

Blackfly ecology and *Onchocerca volvulus* transmission in three formerly hyperendemic foci in Uganda, Tanzania and Cameroon

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

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Kriebelmug ecologie en *Onchocerca volvulus* transmissie in
drie voorheen hyperendemische gebieden in Oeganda,
Tanzania en Kameroen

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Cover picture – Mahenge resident, accompanying research-team, taking an interest in immature blackfly stages present on decaying vegetation in a local river. Photograph by Adam Hendy, Tanzania, 2015.

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Summary

Human onchocerciasis or 'river blindness' is a chronic and debilitating disease caused by repeated infection with *Onchocerca volvulus*, a parasitic filarial worm transmitted by blackflies (Diptera: Simuliidae). Around 99% of an estimated 25.7 million infections occur in sub-Saharan Africa. Current interventions mainly rely on mass drug administration through annual community directed treatment with ivermectin (CDTI) to control the disease. The drug only temporarily sterilises adult parasites and must therefore be taken for the reproductive lifespan of the worms (12 - 15 years) in order to suppress transmission. The World Health Organization (WHO) currently aims to eliminate onchocerciasis by 2025, but it is not known whether this can be achieved through annual CDTI alone. This thesis aimed to provide a detailed study of the blackfly vectors and *O. volvulus* transmission in three formerly hyperendemic foci in Uganda, Tanzania and Cameroon, under long-term control either with annual CDTI, or vector control in combination with biannual CDTI.

An evaluation of Esperanza Window Traps (EWTs) for the collection of human biting (anthropophilic) blackflies was conducted in Uganda and Tanzania (Chapter 2). Blackfly collections are necessary to evaluate the progress of CDTI-based programmes towards eliminating the disease, but current methods rely on the use of human bait which presents ethical problems due to risk of exposure to vector-borne pathogens. Results showed that EWTs collected numbers of *Simulium damnosum* s.l. (the main vector of *O. volvulus* in sub-Saharan Africa) comparable with vector collectors in northern Uganda, but performed poorly in Tanzania. Breeding site surveys and adult blackfly collections were also carried out in all three study countries between 2014 and 2017 (Chapters 3 – 5). Anthropophilic *Simulium damnosum* s.str. and *Simulium bovis* were collected in low numbers in northern Uganda where onchocerciasis control through biannual CDTI and vector control has been taking place since 2012. *Onchocerca volvulus* was not detected in any of the human biting *S. damnosum* s.l. (133 flies) or *S. bovis* (602 flies) screened for infection, although the bovine parasites *Onchocerca ochengi* and *Onchocerca* sp. 'Siisa' were present. Anthropophilic blackflies collected in Tanzania included 'Nkusi J' and *Simulium kilibanum* cytoforms of the *S. damnosum* complex, and also *Simulium nyasalandicum*. 'Nkusi J' appeared to be the predominant cytoform, and out of 12,452 *S. damnosum* s.l. pool screened, an estimated 0.57% (95% CI 0.43% – 0.74%) carried infective L3 stage *O. volvulus* larvae. Infection rates in blackflies appeared similar to pre-control levels, despite annual CDTI commencing in 1997. In Cameroon, a new chromosomal variant of *Simulium squamosum* E was found breeding along the lower Mbam River. Despite CDTI having taken place annually since 2000,

dissection of 9,281 out of 93,563 blackflies collected on human bait showed that high rates of *O. volvulus* transmission were still occurring at riverside sites.

Whereas blackfly collections in northern Uganda were insufficient to demonstrate interruption of *O. volvulus* transmission according to WHO guidelines, the results are encouraging for the use of integrated approaches to control onchocerciasis. In Tanzania and Cameroon, where control has relied upon CDTI alone, *O. volvulus* transmission is continuing at unacceptable levels despite >15 years of annual ivermectin treatment.

Samenvatting

Onchocerciasis of rivierblindheid is een chronisch slopende ziekte veroorzaakt door infecties met de parasitaire nematode, *Onchocerca volvulus*, overgedragen door kriebelmuggen (Diptera: Simuliidae). Het merendeel (99%) van de geschatte 25.7 miljoen infecties vinden plaats in sub-Saharische landen in Afrika. Controle van deze ziekte verloopt momenteel via jaarlijks georganiseerde behandelingen met ivermectin (community directed treatment with ivermectin, CDTI), een geneesmiddel dat door sterilisatie van de volwassen worm de voortplanting tegengaat. Deze onderbreking van de transmissie is echter tijdelijk en het medicijn moet genomen worden gedurende de volledige reproductieve levensduur van de wormen (12-15 jaar). De Wereldgezondheidsorganisatie (WHO) streeft momenteel naar het elimineren van onchocerciasis tegen 2025. Het blijft nog de vraag of dit zal bereikt worden met de jaarlijkse toedieningen via CDTI. Dit proefschrift heeft tot doel een gedetailleerde studie weer te geven van zowel de vectoren, Simuliidae, als de transmissie van *O. volvulus* in drie voormalig hyper endemische gebieden die reeds onder langdurige controle staan door jaarlijkse toedieningen van ivermectin of tweejaarlijkse toedieningen van het geneesmiddel in combinatie met vectorbestrijding.

In Uganda en Tanzania werden Esperanza Window Traps (EWTs) geëvalueerd voor het verzamelen van bijtende, antropofiele kriebelmuggen (Hoofdstuk 2). Deze collecties zijn nodig om de progressie na te gaan van het ingrijpen via CDTI, gericht op de eliminatie van de Simuliidae. Momenteel wordt nog steeds menselijk lokaas gebruikt (vrijwilligers), met alle ethische implicaties tot gevolg. Resultaten tonen aan dat de aantallen van *Simulium damnosum* s.l. (de belangrijkste vector van *O. volvulus*), gecollecteerd met de EWTs, vergelijkbaar zijn met de vectorcollecties op vrijwilligers in het noord Uganda, maar dat resultaat is echter niet waarneembaar in Tanzania. Tussen 2014 en 2017 werden drie landen opgenomen in de studiegroep voor onderzoek van broedplaatsen en collecties van volwassen Simuliidae: Uganda (Mid North; Hoofdstuk 3), zuidoost Tanzania (Mahenge; Hoofdstuk 4) en centraal Kameroen (Bafia Health District; Hoofdstuk 5). Mensen bijtende *Simulium damnosum* s.str. en de dierlijke variant *Simulium bovis* werden in kleine aantallen gevangen in noord Uganda, waar onchocerciasis onder controle wordt gehouden door tweejaarlijkse toediening van CDTI én vector controle plaatsvindt sinds 2012. *Onchocerca volvulus* werd bovendien niet aangetroffen in de antropofiele *S. damnosum* s.l. (133 exemplaren) of de zoöfiele *S. bovis* (602 exemplaren). Na screening voor andere infecties werden echter wel runderparasieten *Onchocerca ochengi* en *Onchocerca* sp. 'Siisa' aangetroffen. Antropofiele kriebelmuggen verzameld in Tanzania bevatten 'Nkusi J' en *Simulium kilibanum* cytovormen van het *S. damnosum* complex, evenals *Simulium*

nyasalandicum. 'Nkusi J' bleek de overheersende cytovorm te zijn. Uit een pool van 12.452 *S. damnosum* s.l. screenings, bevatte een geschatte 0,57% (95% CI 0,43% - 0,74%) de infectieuze L3-fase van de *O. volvulus* larven. De infectie graden blijken hiermee vergelijkbaar met de niveaus vóór de bestrijding, en dit ondanks de jaarlijkse toedieningen via CDTI, reeds gestart in 1997. In Kameroen werd een nieuwe chromosomale variant van *Simulium squamosum* E aangetroffen op een broedplaats langs het lagere gelegen deel van de Mbam Rivier. Hoewel ook hier reeds in 2000 gestart werd met jaarlijkse toediening door CDTI, tonen dissecties van 9.281 kriebelmuggen op een totaal van 93.563 gevangen exemplaren (via menselijke vrijwilligers) aan dat er nog steeds hoge aantallen van *O. volvulus* transmissies plaatsvinden in de gebieden langs de rivier.

In noord Uganda waren de vangsten onvoldoende naar de WHO-richtlijnen om aan te tonen dat de *O. volvulus* transmissie werd onderbroken. De resultaten zijn echter bemoedigend voor het gebruik van de geïntegreerde toepassingen, namelijk de CDTI gecombineerd met vectorcontrole, ter bestrijding van onchocerciasis. In Tanzania en Kameroen daarentegen, waar de bestrijding enkel plaatsvindt door jaarlijkse CDTI, vindt transmissie van *O. volvulus* nog steeds plaats op onaanvaardbare niveaus, zelfs na meer dan 15 jaar ivermectin behandelingen.

List of acronyms

ABR	Annual Biting Rate
APOC	African Programme for Onchocerciasis Control
ATP	Annual Transmission Potential
CDTI	Community Directed Treatment with Ivermectin
CMFL	Community Microfilarial Load
DBR	Daily Biting Rate
DDT	Dichlorodiphenyltrichloroethane
DRC	Democratic Republic of Congo
ESPEN	Expanded Special Programme for Elimination of Neglected Tropical Diseases
EWT	Esperanza Window Trap
HD	Health District
HLC	Human Landing Collection
MBR	Monthly Biting Rate
MDA	Mass Drug Administration
MOH	Ministry of Health
MTP	Monthly Transmission Potential
OCP	Onchocerciasis Control Programme
OEPA	Onchocerciasis Elimination Programme for the Americas
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR (or real-time PCR)
rDNA	Ribosomal DNA
REMO	Rapid Epidemiological Mapping of Onchocerciasis
VCO	Vector Control Officer
WHO	World Health Organization

CHAPTER 1

Introduction

Background

Human onchocerciasis, otherwise known as ‘river blindness’, has severely affected millions of people living in fertile riverine areas of tropical sub-Saharan Africa for centuries [1]. It is a chronic and debilitating disease caused by repeated infection with *Onchocerca volvulus* (Nematoda: Filarioidea), a parasitic worm transmitted through the bites of blood feeding blackflies (Diptera: Simuliidae) [2]. The colloquial name stems from the riverine breeding habitats of these parasite-transmitting (vector) flies, and the characteristic ocular lesions that can lead to irreversible blindness among those chronically infected [3, 4]. Other clinical symptoms can include intense itching, disfiguring skin lesions, and potentially growth arrest and epilepsy [5-8]. Some of the poorest rural communities in Africa are affected, often in areas where subsistence farming is of vital economic importance, but where onchocerciasis results in decreased agricultural productivity [9]. The disease historically resulted in many villages being abandoned prior to the implementation of large-scale onchocerciasis control programmes which began in the 1970s [1, 10, 11]. Despite the unquestionable success of two of these programmes (the Onchocerciasis Control Programme in West Africa and the African Programme for Onchocerciasis Control), it was estimated in 2008 that 25.7 million people were still infected with the parasite, of which 746,000 were visually impaired, 265,000 were blind, and 4.2 million suffered from severe itching [1].

Epidemiology

Onchocerca volvulus is thought to have originated from an ancestral bovine parasite that was probably introduced to humans during the domestication of cattle in Africa [12, 13]. The disease currently affects 31 countries in sub-Saharan Africa, as well as a small number of isolated foci in Yemen and Latin America (Fig 1) [14]. The parasite is thought to have been introduced to the New World tropics via the slave trade, where the presence of compatible vectors enabled a transmission cycle to establish [15, 16]. However, the recent success of the Onchocerciasis Elimination Programme for the Americas (OEPA) has led to disease interruption or elimination in 11/13 formerly endemic foci [17]. Elimination is defined by the World Health Organization (WHO) as the reduction to zero of the incidence of infection in a defined geographical area, while eradication is the permanent reduction to zero of the global incidence of infection [14].

Onchocerciasis is now of primary clinical significance in sub-Saharan Africa where 99% of cases occur [1]. It is commonly thought that two or more different strains of the parasite are endemic in savannah and forest habitats, with those in the former being responsible for the most severe ocular manifestations of the disease [18-21]. 'Blinding' onchocerciasis was historically associated with blackfly vectors found among the large river basins and expansive savannahs of West Africa [11]. In the forests of western and central Africa, infection is more closely associated with severe itching and skin disease [22]. A spectrum of clinical conditions exists in East Africa [5, 23-26], where the disease occurs in discrete foci, often associated with montane habitats that are interspersed within otherwise transmission free areas [27, 28].

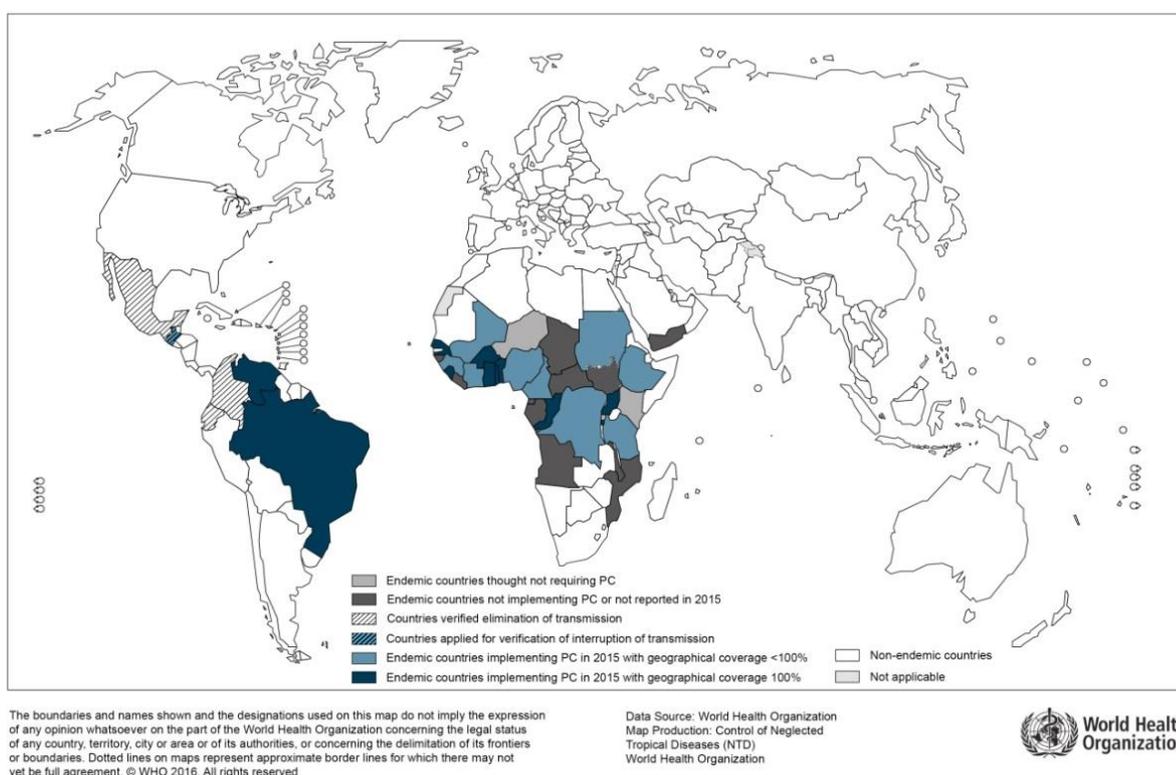


Fig 1. Worldwide distribution of onchocerciasis and status of preventive chemotherapy (2015). *World Health Organization* [2].

Parasite and disease

Onchocerca phylogeny

Around 28 species of *Onchocerca* filarial nematodes have been described, most of which are parasites of large ungulates [12]. Two exceptions are *Onchocerca lupi*, which is a parasite of dogs (although human zoonotic cases have been reported [29]) and *O. volvulus*, for which humans are the only definitive hosts [12]. Phylogenetic work supports the existence of an African bovine-human lineage in which *O. volvulus* forms a monophyletic clade with the bovine parasites *Onchocerca ochengi*, *Onchocerca* sp. 'Siisa', *Onchocerca dukei*, and an

unknown species (*O. sp.*) collected from an African bushbuck (Bovinae) (Fig 2.). Both *O. volvulus* and *O. ochengi* exhibit great morphological homogeneity, and along with *O. sp.* 'Siisa', they share a common blackfly vector in *Simulium damnosum sensu lato* (s.l.) [30].

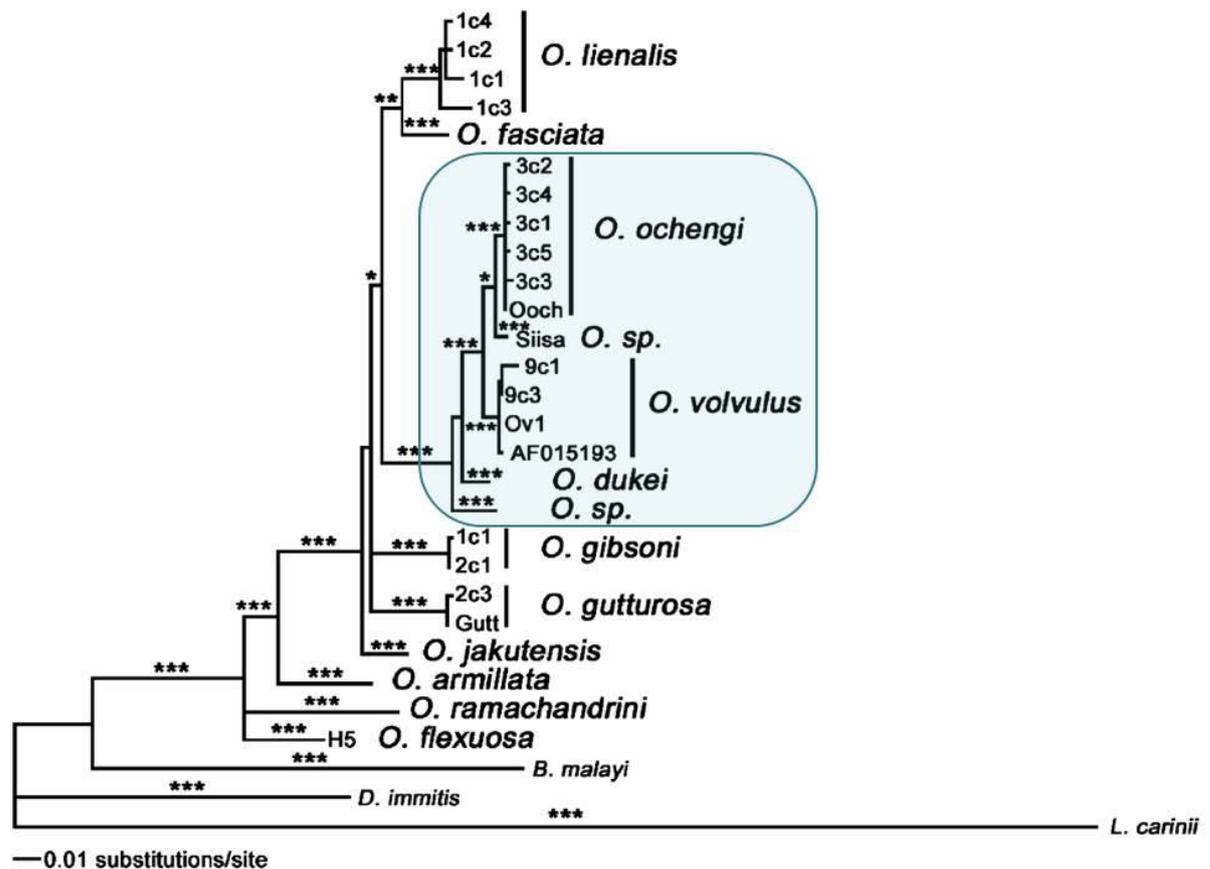


Fig 2. Phylogeny of some *Onchocerca* species based on a concatenated analysis of mitochondrial 16S and 12S rDNA gene sequences, highlighting the African bovine-human monophyletic clade. *Reproduced from Krueger et al.* [12].

Parasite lifecycle and pathogenesis

Onchocerciasis was first discovered to be a vector-borne disease by Blacklock (1926) whose work in Sierra Leone showed that *O. volvulus* larvae (microfilariae) develop to transmissible (infective) stages in human biting blackflies [31]. A blackfly ingests skin-circulating microfilariae while taking a blood meal from an infected person (Fig 3) [32]. Once ingested, some microfilariae penetrate the peritrophic matrix and blackfly midgut, before migrating via the haemocoel to the thoracic flight muscles where they develop through several stages (L1, L2 and L3) in around 7-10 days [3, 32]. The mature L3 stage parasites then migrate to the head and mouthparts of the blackfly, where they are potentially transmitted during a subsequent blood meal [32]. Having entered the human host, the parasites undergo further development before establishing a new infection [6]. Adult female (30 – 80cm) and male (3 – 5 cm) worms are found inside thick, subcutaneous nodules (onchocercomas), or within

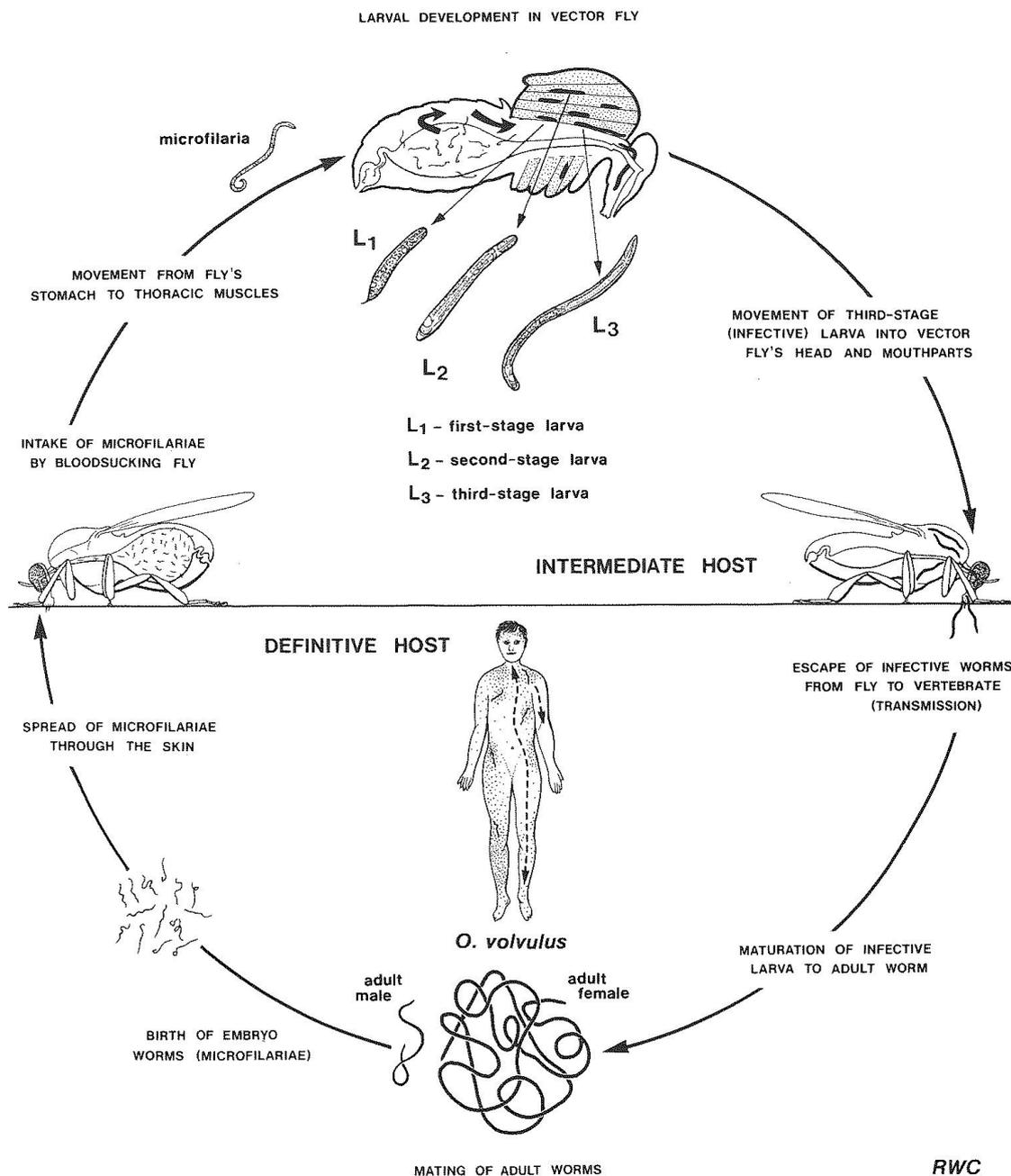


Fig 3. The lifecycle of *Onchocerca volvulus*. Reproduced from Crosskey [3].

deeper connective tissues near muscles bones and joints [6, 33]. They may live for up to 15 years and have a reproductive lifespan of 9 – 11 years [6]. While females remain entangled inside the nodules, male worms migrate between nodules, inseminating females along the way [34]. Around 9 – 18 months after initial infection, fertile females produce several thousand microfilariae each day, which can live for up to two years in the skin [33, 35]. It is the dissemination of millions of these larvae throughout the body of a chronically infected person that causes the main dermal, lymphatic and ocular complications of onchocerciasis [34, 36].

In West Africa, it has been shown that high pre-control intensities of infection were associated with the most severe forms of ocular disease, which can result in irreversible blindness [37]. Infection intensity is measured by the community microfilarial load (CMFL), defined as the geometric mean number of microfilariae per skin snip (mf/ss) in adults aged >20 years [38]. CMFLs exceeding 5-10mf/ss are considered to constitute a public health problem [39].

Human immunity

The human immune response to *O. volvulus* infection involves both Th1 and Th2 cell-mediated pathways [40]. The Th2 response is thought to drive protective immunity against *O. volvulus* L3 larvae and microfilariae, while the Th1 response is induced by the presence of endosymbiotic *Wolbachia* bacteria inside the parasite [41]. However, it has been shown that in *O. ochengi* animal models, successful parasite infection and the onset of patency coincides with downregulation of both Th1 and Th2 associated cytokines [41]. The ocular lesions in human onchocerciasis are thought to be caused by host inflammatory responses to dying microfilariae and the release of *Wolbachia*, which induces neutrophil and macrophage infiltration and causes corneal edema and opacity [40, 42]. The immune responses involved in chronic itching and skin lesions may also be a response to the release of *Wolbachia*, but the eventual clinical outcome may be influenced by host genetics [36, 40].

Onchocerciasis treatment

Onchocerciasis is currently treated with the anthelmintic drug, ivermectin (Mectizan®) [43]. Ivermectin is not a macrofilaricide, but has microfilaricidal properties and also temporarily inhibits production of microfilariae for several months after treatment [44]. This alleviates intense skin itching, halts progression towards blindness, and also reduces transmission when used in mass drug administration (MDA) programmes [43]. However, the effects are only temporary and ivermectin should therefore be taken periodically (at least once a year) for the duration of parasite infection. Due to the lengthy treatment regimen and concerns about possible resistance [45], there is a need to identify alternative or complimentary therapies.

In the past, both suramin and diethylcarbamazine have been used to treat onchocerciasis, although these are no longer recommended due to their high toxicity and/or risk of severe adverse events [40, 46]. Several potential treatments targeting *Wolbachia* are therefore being investigated with the aim of exploiting the obligatory symbiotic relationship between the bacteria and parasite [40, 47]. Among these, doxycycline (a tetracycline antibiotic) has emerged as an important second-line therapy that can achieve long-term sterilisation of adult worms or can be used as a macrofilaricide [40]. Trials in Ghana showed that 100mg

doses administered daily for 6 weeks resulted in *Wolbachia* depletion of more than 90% and also inhibited microfilarial production for up to 24 months post-treatment [41]. Following daily administration of 200mg doses of doxycycline for 6 weeks, the effects were macrofilaricidal, killing >60% adult worms present at the time of treatment (although new infections were established thereafter) [48]. Such intensive treatment regimens with tetracyclines are considered likely to encounter problems with logistics and adherence, although Wanji *et al.* reported 97.5% of 13,000 people adhered to a 6-week doxycycline regimen using a community-based treatment approach [49]. Therapies involving various combinations of doxycycline, minocycline and albendazole have also been trialled to identify macrofilaricidal treatments with shorter regimens than are required for doxycycline alone [50]. Elsewhere, short duration (1-2 weeks) rifampicin treatments have recently been shown to reduce *Wolbachia* by >90% in *O. ochengi* animal models [51].

Vector biology

African vectors

Simulium damnosum s.l. is the major blackfly vector of *O. volvulus* in Africa where it is responsible for around 90% of transmission. It can be found breeding in rivers and streams from south of the Sahel to the southern tip of the continent (Fig 4) [3]. However, the distribution of onchocerciasis is limited by the anthropophilic range of its vectors, which only extends as far south as southern Malawi [52]. Blackflies of the subgenus *Lewisellum* (including *Simulium neavei*, *Simulium woodi* and possibly *Simulium nyasalandicum*) are responsible for most of the remaining 10% of transmission [3, 53].

Like all blackflies, the vectors of *O. volvulus* develop through four lifecycle stages: the egg, larva, pupa and adult [3]. The first three stages are aquatic and are generally found in fast flowing waters in rivers and streams, while the final stage is terrestrial/aerial. Aquatic stages are well adapted to surviving their harsh environments. Eggs are often embedded on substrates (stones, rocks, trailing or dead and decaying vegetation, human-made objects) in suitable riverine habitats by ovipositing (egg-laying) females. Larvae anchor themselves to substrates by means of posterior abdominal hooks that are embedded in a pad of hardened silk secreted by their large salivary (silk) glands. With the aid of paired cephalic fans, larvae filter feed on a diet which includes detritus, dissolved organic matter, bacteria and diatoms. Blackflies face downstream and remain firmly anchored to their substrate during pupation, before adults emerge enveloped in gaseous bubbles. Neonate adults then either climb partially submerged vegetation or rapidly ascend to the water surface before taking flight [3].

The adult flies are anautogenous, meaning they require a blood meal to mature their first and each subsequent batch of eggs [3]. Both male and female blackflies feed on plant nectar which contains carbohydrates essential for flight, but only the female flies blood feed. Biting takes place mainly outdoors and during the daytime, often occurring in early morning and late afternoon peaks [3, 54]. Biting activity is strongly influenced by environmental conditions (light intensity, temperature, wind, rain), and diurnal rhythms may vary according to species and season [3, 23].

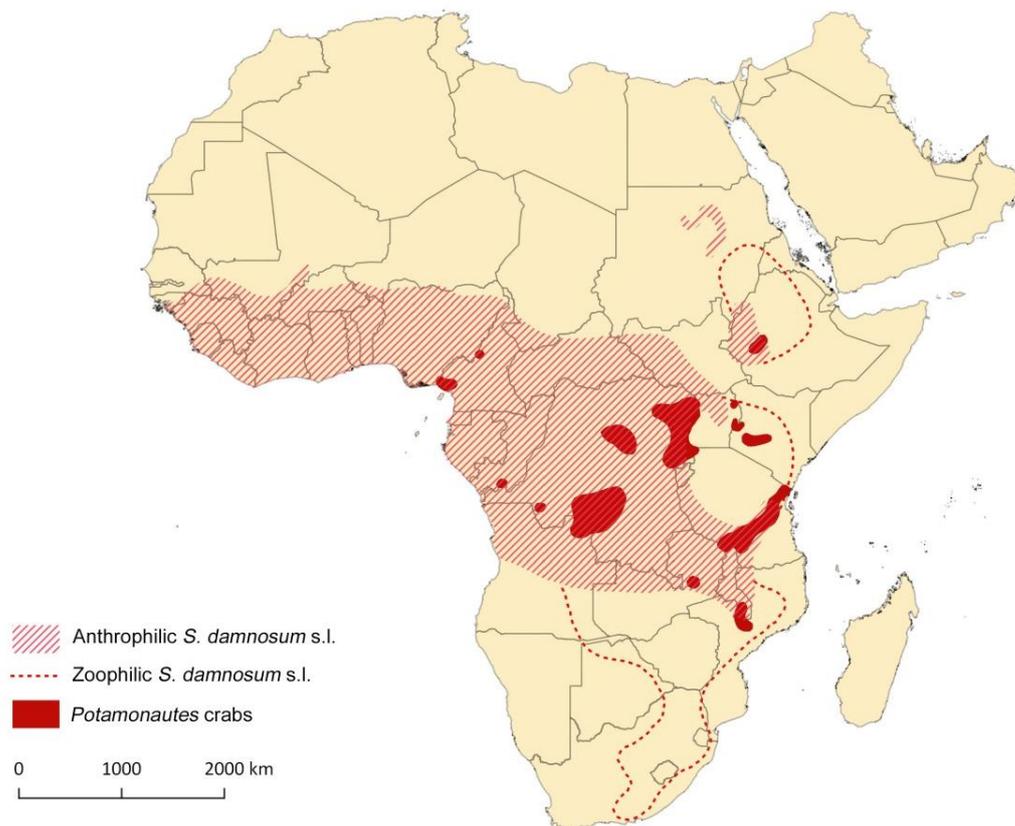


Fig 4. Distribution of anthropophilic and zoophilic *S. damnosum* s.l. in sub-Saharan Africa, and *Potamonautes* crabs, carriers of *S. neavei* group blackflies in central and East Africa. Based on maps by Crosskey [3].

Simulium damnosum s.l.

