.709450	465m	17	2589	152.3	2164	15	15	7	0.43%	0.16%	0.92%
13 Jun–01 Jul	Mzelezi	−8.886916	36.732083	333m	17	1812	106.6	1423	11	11	6	0.62%	0.21%	1.43%
13 Jun–25 Jun	Sali	−8.974883	36.685466	876m	12	672	56.0	614	6	6	5	1.65%	0.46%	4.23%
13 Jun–25 Jun	Lukande	−8.805533	36.830566	355m	12	407	33.9	304	4	2	4	–	–	–
				Total	109	16911	–	12452	104	97	51	Overall Infection Rate	0.57%	0.43% 0.74%
Simulium nyasalandicum														
13 Jun–25 Jun	Sali	−8.974883	36.685466	876m	12	16	1.3	15	1	1	0	–	–	–
13 Jun–01 Jul	Mgolo	−8.920950	36.709450	465m	17	13	0.8	12	1	0	0	–	–	–
13 Jun–01 Jul	Msogezi	−8.630350	36.641916	603m	17	3	0.2	3	1	0	0	–	–	–
				Total	46	32	–	30	3	1	0	Overall Infection Rate	–	– –

* Number of corresponding head and body pools.

† Infection rates in bodies were not estimated due to high proportion of positive pools.

inversion 2L-5 and produced 310 + 380 (+450) bp ITS1 amplicons. Some of these specimens differed from 'Nkusi J' by the presence of a 450 bp amplicon, and may represent genetic variants of this cytoform. Larvae were present in the Mbalu and Luli rivers north of Mahenge, and although chromosomal data from south of Mahenge were limited, larvae present in the Mzelezi and Msingizi rivers produced similar ITS1 banding patterns. The 310 + 380 (+450) bp pattern was also observed in 38/57 adult female blackflies from all catch sites. This included Sali at 876m, where *S. damnosum* s.l. larvae were not found. The absence of these larvae at Sali may reflect the behaviour of 'Nkusi J' in the Uluguru Mountains, where it only breeds in rivers between 100–500 m, yet bites across the full altitudinal range (Krüger et al., 2006b). 'Nkusi' does, however, breed at higher altitudes elsewhere (Krüger et al., 2006b; Maegga et al., 2010).

The study also confirms the existence of both *S. kilibanum* and 'Turiani' cytoforms in Mahenge. Identification of *S. kilibanum* was based on the presence of 2L-st/st, 2L-5/st and 2L-5/5 karyotypes and accompanying single 310 bp ITS1 amplicons. Two molecular forms of *S. kilibanum* have previously been reported by Krüger (2006). *Simulium kilibanum* 'T' (310 (+340) bp), which occurs in southern Tanzania and Malawi but does not transmit *O. volvulus*, and *S. kilibanum* 'U' (290 bp), which is a vector in western Uganda (Krüger, 2006; Mustapha et al., 2005; Vajime et al., 2000; Krüger et al., 1999). The ITS1 profiles of 16/57 *S. damnosum* s.l. collected on human bait produced 310 (+450) bp amplicons, more closely resembling *S. kilibanum* 'T' than 'U'. This ITS1 profile was present in adult blackflies collected at each of the seven routine catch sites. Again, 450 bp amplicons were present in some specimens, but they appeared weaker than in 'Nkusi'. It is impossible to tell the taxonomic implications of these additional amplicons based on current data, although given the apparent diversity of cytoforms present in Mahenge, there may be occasional hybridisation. DNA sequence analyses of ITS1 amplicons and additional gene fragments such as ITS2 or mitochondrial genes, which are known for their phylogenetic information, might help to clarify relationships between molecular variants of these cytoforms (Krüger and Hennings, 2006). Mahenge may represent a cytogenetic 'melting pot', similar to a situation in an area of western Uganda where a highly polymorphic *S. kilibanum* population has been reported (Krüger, 1998).

'Turiani' was identified based on the diagnostic sex-linked heterozygous inversion 3S/1 which was present in two males, both of which produced 270 bp ITS1 amplicons. Females with standard karyotypes present in the Mzelezi and Msingizi rivers also produced 270 bp amplicons and probably represent the same cytoform. No flies collected on human bait produced 270 bp amplicons, suggesting that 'Turiani' in Mahenge, like elsewhere in Tanzania, is probably zoophilic (Raybould and White, 1979; Krüger, 2006; Häusermann, 1969).