The breeding sites of *S. damnosum* s.l. range from large seasonal rivers in dry savannah habitats of West Africa, to relatively small perennially flowing rivers in forests and highland areas of central and East Africa (Figs 5A and 5B) [3]. Breeding is often restricted to the main rivers and seldom occurs in smaller streams or tributaries [3]. Female flies oviposit on rocks and trailing vegetation in fast flowing sections of watercourses [55, 56]. Around 1-3 days after egg-laying, larvae hatch and develop through seven instars (moult) over the course of



Fig 5. A) Typical breeding habitat of *S. damnosum* s.l. in northern Uganda; B) *S. damnosum* s.l. breeding habitat in smaller montane river in Tanzania; C) typical *S. neavei* group breeding habitat in heavily shaded montane river in Tanzania.

about 7-12 days before they pupate (Fig 6) [28, 32]. Adults emerge from pupae 2-5 days later, after which mating can occur almost immediately [32, 57]. Female blackflies are thought to only copulate once during their lifetime, which is sufficient to fertilise all their eggs regardless of the number of batches produced [3]. Feeding also usually takes place on the day of adult emergence. Over the following days and weeks, females undergo gonotrophic cycles of blood feeding, resting (allowing eggs to develop) and laying eggs. Each cycle takes roughly 3-4 days to complete and continues for the lifespan of the adult female fly [3, 58].

The duration of blackfly development from egg to adult varies between species and is strongly influenced by water temperature [3]. The warm tropical habitats, in which *S. damnosum* s.l. vectors thrive, ensure that life cycles are completed quickly and upwards of 20 generations may occur annually among flies associated with perennial West African rivers. Even in seasonal rivers, *S. damnosum* s.l. may complete more than 15 generations each year [3].

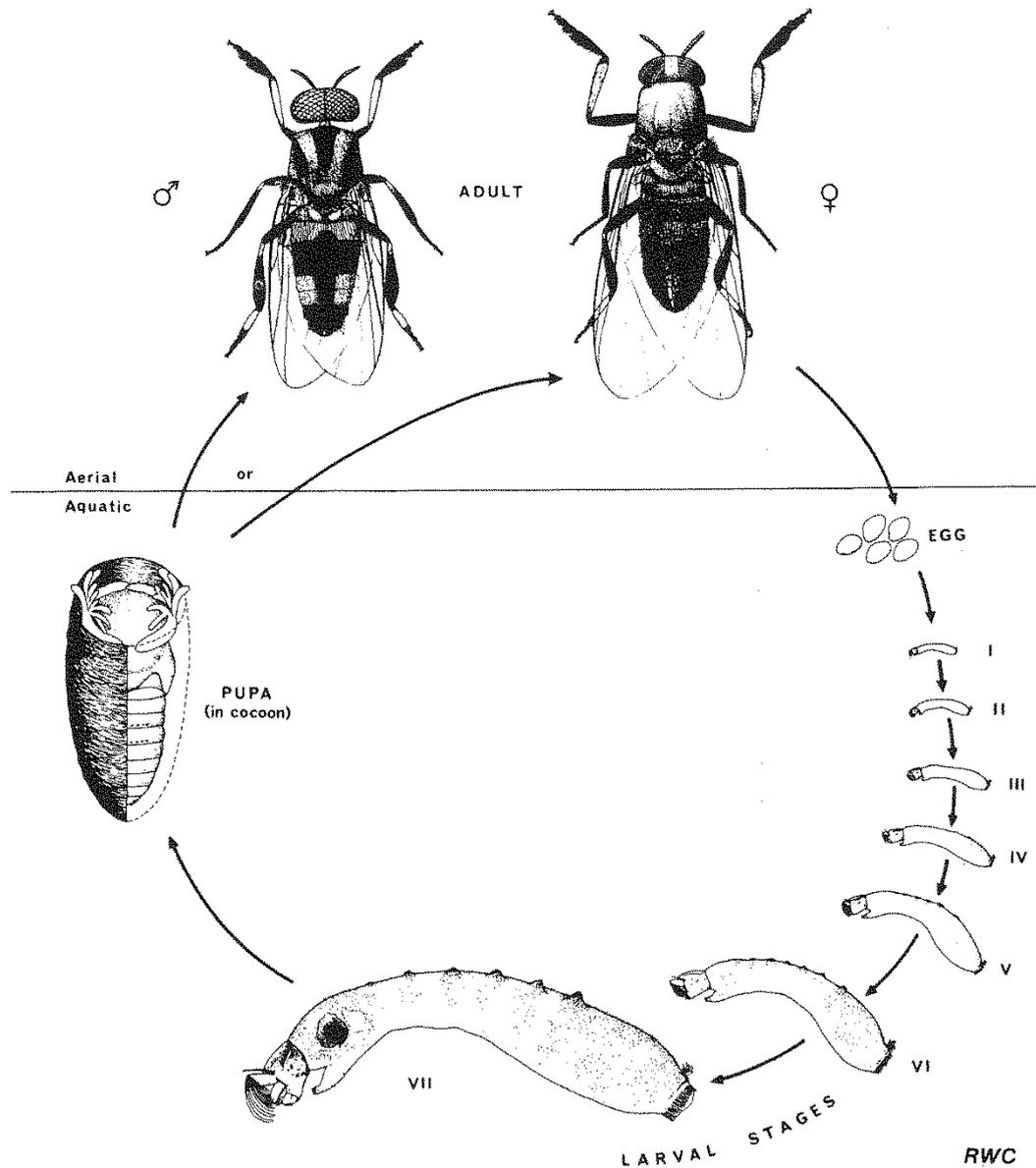


Fig 6. Lifecycle showing the eggs, seven larval instars, pupa and adults of *Simulium damnosum* s.l., the main vector of *Onchocerca volvulus* in sub-Saharan Africa. Reproduced from Crosskey [3].

Simulium neavei group

Vectors among the subgenus *Lewisellum* (referred to as the '*Simulium neavei* group' sensu McMahon 1957 [59]) are generally restricted to forest and highland areas of central and East Africa where they are responsible for *O. volvulus* transmission in multiple foci (Fig 4) [28]. These species possess a unique developmental cycle involving an obligate phoretic relationship with freshwater *Potamonautes* spp. crabs [60]. Phoresy can be defined as the attachment and transport of an organism of one species (the blackfly) on the body of another species (the crab), without the relationship being parasitic [3]. Species of the

S. neavei group are mostly found breeding in heavily shaded, small to medium-sized perennial forest streams and their presence is dependent on dense vegetation cover (Fig 5C) [32]. Consequently, deforestation can result in populations decreasing or disappearing, but may also lead to the creation of suitable habitats for other *O. volvulus* vectors [32, 52, 61, 62]. The duration of larval and pupal development is considerably longer than for *S. damnosum* s.l. [32]. Observations in Tanzania showed that larvae remained on crabs for 26 – 68 days, while pupae remained for 8 – 10 days [63]. The rate of development is limited by the availability of food, and opportunities to filter feed are scarcer as crabs only spend time in flowing water for part of the day [3]. These lengthy development times can be exploited in vector control programmes as the commonly used insecticide, temephos, is only effective against larval (feeding) stages of blackflies, and does not kill eggs or pupae [32, 63]. It must therefore be applied to breeding sites at intervals of no longer than duration of larval development to effectively suppress blackfly populations.

Host preferences

Blackflies feed on warm blooded vertebrates, including humans, but no species is exclusively anthropophilic and not all species attracted to humans bite [15]. The degree of zoophily is therefore an important factor affecting the vector competence of blackflies. In addition to humans, *S. damnosum* s.l. and *S. neavei* group species feed on domesticated animals including livestock, although in reality there has been little quantitative work on their zoophilic habits, particularly with regard to wild mammals [3, 15]. Lamberton *et al.* recently used DNA profiling methods to investigate rates of human blood feeding among *S. damnosum* s.l. in Ghana, showing that other hosts included pigs, cattle, sheep, dogs and goats [64]. The catholic host choice of blackflies means that non-human *Onchocerca* parasites, including *O. ochengi*, are commonly found in human biting flies [65].

Physiological age (parity rates)

The development of eggs following a blood meal can be used to infer the physiological age of blackfly populations. Changes to the physical structure and appearance of the ovaries following egg laying makes it possible to distinguish between flies that have not laid eggs (*nulliparous flies*) and those that have laid eggs at least once (*parous flies*) [3, 58]. Parity rates are of particular importance when investigating *O. volvulus* transmission and disease epidemiology, as only flies that have laid eggs at least once may possess the infective L3 stages. Therefore, if parity rates in blackfly populations are high, there will be more parasite transmission than if parity rates are low, assuming that all other factors are the same. Age structures of blackfly populations vary spatially and temporally, and variations in parasite transmission occur as a result [65-67].

Vector taxonomy and species complexes

Species complexes

There is immense structural homogeneity among the 2000+ currently described species of blackfly, including the African vectors of *O. volvulus* [68]. Two frequently used terms to describe closely related species are 'species-group' and 'species complex'. 'Species group' is used to describe several similar and closely related species that can be separated based on differences in their morphological characteristics [3, 69], whereas 'species complex' describes closely related species which are morphologically indistinguishable, but reproductively isolated [3]. *Simulium damnosum* s.l. is a complex of approximately 60 named 'cytospecies' and 'cytotypes', which have been described on the basis of differences in their larval polytene chromosomes [15, 68, 70, 71]. These chromosomes are present in all Diptera, but are particularly well developed in the late-instar larvae of blackflies [72]. Cytospecies are real species that are reproductively isolated and biologically distinct, whereas cytotypes are chromosomally distinct populations of unconfirmed taxonomic status. Collectively, cytospecies and cytotypes are known as 'cytoforms' [71]. Each cytoform differs in its distribution, ecology, behaviour and ability to transmit parasites [15]. Being able to accurately identify members of the complex is therefore necessary to understand disease epidemiology.

Morphotaxonomy

The *S. damnosum* complex can be easily identified by its external morphology (Figs 7 and 8A). Scales are present on the larval prothoracic proleg and larval stages also possess dorsal abdominal tubercles which may vary in size, but are generally larger in forest cytoforms [3]. Pupae can be recognised by the structure of the respiratory organ (gill), which is described as 'banana-like', while adult flies are characterised by their swollen fore-tarsi, each of which has a dark crest of hair (Fig 7) [3, 73]. However, while wing tuft colours of female flies are sometimes used to separate West African cytoforms [74], in reality, there are few reliable morphological characteristics to distinguish members within the *S. damnosum* complex.

Cytotaxonomy

The fundamental purpose of blackfly cytotaxonomy is to recognise and differentiate members of species complexes [3]. Each cytoform of the *S. damnosum* complex is described with reference to *Simulium kilibanum* (=Nyamagasani), which is phylogenetically central and the arbitrarily chosen chromosomal standard [75, 76]. Cytotaxonomy relies upon visualisation of the 'giant' polytene chromosomes present in tissues of the larval salivary

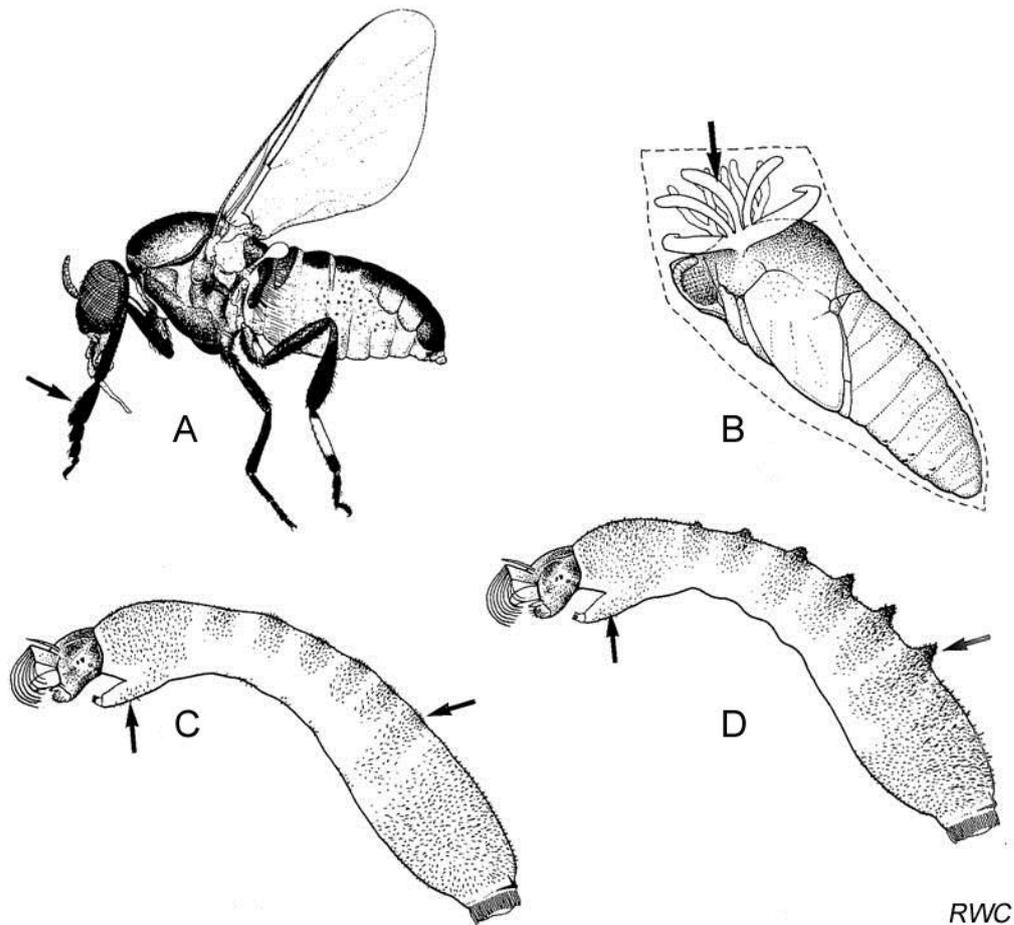


Fig 7. Morphological characteristics of the *Simulium damnosum* complex. A) Adult female fly, with arrow showing enlarged fore tarsi and hair crest; B) pupa, with arrow showing 'banana-like' respiratory organ (gill); C) larva without pronounced abdominal tubercles, common in some savannah cytoforms D) larva with large abdominal tubercles, common in some forest cytoforms. Arrows in C & D showing location of scales on prothoracic proleg, and presence/absence of abdominal tubercles. *Reproduced from Crosskey [3].*

glands (Fig 8B), which grow by cellular enlargement rather than an increase in cell number [72]. These cells exhibit a haploid number (usually $n=3$) of intimately paired chromosomes which appear very large due to repeated cycles of DNA replication without cell division. As a result, blackfly chromosomes may possess 512 – 2,048 parallel strands of DNA that appear as a series of dark and light transverse bands possessing enormous morphological detail (Fig 9) [72, 77].

Cytoforms are distinguished from one another on the basis of chromosomal rearrangements. Inversion rearrangements, which are essentially 180° reversals of banding patterns, are the most common type [72]. They may be interspecific and 'fixed', in which case they only occur homozygously, or they may be intraspecific and 'floating' (polymorphic), in which case they can occur heterozygously. Other inversions, including sex-linked rearrangements, are described thoroughly by Adler *et al.* [72].

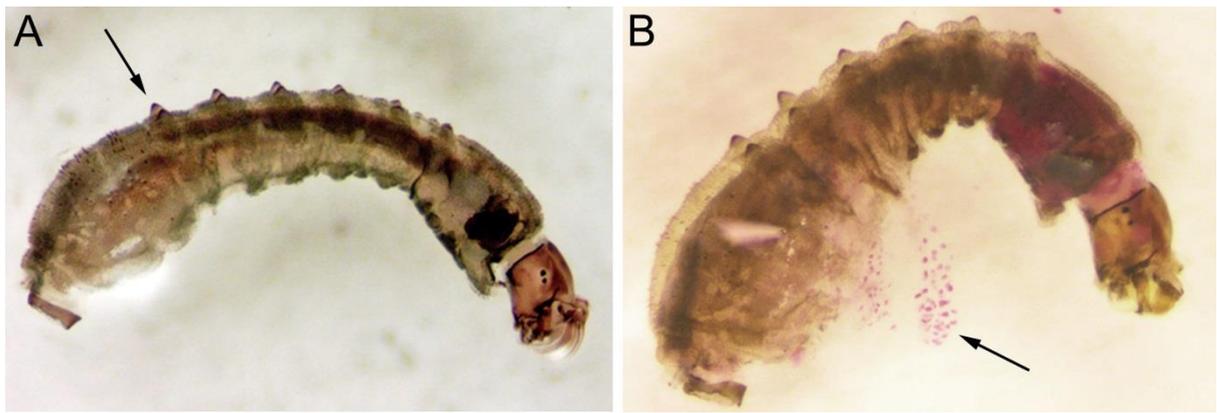


Fig 8. A) *Simulium damnosum* complex larva with arrow showing large abdominal tubercles (Tanzania, Mahenge); B) *Simulium damnosum* complex larva with arrow showing stained polytene chromosomes of salivary glands dissected from the abdomen.

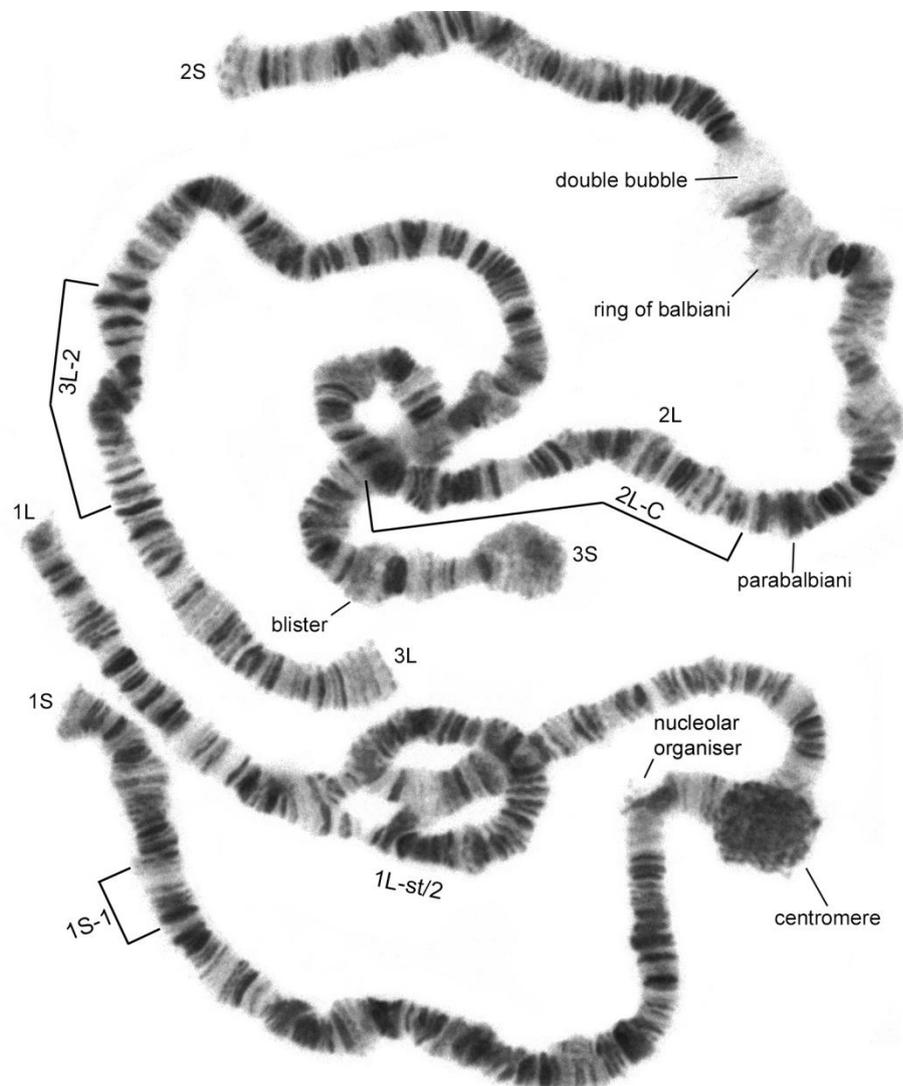


Fig 9. Full chromosome complement of *Simulium damnosum sensu stricto* (Uganda, Nile, Karuma Falls) showing some common landmarks and illustrating chromosome arms (1S = short arm of chromosome 1, 1L = long arm of chromosome 1 etc.), homozygous inversions 1S-1, 2L-C and 3L-2, and heterozygous inversion 1L-st/2. Brackets show limits (breakpoints) of the inversions.

There is a specialised though not always consistent system of nomenclature associated with blackfly cytotaxonomy. In summary, chromosomes are numbered 1, 2 and 3 in order of their decreasing size (Fig 9). Each has a long (L) and a short (S) arm either side of a sub-median centromere [3, 72]. The full chromosome complement is divided into 100 approximately equal sections (not shown in Fig 9). Section 1 occurs at the beginning of the short arm of chromosome 1 (the section furthest away from the centromere), while section 100 occurs at the end of the long arm of chromosome 3 [72]. These divisions are useful for orientation and describing chromosome characteristics. Markers such as the nucleolar organiser, ring of balbiani, double bubble and blister are also useful for this purpose (Fig 9). Each inversion is described by a number (or occasionally a letter) in addition to the chromosome arm in which it is found. For example, '3L-2' represents an inversion arbitrarily numbered '2' by Dunbar [78], which is present in the long arm of chromosome 3 and is found in various *S. damnosum* subcomplex and *Simulium sanctipauli* subcomplex cytoforms [71, 72]. For the purpose of this work, the nomenclature follows that of Krüger [75]. Inversions that are fixed within populations are assigned hyphens e.g. 3L-2, while those that are polymorphic are assigned a forward slash e.g. 3S/1. Using inversion 2L-5 as an example, the homozygous standard, heterozygous and homozygous inverted configurations of individual specimens are expressed as 2L-st/st, 2L-st/5 and 2L-5/5, respectively.

Molecular identification

Polytene chromosomes can also be found in the Malpighian tubules (an excretory organ) of adult blackflies, but are generally not well developed or easy to read [79]. The identity of *S. damnosum* complex cytoforms biting humans is therefore usually inferred based on the identification of larvae breeding in nearby rivers. However, this is not always a reliable method as some flies migrate long distances from their breeding sites [80], and it is also common to find multiple cytoforms breeding together (in sympatry) in the same rivers [3]. As a result, identification by cytotaxonomy is being increasingly supplemented with DNA-based methods [81, 82]. PCR amplicon size polymorphisms of the blackfly internal transcribed spacer 1 (ITS1) rDNA can be used, in combination with cytotaxonomy, to identify many of the ≈26 East African cytoforms [75].

Onchocerca – Simulium complexes

The epidemiology of onchocerciasis is complicated not only by the diversity of cytoforms and their differing roles in transmission, but also the interactions between parasites, vectors and definitive hosts. It is generally stated that severe ocular complications associated with onchocerciasis are more common in savannah habitats, while skin and lymphatic system conditions are more common in forest habitats [22, 33, 83]. In order to explain these

patterns of disease pathology, Duke *et al.* proposed the existence of compatible *Onchocerca* – *Simulium* complexes [18]. They conducted a series of cross-transmission experiments between parasites and vectors from different bioclimatic zones in Cameroon [18, 84-87]. These demonstrated that forest parasites developed well in *S. damnosum* from the forest and Guinea-savannah bioclimatic zones, but showed little or no development in *S. damnosum* from the Sudan-savannah [18]. Conversely, parasites from the Sudan-savannah developed well in *S. damnosum* from the corresponding bioclimatic zone, but developed poorly in *S. damnosum* from the Guinea-savannah and forest zones [18]. The idea that compatible *S. damnosum* forms and parasite strains exist gained credibility when *S. damnosum* was discovered to be a complex of sibling species [78, 88], and molecular studies have since appeared to support this [19, 89]. However, others have questioned whether the hypothesis is too simplistic, citing examples of high rates of blindness occurring in forest-savannah transition zones in West Africa [20]. In addition, the two strain hypothesis cannot sufficiently explain the parasite genetics and pathologies encountered in some East African foci. For example, in Sudan and South Sudan, the clinical picture does not resemble the blinding disease encountered in the West African savannahs [26, 90, 91]. Cheke and Garms [20] speculated that a multitude of factors are likely to be involved in determining the clinical outcome, possibly including: the intensity and rate of transmission, the pathogenicity of local strains (which may be related to variation in endosymbiont fauna such as *Wolbachia*), variations in host response related to race nutrition and immunity, or concomitant infections with other organisms. They also mention a possible role of immunological reactions stimulated by contact with other *Onchocerca* species, such as *O. ochengi* [20]. Elsewhere, it has been proposed that the immunomodulatory effects of blackfly saliva contribute to clinical outcome [92]. Whatever the reasons for the varying pathologies, the early observations that blindness rates were higher in the savannahs of West Africa, where *Simulium damnosum sensu stricto* (s.str.) and *Simulium sirbanum* were major disease vectors, had immense practical implications for onchocerciasis control.

Onchocerciasis control

Early attempts

In the absence of a safe and suitable macrofilaricidal drug, early attempts at onchocerciasis control focussed on controlling its vector [93]. These efforts have been thoroughly reviewed by Brown [94] and Davies [93]. Most interventions were relatively small scale, often focusing on vegetation removal or the use of insecticides to treat blackfly breeding sites [93-96]. Early successes involved the application of the organochlorine DDT to watercourses, which eliminated *S. neavei* from foci in Kenya [94, 95]. The same insecticide was ultimately used to

eradicate *S. damnosum* s.l. from the Nile at Jinja (Uganda), after several attempts had provided only temporary alleviation [97]. However, the use of DDT and other chlorinated hydrocarbons was short-lived due to their toxicity towards non-target organisms [23, 93, 95]. It became apparent during these early interventions that onchocerciasis control by vector control was feasible in discrete (isolated) areas, particularly in East Africa where *S. neavei* was present. However, larger foci or those at risk of blackfly reinvasion would require longer, sustained periods of intervention [94, 96].

The OCP (1974 – 2002)

The severity of blinding disease and the belief that two strains of parasite existed led to the establishment of the Onchocerciasis Control Programme in West Africa (OCP) in 1974 [98]. The programme commenced operational activities in 1975 and initially covered seven countries in the Volta River Basin. By 1986, it had expanded to 11 countries (Benin, Burkina Faso, Côte d'Ivoire, Ghana, Guinea, Guinea Bissau, Mali, Niger, Senegal, Sierra Leone & Togo) and aimed to protect around 30 million people from the disease [1, 11, 98]. The OCP not only had a dual mandate of eliminating onchocerciasis as a public health problem and as an obstacle to socioeconomic development, but it also aimed to ensure sustainability and avoid future disease recrudescence [10, 98]. The initial approach was to control blackfly vectors by the weekly application of larvicides to the breeding sites of savannah sibling-species. These were mainly breeding sites of *S. damnosum* s.str. and *S. sirbanum*, but also of *Simulium squamosum* and *S. sanctipauli* where they occurred sympatrically with savannah species [19, 93, 98, 99]. The larvicide of choice for the OCP was the organophosphate, temephos [11]. Unlike DDT, temephos biodegrades rapidly and has low toxicity towards non-target organisms [100]. Weekly river treatments were necessary due to the fast development of *S. damnosum* s.l. in the warm (22 – 35°C) West African rivers and because temephos has no effect against the non-feeding egg and pupal stages of blackflies [100]. The practical intention of the OCP was to suppress blackfly biting (eradication was never considered feasible), and consequently transmission, for the duration necessary to eliminate the human parasite reservoir [98]. Such a large scale and lengthy vector control programme was unprecedented and the only feasible means of implementing control activities across such a large area was by air [93, 100]. At its peak, the OCP covered an area $\approx 1,300,000\text{km}^2$, a population of 78 million, and treated 50,000km of river each week with insecticides using helicopters and fixed-wing aircraft [11, 100, 101].

Implementing such an ambitious programme was not without difficulties. An early and fundamental problem was with vector reinvasion [100]. It was believed that *S. damnosum* s.l. occupying the forests beyond the southern limits of the original OCP zone would not be

able invade the savannahs, and that the OCP boundaries were sufficient to prevent reinvasion by savannah cytospecies from uncontrolled areas [98, 100]. However, during the first rainy season of operations, densities of biting flies similar to pre-control levels were appearing in well controlled rivers in the west of the OCP zone [80, 93]. There was no evidence of treatment failure or the presence of uncontrolled breeding sites that could explain the phenomenon. It was therefore thought that biting flies were appearing from outside the OCP boundaries [80, 98]. Blackflies arrived in waves in what appeared to be wind assisted migrations which were closely associated with the northward movement of the Inter Tropical Convergence Zone [80]. Many of the arriving flies appeared to have taken several blood meals en route, resulting in high percentages (sometimes >15%) carrying infective L3 stage larvae indistinguishable from *O. volvulus* [80, 102]. Cytotaxonomy was used to identify the migrating cytospecies as *S. damnosum* s.str. and *S. sirbanum*, enabling potential sources of reinvasion to be identified [80]. The experimental treatment of breeding sites in rivers in Côte d'Ivoire, up to ≈400km south west of the areas being reinvaded, resulted in the alleviation of the problem in the OCP area [80]. Other sources of reinvasion were subsequently identified and dealt with in a similar way [80, 93]. Reinvasion was an important occurrence in the OCP that ultimately led to the programme boundaries being redefined [103].

Whereas large-scale, long-distance migration/reinvasion was the most serious problem encountered during the early years of the OCP, a second critical event occurred in 1980 when resistance to temephos emerged [98, 103]. This was initially limited to a forest cytoform of the *S. sanctipauli* subcomplex breeding in the lower Bandama River in Côte d'Ivoire [98]. The response was to switch insecticides to chlorphoxim, and then to *Bacillus thuringiensis* H-14 after resistance to the former also quickly developed [98]. The chlorphoxim-resistant population reverted to normal susceptibility within several months of removing the insecticide [93]. Despite frequent insecticide switching, resistance spread throughout the range of the *S. sanctipauli* subcomplex and by 1986 resistant populations were found in Burkina Faso, Ghana, Mali and Guinea [93, 98]. Resistance among the forest cytoforms was initially of limited concern as they were not considered to be epidemiologically relevant to blinding onchocerciasis [103]. However, resistance to organophosphates soon emerged in the migrating *S. damnosum* s.str. and *S. sirbanum* populations, probably through hybridisation with the sympatric forest cytoforms [93]. The spread of resistance was inevitable, but was carefully managed by the OCP through the alternation of seven larvicides including pyrethroids and carbamates, in addition to those already used [11, 103]. The choice of larvicide was dependent on cytoform susceptibility, river discharge and season [103, 104]. Cytotaxonomy was ultimately crucial to identifying

and resolving the problems of reinvasion and insecticide resistance, and the knowledge gained enabled the OCP to adopt a more selective approach to vector management as the programme progressed [93, 103, 104].

Ivermectin, APOC and beyond (1987 – present)

A seminal moment in onchocerciasis control came in 1987 with the development and licensing of ivermectin (Mectizan®) for treatment of the disease [105]. The vector control strategy of the OCP was an undeniable success and was effective against suppressing blackfly populations and preventing new cases of disease, but it did little to help those already infected [36, 101]. While ivermectin is not a macrofilaricide, it temporarily sterilises adult worms and slowly eliminates microfilariae from the skin and eye [44, 106]. It consequently reduces *O. volvulus* uptake by blood feeding blackflies and therefore reduces transmission [107]. Importantly, ivermectin has an excellent safety profile making it suitable for mass drug administration in most areas [108].

A second critical moment came later the same year (1987) when the drug manufacturer Merck and Co. Inc. committed to providing ivermectin free of charge “to all who needed it, for as long as needed”, through the Mectizan® Donation Program [105, 109, 110]. As a result, ivermectin was introduced to the OCP area to control ocular morbidity in 1988 [11]. It not only changed the OCP strategy to an integrated chemotherapy and vector-based approach, but also revolutionised the future of onchocerciasis control [44]. This led to the establishment of the African Programme for Onchocerciasis Control (APOC) which launched in December 1995 in response to the growing awareness of the clinical and psychosocial impact of onchocercal skin disease [111-113]. APOC was formed to eliminate onchocerciasis as a public health problem in 19 (eventually increasing to 20) endemic countries outside the OCP, where control by larviciding was previously not thought to be practical or cost effective [114, 115]. It aimed to establish within 12-15 years a sustainable community-based ivermectin treatment programme, supplemented with vector control in selected foci [114]. The strategy was to treat all mesoendemic (>40% microfilarial prevalence) and hyperendemic (>60% microfilarial prevalence) areas in each country that were identified by the method of Rapid Epidemiological Mapping of Onchocerciasis or REMO [116, 117]. Chemotherapy was by mass drug administration (MDA) through annual community directed treatment with ivermectin (CDTI) [118]. Community ownership of the ivermectin projects empowered populations to make key decisions about how and when ivermectin was distributed [114]. This was not only essential to the immediate success of the CDTI projects, but it was also necessary to sustain the treatment beyond the APOC mandate. Sustainability will ultimately be crucial in determining the legacy of the programme [114].

Both the OCP and APOC have achieved unprecedented success in controlling onchocerciasis as a public health problem. The former is considered “one of the most successful public health initiatives ever waged in the developing world” [119]. Upon its closure in 2002, an estimated 600,000 cases of blindness had been prevented, 18 million children had been born in areas free from risk of disease, and 25 million hectares of land were considered safe for resettlement [36]. In addition, following an extension of the APOC mandate, it had expanded its reach from 1.5 million people treated with ivermectin in 1997 to 112 million people treated in 180,000 communities in 2014 [115]. APOC eventually closed in December 2015 and the CDTI projects are now the responsibility of respective countries [120]. However, ivermectin is still freely available through the Mectizan® Donation Program and technical support is provided through the WHO Expanded Special Project for Elimination of Neglected Tropical Diseases (ESPEN) [121].

When APOC was founded with the objective of controlling onchocerciasis as a public health problem, it was considered unlikely that annual ivermectin alone could eliminate the disease [115, 122]. However, it was shown that transmission had fallen to very low levels in areas of the former OCP that received treatment without vector control after 10 – 12 years [39]. While this eliminated the public health problem, it was still deemed unlikely that annual ivermectin could eventually eliminate the parasite [39]. Nevertheless, subsequent studies in Mali and Senegal showed that transmission had fallen below WHO thresholds for elimination (see below) after 15 – 17 years of annual or biannual (twice yearly) treatment [14, 123]. In each of the three foci studied, there was no sign of recrudescence 5 years after the last treatments [38, 117]. These results support computer simulations made using the ‘ONCHOSIM’ program, which suggest that elimination is feasible based exclusively on ivermectin MDA [124]. However, success depends heavily on achieving sustained high treatment coverage, especially in foci with high pre-control community microfilarial loads (CMFLs) [124]. Based on these findings and others, the WHO has now set the ambitious aim of achieving operational elimination of onchocerciasis by 2025 [1, 121, 125]. This is defined as “the reduction of onchocerciasis infection and transmission to the extent that interventions can be stopped, but post-intervention surveillance is still necessary” [126].

Evaluation of control programmes

In order to obtain certification of elimination, the WHO requires ivermectin-based interventions to be conducted in three phases (Box 1) [14]. The first is the ‘Treatment Phase’, which requires a minimum 12 – 15 years of periodic (at least annual) MDA with ≥80% therapeutic coverage of the eligible population [14]. Entomological and serological evaluations are then needed to establish whether treatment has sufficiently suppressed *O.*

volvulus transmission before a focus can progress to ‘Phase 2’. Entomological evaluations have several advantages over parasitological evaluations in humans: they are well accepted by communities and preferred to skin snips; infection rates in blackflies are rapid, sensitive indicators of changes in CMFL which result from ivermectin distribution; infection rates correlate well with ivermectin coverage among the human population; and, they are more sensitive than skin snips when infection intensity is low [117].

Box 1. Three phases of ivermectin MDA programmes

Phase 1 – Treatment phase

The first phase, the intervention or treatment phase, is characterized by regular ivermectin treatment with a minimum requirement of 80% therapeutic coverage. This phase typically lasts at least 12–15 years, corresponding to the reproductive lifespan of the adult worm when exposed to drug pressure. Once this stage has been reached an entomological and serological evaluation (Ov-16 antibody test) should be conducted to verify interruption of transmission.

Phase 2 – Post-treatment surveillance

The second phase immediately follows the intervention or treatment phase and is therefore called “post-treatment surveillance”. This phase typically lasts 3–5 years and requires annual entomological and serological evaluation to verify that interruption of transmission continues in the absence of ivermectin.

Phase 3 – Post-elimination surveillance

The third phase starts at the end of the 3–5 years of post-treatment surveillance and is known as “post-elimination surveillance”. It follows the confirmation of the initial assessments at the end of phase 2, thereby providing strong evidence that transmission has been permanently interrupted (eliminated) in a focus. Post-elimination surveillance should continue periodically through entomological evaluation until any risk of recrudescence or reintroduction can be excluded.

Adapted from WHO guidelines (14).

The Phase 1 entomological evaluation requires the collection and ‘pool screening’ of a minimum 6,000 human biting (anthropophilic) blackflies from across a transmission zone (or focus). A human landing collection (HLC) is the preferred method of acquisition [117, 127]. However, this potentially exposes participants to vector borne pathogens which has ethical implications [128]. PCR pool screening uses parasite-specific DNA probes to estimate (with 95% confidence intervals) the percentage of blackflies carrying infective L3 stage *O. volvulus* larvae [69, 129]. The assessment requires that the upper limit of the 95% CI shows an L3 infection prevalence of less than 0.1% (<1/1000) if only parous flies are tested, or less than 0.05% (<1/2000) if all flies are tested, assuming a parity rate of 50%. An annual transmission potential (ATP) of <20 is also thought to be insufficient to sustain transmission based on data from Latin America, although further clarification is needed in African settings [14].

Blackfly collection methods

Human landing collections

The use of human landing collections (HLCs) and other trapping methods for the collection of adult blackflies was comprehensively reviewed by Service in 1977 [130]. HLCs involve the collection of blackflies attracted to, and settling on, the uncovered skin of a ‘human bait’,

before they have an opportunity to bite (Fig 10A). The method provides the most direct and only certain means of collecting species or cytoforms that are biting humans, but it is limited by the varying attractiveness of humans to anthropophilic blackflies [130, 131]. Differences in work ethic and collecting ability are also likely to bias collections [130]. To an extent, this can be overcome by involving several participants at each collection site and rotating collection duties, although it is impossible to negate all confounding factors [127]. The benefits of having a standardised trap are therefore clear, although for the purpose of evaluating onchocerciasis control programmes it is of most importance that traps collect appropriate numbers of the *O. volvulus* vectors biting humans.

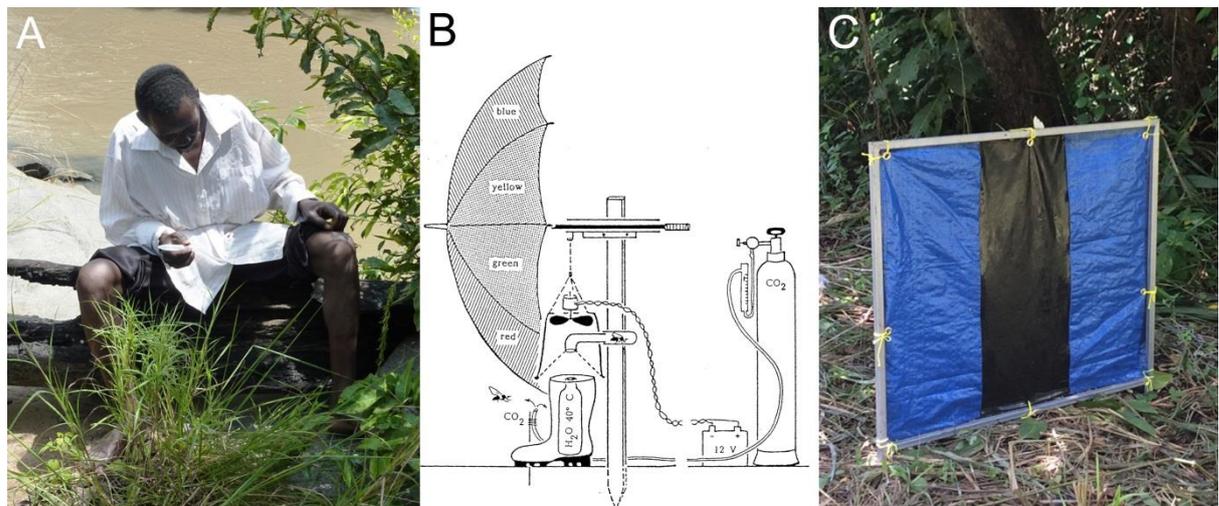


Fig 10. Various blackfly collection methods. A) Standard human landing collection; B) diagram of the Magic Flyboy [132] showing umbrella (visual stimulus), rubber boots filled with warm water (thermal stimulus) and CO₂ tank; C) an unbaited Esperanza Window Trap.

Light traps

In the time since Service's review [130], there have been few studies dedicated to the evaluation and development of trapping tools for the collection of vector blackfly species. Monks Wood light traps were successfully used to collect *S. sirbanum* over seven trapping nights during a rainy season in northern Ghana [54]. Most of the 5,404 females were collected in the first 2-3 hours after nightfall, and of those examined (4,406), 57% were gravid and just three were engorged. The traps were therefore considered unlikely to be of use for the collection of host-seeking blackflies [54]. Service [133] also used Monks Wood light traps to collect 14,644 female *S. squamosum* s.l. during four trapping nights in Ghana. The traps were only effective when placed adjacent to large breeding sites, where approximately 12% of flies were gravid and the remainder appeared to have recently oviposited [133]. Further attempts to collect ovipositing flies using Monks Wood light traps in Ghana yielded 172 *S. damnosum* s.l. representing ≈1% of the total catch made using multiple methods (including Bellec oviposition traps, human and cow baited tents, and a

standard HLC) [134]. Whereas it appears that light traps can be useful for the collection of gravid blackflies or those that have recently oviposited, reproducing successful collections may be difficult, particularly if breeding densities are low.

Sticky traps

Walsh [135] found that *Simulium damnosum* s.l. collections were generally 10 – 20 times lower on 1m² unbaited sticky traps compared to human landing collections in northern Ghana, and it was considered that they offered little promise for monitoring population fluctuations. Traoré *et al.* collected 2,045 *S. damnosum* s.l. (99% of which were *Simulium soubrense/Simulium sanctipauli*) on transparent rectangular (100cm x 50cm) sticky traps in Côte d'Ivoire while investigating blackfly dispersal from breeding sites along a watercourse [136]. The majority (~95%) of flies collected were non-gravid females, but parity rates were not given and it is difficult to determine how useful similar collections might be for monitoring *O. volvulus* transmission. Attempts to collect the *O. volvulus* vector *Simulium ochraceum* s.l. in Mexico using sticky silhouettes and flight inception traps were largely unsuccessful [137].

Biconical traps

Challier-Laveissiere biconical traps, designed for the collection of tsetse flies (*Glossina* spp.), have been used to collect *Simulium yahense* and *S. sanctipauli* s.l. during short studies in Liberia [138, 139]. A modified trap positioned near a breeding site on the St. Paul River collected 302 *S. damnosum* s.l., while 137 were collected in an unmodified trap and 86 were collected on human bait [138]. Cheke and Garms [139] attempted to enhance biconical trap catches of *S. yahense* and *S. sanctipauli* by baiting them with octenol, acetone and a mixture of phenols known to be attractive to *Glossina*. The maximum daily catch in an unwashed acetone baited trap was 2,123 (283.1 flies/hour), compared with 75.8 flies/hour on human bait at the same location. Results showed that collections appeared to be enhanced by contamination through trap handling, rather than by the acetone-bait. In addition, *S. yahense* appeared to enter traps less readily than *S. sanctipauli* [139]. Lamberton *et al.* also attempted to use modified biconical traps for the collection of *S. damnosum* s.l. in Ghana, but failed to collect a single specimen during a two week collection period [140]. This again raises questions about reproducibility using methods that have not been designed specifically for blackflies.

Other methods

Electric nets were also used during the above mentioned Ghanaian study by Lamberton *et al.* [140], although they only collected a single *S. damnosum* s.l. specimen in the two week

collection period. In contrast, human-baited tents collected 2,207 *S. damnosum* s.l. during three visits to Ghana between 2009 and 2011, while human landing collections yielded 6,142 blackflies over the same period [140]. Rodriguez-Perez *et al.* had some success using BG-Sentinel traps (developed for the collection of day-biting *Aedes* mosquitoes) for the collection of *S. ochraceum* s.l. in Mexico, but did not state the overall numbers collected [137].

Novel traps

Attempts to develop traps specifically for the collection of anthropophilic blackflies have been limited in recent years. Renz and Wenk [132] designed an elaborate umbrella-fan trap for the collection of host-seeking blackflies named the “Magic Flyboy” (Fig 10B). Using visual (an umbrella), chemical (CO₂) and thermal stimuli (hot water in rubber boots), the trap caught between 60% and 100% of a corresponding HLC in a Cameroonian rainforest where *S. squamosum*, *S. yahense* and *Simulium mengense* were present. Magic Flyboy collections were even higher in the savannahs where *S. damnosum* s.str. and *S. sirbanum* were present, and a total of 4,591 blackflies were collected during 60 trapping hours overall [132].

The most recently designed trap for the collection of host-seeking blackflies is the Esperanza Window Trap (EWT) (Fig 10C). The EWT is a blue and black target trap that was originally developed for the collection of *S. ochraceum* s.l. in Mexico [137], and has subsequently been trialled for the collection of *S. damnosum* s.l. in Burkina Faso (where *S. damnosum* s.str. and *S. sirbanum* were present) [141, 142]. In each study, EWTs were found to be as effective as HLCs for the collection of vector blackfly species when baited with host odours in the form of worn clothing or a synthetic lure, and CO₂ [137, 141, 142]. The EWT represents a promising new tool for vector blackfly collections, and its development and efficacy are discussed in further detail in Chapter 2.

Thesis outline

Rationale

Many of the CDTI projects established during the lifetime of APOC have now been treating communities >15 years and there is an increasing need to evaluate the impact of both chemotherapeutic and vector-based interventions on parasite transmission [14]. A better understanding of blackfly ecology may also help explain disease epidemiology within and between some of these foci [20]. A recently published multi-country study, conducted in 54 former APOC ‘areas’ between 2008 and 2014, demonstrated that 23 were progressing more quickly than predicted towards elimination [115, 120]. A further 23 were progressing at the expected rate, while eight were progressing more slowly than predicted [115]. However,

while these data are important, assessments were based on human skin snip surveys which are not likely to be as sensitive as entomological assessments at this stage of interventions [14, 117]. At present, there remains considerable doubt as to whether ivermectin treatment alone can interrupt transmission in foci with high pre-control prevalence and intensity of infection [20, 124].

Study areas

The following work is based on entomological studies conducted in three foci in the former APOC countries of Uganda, Tanzania and Cameroon. Each focus was shown to be hyperendemic for onchocerciasis prior to the implementation of interventions [8, 115, 143]. The potentially anthropophilic blackflies present in each study country are shown below (Table 1).

Genus	Subgenus	Species	Author	Year	Country	Human Biting	Reference
<i>Simulium</i>	<i>Anasolen</i>	<i>dentulosum</i>	Roubaud	1915	Ug, Tz, Ca	✓	[73]
<i>Simulium</i>	<i>Byssodon</i>	<i>griseicolle</i>	Becker	1903	Ug, Ca	✓	[144]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>damnosum</i> s.l.*	Theobald	1903	Ug, Tz, Ca	✓	
<i>Simulium</i>	<i>Edwardsellum</i>	<i>damnosum</i> s.str. [†]	Dunbar	1966	Ug, Ca	✓	[100]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>kilibanum</i> [†]	Gouteux	1977	Ug, Tz	✓	[145]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>kipengere</i> [†]	Krüger <i>et al.</i>	2006	Tz	?	[146]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>mengense</i> [†]	Vajime & Dunbar	1979	Ca	✓	[147]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>plumbeum</i> [†]	Krüger	2006	Tz	?	[146]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>sirbanum</i> [†]	Vajime & Dunbar	1975	Ca	✓	[100]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>squamosum</i> [†]	Enderlein	1921	Ca	✓	[147]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>thylense</i> [†]	Vajime <i>et al.</i>	2000	Tz	✓	[62]
<i>Simulium</i>	<i>Edwardsellum</i>	<i>yahense</i> [†]	Vajime & Dunbar	1975	Ca	✓	[147]
<i>Simulium</i>	<i>Lewisellum</i>	<i>neavei</i>	Roubaud	1915	Ug	✓	[69]
<i>Simulium</i>	<i>Lewisellum</i>	<i>nyasalandicum</i>	De Meillon	1930	Tz	✓	[53]
<i>Simulium</i>	<i>Lewisellum</i>	<i>ovazzae</i>	Grenier & Mouchet	1959	Ug, Ca	✓	[148]
<i>Simulium</i>	<i>Lewisellum</i>	<i>woodi</i>	De Meillon	1930	Tz	✓	[149]
<i>Simulium</i>	<i>Meilloniellum</i>	<i>adersi</i>	Pomeroy	1922	Ug, Tz, Ca	✓	[149]
<i>Simulium</i>	<i>Metomphalus</i>	<i>bovis</i>	De Meillon	1930	Ug, Tz, Ca	✓	[150]
<i>Simulium</i>	<i>Metomphalus</i>	<i>vorax</i>	Pomeroy	1922	Ug, Tz	✓	[151]

Table 1. List of potentially anthropophilic blackfly species and *S. damnosum* complex* cytospecies[†] present in each study country, compiled from Adler and Crosskey [68]. Ug = Uganda, Tz = Tanzania, Ca = Cameroon.

Of the three countries, Uganda has a unique place in the history of onchocerciasis control. The former Victoria Nile focus near Jinja is the type locality of *S. damnosum* Theobald (1903), while *S. neavei* Roubaud (1915) was first described from specimens collected in the former western Ankole district [69, 93]. The Victoria Nile is the only focus in the history of onchocerciasis control where it is certain that vector control has led to the eradication of human biting *S. damnosum* s.l. [69, 93, 152]. In recent years, the country has adopted an integrated approach to onchocerciasis control by combining either annual or biannual

ivermectin treatment with vector control where applicable [153]. The Ugandan study focuses on the 'Mid North' area of the 'Madi-Mid North' transmission zone, which includes districts bordering South Sudan in the north of the country [24]. The extent of the onchocerciasis problem in the Mid North was only recently realised following the end of several decades of civil war, and while *S. damnosum* s.str. is thought to be involved in parasite transmission, little is otherwise known about the vectors [154]. The Mid North represents an additional challenge in Uganda's progress towards elimination, and control through biannual CDTI and intermittent vector control has therefore been taking place since 2012 [155].

The study sites in Tanzania and Cameroon rely exclusively on annual ivermectin treatment to control onchocerciasis [156, 157]. Both were included in the recent epidemiological evaluation of onchocerciasis foci referred to above [115]. The Mahenge onchocerciasis focus is situated in Ulunga district, south eastern Tanzania. It was generally thought to be mesoendemic [158], although pre-CDTI evaluations demonstrated that the disease was hyperendemic [156]. Mass drug administration through CDTI commenced in 1997 [156], although coverage >65% has only been achieved since 2003 (Ministry of Health, unpublished data). Mahenge was outperforming ONCHOSIM modelled estimates in its progress towards elimination according to results of skin snip evaluations carried out in 2009 (estimated microfilarial prevalence = 43.8%; actual prevalence = 8.3%) [115]. The only entomological studies known from Mahenge took place in the 1960s, and knowledge of onchocerciasis vectors has changed considerably since [23, 55]. It is thought that a member of the *S. damnosum* complex and possibly the *S. neavei* group are involved in transmission, but the cytoforms and species, and their relative roles in transmission are not clearly defined [23].

In contrast to Tanzania, the study site situated in Bafia Health District (HD) in *Région du Centre*, Cameroon has underperformed when compared with modelled estimates (estimated microfilarial prevalence = 31.0%; actual prevalence = 52.3%) [115]. Annual ivermectin treatment has taken place since 2000 and therapeutic coverage has been >65% since 2002 [157]. However, high pre-control CMFLs ranging from 20.84 – 114.5 were reported from four villages surveyed between 1991 and 1993 [157]. A year-long entomological study was conducted in 1993/94 prior to the implementation of CDTI which provides important baseline data about potential vectors, blackfly biting rates and parasite transmission [67]. The high intensity of infection in Bafia HD has been associated with severe onchocerciasis-related pathologies [8].

Objectives

This study aimed to provide a detailed investigation of the ecology of anthropophilic blackflies and the status of *O. volvulus* transmission in three formerly hyperendemic disease foci under long-term control with either annual CDTI, or vector control in combination with biannual CDTI. The main objectives are provided below, while the specific objectives are outlined in each chapter:

- I. To identify the anthropophilic blackfly species and *S. damnosum* complex cytoforms present in each focus and investigate their relative roles in parasite transmission.
- II. To evaluate the status of *O. volvulus* transmission in each onchocerciasis focus following relevant WHO guidelines [14, 117].
- III. To document the presence and development of non-human *Onchocerca* spp. to infective (L3) stages in anthropophilic blackflies.
- IV. To evaluate the efficacy of a recently developed blackfly trap and assess its suitability as an alternative to human landing collections for the collection of anthropophilic blackflies.

Chapter summary

Chapter 2. Esperanza Window Traps for the collection of anthropophilic blackflies (Diptera: Simuliidae) in Uganda and Tanzania. (Objectives I & IV)

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Human landing collections (HLCs) are currently the gold standard method for the collection of anthropophilic blackflies, but they potentially expose participants to vector-borne pathogens. As onchocerciasis control programmes approach elimination, there is a need to evaluate parasite transmission. A novel trap named the ‘Esperanza Window Trap’ (EWT) was developed by a consortium of researchers from the USA, Mexico and Africa as a possible viable alternative to HLCs for the collection of anthropophilic blackflies. So far, the EWT has been tested with encouraging results in Mexico and Burkina Faso, where anthropophilic *Simulium ochraceum* and *S. damnosum* s.str were collected. However, at the final APOC meeting in 2015, there was considered a need to evaluate the traps in areas where different cytoforms transmit *O. volvulus*. This chapter documents a comparative and systematic evaluation of EWTs with HLCs for the collection of anthropophilic blackflies at multiple locations in the savannah of northern Uganda and a montane habitat in south eastern Tanzania.

Chapter 3. Transmission of *Onchocerca* spp. by human and cattle biting blackflies in northern Uganda. (Objectives I, II & III)

Unpublished.

There is little known about the anthropophilic blackfly species and *Onchocerca* parasites present in northern Uganda, where the extent of onchocerciasis only became apparent following the end of two decades of civil war (1986 – 2006). The discovery of ‘blinding’ disease in three districts bordering South Sudan represents an additional challenge for the Ministry of Health, which aims to eliminate the disease by 2020. The instability caused by war provides a complex epidemiological backdrop to parasite transmission and blackfly behaviour in the region. This chapter provides a detailed survey of blackfly breeding throughout the major rivers and tributaries in the Madi-Mid North onchocerciasis focus. The behaviour of human and cattle-biting blackflies is documented along with parasite transmission in areas where vectors appear to show little preference for host choice. The study took place during the early stages of an integrated control programme that was implemented in 2012.

Chapter 4. The blackfly vectors and transmission of *Onchocerca volvulus* in Mahenge, south eastern Tanzania. (Objectives I & II)

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The Mahenge Mountains onchocerciasis focus in south eastern Tanzania was one of the first in the country to commence CDTI in 1997. This followed several years of ivermectin treatment through a vertical programme of mass drug administration which began in 1994. Periodic clinical and parasitological evaluations were carried out before and during the CDTI programme. However, despite >20 years of chemotherapy, entomological evaluations of parasite transmission have not yet taken place. This chapter documents the first survey of blackfly vectors in Mahenge since the 1960s. Work was conducted during two periods in January 2015 and June/July 2016. Breeding sites were surveyed in perennial rivers throughout the focus, and human landing collections of adult blackflies were made intensively during periods of peak parasite transmission. Laboratory pool screening of blackflies provides important data for comparison with the most recent parasitological surveys among humans.

Chapter 5. *Onchocerca volvulus* transmission in *Région du Centre*, Cameroon, following 16 years of annual CDTI. (Objectives I & II)

Unpublished.

Three of the eight former APOC areas recently reported to be underperforming in their progress towards onchocerciasis elimination are situated in Cameroon. The country historically has some of the highest community microfilarial loads ever documented. In villages near Bafia along the lower Mbam River, a positive correlation has been shown between the intensity of *O. volvulus* infection and the occurrence of epilepsy. The same area was also associated with severe onchocerciasis-related pathologies prior to the implementation of CDTI in 2000. Whereas CDTI has dramatically reduced CMFLs, recent studies have documented poor adherence to ivermectin among young people, while serological evidence suggests that transmission is ongoing. This chapter provides a detailed year-long survey of blackfly biting and *O. volvulus* transmission at several sites similar to those included in another 12-month study conducted in 1993/94. The results enable a direct comparison to be made with the pre-CDTI entomological work conducted previously.

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CHAPTER 2

Esperanza Window Traps for the collection of anthropophilic blackflies (Diptera: Simuliidae) in Uganda and Tanzania

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Abstract

There is an increasing need to evaluate the impact of chemotherapeutic and vector-based interventions as onchocerciasis affected countries work towards eliminating the disease. The Esperanza Window Trap (EWT) provides a possible alternative to human landing collections (HLCs) for the collection of anthropophilic blackflies, yet it is not known whether current designs will prove effective for onchocerciasis vectors throughout sub-Saharan Africa.

EWTs were deployed for 41 days in northern Uganda and south eastern Tanzania where different *Simulium damnosum* sibling species are responsible for disease transmission. The relative efficacy of EWTs and HLCs was compared, and responses of host-seeking blackflies to odour baits, colours, and yeast-produced CO₂ were investigated. Blue EWTs baited with CO₂ and worn socks collected 42.3% (2,393) of the total *S. damnosum* s.l. catch in northern Uganda. Numbers were comparable with those collected by HLCs (32.1%, 1,817), and higher than those collected on traps baited with CO₂ and BG-Lure® (25.6%, 1,446), a synthetic human attractant. Traps performed less well for the collection of *S. damnosum* s.l. in Tanzania where HLCs (72.5%, 2,432) consistently outperformed both blue (16.8%, 563) and black (10.7%, 360) traps baited with CO₂ and worn socks. HLCs (72.3%, 361) also outperformed sock-baited (6.4%, 32) and BG-Lure®-baited (21.2%, 106) traps for the collection of anthropophilic *Simulium bovis* in northern Uganda. Contrasting blackfly distributions were observed on traps in Uganda and Tanzania, indicating differences in behaviour in each area.

The success of EWT collections of *S. damnosum* s.l. in northern Uganda was not replicated in Tanzania, or for the collection of anthropophilic *S. bovis*. Further research to improve the understanding of behavioural responses of vector sibling species to traps and their attractants should be encouraged.

Introduction

In 1966, the World Health Organization (WHO) acknowledged a need to develop new sampling techniques to replace human landing collections (HLCs) for the collection of blackfly (Diptera: Simuliidae) species involved in the transmission of *Onchocerca volvulus*, the parasitic filarial nematode responsible for human onchocerciasis [1]. Despite a comprehensive review of adult blackfly collection methods by Service in 1977 [2], subsequent research efforts to meet the needs outlined by the WHO have been limited [3-9]. The primary concern is for the development of a trap to replace HLCs to monitor progress towards onchocerciasis elimination, but an effective trap might also be deployed as a control mechanism in itself to reduce vector populations in support of mass drug administration. The recent development of the Esperanza Window Trap (EWT), used successfully for the collection of host-seeking anthropophilic blackflies in Mexico and Burkina Faso, has provided the possibility of one such viable method [7, 10-13].

Control and surveillance

Following the implementation of the Mectizan® (ivermectin) Donation Program in 1987, methods of onchocerciasis control switched from vector-based interventions to mass drug administration through community directed treatment with ivermectin (CDTI) [14]. Whereas it has been established that ivermectin treatment can eliminate the disease in certain endemic foci, the conditions under which CDTI alone is effective have not been fully explored [15-17]. It is therefore essential that methods for monitoring entomological and parasitological indices of onchocerciasis transmission are available in intervention and post-intervention settings as countries work towards elimination [18, 19]. For EWTs to be effective in evaluating the impact of chemotherapeutic and vector-based programmes, they should collect appropriate numbers of the same vector populations as those biting humans. They should also collect vectors with the same age structure (parity rates) as those biting humans, or collect them in a condition that enables age structures to be calibrated.

The current WHO guidelines for entomological evaluation of *O. volvulus* transmission in CDTI settings require that HLCs are used for the collection of anthropophilic blackflies [20, 21]. The method is robust, sensitive, and well accepted by communities, and is therefore preferable to more invasive methods of *O. volvulus* surveillance such as Ov-16 serology testing in children [21]. However, human participants collecting biting flies are potentially exposed to a range of vector-borne pathogens, although with appropriate training, the risk is generally considered no higher than for others living in disease endemic areas. Despite

this, obtaining the necessary ethical approval can often delay the implementation of research and surveillance programmes [22].

Available traps

Attempts to develop new, or to utilise or modify existing traps for the collection of host-seeking, anthropophilic blackflies, have been met with mixed or limited success [2]. Light traps [3, 4], sticky traps and silhouettes [23-26], BG-Sentinel traps [7], modified Challier-Laveissiere tsetse traps [5, 6], and other novel traps [27] have been successfully used to collect blackflies in various physiological states, yet repeating collections using these methods has sometimes proved difficult [8, 9].

Visual attraction

Early investigations into the response of blackflies to long-range visual and olfactory stimuli, including colour, shape, and CO₂, were mainly confined to Nearctic species including *Simulium venustum* and *Simulium vittatum* [28-32]. Several studies indicate that host-seeking blackflies generally prefer to land on darker colours and matt surfaces [30, 31, 33], and it is also thought low UV reflectance and strong contrast of traps against their background is important in attraction [28, 32, 34]. Comparatively little research has been dedicated to similar investigations for *Simulium damnosum sensu lato* (s.l.), the principal vector of *O. volvulus* in Africa. The limited data that exists is consistent with colour-choice experiments for other blackflies, in that host-seeking *S. damnosum* s.l. appear to be attracted to dark colours [5, 24, 25, 35]. However, results of behavioural studies should be interpreted cautiously, and Walsh stresses that they should not be generalised for species other than those being investigated [25, 28]. This is likely to be especially relevant when studying *S. damnosum* s.l., a complex of sibling species composed of at least 55 morphologically indistinguishable cytospecies and cytoforms of unknown taxonomic status, each with unique ecological and behavioural traits [36, 37].

Olfaction

Simulium damnosum s.l., like other haematophagous Diptera, are attracted to CO₂ and host odours [38, 39]. CO₂ is a powerful mediator of host-seeking behaviour which can greatly enhance blackfly collections [23, 24], yet the biological mechanisms of blackfly attraction to olfactory and visual stimuli are poorly understood [38]. Following experiments in a Cameroonian rainforest, Thompson (1976) demonstrated that the presence of 'exhaled breath', industrial CO₂, and worn clothing, improved trap collections [24, 40]. He concluded that chemicals present in human sweat are likely to be important in attracting *S. damnosum* s.l. [40], and that visual and olfactory cues are of greatest importance in attracting savannah

and forest sibling species respectively [24]. More recently, EWTs and BG-Sentinel traps baited with worn shirts, trousers (pants) and synthetic chemicals (BG-Lure® and octenol) have been shown to be more effective in attracting blackflies than unbaited traps [7]. Young *et al.* have since used gas chromatography and electroantennography to identify chemicals present in human sweat which are potentially attractive to *S. damnosum* s.l. in Burkina Faso and *Simulium ochraceum* s.l. in Mexico [13]. They then demonstrated that EWTs baited with candidate compounds collected 2-3 times the number of these species in the field compared to traps baited with CO₂ alone, although the authors acknowledge that catch numbers were low and that further research is needed [13].

Esperanza Window Traps

In 2013, Rodriguez-Pérez *et al.* published results of the development and trial of the EWT in Mexico, which involved investigating the attractiveness of coloured fabrics, CO₂ sources, and host odours to *S. ochraceum* [7]. EWTs constructed using blue fabric outperformed those made with red, yellow and black fabrics when baited with either industrial CO₂ released at 150-200mL/min, or CO₂ produced by mixing sugar, yeast (*Saccharomyces cerevisiae*) and water (quantities not specified). There was no statistically significant difference in the number of blackflies collected on traps regardless of the CO₂ source. With the addition of host odours in the form of a worn shirt or BG-Lure®, CO₂-baited blue EWTs approached the attractiveness of HLCs in one of two trials. In the second trial, the baited EWT was only half as effective as the HLC.

Toé *et al.* further developed the EWT in Burkina Faso for the collection of *Simulium damnosum sensu stricto* (s.str.) and *Simulium sirbanum*, but used black traps baited with BG-Lure® and yeast-produced CO₂ as the basic design [11]. EWTs of differing heights were first compared. 'Short' traps, standing within 15cm of the ground were more effective than 'tall' traps, although the difference was only statistically significant at one of two sites investigated. The addition of a vertical blue stripe to the black background further enhanced collections, but again, this was only statistically significant at one of the two sites. Short, striped EWTs baited with CO₂ and BG-Lure® caught similar numbers of *S. damnosum* s.l. as those baited with CO₂ and worn trousers. In a final experiment, EWTs baited with CO₂ and worn trousers collected numbers comparable with HLCs, whereas those baited with worn trousers alone collected numbers similar to unbaited traps. The authors also reported the collection of *Simulium adersi* and *Simulium schoutedeni* from the traps, and questioned the importance of fermentation products other than carbon dioxide in the attraction of vector flies [11].

Rationale and objectives

The various sibling species of the *S. damnosum* complex are behaviourally and ecologically unique in traits such as breeding habitats, dispersal capabilities, degree of anthropophily, and their capacity to transmit disease [37]. It is not yet known whether different sibling species will respond differently to EWTs, and whether current trap designs will prove to be effective for *S. damnosum* s.l. collections throughout onchocerciasis affected areas of sub-Saharan Africa. This study therefore aimed to compare the relative efficacy of EWTs with HLCs for the collection of anthropophilic blackflies in onchocerciasis transmission zones of Uganda and Tanzania, where different sibling species of the *S. damnosum* complex are responsible for disease transmission. Responses of host-seeking blackflies to odour baits, colour schemes, and yeast-produced CO₂ were also investigated.

Materials and methods*Study area*

Experimental work took place for a total of 41 days at five locations in Uganda (26 days), and one in Tanzania (15 days), between 28 June 2015 and 19 September 2016 (Table 1).

Table 1. Blackfly collection locations and distance from nearest known breeding sites. Alt. = Altitude, Dist. = Distance.