'Sebwe' and 'Ketaketa' subcomplex cytoforms were not identified chromosomally during the current study, although material suitable for chromosome preparations from rivers south of Mahenge was limited. However, ITS1 amplicons from 4/105 larval and adult specimens showed banding patterns that did not correspond to the cytoforms 'Nkusi', *S. kilibanum*, or 'Turiani'. Two of the four specimens, one larva and one adult from the Mzelezi River, produced ≈380 + 450 bp amplicons, while the two adults caught at Mgolo produced ≈340 + 380 bp amplicons. These banding patterns could potentially represent members of the 'Ketaketa' subcomplex which are known to exhibit multiple ITS1 bands ranging in size from 250 to 380 bp (Häusermann, 1969; Krüger et al., 2006a). Another possible identity is *S. thyolense*, which is anthropophilic in neighbouring Tukuyu and Ruvuma foci and produces 340 (+380) bp amplicons, although it has not previously been reported from Mahenge (Krüger, 2006; Maegga et al., 2010). However, without additional chromosomal evidence it is not possible to determine whether the unidentified specimens represent either of these cytoforms.

4.2. Simulium neavei group

Simulium nyasalandicum was the only other blackfly species collected on human bait during the study. Its distribution in Tanzania is widespread, and the species is anthropophilic in the Nguru Mountains where it may be a vector (Raybould and White, 1979; Lewis and Raybould, 1974). There is also evidence of anthropophily in the Usambara Mountains, Kilosa, Ruvuma and Tukuyu foci (including Njombe) (Raybould and White, 1979; Lewis and Raybould, 1974; Maegga et al., 2010; Pedersen and Maegga, 1985). During the current study, *S. nyasalandicum* was collected at Sali (876m), Mgolo (465m) and Msogezi (459m), although not in the adjacent village of Mdindo. This correlates well with the distribution reported by Häusermann, who collected biting females at Sali and occasionally Mahenge (Häusermann, 1969, 1966). The heavily forested areas around the Mbezi River in Sali are likely to provide ideal breeding habitats for this species. Msogezi village is also situated on the edge of Myoe, a forest reserve which rises from 800 to 1300 m and is the source of multiple rivers, including the Mwezeza, which feeds the Luli (Lovett and Pocs, 1993). It is possible that suitable breeding habitats exist within the reserves that enable *S. nyasalandicum* to bite on the forest fringes. However, the role of *S. nyasalandicum* in *O. volvulus* transmission is still likely to be minimal. The species is highly zoophilic elsewhere in Tanzania (Raybould, 1969), and human landing rates averaged just 0.7 flies/day over a combined 46 days at three collection sites during the current study. In addition, Häusermann reported that *S. nyasalandicum* was always rare on *Potamonautes* spp. crabs in Mahenge, and only showed limited evidence for the development of *O. volvulus* (Häusermann, 1966).

4.3. Onchocerca volvulus transmission

Onchocerca volvulus was detected in pools of bodies and heads of *S. damnosum* s.l. caught at each of the routine collection sites and an estimated 0.57% (95% CI 0.43% - 0.74%) of all pool screened flies carried infective L3H parasites. Infection rates were clearly above the 0.05% threshold for interruption of transmission (World Health Organization, 2016). They also appeared similar to those recorded by Häusermann who found L3H in 0.61% of 7340 flies dissected between March and July 1967. However, it is necessary to note that the methods of calculating transmission indices between the two studies were very different and the current study did not take into consideration parity rates among pool screened flies (Häusermann, 1969). It is therefore difficult to fully understand the epidemiological significance of these data. Nevertheless, the infection rates were higher than expected considering that CDTI had been taking place for 19 years and skin snip evaluations carried out in 2009 revealed a mean village microfilarial prevalence of 8.3% and a maximum community microfilarial load of 2.2 (Tekle et al., 2016). This evaluation by Tekle et al. was conducted 11–12 months after the previous ivermectin treatment round and immediately prior to the next, when skin microfilarial densities should be at their peak (Tekle et al., 2016). The observed microfilarial prevalence was significantly lower than the ONCHOSIM predicted mean of 43.8%, indicating that progress towards elimination was faster than expected (Tekle et al., 2016). While skin snip microscopy is an acceptable method for evaluating the progress of CDTI programmes, the method is susceptible to the effects of ivermectin and may lack sensitivity if disease prevalence is low (Tekle et al., 2016; World Health Organization, 2016). In contrast, entomological evaluations are highly sensitive indicators of changes in community microfilarial load that correlate well with ivermectin coverage (World Health Organization, 2001).