Country	District	Location	Coordinates		Alt.	Date	Nearest Known Breeding Sites	Dist.
Uganda	Lamwo	Apyeta Bridge	N 03°18.005'	E 032°21.705'	691m	Jul 2015	Achwa River	0km
		Beyogoya	N 03°17.648'	E 032°29.708'	845m	Jul 2015	Achwa River	7.5km
	Moyo	Gwere Luzira	N 03°39.827'	E 031°48.056'	980m	Jul 2015	Nile (S. Sudan)	16km
		Pamulu	N 03°40.758'	E 031°49.452'	1066m	Jul 2015	Nile (S. Sudan)	13km
	Nwoya	Ayago Bridge	N 02°25.907'	E 032°0.452'	897m	Jun 2015	Ayago River	11km
			N 02°25.907'	E 032°0.452'	897m	Aug 2015	Ayago River	11km
N 02°25.974'			E 032°0.454'	898m	Sep 2016	Ayago River	11km	
Tanzania	Ulanga	Chikuti	S 08°36.175'	E 036°44.072'	459m	Jun 2016	Mbalu River	5km

Collections were made in the districts of Lamwo, Moyo and Nwoya in the Madi-Mid North onchocerciasis transmission zone of northern Uganda. Savannah grassland predominates and *S. damnosum* s.str. is thought to be the principal vector of *O. volvulus* [41, 42]. Small numbers of *S. sirbanum* also breed along the Pager River northeast of Kitgum [43]. In addition, a member of the *Simulium bovis* species-group also forms a significant proportion of the anthropophilic blackfly population in the Mid North [44]. Both *S. damnosum* s.l. and *S. bovis* occupy similar breeding habitats [45, 46]. In Lamwo district, these are mainly along the larger rivers including the Achwa (Aswa) and Pager [47, 48]. In Moyo, there is thought to be little local breeding of *S. damnosum* s.l., and it is likely that biting blackflies migrate from a series of rapids along the Nile in neighbouring South Sudan [43, 49]. The Murchison Nile

forms the southern boundary of Nwoya district and is a major source of blackfly breeding [49]. There are historical reports of *S. damnosum* s.l. breeding along the Ayago River, a tributary of the Nile, and the Kibaa and Murchison River tributaries have also been cited as possible sources of infestation [49, 50]. Rainfall lasts from April to November, with peaks occurring early and late in the rainy season. The climate is hot and dry from December to March [51].

Collections in Tanzania were made at Chikuti on the north side of the Mahenge Mountains in the Mahenge onchocerciasis transmission zone of Ulanga district. The area is characterised by Precambrian limestone, and the presence of riverine, dry lowland and submontane forests [52]. The mountains are drained by numerous stony streams and rivers that are favourable to blackfly breeding [53]. Again, the principal vector of onchocerciasis is *S. damnosum* s.l. [35]. The cytoforms present in Mahenge are 'Nkusi', *Simulium plumbeum* (= 'Hammerkopi' and 'Ketaketa'), 'Sebwe' and 'Turiani' [35, 54, 55]. 'Nkusi' is thought to be the predominant anthropophilic species, and *S. plumbeum* may have a limited role in human biting. Both 'Sebwe' and 'Turiani' are zoophilic [35, 54]. *Simulium nyalalandicum* (originally reported as *S. woodi*) also contributes to biting in small numbers, mainly in the south of the transmission zone [35, 56]. Rainfall lasts from November to May, and peaks between March and May. The dry season lasts from June to October [35, 52].

Basic trap design

Traps were constructed using locally-sourced materials. Frames were composed of a light-gauge steel and trap faces measured approximately 1m² (Fig 1). Traps stood on 0.25m sharpened legs which were easily pushed into the ground. The basic design included a blue tarpaulin screen that was hung tightly inside the frame. Blue was chosen as the base-colour as blue traps yielded the greatest number of blackflies during collections by Rodriguez-Pérez *et al.* in Mexico [7]. A black central stripe ⅓ the width of the blue screen was painted onto the trap using a matt black emulsion (Sadolin Paints (U) Limited, Uganda) during initial experiments in Uganda in 2015. The paint was allowed to dry for two days before traps were deployed. During subsequent collections in Tanzania and Uganda (2016), the black paint was replaced with black tarpaulin which was sewn together with the blue tarpaulin to form the screen. A CO₂ outlet and host odour attractants were attached to the top corners of the EWT frame (Fig 1). Traps were covered with a black plastic sheet when not in use.

Adhesives

Tangle-TrapTM insect trap coating paste (Contech, Victoria, BC, Canada) was used to coat EWTs in Uganda. It was not possible to acquire the same product for trapping work in Tanzania due to manufacturing problems. EWTs in Tanzania were therefore coated with

Temmen-Insektenleim (Temmen GmbH, Hattersheim, Germany). Both products were thinned using $\approx 150\text{mL}$ locally purchased white spirit (Sadolin Paints (U) Limited, Uganda), before being applied to traps at least 24h prior to their deployment.

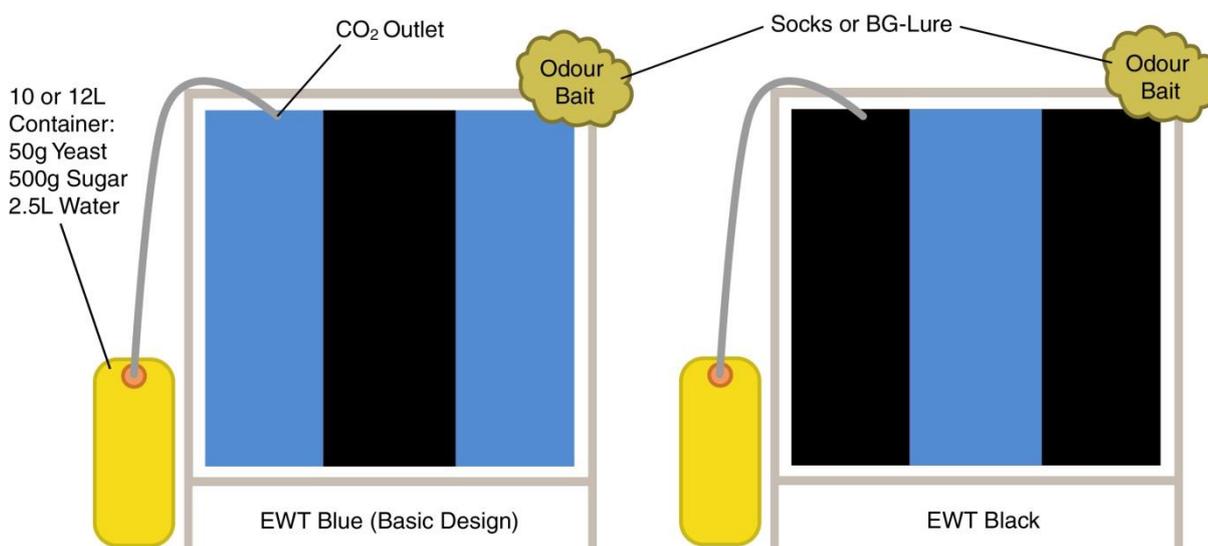


Fig 1. Blue and black trap designs showing position of CO₂ and odour baits. Blue screens with a black vertical stripe (basic design) were used for all trapping experiments in Uganda. Black screens with a blue vertical stripe were additionally used in Tanzania.

CO₂ production

A sugar-yeast based source of carbon dioxide was produced in the field following methods outlined by Smallegange *et al.* [57]. However, quantities of ingredients were adjusted to provide sufficient CO₂ output ($>80\text{mL}/\text{min}$ for at least 11 hours) following incubation at 30°C during preliminary laboratory experiments (Fig S1 [see Supplementary Information]). Dry baker's yeast (50g), sugar (500g) and water (2.5L) were mixed in 10L (Uganda) or 12L (Tanzania) containers immediately prior to blackfly collections commencing. PVC tubing extended from a hole in the container to an outlet at a top corner of the EWT. Containers were briefly shaken before being placed next to traps. Fresh sugar-yeast mixtures were prepared each day by community members assisting with blackfly collections.

Host odour attractants

Traps were either baited with host odours emanating from a pair of worn socks, or BG-Lure® (Biogents AG, Regensburg, Germany), a synthetic mosquito attractant containing chemicals found on human skin (ammonia, lactic acid, and caproic acid) [58]. Worn socks were provided by villagers in exchange for a new pair of socks, and were tied to the top corner of the EWT opposite the CO₂ outlet and replaced every three days. Worn socks have been shown to be effective for up to 8 days for the collection of mosquitoes [59].

Human landing collections

HLCs were made by trained community-based participants following standard methods [20]. A team of two people worked alternate hours between 07:00 and 18:00, collecting blackflies landing on their exposed legs. Flies were collected in individual tubes and hourly catches were recorded.

Specimen preservation and identification

Blackflies were removed from EWTs using forceps after applying a drop of white spirit to specimens in order to partially dissolve the adhesive. A 10x magnification hand lens was used to verify identification of insects where necessary. All blackflies were preserved in >95% ethanol and were identified in the laboratory using morphological keys in Freeman & De Meillon [60]. The member of the *S. bovis* species-group present in northern Uganda was identified based on the morphology of male pupae collected at Apyeta Bridge in 2015. To confirm identification, specimens were compared with reference material at the Natural History Museum, London, UK. The identity of adult *S. bovis* group flies collected on traps and by HLC was inferred based on the pupal identifications. Biting flies other than blackflies were removed from traps and preserved during collections made in 2016 only.

Study design

Odour baits. Blackfly collections were made for 21 days at five locations in Lamwo, Moyo and Nwoya districts of northern Uganda between June and August 2015, to compare the efficacy of EWTs (basic design) baited using CO₂ and either worn socks or BG-Lure[®], with HLCs. At each location, precise vector collection sites were identified with the assistance of community members according to where blackfly biting was already known. A day was spent training participants in HLC methods and also to prepare CO₂ mixtures for baiting traps. Three collection sites were selected at each location for the deployment of 1) a team of two people to make HLCs, 2) two EWTs baited with CO₂ and BG-Lure[®] (EWT BG-Lure[®]), and 3) two EWTs baited with CO₂ and worn socks (EWT Socks). EWTs were placed in pairs, at right-angles to one another, in an attempt to maximise their visibility. HLC and EWT collections were made simultaneously between 07:00 and 18:00 for a minimum of three days (or in multiples of three days) at each location. Collection sites were at least 30m apart and HLCs and EWTs were rotated daily in a 3x3 randomised Latin square design in order to minimise interference and collection site bias respectively. Blackflies were removed from EWTs each day at approximately 11:00, 14:00 and 17:00 to minimise the impact of desiccation on specimen quality. Daily blackfly catches were compared for each method.

Colour schemes. Blackfly collections were made for 15 days at a single location near Chikuti village on the northern side of the Mahenge Mountains in Tanzania in June 2016, to compare the efficacy of EWTs of different colour schemes, with HLCs. Three collection sites were selected in a cultivated field approximately 0.5km from the village centre. Collection methods included 1) a team of two people to make HLCs, 2) two blue EWTs with a black central stripe (EWT Blue), and 3) two black EWTs with a blue central stripe (EWT Black). The EWT Black was similar to the design previously used by Toé *et al.* in Burkina Faso [11]. Each EWT was baited with CO₂ and worn socks as previously described. Again, EWTs were placed in pairs, at right-angles to one another. HLC and EWT collections were made simultaneously between 07:00 and 18:00 each day and blackflies were removed from EWTs at approximately 10:00 and 17:00. Collection sites were at least 50m apart and HLCs and EWTs were rotated daily in a 3x3 randomised Latin square design. Daily blackfly catches were compared for each method.

Yeast-produced CO₂. Blackfly collections were made for 5 days at Ayago Bridge in Uganda in September 2016, to compare the efficacy of EWTs (basic design) baited with either a freshly prepared sugar-yeast mixture (EWT CO₂+), or a mixture that had been prepared 5 days in advance and was no longer producing CO₂ (EWT CO₂-). No other odour baits were used in this experiment. Provisional laboratory observations demonstrated that CO₂ production was <80mL/min after exposing sugar-yeast mixtures to continuous temperatures of 25°C, 30°C and 35°C for 12h (Fig S1). The amount of gas produced after 5 days would therefore be negligible. Two collection sites were prepared approximately 50m apart by clearing vegetation adjacent to the Ayago River. One trap was placed at each site and collections were made between 07:00 and 18:00 each day. Blackflies were removed at approximately 11:00, 14:00 and 17:00 each day and traps were rotated daily as in previous experiments. Daily blackfly catches were compared for each method.

Blackfly distribution. In response to observations that *S. damnosum* s.l. were attracted to the lower parts of EWTs during odour bait experiments in Uganda in 2015, attempts were made to quantify blackfly distribution on traps during subsequent colour and CO₂ experiments in Uganda and Tanzania in 2016. Small holes were made in EWT screens to divide the surface into nine approximately equal squares. The number of blackflies removed daily from each square was recorded for each trap type. Counts from corresponding squares on each side of the trap were combined. Blackflies were preserved daily according to trap type, rather than for each square. Reported blackfly counts on each square are therefore for all blackfly species and not individual species.

Statistical analysis

In all experiments, blackfly count was the response variable and was modelled as a function of trap type, the main covariate of interest. Location, collection site and rainfall were included as additional covariates. A generalized linear framework with a negative binomial distribution was used to take into account the overdispersion observed in the count data. The Akaike Information Criterion was used to select the most appropriate model for each data set, and models were verified by means of diagnostic plots. When more than one anthropophilic blackfly species was active at a study location, data for each species were analysed separately. Data were excluded from analysis for a particular species if blackfly collections were low (<5/day using all methods), or if the species was absent. The negative binomial model was also used to analyse the distribution of blackflies on traps, and to investigate interactions between blackfly attachment on columns and rows. Heat maps of blackfly attachment to traps were produced using log transformed data to improve graphical representation of blackfly distribution. Analyses were performed within the R version 3.3.2 statistical computing environment [61].

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 (960/14, 1089/16); the Higher Degrees, Research and Ethics Committee, Makerere University School of Public Health, Kampala, Uganda (2014/244); and the Medical Research Coordinating Committee at the National Institute for Medical Research, Dar es Salaam, Tanzania (NIMR/HQ/R.8a/Vol.IX/2212). Formal approval to conduct studies in Uganda was granted by the Uganda National Council for Science and Technology (HS 1701). All participants were adults over the age of 18 years who provided written informed consent.

Results

A total of 13,152 female blackflies (*Simulium* spp.) were collected during the study using all methods (Table 2). Of these, 10,652 were preserved and identified. The remaining 2,500 were discarded when catch numbers were either too high to remove and preserve all specimens, or the species composition was known to be >99% *S. damnosum* s.l. based on previous collections. No male blackflies were caught by HLCs or EWTs during the study. In 2015, *S. damnosum* s.l. comprised >99.9% (5,656/5,663) of all blackflies collected in Moyo and Nwoya districts of northern Uganda, but only 1.4% (7/506) of those collected in Lamwo district. The remaining 98.6% (499/506) were identified as *S. bovis sensu* De Meillon (1930) [60]. In 2016, a further 3,476 blackflies were collected on EWTs in Nwoya district, but only 1,201 were preserved. Of these, 99.6% (1,196/1,201) were identified as *S. damnosum* s.l.

and it was presumed that a similar proportion of the 2,275 non-preserved flies were the same species. *Simulium damnosum* s.l. comprised 96.3% (3,161/3,282) of all blackflies preserved and identified from collections made in Tanzania using all methods. Other Simuliidae present in Tanzania included *S. vorax*, *S. adersi*, *S. hirsutum* and a number of small unidentified species.

Table 2. Summary data showing number of blackflies of each species collected using all methods.

Year	Country	District	Location	Trap Days	Total Blackflies	Total Preserved	<i>damnosum</i>	<i>bovis</i>	<i>vorax</i>	<i>adersi</i>	<i>hirsutum</i>	Other	Not Preserved
2015	Uganda	Lamwo	Apyeta Bridge	3	327	327	1	326	0	0	0	0	0
			Beyogoya	3	179	179	6	173	0	0	0	0	0
		Moyo	Gwere Luzira	3	766	766	766	0	0	0	0	0	0
			Pamulu	3	935	935	929	0	0	0	0	6	0
		Nwoya	Ayago Bridge	9	3962	3962	3961	0	0	0	0	1	0
2016	Uganda	Nwoya	Ayago Bridge	5	3476	1201	1196	0	0	0	0	5	2275 ^b
2016	Tanzania	Ulanga	Chikuti	15	3507	3282	3161	0	8	11	5	97	225 ^c
Total				41	13152	10652	10020	499	8	11	5	109^a	2500

^aSmall blackflies unidentifiable morphologically using Freeman & De Meillon [60].

^bSpecimens presumed to be *S. damnosum* s.l. based on known species composition at Ayago Bridge.

^cSpecimens removed from EWT Blue without being preserved on a single collection day when catch numbers were unexpectedly high. Based on the frequency distribution of the observed specimens it was estimated that 194 of the 225 specimens were *S. damnosum* complex.

Odour baits

Pairs of traps baited with CO₂ and worn socks (EWT Socks) were as effective as the HLC for the collection of *S. damnosum* s.l. in northern Uganda, while pairs of traps baited with CO₂ and BG-Lure® (EWT BG-Lure®) were the least effective overall (Fig 2A). However, there was a significant interaction effect of trap type and location on blackfly collections ($p=0.002$). The EWT Socks outperformed the HLC and EWT BG-Lure® at Ayago Bridge and Gwere Luzira, whereas the reverse was true at Pamulu. After 15 trap days, the EWT BG-Lure® collected 25.6% (1,446), the EWT Socks 42.3% (2,393), and the HLC 32.1% (1,817) of the total *S. damnosum* s.l. catch (Table 3).

There was a significant effect of trap type on the number of *S. bovis* collected in Lamwo district ($p=0.008$), and there was no interaction effect of trap type and location on collections ($p=0.58$) (Fig 2B). The HLC clearly outperformed EWTs of both types at Apyeta Bridge and Beyogoya ($p<0.001$), and there was weak evidence to suggest the EWT Socks was the least effective trap overall ($p=0.074$). After 6 trap days, the EWT BG-Lure® collected 21.2% (106), the EWT Socks 6.4% (32), and the HLC 72.3% (361) of the total *S. bovis* catch (Table 3).

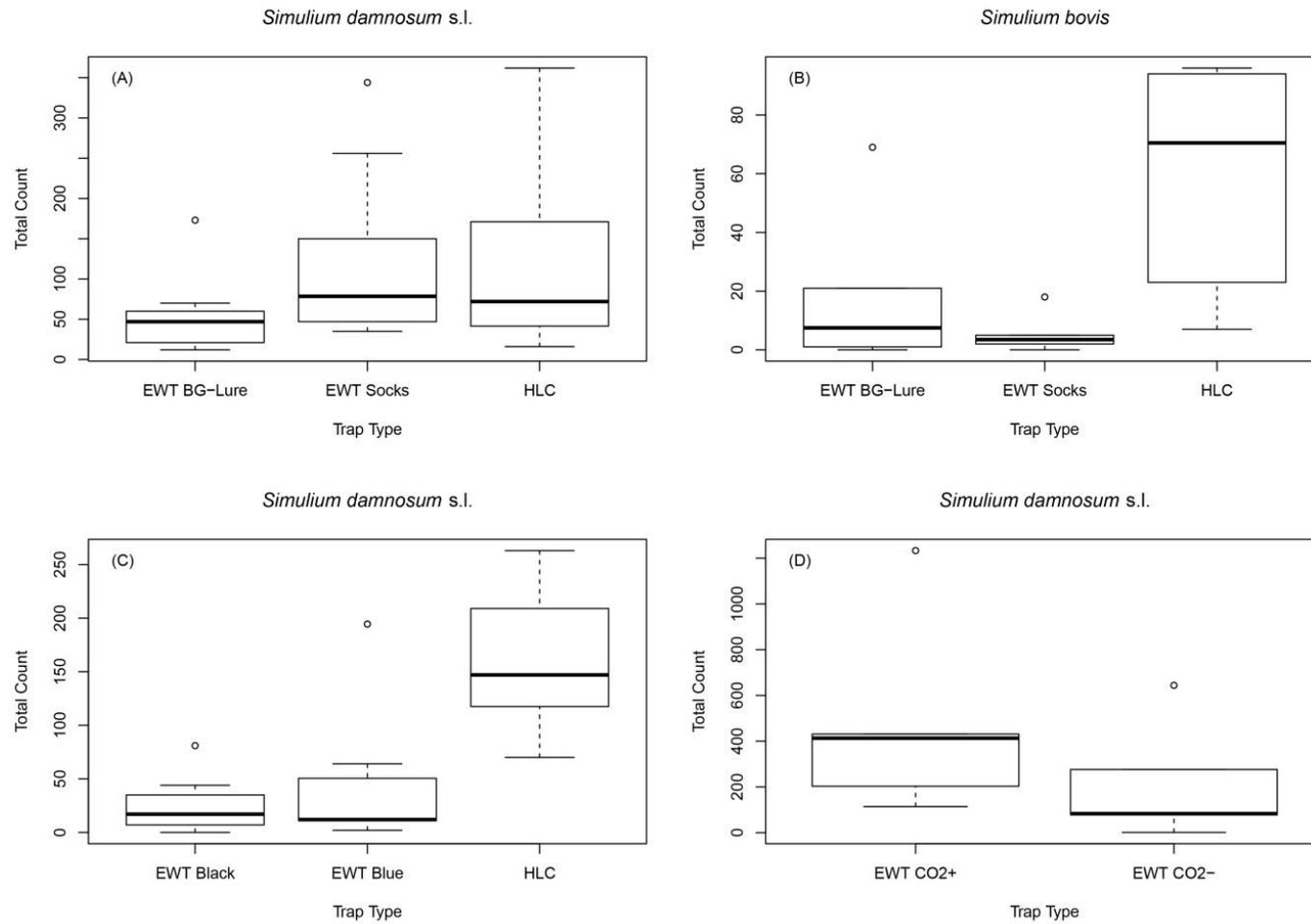


Fig 2. Median values and interquartile ranges of daily *S. damnosum* s.l. and *S. bovis* collections made using EWTs and HLCs. (A) *S. damnosum* s.l. collections made using BG-Lure® and sock-baited EWTs in northern Uganda, 2015; (B) *S. bovis* collections made using BG-Lure® and sock-baited EWTs in northern Uganda, 2015; (C) *S. damnosum* s.l. collections made using black and blue EWTs in Tanzania, 2016; (D) *S. damnosum* s.l. collections made using fresh (CO₂+) and pre-prepared (CO₂-) sugar-yeast sources of CO₂ in northern Uganda, 2016.

Table 3. Summary data of *S. damnosum* s.l. and *S. bovis* collections for each trap type. The EWT BG-Lure® and EWT Socks were additionally baited with CO₂ as stated in the methods.

Year	Country	Species	Trap Days	Trap Type	Median	IQR	Min.	Max.	Total	% Total
2015	Uganda	<i>S. damnosum</i> s.l.	15	EWT BG-Lure®	47	39	12	173	1446	25.6
				EWT Socks	78.5	97.5	35	344	2393	42.3
				HLC	72.0	129.5	16	362	1817	32.1
2015	Uganda	<i>S. bovis</i>	6	EWT BG-Lure®	7.5	20	0	69	106	21.2
				EWT Socks	3.5	3	0	18	32	6.4
				HLC	70.5	71	7	96	361	72.3
2016	Uganda	<i>S. damnosum</i> s.l.	5	EWT CO ₂ +	413	228	114	1233	2394	68.9
				EWT CO ₂ -	83	198	1	644	1082	31.1
2016	Tanzania	<i>S. damnosum</i> s.l.	15	EWT Black	20	32	5	95	360	10.7
				EWT Blue	19	42	2	194	563	16.8
				HLC	147	91.5	70	263	2432	72.5

Colour schemes

More than 99% of blackflies recovered from EWTs in Uganda were morphologically indistinguishable from those collected by HLC. This was not the case in Tanzania where *S. damnosum* s.l. comprised 100% of the catch by HLC, but only 86.3% (360/417) and 85.6% (563/658) of the catch on the EWT Black and EWT Blue traps respectively. There was a significant effect of trap type on *S. damnosum* s.l. collections at Chikuti ($p < 0.001$) where the HLC clearly and consistently outperformed EWTs of each colour scheme (Fig 2C). There was no overall difference in efficacy between the EWTs, and despite the EWT Blue outperforming the EWT Black at two of the three collection sites, there was insufficient evidence to suggest *S. damnosum* s.l. preferred one colour scheme over another ($p = 0.28$). After 15 trap days, the EWT Black collected 10.7% (360), the EWT Blue 16.8% (563), and the HLC 72.5% (2,432) of the total *S. damnosum* s.l. catch (Table 3).

Yeast-produced CO₂

Rainfall restricted trapping to five days at Ayago Bridge in Uganda during September 2016, although this was sufficient to demonstrate that freshly prepared sugar-yeast mixtures (producing CO₂) enhanced *S. damnosum* s.l. collections ($p < 0.001$) (Fig 2D). After 5 trap days, the EWT CO₂+ collected 68.9% (2,394) and the EWT CO₂- 31.1% (1,082) of the total *S. damnosum* s.l. catch (Table 3). Trap site was a significant explanatory variable ($p < 0.001$) and blackfly activity was noticeably higher at one of the two collection sites. Both sites were situated in areas of cleared bush surrounded by tall vegetation, although the most productive site had greater exposure to sunlight. When exposed to direct sunlight, *S. damnosum* s.l. would primarily land on the shaded side of traps.

Blackfly distribution

The vertical distribution of blackflies (all species) was similar for both the EWT CO₂+ and EWT CO₂- in Uganda where 62.8% and 66.9% of specimens were removed from the bottom rows of respective traps (Table 4). Blackfly numbers decreased with increasing height on the traps ($p < 0.001$) regardless of whether CO₂ was present or absent.

Table 4. Summary data showing blackfly distribution on rows and columns of traps, including mean daily catch and standard errors (SE).

Country	Trap Days	Trap Type	Row	Mean Daily Catch ^a (SE)	% Total	Column	Mean Daily Catch ^a (SE)	% Total
Uganda	5	EWT CO ₂ +	Top	60.8 (24.2)	12.7	Left	227.4 (105.3)	47.5
			Middle	117.4 (49.8)	24.5	Middle	171.8 (65.2)	35.9
			Bottom	300.6 (124.5)	62.8	Right	79.6 (29.4)	16.6
	5	EWT CO ₂ -	Top	15.8 (7.1)	7.3	Left	53 (19.4)	24.5
			Middle	55.8 (27.7)	25.8	Middle	88 (49.3)	40.7
			Bottom	144.8 (82.7)	66.9	Right	75.4 (49.2)	34.8
Tanzania	12	EWT Blue	Top	31.7 (14.9)	60.4	Left	25.3 (9.6)	48.2
			Middle	11.4 (3.8)	21.8	Middle	7.8 (2.0)	14.9
			Bottom	9.3 (2.8)	17.8	Right	19.3 (8.1)	36.9
	12	EWT Black	Top	18.7 (6.1)	58.0	Left	11.9 (2.7)	37.0
			Middle	7.8 (1.4)	24.1	Middle	10.1 (2.3)	31.3
			Bottom	5.8 (0.9)	17.9	Right	10.2 (3.5)	31.6

^aAll blackfly species.

In contrast, blackflies (all species) in Tanzania showed greater attraction to the top row of EWTs ($p < 0.001$) (Table 4). Again, the percentage of blackflies differed little between the traps, with 60.4% and 58.0% being removed on the top rows of the EWT Blue and EWT Black respectively. Blackfly numbers decreased with decreasing height on EWTs of both colour schemes ($p = 0.021$). The horizontal distribution of blackflies on the EWT Blue indicated a preference towards the outer columns where the CO₂ outlet (left) and worn socks (right) were located ($p = 0.002$). There was also a slight preference towards the left column on the EWT Black, although blackflies were otherwise more evenly distributed across columns than on the EWT Blue. Log transformed counts of blackfly distribution are illustrated in Fig 3.

Other biting flies

Only five biting flies other than blackflies were removed from traps in Tanzania and all were Tabanidae of the genera *Haematopota* and *Tabanus* (Table 5). Biting flies were more diverse and abundant at Ayago Bridge in Uganda and included both male and female *Glossina f. fuscipes* and *Glossina pallidipes*. Glossinidae were identified to species using morphological and molecular methods in the laboratory of Prof Stephen Torr (Liverpool School of Tropical Medicine, UK). *Stomoxys calcitrans* and several unidentified *Haematopota* and *Tabanus*

species were also collected. The biting flies recovered from traps were of sexes exhibiting anthropophilic behaviour for each species.

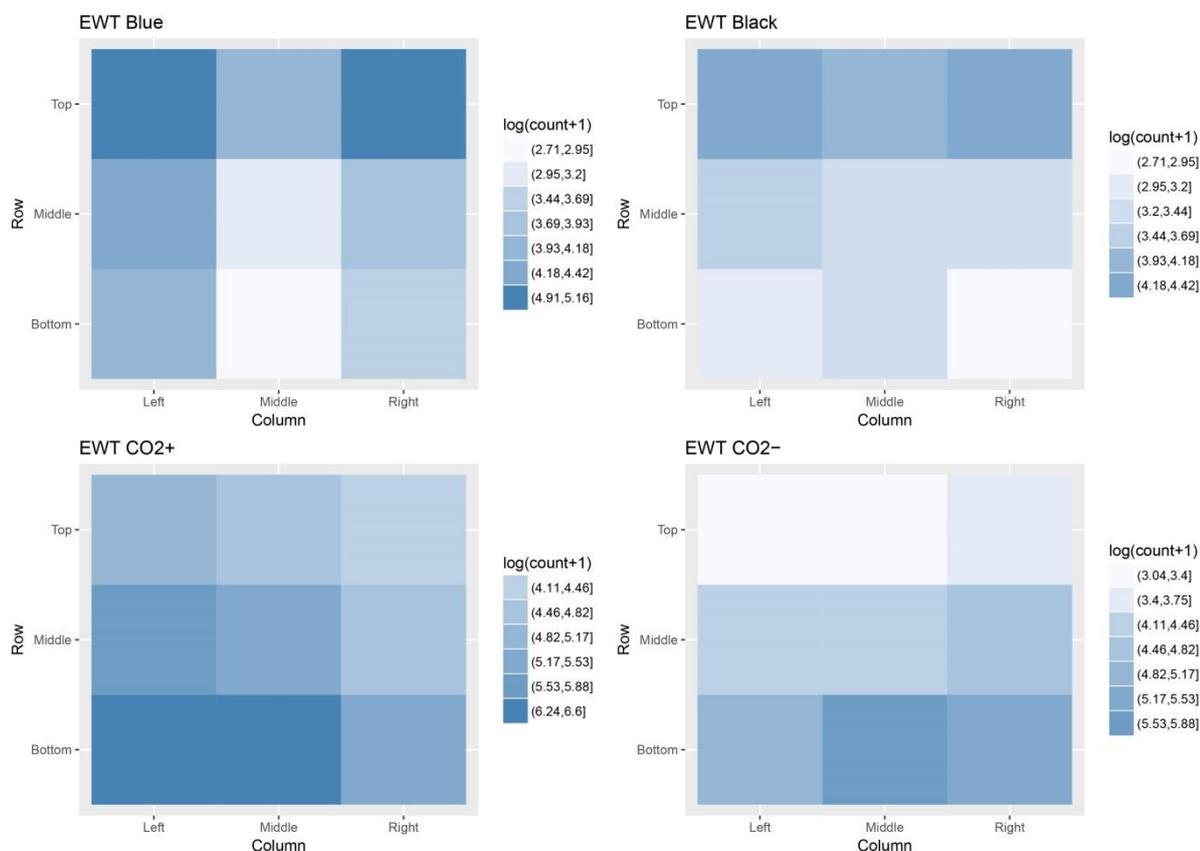


Fig 3. Heat maps illustrating distribution of all blackfly specimens collected on EWTs in Tanzania (EWT Blue and EWT Black) and Uganda (EWT CO₂⁺ and EWT CO₂⁻) in 2016.

Date of Collection	Country	Location	Family	Genus	Species	Sex	Number		
June 2016	Tanzania	Chikuti	Tabanidae	<i>Haematopota</i>	sp.	♀	1		
				<i>Tabanus</i>	sp.	♀	4		
September 2016	Uganda	Ayago Bridge	Glossinidae	<i>Glossina</i>	<i>f. fuscipes</i>	♀	9		
						♂	14		
					<i>pallidipes</i>	♀	3		
						♂	10		
					Muscidae	<i>Stomoxys</i>	<i>calcitrans</i>	♀	4
					Tabanidae	<i>Haematopota</i>	sp.	♀	7
							<i>Tabanus</i>	spp.	♀

Table 5. Species and number of biting flies other than blackflies removed from traps in Tanzania (15 days) and Uganda (5 days) in 2016.

Discussion

Odour baits

It was initially stated that for EWTs to be viable for *O. volvulus* surveillance, they should sample appropriate numbers of the same vector populations as those biting humans.

Whereas pairs of blue EWTs baited with CO₂ and BG-Lure® appeared to be less effective than in previous studies in Mexico and Burkina Faso [7, 11], those baited with CO₂ and worn socks regularly collected numbers comparable with HLCs in northern Uganda. A notable exception was at Pamulu, where the EWT Socks caught the fewest flies. Blackfly activity varied greatly from site to site at each location, and it rained on the day the EWT Socks was positioned at the site with highest activity at Pamulu. The negative impact of rain on trap performance was compounded by the limited number of catching days (3) at this location. There was no rain at Gwere Luzira, so traps were unaffected. In addition, the higher number of trapping days (9) at Ayago Bridge meant the impact of rain on overall trap performance was less apparent than at Pamulu.

In contrast to the success of the Ugandan collections, EWTs baited with CO₂ and worn socks performed relatively poorly compared to HLCs for the collection of *S. damnosum* s.l. in Tanzania. It is not clear why, although given that different *S. damnosum* sibling species were present in the study areas of each country, it seems plausible that they might respond differently to traps. The host-oriented behaviour of Glossinidae has been extensively studied and there is evidence of both interspecific and intraspecific variation in response to host kairomones [62, 63]. Similar differences in behavioural response may exist for the many sibling species of the *S. damnosum* complex, and the recent study of blackfly attraction to human semiochemicals by Young *et al.* should provide a good starting point for further research [13]. In the meantime, the most appropriate odour bait is probably worn clothing, that is easy to obtain and reflects odour profiles of local populations.

EWTs performed poorly for the collection of *S. bovis* in northern Uganda. This is a species that generally feeds on cattle, although frequent human biting has been reported in the past from Nigeria and northern Cameroon [45, 64]. It has been proposed that anthropophily may develop in the absence of its usual bovine host [45]. Pairs of EWTs baited with worn socks collected just 6.4% (32/499) of the total *S. bovis* catch (Table 3). EWTs baited with BG-Lure® performed slightly better, collecting 21.2% (106/499) of the total catch. However, the difference in trap efficacy can probably be explained by the presence of a herd of cattle, rather than attraction to the lures. Of the 106 *S. bovis* collected over six days on traps baited with BG-Lure®, 65.1% (69) were collected on a single day at Apyeta Bridge. On that day, cattle passed within a few metres of the BG-Lure®-baited traps. The observed number of

blackflies was noticeably higher on these traps immediately after the cattle had passed. Whereas flies “carried” by the cattle might have dispersed and enhanced collections on all trap types, the impact was much more evident on those closest to the herd. A similar event occurred at Gwere Luzira where the presence of cattle also coincided with a high (240) *S. damnosum* s.l. catch on sock-baited EWTs. Again, there were noticeable differences in the number of blackflies on these traps before and after the event. Such confounding factors will need to be taken into consideration if attempting to calibrate trap collections with human biting rates. Care will also need to be taken to place traps away from shared animal hosts of human biting blackflies.

Uniformity of experiments would have been improved by standardising the washed status of HLC participants and also the amount of time socks were worn for in advance of trapping. Baiting traps with socks from both HLC participants might also have reduced bias caused by variation in human attractiveness to blackflies [59].

Colour schemes

HLCs consistently outperformed EWTs of each colour scheme in Tanzania. Possible reasons for differences in trap-efficacy observed between countries are discussed in the following sections. As a result of the poor relative performance of traps in Tanzania, there was insufficient evidence to demonstrate that *S. damnosum* s.l. preferred one colour scheme over the other. Further investigations of colour preference among *S. damnosum* sibling species are warranted.

Yeast-produced CO₂

Freshly prepared sugar-yeast mixtures clearly enhanced the number of blackflies collected on EWTs. Despite concerns raised that fermentation products other than CO₂ are likely to attract vector flies other than those seeking a blood meal, the impact appears to have been negligible [11, 57]. Since no male blackflies were collected on traps, despite non-vector species breeding in the adjacent river, it is likely that CO₂ is the most important compound in attraction. However, it should be noted that various Hymenoptera and Diptera were frequently attracted to the jerry can containing the sugar-yeast mixture. Comparing the parity rates and gonotrophic status of HLC and EWT-collected flies would help further clarify whether sugar-yeast mixtures are only attracting host-seeking vectors.

Blackfly distribution

The contrasting distribution of blackflies of all species on EWTs in Uganda and Tanzania appears to indicate differences in *S. damnosum* s.l. behavioural response, although differences in species composition present obvious limitations.

Perhaps the simplest explanation would be to refer to the previously mentioned work of Thompson in Cameroon [24]. If savannah sibling species are more reliant on visual host-seeking cues [24], are naturally inclined to fly close to the ground [38, 65, 66], and tend to land low on their host [65, 66], this could sufficiently explain the distribution of blackflies on traps in Uganda. The percentage of blackflies removed from the bottom (62.8%/66.9%) and middle (24.5%/25.8%) rows of the EWT CO₂+ and EWT CO₂- (Table 4), compares well with a study of savannah *S. damnosum* s.l. in northern Cameroon [66]. Here, Renz and Wenk demonstrated that most flies fed on the ankles (53%/51%) and calves (28%/27%) of standing and sitting volunteers respectively [66]. The percentage of blackflies removed from the top (60.4%/58.0%) and middle (21.8%/24.1%) rows of the EWT Blue and EWT Black at Chikuti in Tanzania shows a considerably contrasting distribution. It could be that the behaviour of sibling species present in the Mahenge Mountains more closely resembles the forest sibling species described by Thompson [24]. It is possible that they are more reliant upon olfactory cues when host-seeking, explaining why greater numbers were removed from the top rows of traps where odour baits were positioned [24].

Host preferences of sibling species present in Mahenge may offer another explanation. It is known that the vertical distribution of haematophagous Diptera can be influenced by their hosts [67, 68]; that no blackfly species is exclusively anthropophilic [37], and that degrees of anthropophily vary among human biting members of the *S. damnosum* complex [69]. Little is known about the respective blood hosts of *S. damnosum* s.l. in Mahenge, although 'Nkusi' is probably responsible for the majority of human biting [35]. It is also known to feed on cattle in addition to humans in western Uganda [70]. The remaining cytoforms, *S. plumbeum*, 'Sebwe' and 'Turiani' are either mainly or entirely zoophilic [35, 54], and zoophilic blackflies can also be specific in their preferred feeding sites on a host [71]. For example, East African *S. vorax* and *S. nyalalandicum* prefer to bite the ears and underside of cattle, respectively [71]. Many ornithophilic blackfly species also prefer to bite the area around the head and neck of their hosts [72, 73]. Studies of Glossinidae have shown that odour-oriented responses attract flies towards their hosts, but final responses are to visual cues [63, 74]. Again, similar mechanisms of host-location might also exist for blackflies [63].

It is not known whether EWTs were sampling the same sibling species as HLCs during studies in Uganda and Tanzania. PCR-based identification of *S. damnosum* s.l. collected using each method might have highlighted any differences in sibling species composition [75]. The use of unbaited EWTs, or EWTs with odour baits positioned at different heights, might have clarified the importance of visual and olfactory cues in each study area. Preserving blackflies according to the area of the trap on which they landed, rather than according to trap type,

would have enabled the distribution of *S. damnosum* s.l. and other species to be represented more accurately. Also, blood meal analyses of flies collected on EWTs or breeding in nearby rivers might have yielded information about host preference.

Absence of males

The lack of male *S. damnosum* s.l. and *S. bovis* on traps might suggest that EWTs specifically target host-seeking females, but this should be considered in relation to the distance of collection sites from breeding sites. Little is known about dispersal distances of male blackflies, although it is generally thought they disperse shorter distances than females [71, 76]. With the exception of adult collection sites at Apyeta Bridge which were adjacent to the Achwa River, those at Pamulu (13km), Gwere Luzira (16km), Beyogoya (7.5km) and Ayago Bridge (11km), were a considerable distance from places of known *S. damnosum* s.l. breeding (Table 1). At Chikuti, they were also 5km from known breeding sites in the Mbalu River.

Other biting flies

It was unsurprising that biting flies other than blackflies were recovered from traps since blue and black target traps are commonly used for the collection of diurnally active haematophagous Diptera, including the genera collected during this study [63]. Given that only blood-feeding sexes of each species were recovered implies that EWTs are attractive to host-seeking flies [77].

Consumables

Ideally, the same adhesive would have been used to coat EWTs in both Uganda and Tanzania, but this was not possible due to manufacturing problems. Both Tangle-Trap™ and Temmen-Insektenleim are clear, odourless adhesives commonly used to trap insects [78, 79]. They do not oxidise to form a surface film and remained sticky throughout the trapping experiments. Adhesives with these physical properties are known to be effective for collecting tsetse and other Diptera [80, 81]. Whereas the use of different products might have had an effect on the relative blackfly catch in each country, it is unlikely that this could sufficiently explain the differences in trap efficacy observed.

Differences in locally-sourced products such as sugar, yeast and container-size almost certainly affected rates of CO₂ production in each country. Temperatures to which sugar-yeast mixtures were exposed are also likely to have had an impact. Concerns about the impact of prolonged exposure to high temperatures on CO₂ production were addressed by conducting semi-field experiments at Gulu University (Gulu, northern Uganda) in September 2016 (Fig S2). Experiments were conducted for four days in mean daily (07:00 – 18:00)

temperatures of up to 36.8°C (min. 20.2°C, max. 46.0°C). Results showed that mean daily CO₂ production did not drop below 173.79mL/min when using sugar-yeast mixtures as previously described. It is therefore also unlikely that differences in trap efficacy observed between countries were caused by effects of high temperatures on CO₂ production. Further field-based research into the effects of consumables and environmental variables on CO₂ production and trap efficacy is needed.

Trap function and limitations

The choice of trap materials and their interactions with the environment affected trap performance and ease of use. The matt black emulsion initially used to paint stripes on the blue tarpaulin screen frequently peeled when removing overnight covers, although this problem was easily overcome by replacing the paint with black tarpaulin during trap construction. The adhesives used were costly if imported and affected specimen quality. It was necessary to apply a drop of white spirit to partially dissolve the glue before removing a specimen as previously recommended by Toé *et al.* [11]. This improved specimen quality, although specimen removal was consequently laborious if catch numbers exceeded 500 blackflies a day, and only a single person was working to remove them. Rodriguez-Pérez *et al.* previously stated that a single person can easily maintain five traps, and this is true providing that catch numbers are relatively low [7]. The prolonged presence of an individual at a trap also served to attract even greater numbers of blackflies. Specimen desiccation was a problem in Tanzania where blackflies were removed from traps twice daily, but was less so in Uganda where specimens were removed three times daily. It was also necessary to frequently clean traps and reapply adhesives following rainfall, which often left soil and detritus covering the base of EWTs. This was particularly important in Uganda where blackflies were mostly found on the lower third of traps.

Trap placement was also important to the success of collections with significant site-to-site variation in blackfly activity frequently encountered. Although no attempts were made to standardise trap placement, sites with partial shade and some direct sunlight appeared to collect most flies. Traps performed poorly in sites that were too exposed, while those placed in heavily shaded areas often caught the fewest flies.

Conclusion

Esperanza Window Trap collections of *S. damnosum* s.l. in Uganda were very encouraging, with pairs of traps baited with yeast-produced CO₂ and worn socks proving to be as efficacious as HLCs. However, successes of the Ugandan collections were not replicated in Tanzania where HLCs clearly and consistently outperformed EWTs of both colour schemes.

Behavioural responses of *S. damnosum* s.l. to EWTs appeared to differ between study countries and this was highlighted by differences in the distribution of blackflies on traps. Responses of *S. damnosum* s.l. to visual and olfactory stimuli should be investigated further in East Africa given the diversity of sibling species present. Further research should also investigate whether EWTs sample the same sibling species as HLCs in areas such as Mahenge where anthropophilic and zoophilic *S. damnosum* s.l. occur sympatrically [35]. Since several non-anthropophilic *Simulium* species were collected on traps, it seems reasonable to assume that non-anthropophilic *S. damnosum* s.l. could also be present. The relatively poor performance of EWTs for the collection of anthropophilic *S. bovis* should raise awareness of potential limitations of EWTs for the collection of anthropophilic blackflies in areas where *S. damnosum* s.l. is not the vector of *O. volvulus*.

Current EWT designs have shown promise for the collection of *S. damnosum* s.l. in Burkina Faso and northern Uganda [11]. Further research and development should be encouraged to improve understanding of behavioural responses of blackflies to traps and their attractants in order to develop them as a tool for onchocerciasis surveillance in sub-Saharan Africa.

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EWT construction & preparation at Gulu University, Uganda.



Applying adhesive to traps at Pamulu, Moyo district.



EWT baited with freshly prepared sugar-yeast mixture, Ayago Bridge, Nwoya district.



S. damnosum breeding site, Ayago River near confluence with the Nile, Nwoya district.

Black EWTs situated on top of small hill, Chikuti, Tanzania.



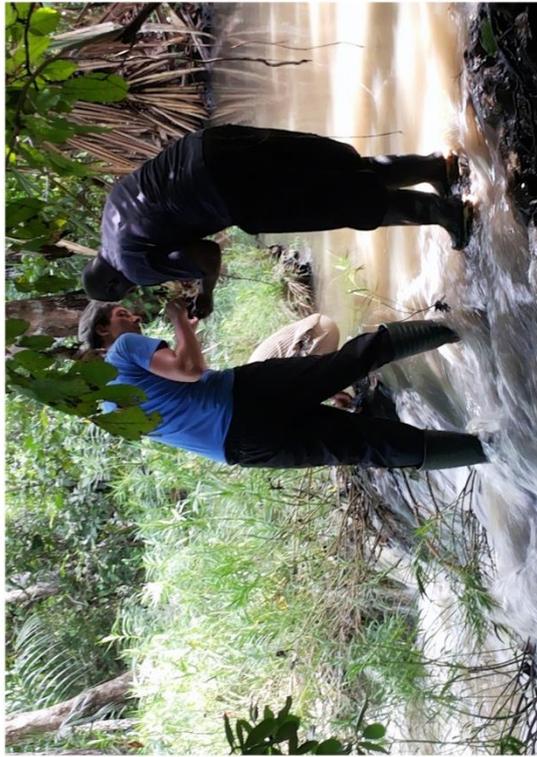
Black EWTs positioned below tree, Chikuti.



Oviposition traps (not documented) at *S. damnosum* breeding site, Mbalu River, Chikuti.



S. damnosum breeding site, Mbalu River, Chikuti.



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CHAPTER 3

Transmission of *Onchocerca* spp. by human and cattle biting blackflies in northern Uganda

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Abstract

The 'Madi-Mid North' transmission zone in northern Uganda is one of the last important onchocerciasis foci in the country. In several of the 'Mid North' districts bordering South Sudan, access to onchocerciasis-affected areas was limited during two decades of civil war, at a time when countrywide onchocerciasis mapping was carried out. Control within these districts is now a priority as the country aims to eliminate the disease by 2020. Annual community directed treatment with ivermectin was therefore introduced in 2009, before a biannual regimen supplemented with vector control commenced in 2012. This study aimed to identify the vectors of *Onchocerca volvulus*, map their distribution, and evaluate transmission, giving particular attention to the Mid North districts of Kitgum, Lamwo and Pader.

Major rivers and tributaries were surveyed for blackfly larvae and pupae in 2014/15. Human landing collections of adult blackflies were made at 20 sites across five districts (Kitgum, Lamwo, Pader, Moyo and Adjumani) in 2015/16. Collections from cattle were also made ad hoc in Lamwo in 2014/15. All collections were made during the rainy season and blackflies were identified by morphology, cytotaxonomy, and ITS1 PCR. Anthropophilic blackflies collected in the Mid North were screened for *O. volvulus* infection using O-150 primers and a triplex real-time PCR. *Simulium damnosum* s.str., *Simulium sirbanum* and *Simulium bovis* were among the species breeding in the Mid North and Adjumani districts. *Simulium damnosum* s.l. and *S. bovis* were collected on human and cattle bait in Lamwo district, and also on human bait in Pader. No anthropophilic blackflies were collected in Kitgum and numbers were low in Adjumani. *Simulium damnosum* s.l. biting rates were high in Moyo, where there was little evidence of local breeding. Human and cattle biting blackflies were negative for *O. volvulus* infection, although *Onchocerca ochengi* and *Onchocerca* sp. 'Siisa' were detected in the heads of human biting blackflies.

The integrated approach to control appears to have had a considerable impact on *O. volvulus* transmission in an area where *S. damnosum* s.l. and *S. bovis* could potentially act as vectors, although it was not possible to incriminate either species. Biting in Moyo appears to result from breeding in South Sudan and cross country collaboration will be important in eventually eliminating the disease.

Introduction

Onchocerciasis in Uganda

Among the blackflies of northern Uganda, *Simulium damnosum* s.l. is renowned for its role in the transmission of *Onchocerca volvulus*, the parasitic nematode that causes human onchocerciasis [1]. However, *Simulium bovis*, which as its name suggests is more commonly found feeding on cattle, also exhibits anthropophilic behaviour in two districts bordering South Sudan [2, 3]. These districts fall within the 'Mid North' area of the 'Madi-Mid North' transmission zone, which is one of the last important onchocerciasis foci in the country. While it was known that onchocerciasis existed in the Mid North, the full extent of the problem only became apparent following the end of two decades of civil war (1986 – 2006) [4].

Onchocerciasis in Uganda was historically associated with approximately 18 foci, many of which were situated along the western border with the Democratic Republic of Congo (DRC) [5, 6]. In these areas transmission was predominantly by *Simulium neavei*, which was also the vector in the only focus in eastern Uganda near Mount Elgon before it was eliminated in 2011 [5, 7]. Members of the *S. damnosum* complex are known to transmit *O. volvulus* along a stretch of the Victoria Nile from Atura to Lake Albert, and *Simulium kilibanum* was the vector in the former Rwenzori focus in western Uganda [5, 8, 9]. Brown also suspected that *S. damnosum* s.l. was breeding along the Albert Nile near Nimule, at the (South) Sudan border [5, 10]. However, the complex is originally known from a 70km series of rapids along the upper Victoria Nile, stretching from below the Owen Falls Dam near Jinja to Lake Kyoga [1, 9]. This is the type locality of the *S. damnosum* complex, and the location of the first successful vector control campaign against *S. damnosum* s.l. [1, 11].

Beginning in 1952, it took several attempts to control *S. damnosum* s.l. through short duration applications of DDT in the stretch of river extending from Lake Victoria, before the species was eventually eradicated in 1973 [1]. Since then, Uganda has been at the forefront of onchocerciasis control in East Africa [12]. Transmission had been interrupted in at least 10 Ugandan foci by 2015 and is suspected to have been interrupted in several more, although concerns exist about cross-border transmission in some foci bordering the DRC and South Sudan [6, 7, 12-15]. Uganda is one of only three endemic countries, alongside Tanzania and Equatorial Guinea, to supplement ivermectin chemotherapy with vector control in certain areas [16]. In addition, the Ministry of Health (MOH) adopted a strategy of biannual (twice yearly) ivermectin treatment in several foci in 2007 [17]. This intensive approach to disease control makes interruption of transmission in Uganda by 2020 a realistic possibility, although elimination is likely to take longer [12]. However, the discovery

of hyperendemic onchocerciasis in areas of northern Uganda that had previously been masked by civil war, presents an additional challenge [4, 18].

The Madi-Mid North focus

In 1993, countrywide rapid epidemiological mapping of onchocerciasis (REMO) revealed the presence of the disease in the northern districts of Nwoya and Oyam, north of the Murchison Nile, and Gulu and Amuru to the west of the Achwa River [19]. Surveys in districts east of the Achwa (now Kitgum, Lamwo and Pader and the north of Lira) were initially limited by insecurity, and the extent of the problem was therefore not realised until mapping took place in 2008 [4, 12, 19]. The districts listed above collectively formed the large 'Mid North' focus, thought to potentially extend into South Sudan [12, 18]. The adjacent West Nile districts of Moyo and Adjumani formed the contiguous 'Madi' focus, and the two foci have recently been recognised as a single transmission zone (Madi-Mid North) by the Ugandan Onchocerciasis Elimination Expert Advisory Committee [11, 12, 19].

The *O. volvulus* vectors in the Madi-Mid North districts are thought to be members of the *S. damnosum* complex [18]. Whereas descriptions of the cytoforms and their distribution have not been formally documented, unpublished data show that *S. damnosum* s.str. formed ≈95% of the *S. damnosum* s.l. breeding populations in the Mid North in 2012 [11]. Small numbers of *Simulium sirbanum* and 'Nkusi' cytoforms were also reportedly present in some parts of Kitgum and Lamwo districts [3, 11]. Additionally, large numbers of anthropophilic *S. bovis* have also been collected in Kitgum and Lamwo, where they are thought to represent approximately half the population of human biting blackflies [2, 3].

As well as transmitting *O. volvulus*, members of the *S. damnosum* complex are also vectors of several African bovine *Onchocerca* species including *Onchocerca ochengi*, for which northern Uganda (Gulu) is the type locality [20, 21]. The two species are very similar and are difficult to distinguish morphologically due to overlapping lengths of L3 stage parasites [22, 23]. The sympatric occurrence of *O. volvulus* and *O. ochengi* can therefore cause problems when estimating transmission potentials during control programmes, for which accurate parasite identification is essential [23, 24]. However, the two species can be differentiated by molecular methods, but this usually entails a rather laborious process of identification using O-150 PCR combined with Southern blotting and DNA hybridisation [25]. While *S. damnosum* s.l. is able to transmit both *O. volvulus* and *O. ochengi*, it is not known whether the comparatively small *S. bovis* can transmit either. In Nigeria, Crosskey found infective stage parasites in thoraces of human biting *S. bovis* that were morphologically indistinguishable from *O. volvulus* [26]. He also reported that no cattle were present within 10 miles of collections, commenting that it was unlikely *O. ochengi* was the observed

parasite. *Simulium bovis* was shown to be an efficient vector of the smaller bovine parasite, *Onchocerca dukei*, in an area of northern Cameroon where it was also anthropophilic, but there was no evidence to suggest it could transmit *O. volvulus* or *O. ochengi* [27].

Control in the Mid North districts

The control of onchocerciasis in the Mid North districts of Kitgum, Lamwo, Pader and Lira began with annual community directed treatment with ivermectin (CDTI) in 2009 [28]. This changed to a biannual strategy in 2012 (reported as 2013 by Burton [29]), while at the same time an aerial and ground larviciding vector control campaign was implemented [2, 29]. The intensive control operation was partly due to the ambitious approach Uganda has towards onchocerciasis elimination [12], and partly in response to a reported association between *O. volvulus* infection and an epidemic of childhood epilepsy known as nodding syndrome [29-32]. The latter condition, which also only became apparent after the war, has devastated affected families and communities in the Mid North [33, 34].

Rationale

Since the discovery of hyperendemic onchocerciasis in the Mid North, little has been reported about the blackfly vectors involved in parasite transmission. This study therefore aimed to investigate the distribution of anthropophilic blackflies in the Madi-Mid North focus; to identify the vector(s) of *O. volvulus*, and evaluate the extent of transmission. Particular attention was given to collections in the Mid North districts of Kitgum, Lamwo and Pader, where an integrated chemotherapeutic and vector-based approach to disease control had been implemented.

Materials and methods

Study area

The study took place between April 2014 and November 2016 in the Madi-Mid North districts of Kitgum, Lamwo and Pader (Acholi sub-region), and Adjumani and Moyo (West Nile sub-region) (Fig 1, Fig S3). Much of the area is characterised by dry savannah grassland with little variation in geographic relief, although upland areas in Moyo rise to above 1500m [35]. The largest rivers include the Albert Nile, which intersects Moyo and Adjumani districts before crossing the border into South Sudan, and the Achwa (Aswa), which is a major tributary of the Nile responsible for draining much of the north eastern highland and northern plateau of Uganda [36]. The Agago and Pager rivers flow into the Achwa along the western borders of Pader and Lamwo districts. The Nyimur River flows through Lamwo and into South Sudan before joining the Achwa towards its confluence with the White Nile. The larger rivers in the Mid North were sites of extensive *S. damnosum* s.l. breeding prior to the

implementation of control interventions [4, 37]. Blackfly biting in the Mid North occurs during the long rainy season which lasts from April to October [38]. The northern districts receive around 750 – 1500mm of annual rainfall [35]. The dry season, which lasts from November until March, can be severe. Drought tolerant crops are therefore cultivated and include finger millet, simsim (sesame), cassava and sorghum [39]. The majority of the population rely on small scale agriculture as a primary source of income [40]. Ninety percent of farmers are engaged in crop production, while a small percentage rear livestock, including Ankole and Zebu cattle in the Mid North [39, 40].

Collection and preservation of blackflies

Breeding site surveys were carried out along the major rivers and their tributaries in April, September and October 2014, and from June until August 2015. For comparison, additional collections were made from sites on the Albert Nile near Karuma Falls in September 2014, and also from the adjoining Ayago River in Murchison Falls National Park in August 2015. Potential breeding sites were identified with the assistance of a Vector Control Officer (VCO) in each district. Where present, blackfly larvae and pupae were collected from rocks and trailing vegetation and were fixed in three changes of Carnoy's fixative (\approx 3: 1 ethanol: glacial acetic acid) for cytotoxic study. Pupae were collected and preserved in the same way, but were subsequently transferred to absolute ethanol in the laboratory. Additional data from breeding site surveys conducted in northern Uganda in 2012 and 2013 were provided by Post (unpublished distribution data) (Table S4) [11].

Human landing collections of adult blackflies were made by trained community-based participants as described in Chapter 2 [3]. Teams of two people worked alternate hours, under the supervision of a village health team member, to collect blackflies landing on their exposed legs between 07:00 and 18:00 each day. Flies were collected in individual tubes and hourly catches were recorded. Collections were attempted at 20 locations for a combined 79 days in July and August 2015 and from September to November 2016. Collections of cattle biting blackflies were also made *ad hoc* at two locations in Lamwo district in April 2014 and July and August 2015. Adult flies were preserved in absolute ethanol.

All preserved specimens were kept in the dark at ambient temperature until they were stored at 4°C (or -20°C for specimens in Carnoy's) in the laboratory within two weeks of their collection.

Identification of blackflies

Breeding blackflies were mainly identified by the morphology of pupal respiratory organs using the taxonomic key in Freeman and De Meillon [41]. Larvae of the *S. damnosum*

complex were also identified morphologically by the presence of dorsal abdominal tubercles and scales on the prothoracic proleg [42]. Cytotaxonomy was used to identify the *S. damnosum* complex cytoforms present. Some late-instar larvae first had their heads and thoraces removed, which were preserved individually in absolute ethanol for subsequent ITS1 analysis. Salivary glands were then dissected from abdominal cavities of associated specimens and chromosomes were prepared following a Feulgen-staining method outlined by Adler *et al.* [43]. Larvae were identified with reference to chromosome maps and descriptions in Vajime and Dunbar [44], Boakye [45] and Post [11]. Nomenclature follows Krüger [46].

Adult blackflies collected on humans and cattle were also identified morphologically either to species-group or species complex using the key in Freeman and De Meillon [41]. The member of the *Simulium bovis* species-group was identified by the morphology of male pupae as described in Chapter 2, and the identity of the species biting humans and cattle was inferred based on this.

Attempts were made to differentiate anthropophilic species breeding and biting throughout the study area based on amplicon size polymorphisms of ITS1 rDNA of larvae, pupae and adults as described by Krüger [46]. This involved DNA extraction of either a whole or part of a specimen using QIAGEN DNeasy Blood & Tissue Kits (Qiagen, N.V.) according to the manufacturer's instructions. DNA extracts were amplified using ITS1 Fw and ITS1 Rev primers (Table 1), following a modified protocol based on methods outlined by Tang *et al.* [47]. Reactions were carried out in 25µL total volumes containing 10pmol of each primer, 5µL 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 60 secs, 45°C for 60 secs, and 72°C for 60 secs, 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.

Identification of Onchocerca species

Heads and bodies of blackflies collected on humans and cattle were screened either individually or in small pools (≤ 10) to investigate the presence and development of *Onchocerca* parasites, particularly *Onchocerca volvulus* (sample preparation is described in detail in Chapter 4). Microfilariae, L1 and L2 stage parasites are generally found in blackfly bodies (midgut and thorax), while infective L3 stages are generally found in heads (L3H). DNA was extracted from individual or pooled samples as described for ITS1 analysis. DNA extracts of anthropophilic blackflies were screened for *Onchocerca* infection, using S3/S4 primers that target the O-150 tandem repeat region of *O. volvulus* and *O. ochengi*, following

published methods (Table 1) [25]. Samples known to be positive for *O. volvulus/ochengi* served as positive controls.

Table 1. Primers and hybridisation probe sequences and concentrations used in the ITS1, O-150 and triplex PCRs. Ov = *Onchocerca volvulus*, Oo = *Onchocerca ochengi*.

PCR	Primers	Sequence (5' – 3') and Modification	Concentration (nM)
ITS1	ITS1 Fw	TACTATCTTATTTCCACAA	400
ITS1	ITS1 Rev	CCCCTGTCTAGATGTTAT	400
O-150	S3	ATCAATTTTGCAAATGCG	400
O-150	S4	AATAACTGATGACCTATGACC	400
Triplex	OvOo ND5 forward	GCTATTGGTAGGGTTTGCAT	300
Triplex	OvOo ND5 reverse	CCACGATAATCCTGTTGACCA	300
Triplex	Ov probe	Fam-TAAGAGGTTATTGTTTATGCAGATGG-BHQ1	100
Triplex	Oo probe	Hex- TAAGAGATTGTTGTTTATGCAGATAGG-BHQ1	100
Triplex	16S rDNA forward	AATTACTCCGGAGTTAACAGG	500
Triplex	16S rDNA reverse	TCTGTCTCACGACGAACTAAAC	500
Triplex	16S rDNA probe	Cy5-TACAACATCGATGTAGCGCAGC-BBQ-650	150

A triplex real-time PCR (qPCR) was used to differentiate *O. volvulus* from *O. ochengi* in an area where both parasites are likely to be transmitted by *S. damnosum* s.l. [4, 21]. The triplex method differentiates the two species based on differences in respective ND5 genes and is described in detail in Chapter 4. It also includes genus-specific 16S rDNA primers and hybridisation probes used to identify other *Onchocerca* spp. that may be present (Table 1). Positive bodies (or pools of bodies) were interpreted as being infected with microfilariae or developing *O. volvulus* larvae, whereas positive heads indicated the presence of mature, potentially transmissible parasites.

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 (960/14, 1089/16) and the Higher Degrees, Research and Ethics Committee, Makerere University School of Public Health, Kampala, Uganda (2014/244). Formal approval to conduct studies in Uganda was granted by the Uganda National Council for Science and Technology (HS 1701). All participants were adults over the age of 18 years who provided written informed consent.

Results

Blackfly species and their distribution

Breeding of *S. damnosum* s.l. was largely confined to the main rivers in Kitgum, Lamwo and Pader districts (Fig 1, Table S4). Access to breeding sites along the Achwa was mainly from the Awere, Achwa and Apyeta bridges, all of which were *S. damnosum* s.l. positive on at least one occasion between 2012 and 2015. The breeding site at Te Lute (Achwa) was 7.5km

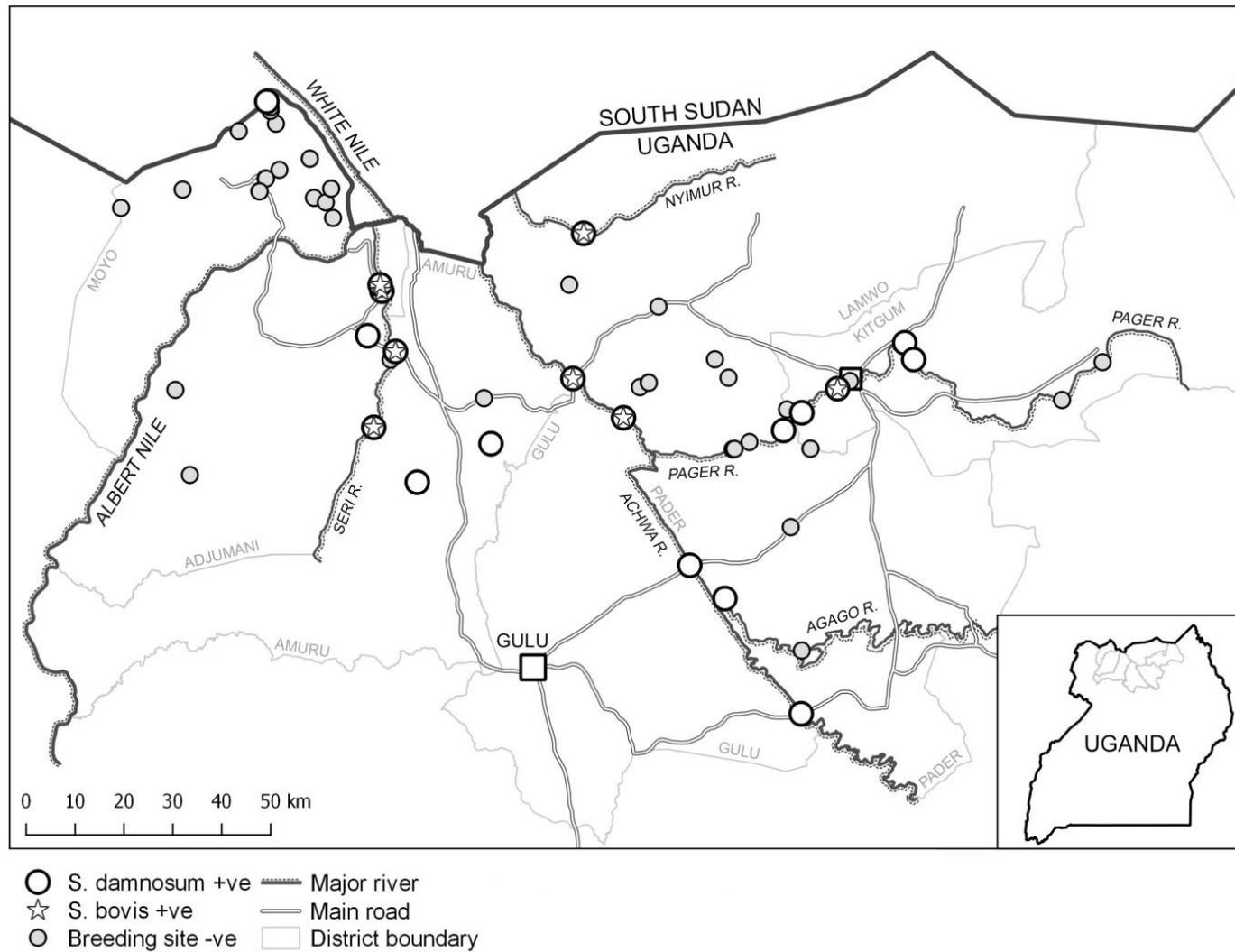


Fig 1. Map of northern Uganda showing *S. damnosum* s.l. and *S. bovis* breeding sites from collections made in 2012/13 and 2014/15. Only unique sites of collection are shown (i.e. repeat visits to the same site are not represented by more than a single marker).

from the nearest village and only accessible by foot, but was a very productive habitat for both *S. damnosum* s.l. and *S. bovis*. Both species were also collected at Apyeta Bridge (2012 and 2015), and the Nyimur River (2012) close to the South Sudan border. Prospections of tributaries in these districts did not yield *S. damnosum* s.l., only non-anthropophilic blackflies. The only two sites surveyed along the Pager River, east of Kitgum Matidi (July 2015), were also *S. damnosum* s.l. negative. At both points, the river was smaller, flowing gently, and was not suitable for breeding (although this may change later in the rainy season). In Amuru and Adjumani districts, *S. damnosum* s.l. was collected from the smaller Unyama, Aiyuge, Ayugi, Seri (all 2013) and Nyeguy (2014) rivers. *Simulium bovis* was also collected at multiple sites along the Seri and Ayugi rivers in 2013, but this species was absent when the rivers were surveyed in 2014/15. In Moyo, the majority of watercourses are small mountainous streams that do not support *S. damnosum* s.l. breeding. Of all the sites surveyed between 2013 and 2015, this species was only collected by Post in 2013 from a single site on the Ayo River, close to the South Sudan border [11].