Ivermectin has been shown to effectively suppress the uptake of skin microfilariae by blackflies for up to 6 months after treatment (Cupp et al., 1986). The current entomological study took place approximately 5 months after the previous CDTI round, and while it is likely that the effects of ivermectin had diminished at the time of blackfly collections,

the observed rates of infection may also indicate problems with drug coverage and/or adherence. The reported therapeutic coverage of ivermectin in Ulanga district was above 65% (mean 76%) for the years 2003–2015 (Tanzania Ministry of Health, unpublished data). However, in two Cameroonian foci with similarly high therapeutic coverages (> 65% for the years 2002–2014), poor drug adherence is thought to have contributed to persisting levels of mesoendemicity (Kamga et al., 2016).

Despite the apparent difficulties in comparing these past and present infection rates, this study confirms several recent observations that the current strategy of annual CDTI is unlikely to interrupt *O. volvulus* transmission in all foci, and particularly those with high pre-control disease prevalence (Kamga et al., 2016; Katabarwa et al., 2013; Lamberton et al., 2015). While the annual CDTI strategy may be sufficient to eliminate clinical disease in Mahenge, new infections will continue to occur which will inevitably extend the required treatment duration. Alternative approaches to onchocerciasis control should therefore be considered in underperforming foci. Both biannual CDTI and biannual CDTI supplemented with vector control have recently been effective in interrupting transmission in foci in Uganda and South Sudan, and these strategies should be considered elsewhere (Higazi et al., 2013; Zarroug et al., 2016; Lakwo et al., 2017).

4.4. Limitations

The study was mainly limited by relying exclusively upon pool screening to determine transmission indices. Infection rates in *S. damnosum* s.l. bodies were not calculated as the high percentage (93%) of positive pools would have resulted in unreliable estimates. Analysis of smaller pool sizes would therefore have been more appropriate in this context. It was also not possible to evaluate the relative roles of 'Nkusi J' and *S. kilibanum* 'T' in transmission using the pool screening method alone. There is currently no evidence for the occurrence of mixed *S. damnosum* s.l. vector populations in Tanzania (Krüger, 2006). However, adopting a molecular approach to identifying a subsample of individually dissected flies and parasites might have confirmed whether this is the case in Mahenge. Supplementing molecular analyses with morphological classification of flies according to wing tuft category or colour class might have been appropriate in different circumstances, but there are no reliable morphological characteristics that can be used to separate these two cytoforms (Krüger, 2006, 1998).

A further disadvantage of the pool screening method is that vector collectors may possess skin microfilariae that could contribute to the occurrence of false positive results. However, efforts were made to minimise this possibility by recruiting Community Drug Distributors who, along with National Onchocerciasis Control Programme personnel and influential community leaders, take ivermectin in the presence of community members to demonstrate drug safety at the beginning of each annual treatment round. Vector collectors also received ivermectin before making blackfly collections in order to reduce skin microfilariae should they be infected. As an additional precaution, flies with visibly distended abdomens that may have blood fed on vector collectors were discarded prior to pool screening. While it is difficult to mitigate the risk of generating false positive results entirely, it is likely that the estimated infection rates provide a fair reflection of current *O. volvulus* transmission in Mahenge.

5. Conclusion

Onchocerca volvulus continues to be transmitted by *S. damnosum* s.l. throughout the eastern slopes of the Mahenge Mountains despite 19 years of annual CDTI. Current findings suggest that L3H infection rates are similar to those reported in the 1960s, although a more detailed study will be necessary to fully understand the dimensions and epidemiological significance of the ongoing transmission. This study provides further evidence that the annual CDTI strategy may be insufficient to

eliminate the parasite in formerly hyperendemic foci, and alternative approaches to control should therefore be considered.

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