Cytotaxonomy

Ninety six *S. damnosum* complex larvae collected from 6 sites in northern Uganda (including Karuma) were fully karyotyped (Table 2). All possessed fixed inversions 1S-1, 1L-3, 2L-C and 3L-2, characteristic of the *S. damnosum* subcomplex. Inversion 1L-1 was also fixed, while Inversions 1S-2 and 1S-3 were polymorphic, but not sex-linked, in all populations examined. In addition, 1L-2 was polymorphic in populations at Karuma, Nwoya (Ayago River), Tumangu and Te Lute, but absent in populations at Orima and Adjumani (Nyeguy River).

Date	Location	River	Latitude	Longitude	Altitude	No. Larvae	2L			3L			
							C/C	C/C.8	C.8/C.8	2/2	2.6.110/2.6.110	2.6/2.6.110	2/2.6.110
09/14	Karuma	Nile	2.25450	32.26089	1031m	27	27			27			
09/14	Tumangu	Pager	3.20443	32.75405	859m	24	10	12	2	24			
10/14	Orima	Pager	3.33355	32.99327	946m	17			17	11	5	1	
10/14	Adjumani*	Nyeguy	3.37693	31.98887	656m	4	1		3	1	3		
07/15	Te Lute	Achwa	3.22757	32.45945	717m	8	1	5	2	8			
08/15	Nwoya*	Ayago	2.37078	31.92169	897m	16	16			16			
Total						96	55	17	24	76	14	5	1

Table 2. *Simulium damnosum* s.l. inversions present in chromosome arms 2L and 3L. * = District name.

Larvae collected from Karuma and Ayago River were identified as *S. damnosum* s.str. and possessed the fixed inversion 2L-C, while 2L-8 was absent in both males and females (Fig 2). Populations examined from Tumangu and Te Lute agreed with descriptions of *S. damnosum*

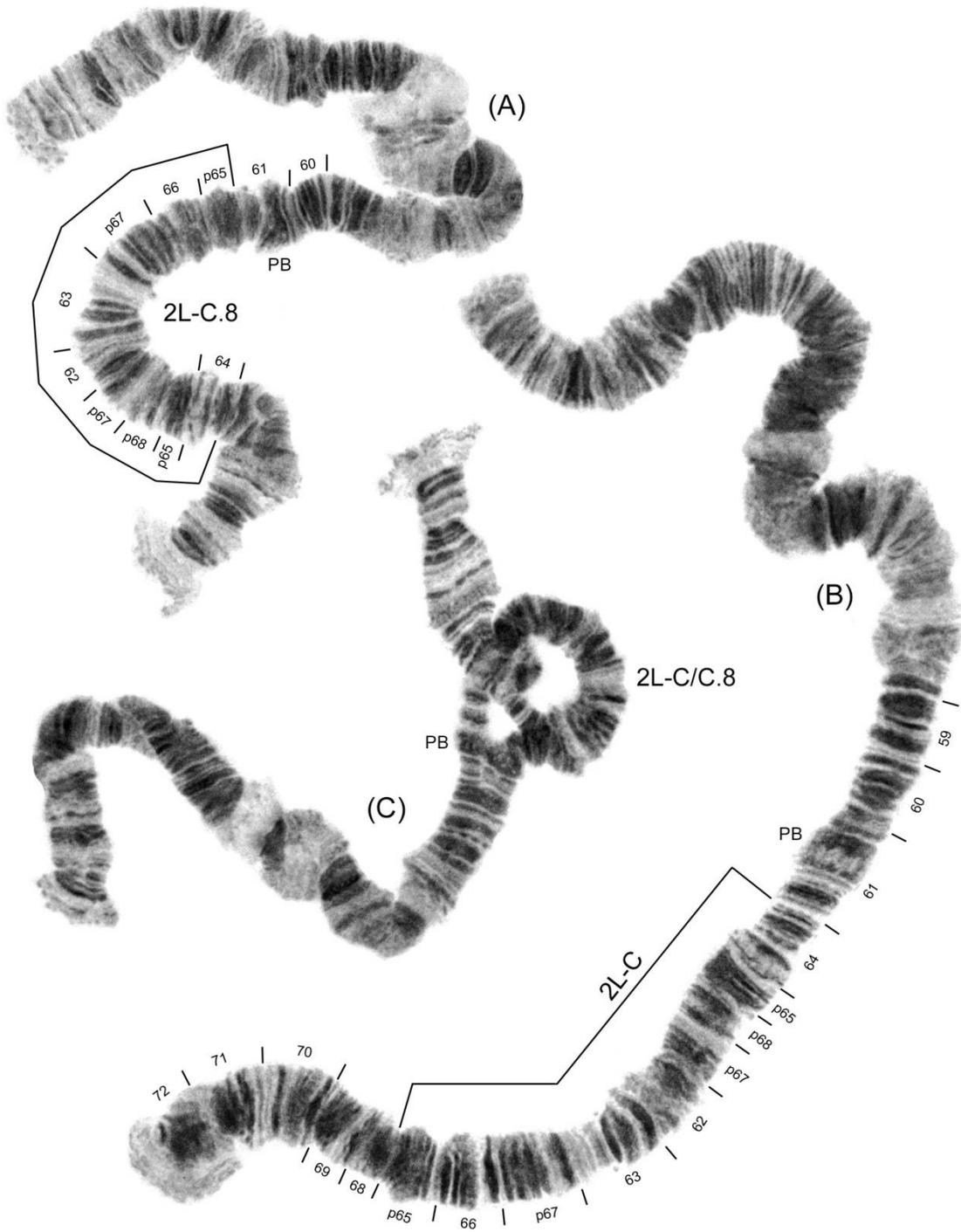


Fig 2. Chromosome 2. Cytotaxonomy of *S. damnosum* s.str. and *S. sirbanum* from central and northern Uganda. (A) *S. sirbanum* (female) collected at Orima (Pager), Kitgum, showing homozygous inversion 2L-C.8; (B) *S. damnosum* s.str. (female) collected at Karuma (Nile), showing homozygous inversion 2L-C which is fixed within the *S. damnosum* subcomplex; (C) *S. damnosum* s.str. (female) collected at Tumangu (Pager), showing heterozygous inversion 2L-C/C.8. 'PB' = parabalbiani.

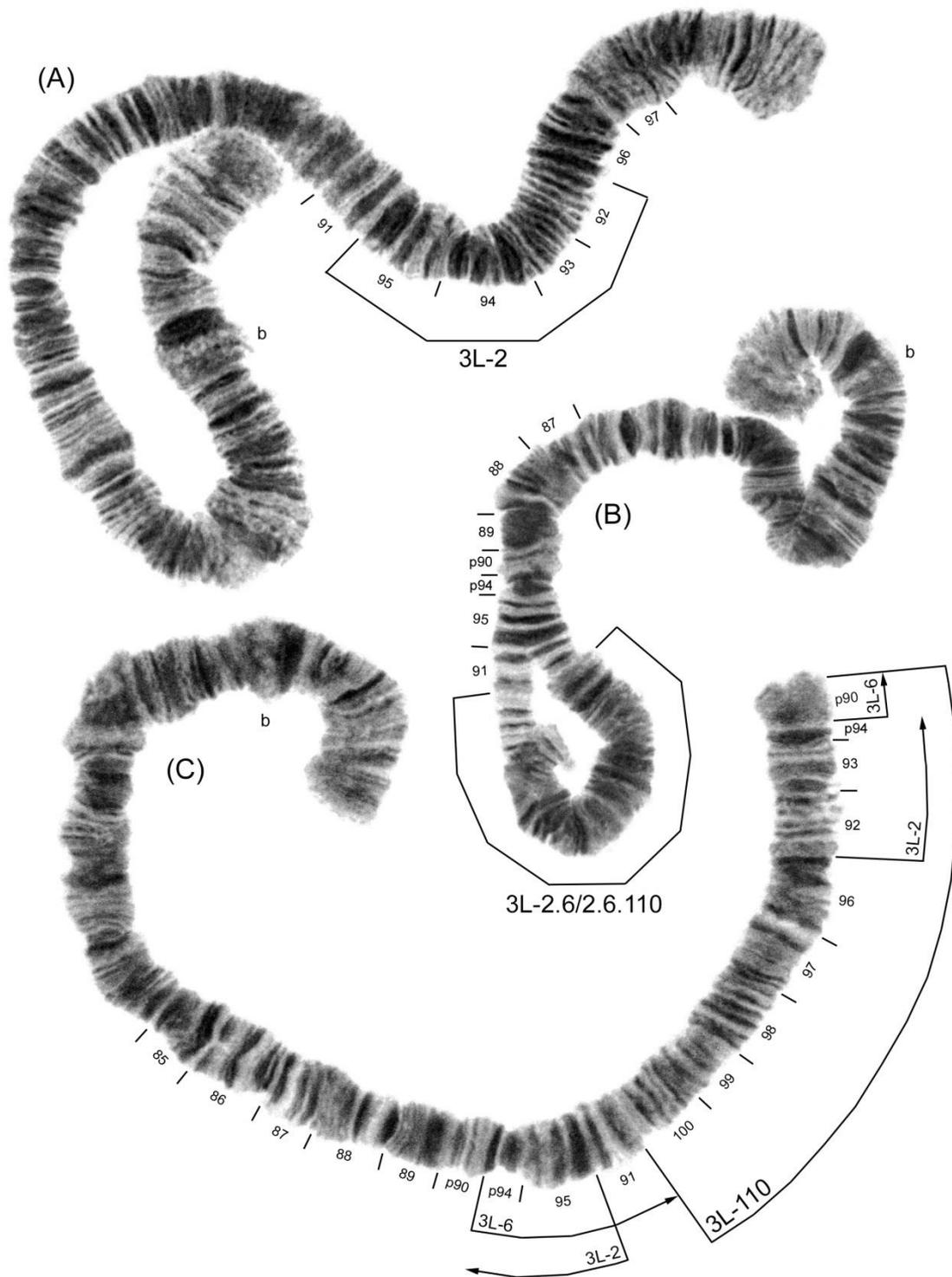


Fig 3. Chromosome 3. Cytotaxonomy of *S. damnosum* s.str. and *S. sirbanum* from central and northern Uganda. (A) *S. damnosum* s.str. (female) collected at Karuma (Nile), showing homozygous inversion 3L-2 which is fixed within the *S. damnosum* subcomplex; (B) *S. sirbanum* (male) collected at Orima (Pager), Kitgum, showing inversions 3L-2.6/2.6.110; (C) *S. sirbanum* (female) collected at Orima (Pager), Kitgum, showing homozygous inversions 3L-2.6.110/2.6.110. 'b' = blister.

s.str. collected by Post [11]. Inversion 2L-8, which is normally absent in *S. damnosum* s.str. in East Africa (or present, but sex-linked in West Africa), was polymorphic, but not sex-linked among populations in the Achwa and Pager rivers (Fig 2). Specimens collected at Orima (Pager) were identified as *S. sirbanum* (s.l.). Inversion 2L-8 was fixed, which is characteristic of this cytoform, while 3L-6 was present in the homozygous form in all but one specimen, which was heterozygous (Table 2, Fig 3). The polymorphic inversion 3L-110 (Fig 3), originally described by Post [11], was present in all specimens examined from Orima. *Simulium sirbanum*, possessing 3L-110, were also present in the small sample collected from the Nyeguy River in Adjumani district. The 'Nkusi' cytoform, which had previously been collected from the Achwa and Nyimur rivers, was not identified chromosomally during the current study [11, 48].

ITS1

ITS1 rDNA was amplified from 122 larval, pupal and adult blackflies that were collected along major watercourses from each district within the study area, and also the Nile at Karuma Falls and the Ayago River. *Simulium damnosum* s.l. mostly produced single or multiple ITS1 amplicons ranging in size from approximately 270 - 320 bp, but there were no consistent banding patterns that enabled cytospecies (*S. damnosum* s.str. and *S. sirbanum*) or cytotypes in different rivers to be differentiated (Fig 4). However, *S. bovis* pupae and adults (identified morphologically) consistently produced single 190bp ITS1 amplicons. This made it possible to identify adult *S. bovis* collected on human bait when specimens were too poorly preserved to identify by morphology.

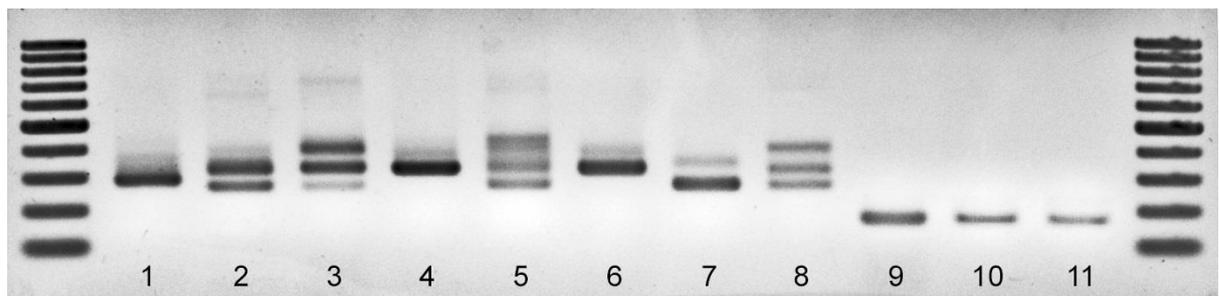


Fig 4. Representative ITS1 banding patterns of blackfly larvae, adults and pupae, visualised alongside 100 bp DNA ladders (Thermo Scientific, Lithuania), and showing variation among *S. damnosum* s.l. (lanes 1 – 8) and shorter 190 bp marker of *S. bovis* (lanes 9 – 11). Lanes: 1 & 2 = *S. damnosum* s.str. larvae, Nile/Ayago confluence; 3 = *S. damnosum* s.l. adult, Pamulu (Moyo); 4 & 5 = *S. damnosum* s.str larvae, Tumangu (Kitgum), Pager River; 6 & 7 = *S. sirbanum* larvae, Orima (Kitgum), Pager River; 8 = *S. damnosum* s.l. adult, Aruu Falls (Pader); 9 = *S. bovis* pupa, Te Lute (Lamwo), Achwa River; 10 = *S. bovis* adult, Aruu Falls (Pader); 11 = *S. bovis* adult, Pabit (Pader).

Human and cattle biting blackflies

A total of 5,579 adult female blackflies were collected during the study (Table 3, Fig 5). Both *S. damnosum* s.l. (4,807) and *S. bovis* (772) were collected biting humans. Of the *S.*

Table 3. Human landing collections of anthropophilic blackflies conducted in 2015 and 2016, showing the total and mean daily catch of *S. damnosum* s.l. and *S. bovis*.

District	Location	River	Latitude	Longitude	Altitude	No. Days	<i>S. damnosum</i>		<i>S. bovis</i>	
							Total	Mean	Total	Mean
Adjumani	Ocesa	Ayugi	3.404194	32.021528	657m	2	2	1.00	0	0
Adjumani	Otika	Ayugi	3.482778	32.012306	636m	2	1	0.50	0	0
Adjumani	Seri Bridge	Seri	3.210111	32.007778	773m	2	0	0	0	0
Kitgum	Adwara	Pager	3.281083	32.853667	916m	3	0	0	0	0
Kitgum	Hotel	Pager	3.235950	32.786750	876m	3	0	0	0	0
Kitgum	Jaipii	Pager	3.328767	33.340867	1019m	3	0	0	0	0
Kitgum	Orima	Pager	3.333552	32.993269	946m	3	0	0	0	0
Kitgum	Otwara	Pager	3.204433	32.754050	859m	3	0	0	0	0
Kitgum	Wang Ayule	Pager	3.260033	33.266400	1003m	3	0	0	0	0
Lamwo	Abam	Pager	3.170750	32.663722	805m	5	32	6.40	41	8.20
Lamwo	Apyeta	Achwa	3.300117	32.361917	651m	7	6	0.86	474	67.71
Lamwo	Beyogoya	Achwa	3.294133	32.495133	845m	7	60	8.57	252	36.00
Moyo	Gwere Luzira	Nile	3.663783	31.800930	980m	9	2240	248.89	0	0
Moyo	Pamulu	Nile	3.679385	31.825775	1066m	9	2434	270.44	0	0
Pader	Aruu Falls	Agago	2.898033	32.646050	964m	4	26	6.50	4	1.00
Pader	Awere	Achwa	2.690107	32.786025	979m	4	4	1	0	0
Pader	Pabit	Achwa	2.975763	32.607475	924m	4	2	0.50	1	0.25
Pader	Puranga Bridge	Achwa	2.607995	32.936470	997m	2	0	0	0	0
Pader	Agago Bridge	Agago	2.853078	33.099615	1007m	2	0	0	0	0
Pader	Agora Bridge	Agago	2.842660	32.963847	994m	2	0	0	0	0

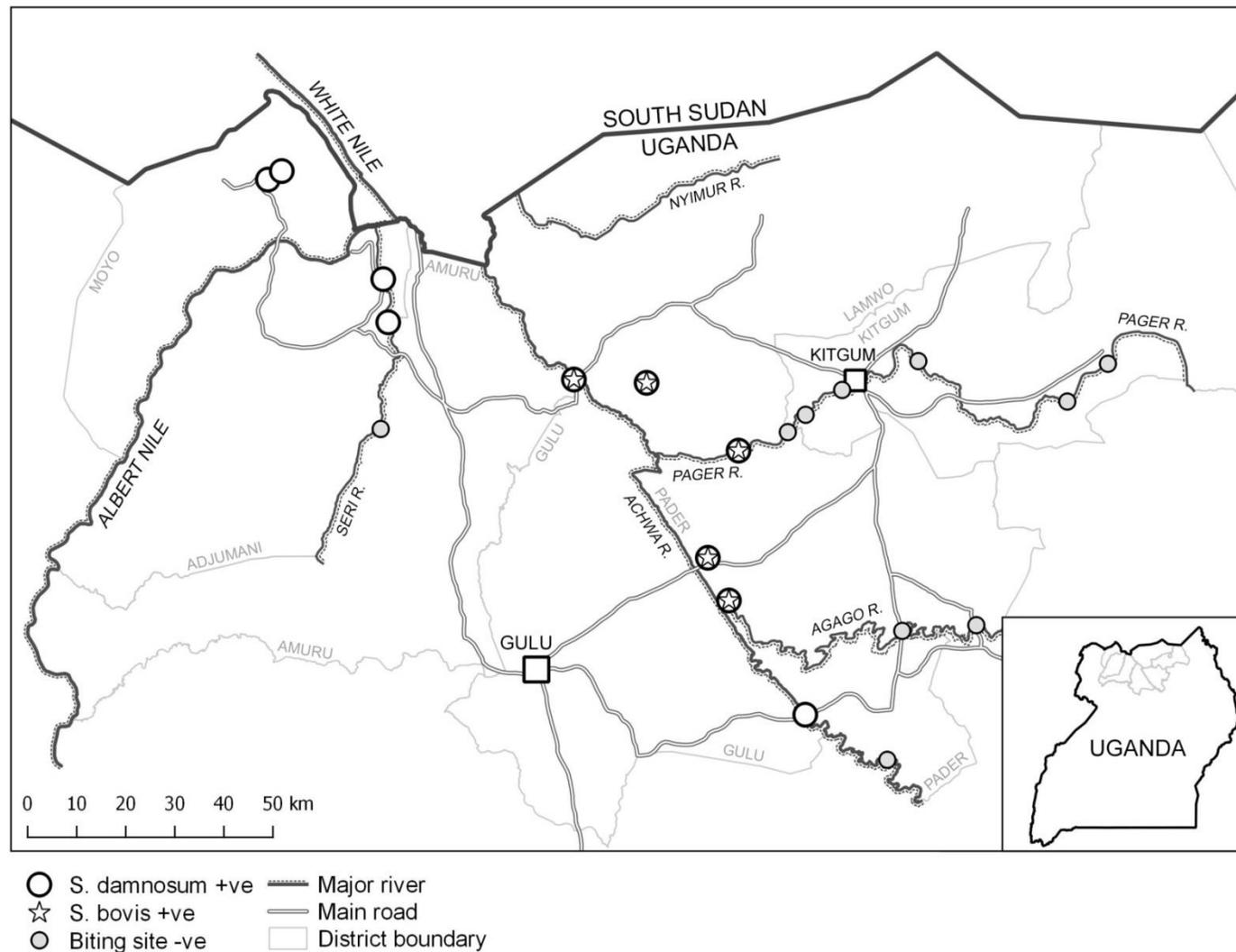


Fig 5. Map showing sites of adult female *S. damnosum* s.l. and *S. bovis* biting based on human landing collections made in 2015 and 2016. All sites positive for *S. bovis* were also *S. damnosum* s.l. positive.

damnosum s.l. collected on human bait, 4,674 (97.2%) were collected in Moyo district, while 130 (2.7%) were collected in the Mid North districts of Kitgum, Lamwo and Pader. In addition, just three (0.1%) *S. damnosum* s.l. were collected during a combined six collection days at the three sites in Adjumani district. *Simulium bovis* was regularly collected at each of the three sites in Lamwo district including at Beyogoya, where the nearest breeding site was 7.5km from the catch site. Neither *S. damnosum* s.l. nor *S. bovis* were collected during a combined 18 days at six sites in Kitgum district, where both species were previously abundant [2, 11]. *Simulium bovis* was also absent from Adjumani, despite reports of it breeding in the Seri and Ayugi rivers in 2013 [11]. This species was, however, identified from collections at Aruu Falls (Agago) and Pabit (Achwa) in Pader district. Specimens were poorly preserved and morphological identification was inconclusive, but blackflies produced 190bp amplicons consistent with other *S. bovis* specimens. Both species were also collected from Zebu and Ankole cattle at Beyogoya and Apyeta Bridge respectively, where farmers reported being frequently bitten.

Onchocerca infection

No pools of human or cattle biting *S. damnosum* s.l. or *S. bovis* tested positive for *O. volvulus* infection (Table 4). Of the limited number of *S. damnosum* s.l. collected on humans, only two pools of bodies tested positive using both the 16S and O-150 primers, although neither was identified as *O. volvulus* or *O. ochengi* by qPCR. A single pool of heads tested positive using the 16S primers only. A pool of eight bodies of *S. damnosum* s.l. collected opportunistically on an oviposition trap at Apyeta Bridge in July 2015 (see Bellec [49] for method) was positive for *O. ochengi* infection. A high percentage of *S. damnosum* s.l. collected on cattle at Beyogoya were 16S+ve and O-150+ve. Many of these infections are likely to have been caused by microfilariae ingested when flies were feeding, as blood-fed flies were not discarded before screening in order to maximise the possibility of finding *O. volvulus*. Even though it was not possible to distinguish between recently ingested microfilariae and older infections, the collections did highlight potential cross reactivity between the O-150 primers and a non-*O. volvulus/ochengi* parasite. Results showed that 39 pools of bodies and heads were O-150+ve, but only 12 were positive for *O. ochengi* infection by qPCR.

Screening of anthropophilic *S. bovis* collected in Lamwo district showed that 30 pools of bodies, but only three pools of heads tested positive using the 16S PCR (Table 4). However, two pools of heads collected at Apyeta Bridge were O-150+ve, of which one pool was positive for *O. ochengi* infection by qPCR. A single specimen collected on human bait at Aruu Falls in Pader tested negative. Of the 100 pools of *S. bovis* collected on cattle in Lamwo, 30

Species	Host	District	Location	Latitude	Longitude	No. Blackflies Screened	No. Pools	Max. Pool Size	Number of Positive Pools							
									16S PCR		O-150 PCR		qPCR Oo		qPCR Ov	
									Bodies	Heads	Bodies	Heads	Bodies	Heads	Bodies	Heads
<i>S. damnosum</i>	Human	Adjumani	Ocesa*	3.482778	32.012306	3	1	3	.	.	-	-
		Lamwo	Abam	3.170750	32.663722	32	3	12	.	.	-	-
			Apyeta Br.	3.300117	32.361917	6	1	6	.	.	-	-
			Beyogoya	3.294133	32.495133	60	16	10	2	1	2	-	-	-	-	-
		Pader	Aruu Falls	2.898033	32.646050	26	3	10	.	.	-	-
			Awere Br.	2.690107	32.786025	4	1	4	.	.	-	-
			Pabit	2.975763	32.607475	2	1	2	.	.	-	-
		Total				133	26		2	1	2	0	0	0	0	0
Bovine*	Lamwo	Apyeta Br.	3.300117	32.361917	8	8	1	1	-	1	-	1	-	-	-	
		Beyogoya	3.294133	32.495133	629	76	10	42	36	24	15	6	6	-	-	
	Total				637	84		43	36	25	15	7	6	0	0	
<i>S. bovis</i>	Human	Lamwo	Abam	3.170750	32.663722	26	6	5	1	-	1	-	-	-	-	
			Apyeta Br.	3.300117	32.361917	369	79	5	14	3	6	2	1	1	-	-
				Beyogoya	3.294133	32.495133	202	47	5	15	-	3	-	1	-	-
			Pader	Aruu Falls	2.898033	32.646050	4	2	3	-	-	-	-	-	-	-
				Pabit	2.975763	32.607475	1	1	1	-	-	-	-	-	-	-
	Total				602	135		30	3	10	2	2	1	0	0	
Bovine	Lamwo	Apyeta Br.	3.300117	32.361917	60	12	5	4	-	1	-	1	-	-		
		Beyogoya	3.294133	32.495133	430	88	5	26	4	5	-	4	-	-		
	Total				490	100		30	4	6	0	5	0	0	0	

Table 4. *Onchocerca* infection in human and cattle biting blackflies collected between 2014 and 2016. 16S primers are *Onchocerca* genus-specific; the O-150 PCR is specific for, but does not distinguish between, *O. volvulus* and *O. ochengi*; the qPCR distinguishes between *O. volvulus* and *O. ochengi* infection. 'No. Pools' = number of pools of heads and number of pools of bodies; 'Oo' = *Onchocerca ochengi*, 'Ov' = *Onchocerca volvulus*; '-' = negative; '.' = not tested; *also includes single fly collected at Otika.

pools of bodies and four pools of heads were 16S+ve. Several of these pools of bodies were O-150+ve or qPCR+ve, but heads were negative.

PCR amplicons of *Onchocerca* ND5 genes extracted from 10 *S. damnosum* s.l. collected on humans and cattle at Beyogoya were sequenced at the University of Bonn, Germany. *Onchocerca* sp. 'Siisa' was detected in two bodies (both were 16S+ve and O-150+ve) and a corresponding head (16S+ve, O-150-ve) of human biting flies, and also in heads and bodies of cattle biting flies. The sequences showed T and C base mismatches with the *O. ochengi* hybridisation probe. These were consistent with *O.* sp. 'Siisa', a close relative of *O. ochengi*, for which Zebu cattle are known hosts [23].

Discussion

Species distribution

There are few published reports of blackfly collections in or near the areas now comprising the Madi-Mid North onchocerciasis focus of northern Uganda. In the 1960s, Dunbar collected *S. damnosum* s.str. and 'Nkusi' cytoforms from the Achwa River at Apyeta Bridge [48]. Lewis had earlier suspected breeding of *S. damnosum* s.l. along the same river in rapids near the Nimule to Juba road bridge in South Sudan, but only found *Simulium arnoldi* [10]. The latter species is closely related to *S. bovis*, which is often found breeding with *S. damnosum* s.l. and appears to be relatively widespread in this part of northern Uganda [41]. Brown also suspected *S. damnosum* s.l. to breed along the Albert Nile near Nimule, but this was not proven [5].

The current data suggest that breeding of *S. damnosum* s.l. and *S. bovis* is restricted to the major rivers in the Mid North, where both species occupy the same breeding sites at several localities. Sympatric breeding of the two species has also been documented in Mvolo in South Sudan, where each appeared to be more abundant than the other at different times of year [10]. Seasonal differences in species composition might explain why collections made from cattle in Lamwo in April 2014 were predominantly *S. damnosum* s.l. (data not shown), while *S. bovis* formed the majority of collections on humans and cattle in June and July 2015. However, more detailed studies would be needed to verify this. In Adjumani, both species were also present at multiple sites surveyed along the Seri and Ayugi rivers in 2013 [11], while in Moyo *S. bovis* was absent and *S. damnosum* s.l. was only collected at a single site (Ayo River), close to the South Sudan border [11]. Moyo district is mostly upland and the majority of watercourses are small, mountainous streams that are not suitable for *S. damnosum* s.l. breeding. However, blackfly biting rates are high, and it is suspected that breeding takes place along a series of rapids in the White Nile across the border in South Sudan [3, 50].

Chromosomal identifications of *S. damnosum* complex larvae collected in 2014/15 mainly agreed with unpublished descriptions by Post [11] in terms of their cytotaxonomy and distribution. Larvae of *S. damnosum* s.str. collected from the Nile and Ayago rivers agreed with standard descriptions of the East African cytoform while those collected in the Achwa, Pager and Nyeguy rivers did not. Notable additions to Post's findings were that *S. sirbanum* did not possess the polymorphic 2L inversion with breakpoints "coincident with the distal breakpoint of 2L-8 and proximal breakpoint of 2L-3", and also that an additional population of *S. sirbanum* was found in the Nyeguy River in Adjumani district [11]. These specimens were chromosomally identical to the *S. sirbanum* collected in Kitgum, although the presence of a single larva possessing 2L-C, but lacking 2L-8, suggests it might be mixed population. The number of *S. damnosum* complex larvae available for cytotaxonomy during the current study was limited by the presence of productive breeding sites, which appeared to have decreased in number considerably since the implementation of vector control measures by the Ministry of Health in 2012. Prior to this, *S. damnosum* s.l. breeding took place extensively along the major rivers in Kitgum, Lamwo and Pader districts (data not shown).

Anthropophilic blackflies

While it is perhaps unsurprising that *S. damnosum* s.l. is active in the dry savannahs surrounding riverine areas in northern Uganda, the discovery in 2012 that approximately half the population of biting flies in Kitgum and Lamwo districts were *S. bovis* was unexpected [2, 11]. This is not a species that regularly bites humans, but it is occasionally anthropophilic [51], and reports of regular human biting in Nigeria and northern Cameroon have already been mentioned [26, 27]. Based on the 190 bp ITS1 amplicons produced by *S. bovis*, it has been possible to show that this species was not only present in Lamwo district, but also among human landing collections at Aruu Falls and Pabit in Pader district (Fig 5). It is not known why *S. bovis* is anthropophilic in northern Uganda, although Crosskey speculated that anthropophily may develop in the absence of its normal animal host [26]. This sentiment was echoed by Krüger, who cited the unpublished observation of Garms that anthropophilic behaviour of the 'Nkusi' cytoform increased in the Itwara focus of western Uganda following the disappearance of large herds of cattle, which was their 'preferred' blood source [52]. Hundreds of thousands of cattle were stolen from the Acholi subregion (which includes Kitgum, Lamwo and Pader districts) during the early years of conflict between the government and the Lord's Resistance Army, which began in the mid-1980s [53]. It would therefore seem plausible that blackfly behaviour might have changed in response to the pressure of finding an alternative blood source.

Human and cattle biting *S. damnosum* s.l. and *S. bovis* continue to be present in Lamwo district, but the absence of anthropophilic blackflies from all sites in Kitgum district is in contrast with previous findings [11]. Whereas collections were only attempted in Kitgum for a few days on each visit (Table 3), regular communication was maintained with the district Vector Control Officer, who reported almost no biting during routine National Onchocerciasis Control Programme collections between July 2015 and November 2016. Very few *S. damnosum* s.l. and no *S. bovis* were collected in Adjumani, and the only sites of sustained *S. damnosum* s.l. biting were in Moyo, where it has already been stated that biting flies probably originate in the White Nile in South Sudan.

Onchocerciasis

The lack of evidence for *O. volvulus* infection suggests that the vector control and ivermectin-based interventions currently being implemented in the Mid North are effectively suppressing transmission. However, insufficient data were obtained to know whether rates of transmission were below thresholds perceived to represent a public health problem [54, 55]. Regardless of this, interventions will need to continue for the reproductive lifespan of adult worms which is usually 12 – 15 years [54]. As elimination approaches, it will be essential to accurately identify L3 stage larvae when calculating transmission potentials. The cross reactivity of O-150 primers with both *O. ochengi* and *O. sp. 'Siisa'* has the potential to distort these indices, and this is something that must be considered in the Mid North where both parasites appear to develop to infective stages in human biting flies. Two pools of *S. bovis* heads collected on human bait at Apyeta Bridge tested positive using O-150 primers and one of these was identified as *O. ochengi* by qPCR. The second pool was not sequenced, but possibly represents 'Siisa'. Since it is generally assumed that a positive head or pool of heads indicates the presence of infective stage parasites [54], it appears that *S. bovis* may act as a vector of, among others, *O. ochengi* and potentially *O. sp. 'Siisa'*. However, the possibility that flies were diverted to a human host when blood feeding on cattle cannot be excluded, in which case, the positive results could have been caused by recently ingested microfilariae. Nevertheless, it is likely that humans in the Mid North are exposed to *O. ochengi* and *O. sp. 'Siisa'* through anthropophilic *S. damnosum* s.l. or *S. bovis*, or both species.

The evolutionary and clinical importance of *Onchocerca* sp. 'Siisa' is not well understood. It was first discovered in a member of the *S. damnosum* complex, likely to be 'Nkusi' cytoform, in the former Itwara onchocerciasis focus in western Uganda [20]. It has since been found in northern Cameroon where Zebu cattle were identified as definitive hosts [23]. At present, there is nothing known about the effects of human exposure to the parasite. However,

'Siisa' is phylogenetically intermediate between *O. volvulus* and *O. ochengi* [20, 23], and both of these parasites exhibit extensive antigenic cross-reactivity [56, 57]. This has been demonstrated experimentally, and studies in Cameroon have also indicated that high densities of cattle in relation to humans may have a considerable zooprophylactic effect which may protect from severe onchocerciasis [58-61]. It is not known whether cattle theft during the early years of war had any impact on clinical onchocerciasis in the Mid North [53], but these events potentially altered human exposure to cattle biting blackflies and their parasites.

Conclusion

At present, onchocerciasis control in the Mid North appears to be progressing well in an area where *S. bovis* breeding takes place along the major rivers in sympatry with members of the *S. damnosum* complex. Both species are anthropophilic, but it was not possible to incriminate either as a vector of *O. volvulus* due to the absence of the parasite. While it is likely that *S. damnosum* s.str. was primarily responsible for transmission, infection of a pool of *S. bovis* heads with *O. ochengi* suggests that it may be able to support the development of a parasite similar in size to *O. volvulus*. Knowing that both *O. ochengi* and *O. sp. 'Siisa'* are present in human biting flies will be important during the evaluation phase of the control programme, particularly since these species appear to cross react with O-150 primers commonly used for *O. volvulus* identification. The absence of breeding blackflies from all but one of the surveyed sites in the Moyo district suggests that high biting rates are the result of flies breeding in the White Nile in neighbouring South Sudan, and collaboration with neighbouring countries will therefore be important to ultimately achieving elimination of the disease.

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River prospection in the Achwa River at Awere Bridge.



S. damnosum breeding site in the Pager River at Tumangu.



S. damnosum and *S. bovis* breeding site in the Achwa River at Te Lute.



S. damnosum breeding site in the Agago River at Aruu Falls.





Checking vegetation downstream from Apyeta Bridge.



S. damnosum and *S. bovis* collections from underside of cattle at Beyogoya.



Pager River at Wang Ayule (July 2015).



S. damnosum breeding site and temephos dosing point, Achwa River at Achwa Bridge.

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CHAPTER 4

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

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Abstract

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. In the meantime, the taxonomy of the *S. damnosum* complex has been revised, and onchocerciasis control through community directed treatment with ivermectin (CDTI) has taken place annually since 1997. An entomological and parasitological evaluation was therefore conducted to evaluate the current status of *O. volvulus* transmission by blackflies in the focus.

Rivers were surveyed to identify sites of *S. damnosum* s.l. breeding in 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 present. 'Nkusi' and *S. kilibanum* were collected on human bait at 7/7 catch sites, while 'Turiani' was not collected on human bait and appears to be zoophilic. *Simulium nyasalandicum*, a member of the *S. neavei* group, was collected in low numbers at 3/7 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%). A single pool of *S. nyasalandicum* bodies was also positive for infection.

Onchocerca volvulus transmission is continuing after 19 years of CDTI. Infection rates are similar to those reported in the 1960s, which may partly be due to the high pre-control prevalence of onchocerciasis. Both 'Nkusi' and *S. kilibanum* are anthropophilic, but their relative roles in transmission are unknown. The role of *S. nyasalandicum* in transmission is likely to be minimal.

Introduction

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) [1]. In sub-Saharan Africa, the disease is endemic in 31 countries, although many are now working towards control and elimination [2-4]. Onchocerciasis epidemiology is largely defined by the presence of suitable vector breeding sites [3]. These can be in fast-flowing rivers, or smaller riverine habitats of freshwater crab (*Potamonautes* spp.) carriers of certain phoretic blackfly species [5]. 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 [6, 7].

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 [8]. Blackflies of the *Simulium neavei* group (*sensu* McMahon 1957 [9]), whose immature stages are associated with freshwater crabs, are responsible for transmission in the Usambara and Nguru Mountains [8, 10]. 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, which are usually identified by fixed or sex-linked inversion differences in their larval polytene chromosomes [7]. The *S. damnosum* complex consists of approximately 60 named cytospecies and cytotypes (chromosomally distinct populations of unconfirmed taxonomic status), collectively known as cytoforms [7, 11]. About 26 of these are known from East Africa [12]. Each cytoform differs in its distribution, ecology, behaviour and ability to transmit parasites, and correct identification is therefore important in understanding disease epidemiology [7, 13]. In East Africa, chromosomal identification can sometimes be supplemented with molecular identification based on PCR amplicon size polymorphisms of the blackfly ITS1 rDNA [12].

The Mahenge focus

The Mahenge onchocerciasis focus is located in Ulanga district, south eastern Tanzania. It was historically one of the most heavily infected areas of the country, and although prevalence was as high as 87% among some communities, the focus was generally thought to be mesoendemic [14-18]. However, more recent pre-control epidemiological surveys demonstrated that the area was hyperendemic [19]. 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 [20]. Cases of nodding syndrome have been diagnosed at Mahenge Epilepsy Clinic and those affected have come from villages throughout the area. However, it has not been determined 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 [16, 21]. 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 [16]. The list was subsequently updated by Raybould and White to include 'Nkusi', 'Sebwe', 'Turiani', 'Hammerkopi' and 'Ketaketa' [8]. In addition, they stated that 'Turiani' was previously misidentified as 'Nyamagasani' (= *S. kilibanum*). Current taxonomic classifications place 'Nkusi', 'Sebwe' and 'Turiani' within the 'Sanje' subcomplex [12], while 'Hammerkopi' and 'Ketaketa' have been synonymised with *Simulium plumbeum* which is classified within the 'Ketaketa' subcomplex [22]. *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 [21, 23]. *Simulium adersi*, *Simulium bovis* (species-group) and *Simulium vorax* have also been collected during larval and pupal surveys [21]. Whereas the latter species are occasionally anthropophilic in Tanzania and elsewhere in Africa, they are not known to be vectors of *O. volvulus* [24-28].

Häusermann dissected 12,416 *S. damnosum* s.l. collected on human bait in the Mzelezi Valley between 1966 and 1967 [16]. He showed that 6.9% (856) had developing *O. volvulus* infections and 0.68% (85) contained infective L3 stage parasites in the head (L3H) [16]. At this time, the prevalence of human onchocerciasis in nearby communities was as high as 65.1% [16]. 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 [21]. However, there was no evidence that they developed to infective stages.

Onchocerciasis control and evaluation

Attempts to control onchocerciasis in Mahenge started in 1994 through a vertical programme of mass drug administration (MDA) with ivermectin [19]. 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 [19]. There have been no attempts at vector control in the area since the late 1960s [16].

The most recent estimates of onchocerciasis prevalence in Mahenge were based on skin snip evaluations carried out in 10 villages in 2009 [2]. 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 much faster than expected [2]. According to the WHO, the anticipated duration of treatment phases of MDA programmes should typically last between 12 – 15 years, and should continue with a minimum 80% annual therapeutic coverage until *O. volvulus* transmission is interrupted [3]. 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 6,000 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%) [3].

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. The objectives of this study were to provide a cytogenetic and molecular update of *S. damnosum* complex cytoforms present in Mahenge, and to evaluate the current state of *O. volvulus* transmission by blackflies following 19 years of annual CDTI.

Materials and methods

Study area

The Mahenge Mountains rise to approximately 1500m 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 [16]. Annual rainfall is between 1000mm and 1500mm, and occurs mainly between November and May [21, 29]. 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, see Results) [16]. Whereas *S. damnosum* s.l. breeding and biting takes place throughout the focus, *S. nyalalandicum* appears to be restricted to higher altitudes and biting has only been reported from areas around Sali and Mahenge [21]. Detailed descriptions of seasonal changes in blackfly breeding and biting are provided elsewhere [16, 21]. The majority ethnic group residing in

the area are the Pogoro, who keep animals including chickens, goats and occasionally pigs. Cattle are rare and in the past were only kept by the missions [16]. In 2012, it was estimated that 515,752 people were living in areas previously either meso- (41 – 59% prevalence) or hyperendemic ($\geq 60\%$ prevalence) for onchocerciasis in Ulanga and Kilombero districts (Tanzania Ministry of Health, unpublished data).

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 cytotoxic 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. Two people from each of the villages surveyed for blackfly breeding were trained in standard human landing collection methods for adult blackflies [30]. Trial catches were conducted for a single day between 07:00 and 18:00 to establish sites of highest blackfly activity, before routine collections were carried out at the most productive sites. Catches were recorded hourly and specimens were preserved daily in absolute ethanol. Collections were not fully supervised, although spot-checks were conducted and regular mobile phone communication was maintained with the collectors. Preserved specimens were 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.

Identification of blackflies

Simulium damnosum complex larvae were identified morphologically by the presence of dorsal abdominal tubercles, and scales on the prothoracic proleg [5]. Late-instar larvae, pupae and adult blackflies were otherwise identified where possible using morphological keys in Freeman & De Meillon [31]. Adults of the *S. neavei* group were identified using keys in Lewis and Raybould [23] and were compared with reference specimens, including Häusermann's [16, 21], at the Natural History Museum, London, UK.

Prior to cytotoxic 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.* [32]. Larvae were identified with reference to chromosome maps in Boakye [33], Proconier and Muro [34], and Krüger [12]. Nomenclature follows Krüger [12].

ITS1 amplicon size polymorphisms of *S. damnosum* complex larvae and adults were interpreted with reference to Krüger [12]. DNA was extracted using QIAGEN DNeasy Blood & Tissue Kits (Qiagen, N.V.) and amplified using ITS1 Fw and ITS1 Rev primers (Chapter 3.) and a modified protocol based on methods outlined by Tang *et al.* [35]. Reactions were carried out in 25µL total volumes containing 10pmol of each primer, 5µL 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 60 secs, 45°C for 60 secs, and 72°C for 60 secs, 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.

Pool screening

Adult *S. damnosum* s.l. were prepared in pools of heads and corresponding bodies according to collection site. Heads were separated from bodies 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 by heating using a FIREBOY safety Bunsen burner (INTEGRA Biosciences, Switzerland) after each use to reduce the risk of contamination. Pooled samples were placed in 2mL 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, the samples were tested for PCR inhibiting factors as described previously [36]. 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 20µL total volumes containing 2 µL template DNA, 1X HotStar Taq Buffer (Qiagen, N.V.), 4.5 mM MgCl₂, 40 mM dNTP, 2.5 units HotStar Taq, and primers and hybridisation probes listed in Chapter 3. Cycling conditions involved Taq polymerase

activation at 95°C for 15 min, followed by 45 cycles at 95°C for 10 secs and 61°C for 30 secs 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 [36].

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 [37] was used to estimate *O. volvulus* infection rates in pools of unequal size, with 95% confidence intervals. Transmission potentials were not estimated due to the short duration of the study.

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). Collectors were from local communities, were already participating in the CDTI programme as community drug distributors, and were receiving annual ivermectin treatment in accordance with the national onchocerciasis control programme. All participants were adults over the age of 18 years and provided written informed consent.

Results

Identification of blackflies

Twenty one out of 23 riverine sites visited in January 2015 and June 2016 were positive for blackfly larvae or pupae (Table S5). *Simulium damnosum* s.l. was present at 12 sites (Fig 1, Table 1). These included rivers near villages where adult blackfly collections were taking place, with the exception of Sali, a relatively isolated mountain community situated above 850m in the south of the focus (Fig 1, Table 1). Blackfly larvae were otherwise abundant in the Mbezi River at Sali and included *S. vorax*, which was identified by the morphology of the pupal respiratory organs dissected from three mature larvae, and a single pupa (Table S5). Pupae of *S. vorax* were also present in the Luli and Mbalu rivers, and *S. adersi* pupae were present in 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

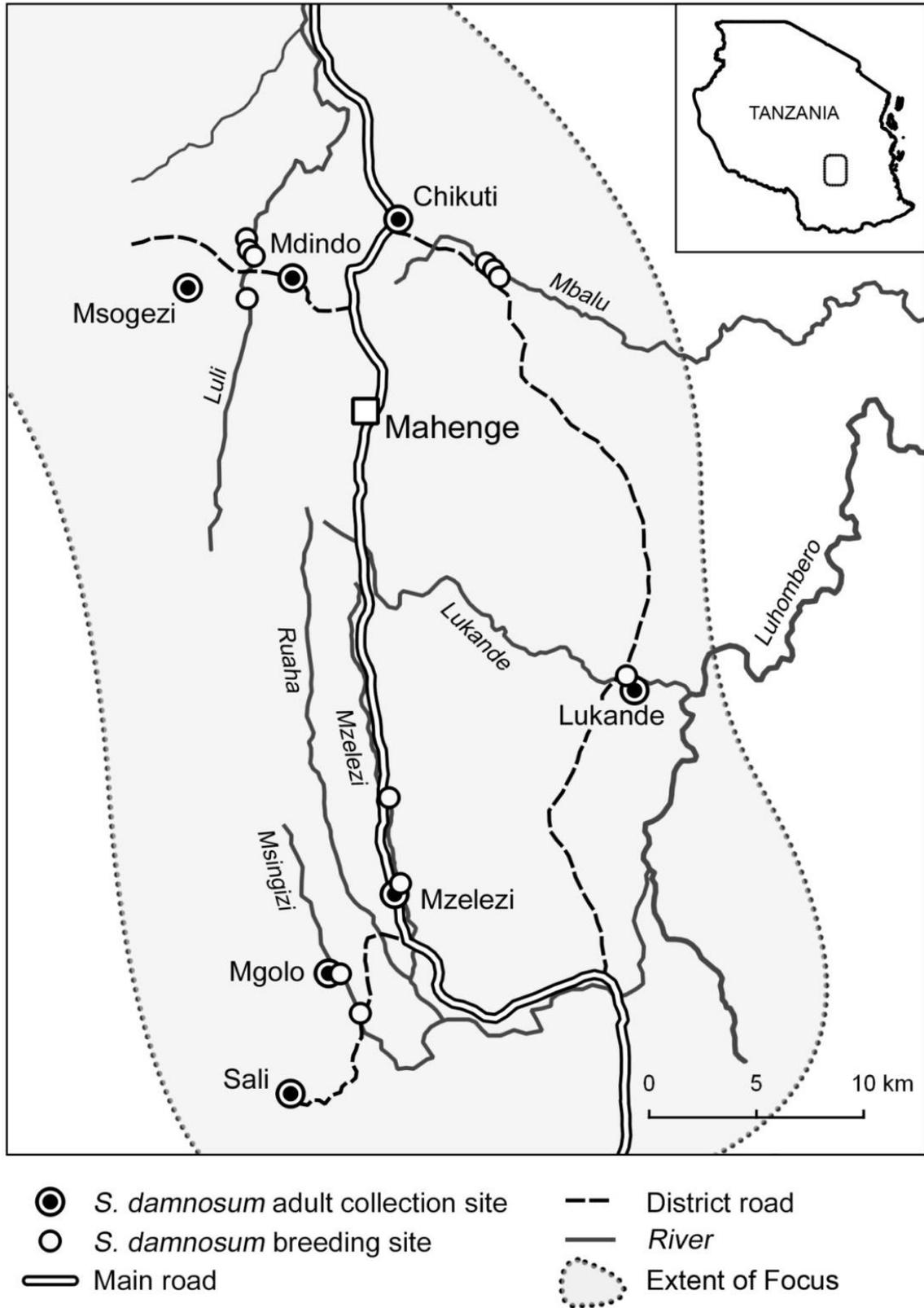


Fig 1. Map of *S. damnosum* s.l. breeding and adult collection sites. The shaded area represents the approximate extent of the onchocerciasis focus on the eastern slopes of the Mahenge Mountains as defined by Häusermann [21]. Inset shows the location of the study area in south eastern Tanzania.

complex (Table 1). 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 chromosomally 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).

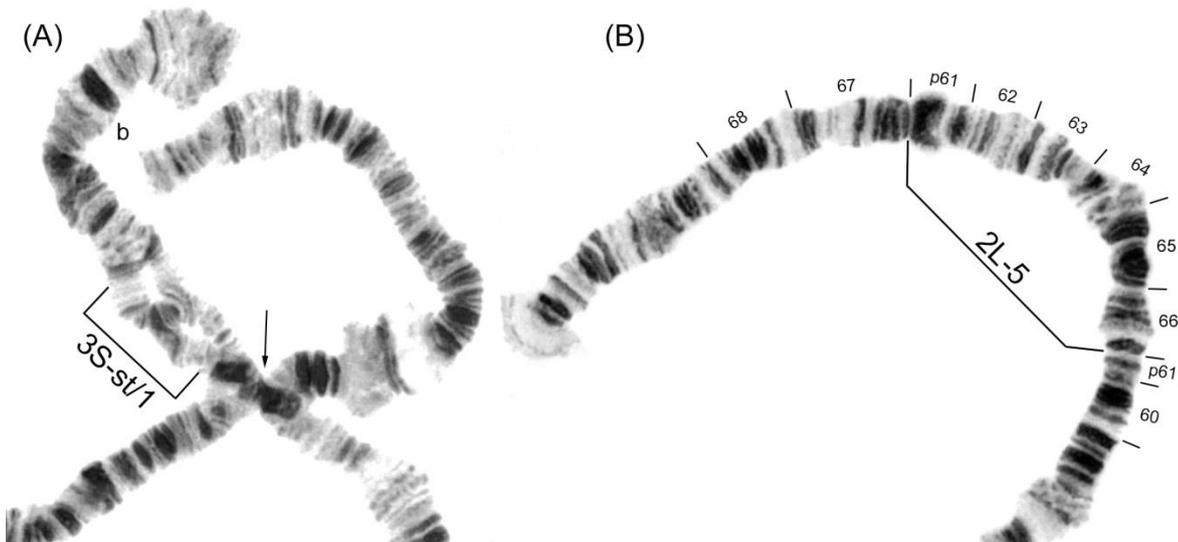


Fig 2. *Simulium damnosum* s.l. chromosomes showing A) male sex-linked heterozygous inversion 3S-st/1, diagnostic for 'Turiani' cytoform, and also ectopic pairing of centromeres 2 and 3 (arrow); B) homozygous inversion 2L-5, which is fixed in 'Nkusi' and polymorphic in *S. kilibanum* and 'Turiani' cytoforms.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

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 [12, 38]. 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 [12]. A 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 2). 'Sanje' cannot be excluded as it also produces a 270 bp amplicon [12], however, given the known presence of 'Turiani' and the lack of chromosomal evidence for Sanje, 'Turiani' is the most likely designation.

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			
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

Table 1. 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.

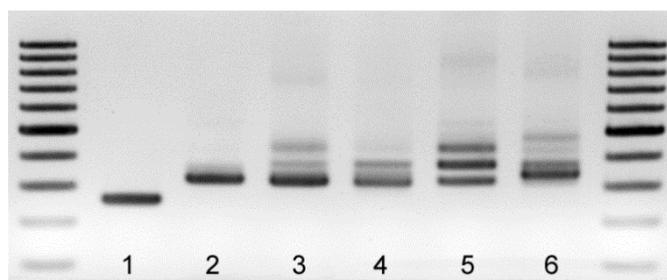


Fig 3. Representative ITS1 banding patterns visualised alongside 100 bp DNA ladders (Thermo Scientific, Lithuania), showing variation among specimens. Interpreted as 1) 'Turiani' 270 bp, 2) *S. kilibanum* 310 bp, 3) *S. kilibanum* 310 + 450 bp, 4) 'Nkusi' 310 + 380 bp, 5) 'Nkusi' 310 + 380 + 450 bp,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

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 2). 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 2). One larva and one adult from the Mzelezi River each exhibited \approx 380 + 450 bp ITS1 amplicons, while two adults caught at Mgolo each had \approx 340 + 380 bp amplicons. No (0/57) adult blackflies collected on human bait produced 270 bp amplicons, and 'Turiani' therefore appears to be zoophilic.

Table 2. 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	

^aCollections combined as the Luli River flows between the villages and adult catch sites were in close proximity.

^bTrial catch sites not included in routine collections.

^cITS1 bands approx. 380 + 450 bp.

^dITS1 bands approx. 340 + 380 bp.

<i>Simulium damnosum</i> s.l.													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 -/+			
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%		
<i>Simulium nyasalandicum</i>																
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 pools of heads and number of pools of bodies.

[†] Infection rate not estimated due to insufficient catch.

Table 3. 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.

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 3). 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 [23].

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 – 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 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 the *S. damnosum* s.l. screened from the Mahenge Mountains contained infective L3H parasites (Table 3). 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*.

Discussion

Simulium damnosum complex

Members of the *S. damnosum* complex previously reported from Mahenge included 'Nkusi', 'Sebwe', 'Turiani' and *S. plumbeum* (= 'Hammerkopi' and 'Ketaketa' cytoforms), and it was thought that 'Turiani' had been misidentified as *S. kilibanum* [8, 22, 34]. 'Nkusi' was believed to be the likely man-biting species given its abundance in the Mzelezi River [8, 16]. The molecular form, 'Nkusi J', is the assumed vector in the Uluguru Mountains, approximately 200km north east of Mahenge, and anthropophilic 'Nkusi' *sensu* Dunbar [39] is present in nearby Kilosa focus where its vectorial status is unknown [8, 34, 38, 40]. 'Nkusi' in Mahenge possessed the fixed 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 – 500m, yet bites across the full altitudinal range [38]. 'Nkusi' does, however, breed at higher altitudes elsewhere [38, 41].

The study 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 [12]. *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 [12, 42-44]. 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 [45]. 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 [46].

'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 270bp 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 [8, 12, 16].

'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 $\approx 380 + 450$ bp amplicons, while the two adults caught at Mgolo produced $\approx 340 + 380$ 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 – 380 bp [16, 22]. 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 [12, 41]. However, without additional

chromosomal evidence it is not possible to determine whether the unidentified specimens represent either of these cytoforms.

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 [8, 23]. It is also anthropophilic in the Usambara Mountains, Kilosa, Ruvuma and Tukuyu foci (including Njombe) [8, 23, 41, 47]. During the current study, the species was collected at Sali (876m), Mgolo (465m) and Msogezi (459m), although not in the adjacent village of Mdindo. The distribution correlates well with that reported by Häusermann, who collected biting females at Sali and occasionally Mahenge [16, 21]. 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 – 1300m and is the source of multiple rivers, including the Mwezeza, which feeds the Luli [29]. It is certainly possible that suitable breeding habitats exist within the reserves and that *S. nyasalandicum* contributes to biting on forest fringes. Still, its role in transmission is likely to be minimal given the very low human landing rates, limited evidence for the development of *O. volvulus*, and past observations that larvae of the species were always rare [21].

Onchocerca volvulus transmission

Positive *O. volvulus* infections were found in the heads of flies from each of the routine collection sites, at altitudes ranging from 333 – 876m. The highest daily *S. damnosum* s.l. landing rates were recorded at Msogezi, Mdindo and Chikuti on the north side of the mountains, and the lowest at Sali and Lukande in the south. However, given the short duration of the study, little can be inferred about differences in biting activity or transmission at these sites. It has already been explained that breeding and biting varies seasonally and at different altitudes in Mahenge [21]. Ivermectin had also recently been delivered to communities at the time of the study, but the extent to which it had been distributed was not clear. Variables such as these could inevitably cause localised differences in biting rates and infection rates in blackflies. Furthermore, transmission potentials would be artificially high if estimated using data from a single month when *O. volvulus* transmission was at its peak. A longer study would therefore be necessary to minimise the impact of such confounding factors.

Pool screening results show that the overall percentage of blackflies carrying L3H parasites (0.57%, 95%CI 0.43% - 0.74%) is above the 0.05% threshold for interruption of transmission [3]. The infection rate is also similar to those recorded by Häusermann in the Mzelezi Valley

in March (0.60%), April (0.50%), May (0.51%), June (0.19%) and July (0.65%), 1967 [16], but lower than pre-control infection rates in the Tukuyu and Uluguru Mountains foci where 1.45% (192/13,238) and 0.88% (48/5,430) of all blackflies dissected were carrying L3H, respectively [47, 48]. Nevertheless, the result is unexpected considering that skin snip evaluations carried out in 2009 showed a mean village microfilarial prevalence of 8.3%, and a maximum community microfilarial load of 2.2 [2]. This evaluation 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. The observed microfilarial prevalence was significantly lower than the predicted mean of 43.8%, indicating a faster than expected progress towards elimination [2]. However, it has also been reported, albeit less formally, that onchocerciasis prevalence in Mahenge was still 46% in 2011 [49]. The skin snip method used in the 2009 evaluation has limitations. It is affected by the timing of CDTI and is known to have low sensitivity, particularly when disease prevalence is low [3]. In contrast, entomological evaluations are sensitive indicators of changes in community microfilarial load that correlate well with ivermectin coverage [50]. This would suggest that ivermectin had either not been distributed at the time of the current entomological evaluation, or that coverage had been suboptimal. Problems with ivermectin adherence have recently been reported from onchocerciasis endemic areas of Cameroon where CDTI has taken place for 15 years and is thought to be contributing to persisting levels of mesoendemicity [51]. However, the reported therapeutic coverage of ivermectin in Ulanga district was >65% (mean 76%) for the years 2003 – 2015 (Ministry of Health, unpublished data). The current infection rates in Mahenge may therefore, at least partly, be explained by the high pre-control prevalence of onchocerciasis that existed compared to other Tanzanian foci, although the accuracy of coverage reports should be verified [2, 15, 19].

Conclusion

Onchocerca volvulus continues to be transmitted throughout the eastern slopes of the Mahenge Mountains following 19 years of annual CDTI. Current *S. damnosum* s.l. infection rates are similar to those reported by Häusermann in the 1960s and may partly be a consequence of the high pre-control prevalence within the focus. It would be useful to know whether the current prevalence of *O. volvulus* infection in the human population reflects the entomological and parasitological findings reported here. Despite one pool of *S. nyalalandicum* bodies being positive for *O. volvulus* infection, it is unlikely to contribute significantly to transmission given its scarcity. Cytotaxonomic and molecular identifications demonstrated that both 'Nkusi' and *S. kilibanum* cytoforms are anthropophilic in Mahenge, although their relative roles in *O. volvulus* transmission are yet to be determined. The molecular profiles of some of these specimens differed from previous reports of 'Nkusi J'

and *S. kilibanum* 'T', which they most closely resembled, by the presence of additional 450bp ITS1 amplicons. 'Turiani', which was present in sympatry with other *S. damnosum* complex cytoforms, appears to be zoophilic. Past reports state that other members of the *S. damnosum* complex are present in Mahenge, and this was indicated by ITS1 profiles of immature and adult blackflies that did not match the previously mentioned cytoforms. To fully understand the diversity and behaviour of the *S. damnosum* complex in Mahenge will require a more detailed study than has been possible here.

Acknowledgements

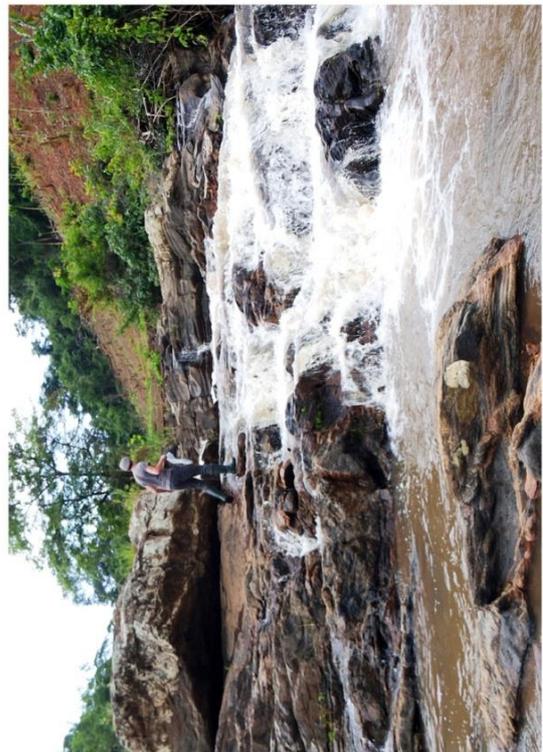
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Northern escarpment of Mahenge Mountains.



Mzelezi River, January 2015.



S. damnosum breeding site in the Luli River at the border of Mbindo and Msogezi.



Inspecting vegetation at human-made structure in the Luli River.

Mbezi River at Sali, January 2015.



Sali village, with Gongo Mountain in the background.



Identifying suitable villages for blackfly collections at Mahenge Hospital, June 2016.



Demonstrating human landing collections at Mzelezi village.



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CHAPTER 5

Onchocerca volvulus transmission in *Région du Centre*, Cameroon, following 16 years of annual CDTI

Authors

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Abstract

The onchocerciasis focus surrounding the lower Mbam and Sanaga rivers was historically the largest in southern Cameroon. Control through annual community directed treatment with ivermectin (CDTI) officially commenced in 2000, and takes place around July each year. However, recent surveys revealed that the area is still mesoendemic and suggest that parasite transmission is ongoing. This study aimed to evaluate the intensity of blackfly biting and *Onchocerca volvulus* transmission along the lower Mbam River near Bafia, 16 years after the formal onset of annual CDTI.

Blackflies were collected using human bait between July 2016 and June 2017. Collections were made for three consecutive days each month at two riverside sites (Bayomen and Nyamongo I), and two inland sites (Egona II and Ondouano) situated in a transect 4.9km and 7.9km away from Nyamongo I, respectively. On a single collection day each month, blackflies were dissected to determine their parity rates and the intensity of *O. volvulus* infection. The remaining samples were preserved for pool screen analysis (only preliminary data are reported here). Of the 93,563 *Simulium damnosum* s.l. collected, 9,281 were dissected and 84,282 were preserved. Monthly biting rates (MBRs) were highest at the riverside sites and decreased with increasing distance from the river. Whereas MBRs were consistently high at Bayomen, biting was distinctly seasonal at Nyamongo I, where peaks occurred from September – October and again from December – March. *Onchocerca volvulus* transmission coincided with periods of high parity rates and occurred almost exclusively at the riverside sites between January and May. Cytotaxonomic analysis of *S. damnosum* complex larvae collected from nearby breeding sites revealed a variant of *Simulium squamosum* E, making it the first time this cytotype has been found east of Lake Volta in Ghana.

While CDTI has had an important clinical impact on onchocerciasis along the lower Mbam River, parasite transmission remains high at riverside sites. The late dry season/early rainy season peak in *O. volvulus* transmission (January - May) is similar to that reported following an entomological survey conducted in 1993/94. This was before ivermectin was introduced. The current timing of CDTI may therefore not be ideal, and communities may benefit from earlier treatment (ca. December), before blackfly parity rates and parasite transmission increase.

Introduction

Five main hyperendemic onchocerciasis foci existed in Cameroon before the community directed treatment with ivermectin (CDTI) strategy was introduced to control the disease [2]. The foci extended across the savannahs in the north of the country, the forest-savannah transition zones in the centre, and the dense humid forests further south [2, 3]. All are associated with fast flowing rivers that provide suitable breeding habitats for *Simulium damnosum* complex blackflies, the only important vectors of this disease in the country [4, 5]. Eight cytoforms of the complex are currently known from Cameroon [5]. Their distribution has been mapped across the north [6], and large parts of the centre, east and west of the country [4]. *Simulium damnosum sensu stricto* (s.str.) and *Simulium sirbanum* are common in the savannah habitats [6], while *Simulium yahense* and *Simulium squamosum* are associated with forest and transitional zones [4, 6]. However, the latter is reported to spread north into the savannah rivers during the rainy season [4]. *Simulium squamosum* is divided into five cytotypes (chromosomally distinct populations of unconfirmed taxonomic status) designated A – E, of which cytotypes A – D are present in Cameroon, while cytotype E occurs west of Lake Volta [4, 7, 8]. *Simulium squamosum* A is the typical form described by Vajime and Dunbar [9]. It is found throughout most of Cameroon except for the Sanaga River, which is the only known breeding locality of *S. squamosum* B [4]. Cytotypes C and D are known from the areas around Mount Cameroon where A and C also appear to interbreed [4, 7]. Another member of the complex, *Simulium mengense*, has a scattered distribution throughout the north where it occupies similar habitats to *S. damnosum* s.str. and *S. sirbanum* [6]. It is also present in rivers around Mount Cameroon and in *Région du Centre*, where it is often found sympatrically with *S. squamosum* [1, 10, 11]. All *S. damnosum* s.l. cytospecies present in Cameroon are known or suspected vectors of *O. volvulus* [12-15].

The onchocerciasis focus surrounding the lower Mbam and Sanaga rivers was historically the largest in southern Cameroon [13]. Disease prevalence was particularly high in villages along the lower Mbam, where infection was associated with severe ocular disease and also high rates of epilepsy [1, 16, 17]. A case control study took place from 1991 – 1992 in villages close to the Mbam River, which showed a statistically significant relationship between community microfilarial loads (CMFL) and epilepsy. An arithmetic mean 288 microfilariae per skin snip (mf/ss) was present in epilepsy cases, and 141 mf/ss in matched controls [17]. A subsequent entomological study was conducted in 1993/94 along two transects perpendicular to the river [1]. Results showed blackfly biting continued throughout the year, although *Onchocerca volvulus* transmission appeared to be seasonal,

occurring mainly between February and May, and peaking around February and March [1]. The *Simulium damnosum* complex blackflies present were only found breeding in the main river, and not the tributaries. Ninety percent of larvae collected were identified as *S. squamosum* s.str. (presumably cytotype A), while the remaining 10% were *S. mengense* [1]. Traoré-Lamizana *et al.* also reported the presence of *S. squamosum* A from the Mbam and Noun rivers north of Bafia [4].

There has been no vector control along the Mbam River, where onchocerciasis is controlled solely through annual CDTI [3, 18]. The first large-scale ivermectin treatments commenced in 1994 in selected villages as part of a clinical trial to evaluate the macrofilaricidal potential of the drug [1, 19]. CDTI was then launched in 1997, but initially encountered problems caused by severe adverse events related to *Loa loa* infection [16]. Treatment coverage during the first years was consequently low and the official start of the CDTI project was in 2000 [3, 16]. Epidemiological studies conducted in 2011 showed that the evaluation area 'Center 1' (which covers Bafia Health District) had a mean village microfilarial prevalence of 52.3% based on 12 villages surveyed, and was progressing more slowly than expected towards elimination [3, 20]. After corrective measures had been made to the CDTI programme, Kanga *et al.* conducted a follow-up survey in 2015 [3]. Their results showed a mean microfilarial prevalence of 41.6% in four villages surveyed, and whereas prevalence was still higher than expected, disease intensity as measured by CMFL had decreased dramatically from pre-control levels [3]. However, as the authors state, microfilaridermia and nodule presence in children <10 years old is evidence of ongoing transmission [3].

Onchocerciasis is still a country-wide public health problem in Cameroon, particularly in villages surrounding the lower Mbam River [3, 21]. This study aimed to verify the identity of the *S. damnosum* complex cytoform(s) breeding in the Mbam near Bafia, and to investigate the seasonal patterns and extent of blackfly biting and *O. volvulus* transmission in the area. Blackfly collections were made almost 23 years after a similar pre-control survey by Barbazan *et al.*, and 16 years after the formal onset of annual CDTI [1, 3].

Materials and methods

Study area

The study was conducted near villages surrounding the perennially flowing Mbam River near Bafia (N 4.75, E 11.23334) in *Région du Centre*, Cameroon (Fig 1). The Mbam originates in the extensive savannah regions in the north of the country and flows through the transitional forest-savannah mosaic surrounding Bafia before joining the Sanaga River as its

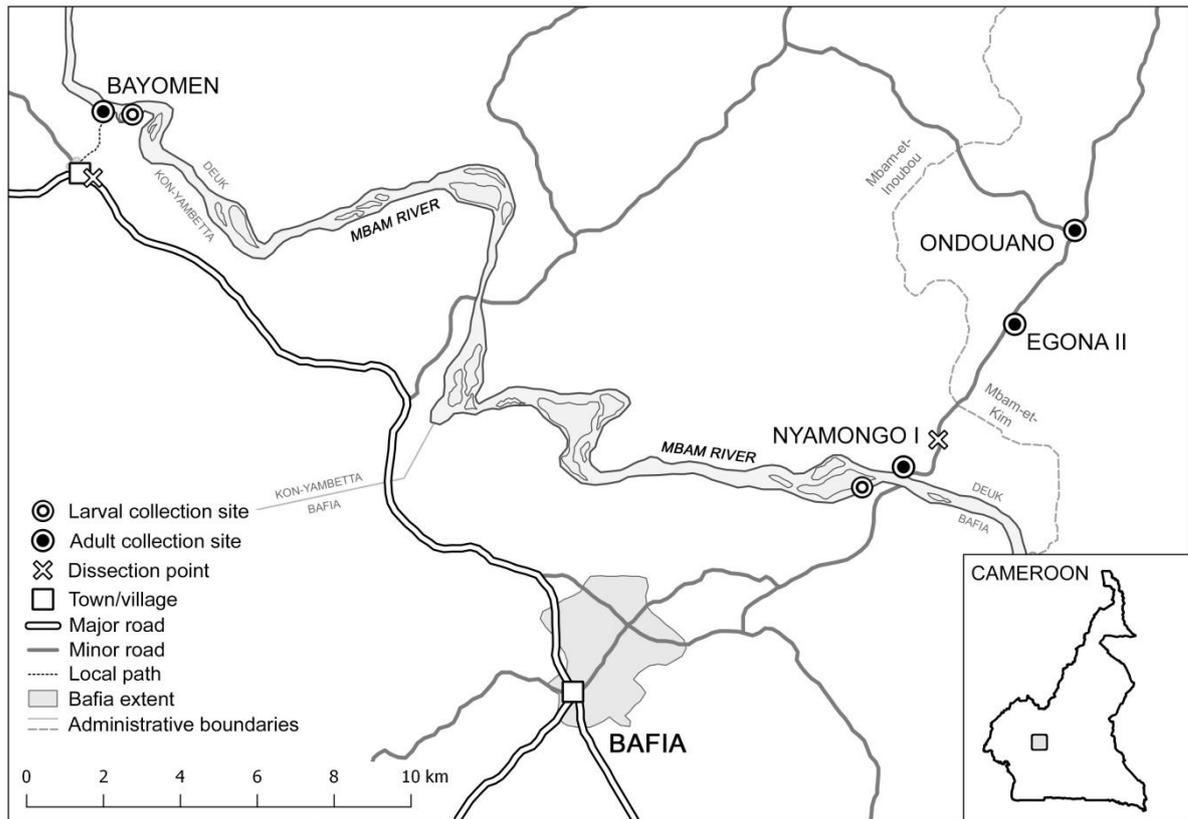


Fig 1. Map of study area showing blackfly collection sites and dissection points in relation to the Mbam River. Inset showing location of study area in Cameroon.

main tributary [22]. When passing close to Bafia, the river is characterised by a series of rapids that provide ideal sites for blackfly breeding [17]. The climate is equatorial and the area receives 1700 – 1850mm in annual rainfall, occurring mainly in two peaks (Fig 2) [23]. The lesser of these takes place from March – June and precedes a brief dry period in July, which is followed by more frequent rains from August – October. River discharge increases throughout the rainy seasons before declining abruptly in November at the start of the long dry season, which lasts until February (Fig 2) [1]. Blackfly biting takes place throughout the year, with seasonal peaks occurring around February – May and September – October. *Onchocerca volvulus* transmission mainly takes place between February and May [1]. The estimated population of Bafia Health District was 226,073 in 2014 [3]. Many people engage in subsistence farming, fishing and sand mining along the Mbam River [3], and nomadic pastoralists ('Bororo' herdsman) migrate to the area annually, at varying times between November and May (M Ronse pers. comm.).

Blackfly collection sites were located either at the riverside (Bayomen, N 4.8785, E 11.11140, \pm 513m; Nyamongo I, N 4.791433, E 11.296467, \pm 431m), or several kilometres away from the riverside (Egona II, N 4.828292, E 11.321831, \pm 465m; Ondouano,

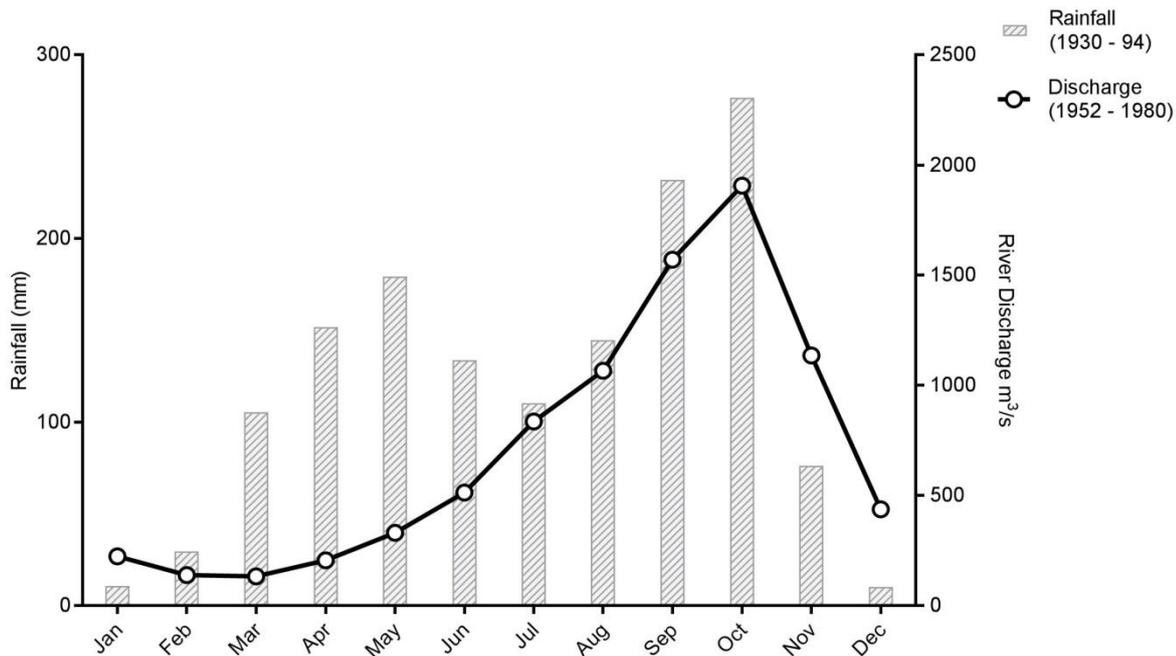


Fig 2. Mean monthly rainfall at Bafia for years 1930-94 [24] and mean monthly river discharge (m^3/s) calculated using data collected at Goura river gauge from 1952-80, approximately 25km S SE of Bafia (N 4.567025, E 11.3674) [25].

N 4.849764, E 11.338832, $\pm 461\text{m}$). The sites at Nyamongo I and Ondouano are at locations similar to those used by Barbazan *et al.* (Fig 1) [1]. The riverside site at Bayomen was frequented by local fishermen and is a well-known site of blackfly biting, while Nyamongo I was the site of a ferry crossing, approximately 1km downstream from major rapids.

Collection and preservation of blackflies

Blackfly larvae and pupae were collected during the dry season from rocks and trailing vegetation in rapids close to Nyamongo I and Bayomen in January 2017 (Fig 1). Specimens were preserved in three changes of Carnoy's fixative ($\approx 3: 1$ ethanol: glacial acetic acid). Those not used for cytotaxonomy (i.e. non-*S. damnosum* complex larvae and all pupae) were transferred to absolute ethanol in the laboratory, where all specimens were stored at -20°C until needed.

Adult blackflies were collected at all four sites simultaneously, for three consecutive days each month, beginning in July 2016 and ending in June 2017. Catches were made between 07:00 and 18:00 each day, by teams of two people recruited from each village who were trained in standard human landing collection methods [26]. Due to anticipated high landing rates, blackflies were collected using aspirators rather than individually in tubes. Aspirators were labelled and changed hourly, and hourly catches were recorded. Adult blackflies were then either dissected in the field (see below) or preserved in absolute ethanol according to the site and date of collection. Preserved specimens were kept in the dark at ambient

temperatures until they were stored at 4°C in the laboratory. Landing rates were interpreted as being representative of exposure to biting, and are therefore referred to as biting rates.

Identification of S. damnosum complex

Simulium damnosum complex larvae were identified morphologically by the presence of dorsal abdominal tubercles and scales on the prothoracic proleg [27]. Adult *S. damnosum* s.l. were identified by their enlarged fore-tarsi bearing crests of dark hair, and the presence of white bands on the hind basitarsi [28]. Prior to cytotaxonomy, heads and thoraces of late-instar larvae were removed from specimens in the laboratory and were stored individually in absolute ethanol for morphological identification. 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.* [29]. Larvae were identified with reference to the cytotaxonomic key in Post *et al.* [14], and chromosome maps in Vajime and Dunbar [9], Boakye [30] and Mustapha *et al.* [10]. Inversion nomenclature follows Post *et al.* [31].

Dissection of adult blackflies

Blackfly dissections took place at 'dissection points' located at Bayomen or Nyamongo I in order to investigate parity rates and intensity of *O. volvulus* infection (Fig 1). Flies from each of the four sites were dissected by trained technicians on one of the three collection days each month. Aspirators containing hourly catches were transported throughout the day to the nearest dissection point, where flies were first identified morphologically. Up to 30 *S. damnosum* s.l. were then dissected per site/hour, with a maximum of ≈ 330 dissected per site/day. Dissections involved anaesthetising blackflies with chloroform and placing each in a drop of saline solution on a microscope slide. Specimens were then dissected for parity, before nulliparous flies were discarded and parous flies were dissected further for *O. volvulus* infection following standard methods [32]. If parasites were present, the numbers and developmental stages (L1 – L3, and L3H) were recorded. L3H parasites were defined as infective (L3) stages present in the heads of blackflies (L3 stages were occasionally found elsewhere in blackfly bodies). Parasites were then air dried on the slide and stored for later molecular confirmation of identification [33]. Blackflies that were not dissected were preserved in ethanol for laboratory pool screening (molecular work is ongoing and only preliminary pool screening results are reported). Pool screening methods are described in detail in Chapter 4.

Statistical analysis

Simulium damnosum s.l. collection and dissection data were used to estimate the monthly and annual biting rates (MBR and ABR) and the monthly and annual transmission potentials (MTP and ATP) at each collection site. These indices were calculated using formulae described by Walsh *et al.* [26].

The Wilson method was used to calculate confidence intervals for all proportions (biting rates, parity rates and L1 – L2 and L3H infection rates), as recommended by Agresti and Coull [34]. The coverage probabilities were close to the nominal confidence levels. All the confidence intervals used later were calculated at 95% significance level. A logistic regression was used to test the effect of seasonality on parity and infection rates between the different collection sites. Blackfly catches were analysed using a negative binomial regression to avoid problems with overdispersion, and to test possible differences across collection sites and seasons. Independence of blackfly counts across months is assumed in both analyses.

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 (1041/15) and the Comité National d’Ethique de la Recherche pour la Santé Humaine (CNERSH), Cameroon (2016/03/677/L/CNERSH/SP). Collectors received appropriate training and were not considered to be at a higher risk of exposure than others living in local communities. All participants were adults over the age of 18 years. They were receiving ivermectin treatment in accordance with the national onchocerciasis control programme, and provided written informed consent.

Results

Identification of S. damnosum complex

Simulium damnosum complex larvae and pupae were collected from large rapids ≈1km upstream from the ferry crossing at Nyamongo I and ≈300m downstream from the collection site at Bayomen (Fig 1). Morphological and cytotoxic identification of larvae revealed the presence of *S. squamosum* s.l. and *S. mengense* (Figs 3 and 4), both of which were previously reported from the area by Barbazan *et al.* [1]. However, the *S. squamosum* did not conform to descriptions of the A, B, C or D cytoforms present in Cameroon [4, 5, 7]. All 49 specimens analysed from Nyamongo I (n=39) and Bayomen (n=10) contained homozygous inversions 1S-1 and 1L-3, which are generally fixed in the *S. squamosum* subcomplex (Fig 3A) [30]. They also possessed a new inversion (1L-57) from sections p34 to

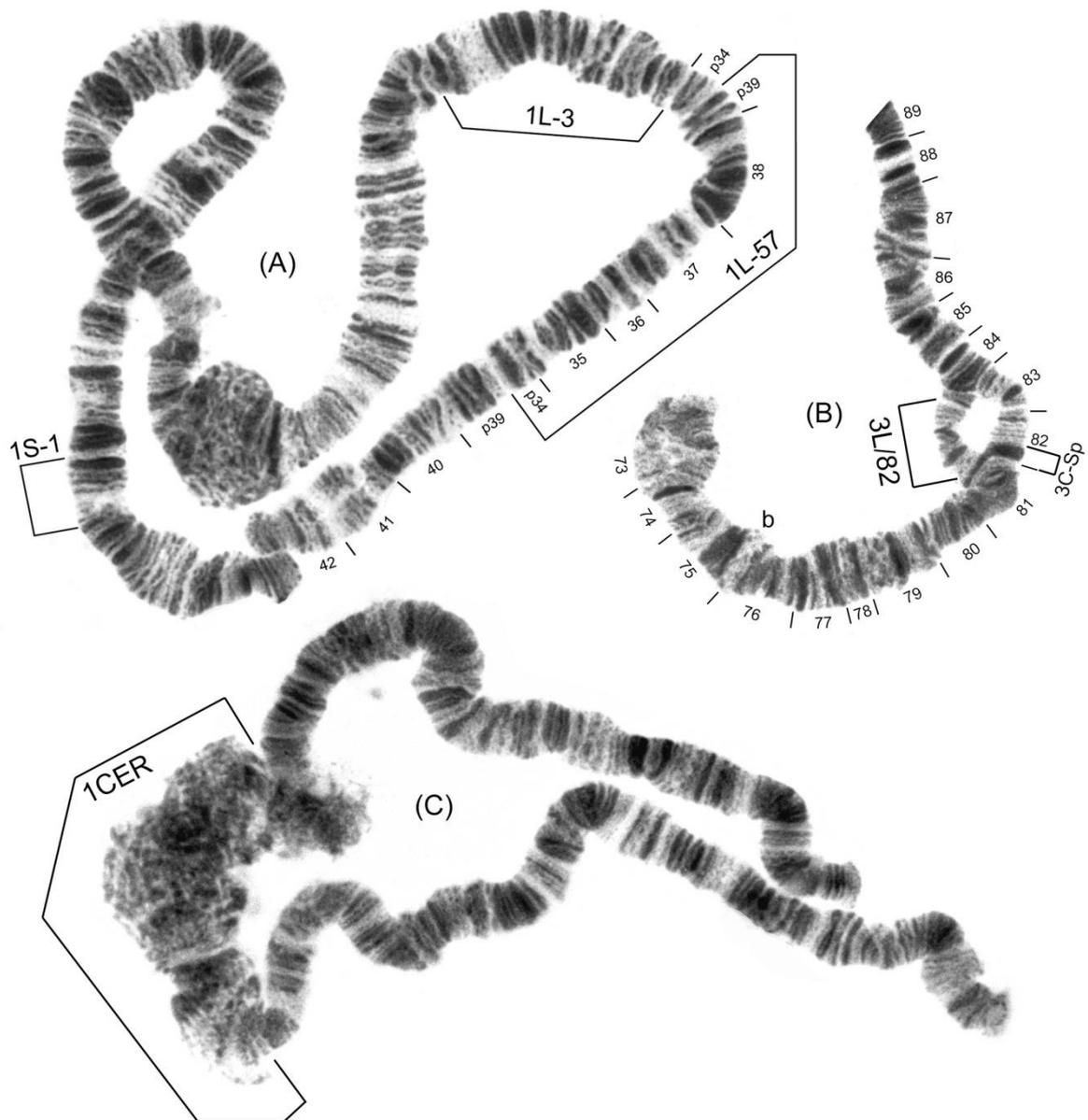


Fig 3. Cytotaxonomy of *S. squamosum* E2 and *S. mengense*. (A) Chromosome 1 of *S. squamosum* E2 showing fixed inversions 1S-1 and 1L-3, and new inversion 1L-57 (fixed in specimens examined); (B) Part of chromosome 3 of an *S. squamosum* E2 male, showing a sex-linked band dimorphism (3C-Sp) and a sex-linked heterozygous inversion (3L/82), present in 19/20 male specimens examined, 'b' = blister; (C) Chromosome 1 of *S. mengense* showing 1CER (expanded centromere).

p39, which was fixed within the population. In addition, 16/17 male specimens from Nyamongo I and 3/3 male specimens from Bayomen possessed a sex-linked band dimorphism (3C-Sp), and a heterozygous inversion (3L/82) near the centromere of chromosome 3 (Fig 3B). This was absent in all 29 females collected from both sites. The involvement of 3C in sex determination among *S. squamosum* is diagnostic for *S. squamosum* E (=type III) [30, 31]. Since only a small number of specimens were examined from a narrow geographic range, the name *S. squamosum* E2 is proposed for this variant possessing 1L-57.

An additional five larval specimens collected at Bayomen agreed with descriptions of *S. mengense*. These were identified by expanded regions associated with the centromere of chromosome 1 (Fig 3C), and also tufts of hair-like scales on the anterior dorsum of the larval thorax of associated specimens (Fig 4) [10, 14, 35]. Adult *S. mengense* have hairs on the subcostal wing vein [10, 35], although no flies retrospectively examined from those collected on human bait between July 2016 and January 2017 at Bayomen (n=254), Nyamongo I (n=74) and Ondouano (n=77) possessed this characteristic. No other morphological characteristics were used to identify cytoforms biting humans.

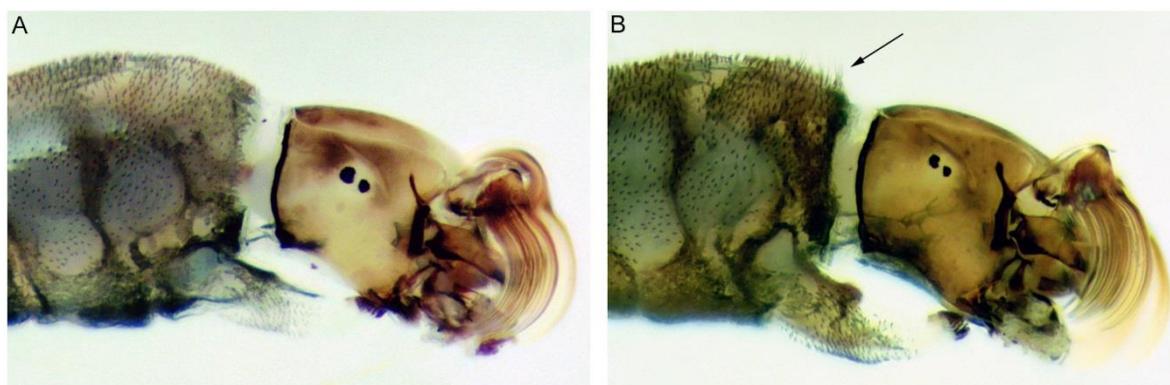


Fig 4. Head and thorax of late-instar larvae. (A) *S. squamosum* E2; (B) *S. mengense*, with arrow showing tuft of hair-like scales on the anterior dorsum of the thorax.

Adult blackfly collections and biting rates

In total, 93,563 adult female *S. damnosum* s.l. were collected on human bait across the four sites, and of these, 9,281 were dissected for parity and *Onchocerca* spp. infection. The remaining 84,282 were preserved in ethanol for pool screening (Tables 1-4). The highest biting rates were recorded at the two riverside sites, although monthly biting rates at Bayomen were significantly higher than at Nyamongo I ($p < 0.001$) (Table 5). Mean daily biting rates (DBRs) at Bayomen remained consistently high ($>1,500$) throughout the year. They decreased in July, December and February, but still remained >700 (Fig 5A). Despite being highest at Bayomen (ABR 606,370), the ABR at Nyamongo I (233,167) was still 2.4 times higher than the ABR of 98,208 reported by Barbazan *et al.* at a similar site in 1993/94 [1]. There were two clear peaks in biting at Nyamongo I (Fig 5A). The first coincided with the months of highest average rainfall in September and October, before rates decreased abruptly in November at the onset of the long dry season. A second increase occurred in December and biting rates remained high until March, before decreasing in April at the onset of the new rainy season. The majority of biting at Nyamongo I therefore occurred during the long dry season. Similar seasonal patterns were observed at Egona II and

Bayomen	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Total
No. Days	3	3	3	3	3	3	3	3	3	3	3	3	36
Total Blackfly Catch	2182	5326	5800	7105	6125	3327	6451	2902	4718	4671	4804	6289	59700
No. Preserved*	1949	4996	5470	6775	5799	3023	6121	2624	4383	4341	4474	5959	55914
No. Dissected	233	330	330	330	326	304	330	278	335	330	330	330	3786
No. Parous	52	56	104	129	117	68	133	114	88	186	192	134	1373
Parous (%)	22.3	17.0	31.5	39.1	35.9	22.4	40.3	41.0	26.3	56.4	58.2	40.6	36.3
No. Flies with L1 - L3 (%)	0 (0)	3 (0.91)	0 (0)	1 (0.30)	1 (0.31)	0 (0)	0 (0)	4 (1.44)	2 (0.60)	8 (2.42)	1 (0.30)	0 (0)	20 (0.53)
No. Flies with L3H (%)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.31)	0 (0)	0 (0)	1 (0.36)	0 (0)	2 (0.61)	1 (0.30)	0 (0)	5 (0.13)
No. L1	0	10	0	0	2	0	0	6	2	17	0	0	37
No. L2	0	10	0	3	0	0	0	8	1	7	0	0	29
No. L3 (Total)	0	0	0	0	1	0	0	4	0	24	10	0	39
No. L3H (Head)	0	0	0	0	1	0	0	4	0	17	10	0	32

Table 1. Summary of blackfly collection and dissection data from catches made at Bayomen between July 2016 and June 2017.

Nyamongo I	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Total
No. Days	3	3	3	3	3	3	3	3	3	3	3	3	36
Total Blackfly Catch	1228	1471	2230	2770	1089	2087	2896	2666	2729	1155	1682	996	22999
No. Preserved*	961	1224	1893	2440	797	1755	2576	2379	2408	970	1440	776	19619
No. Dissected	267	247	337	330	292	332	320	287	321	185	242	220	3380
No. Parous	62	44	50	48	59	51	74	35	90	68	41	76	698
Parous (%)	23.2	17.8	14.8	14.5	20.2	15.4	23.1	12.2	28.0	36.8	16.9	34.5	20.7
No. Flies with L1 - L3 (%)	2 (0.75)	7 (2.83)	6 (1.78)	2 (0.61)	3 (1.03)	1 (0.30)	6 (1.88)	2 (0.70)	7 (2.18)	6 (3.24)	2 (0.83)	1 (0.45)	45 (1.33)
No. Flies with L3H (%)	1 (0.37)	1 (0.40)	1 (0.30)	0 (0)	0 (0)	0 (0)	1 (0.31)	1 (0.35)	4 (1.25)	2 (1.08)	1 (0.41)	0 (0)	12 (0.36)
No. L1	2	12	5	26	7	7	7	5	2	2	0	2	77
No. L2	0	2	20	0	0	0	4	0	3	23	12	0	64
No. L3 (Total)	2	1	1	0	0	0	6	1	14	3	1	0	29
No. L3H (Head)	2	1	1	0	0	0	6	1	14	3	1	0	29

Table 2. Summary of blackfly collection and dissection data from catches made at Nyamongo I between July 2016 and June 2017.

Monthly Biting Rate (MBR)	2016						2017						ABR
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	
Bayomen	22547	55035	58000	73418	61250	34379	66660	27085	48753	46710	49641	62890	606370
Nyamongo I	12689	15200	22300	28623	10890	21566	29925	24883	28200	11550	17381	9960	233167
Egona II	413	1467	6870	16905	1100	4981	12524	9165	16647	7200	8876	3700	89849
Ondouano	176	372	540	4619	150	837	2625	1475	5849	1920	1498	480	20540

Table 5. Estimated monthly (MBR) and annual (ABR) biting rates at the four collection sites calculated following the methods of Walsh *et al.* [26].

Monthly Transmission Potential (MTP)	2016						2017						ATP
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	
Bayomen	0	0	0	0	188	0	0	390	0	2406	1504	0	4488
Nyamongo I	95	62	66	0	0	0	561	87	1230	187	72	0	2360
Egona II	0	0	0	0	0	0	0	0	0	102	0	0	102
Ondouano	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 6. Estimated monthly (MTP) and annual (ATP) transmission potentials at the four collection sites calculated based on dissection data following the methods of Walsh *et al.* [26].

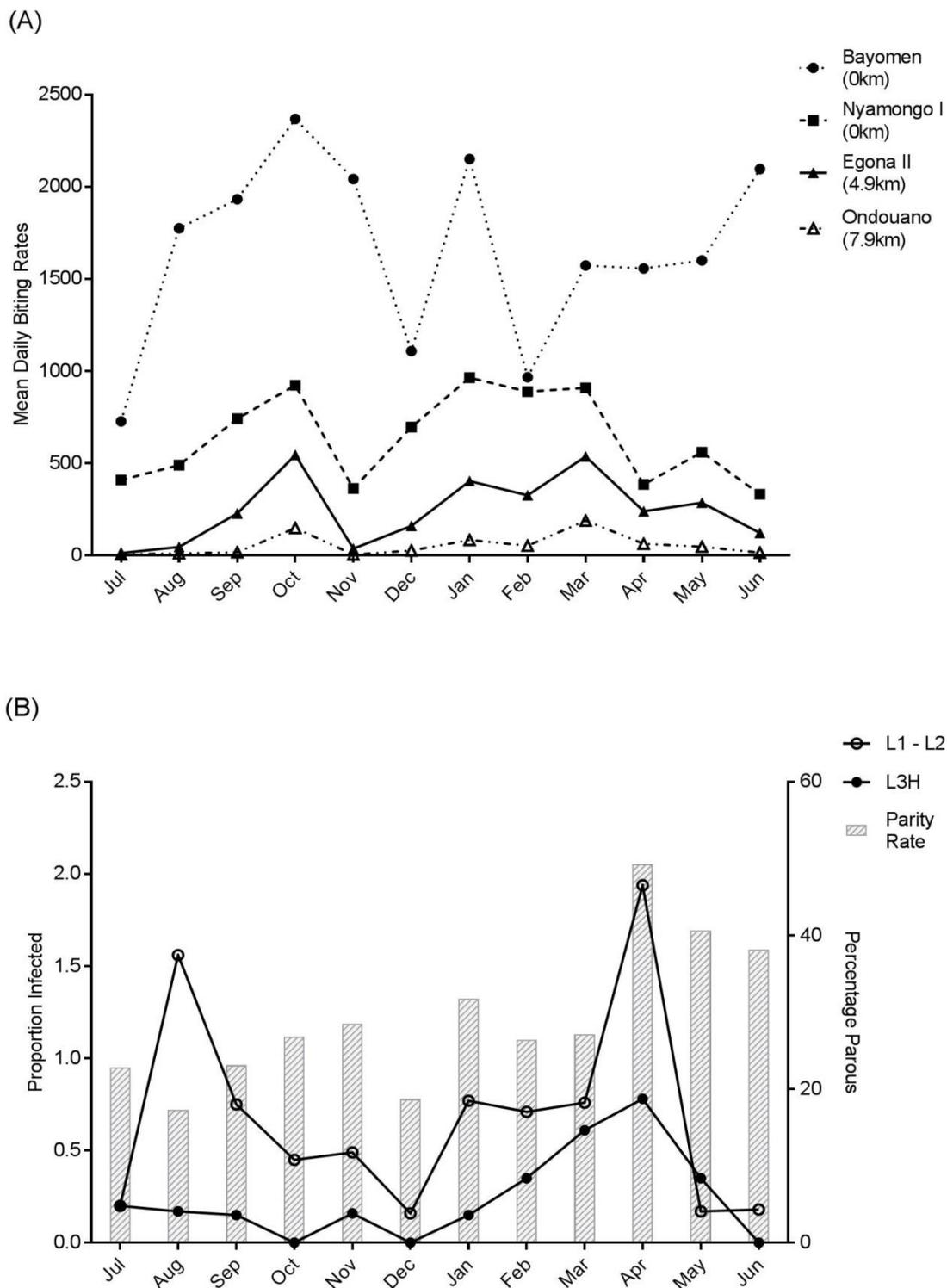


Fig 5. Blackfly biting rates, parity rates and *O. volvulus* infection rates. (A) Mean daily biting rates at the four collection sites, and distance of each site from the river (in parentheses); (B) Combined parity rates and combined infection rates of flies dissected at Bayomen and Nyamongo I riverside sites (2016-17); L1 - L2 = proportion of flies infected with developing parasite stages only, L3H = proportion of flies containing L3 stages in the head.

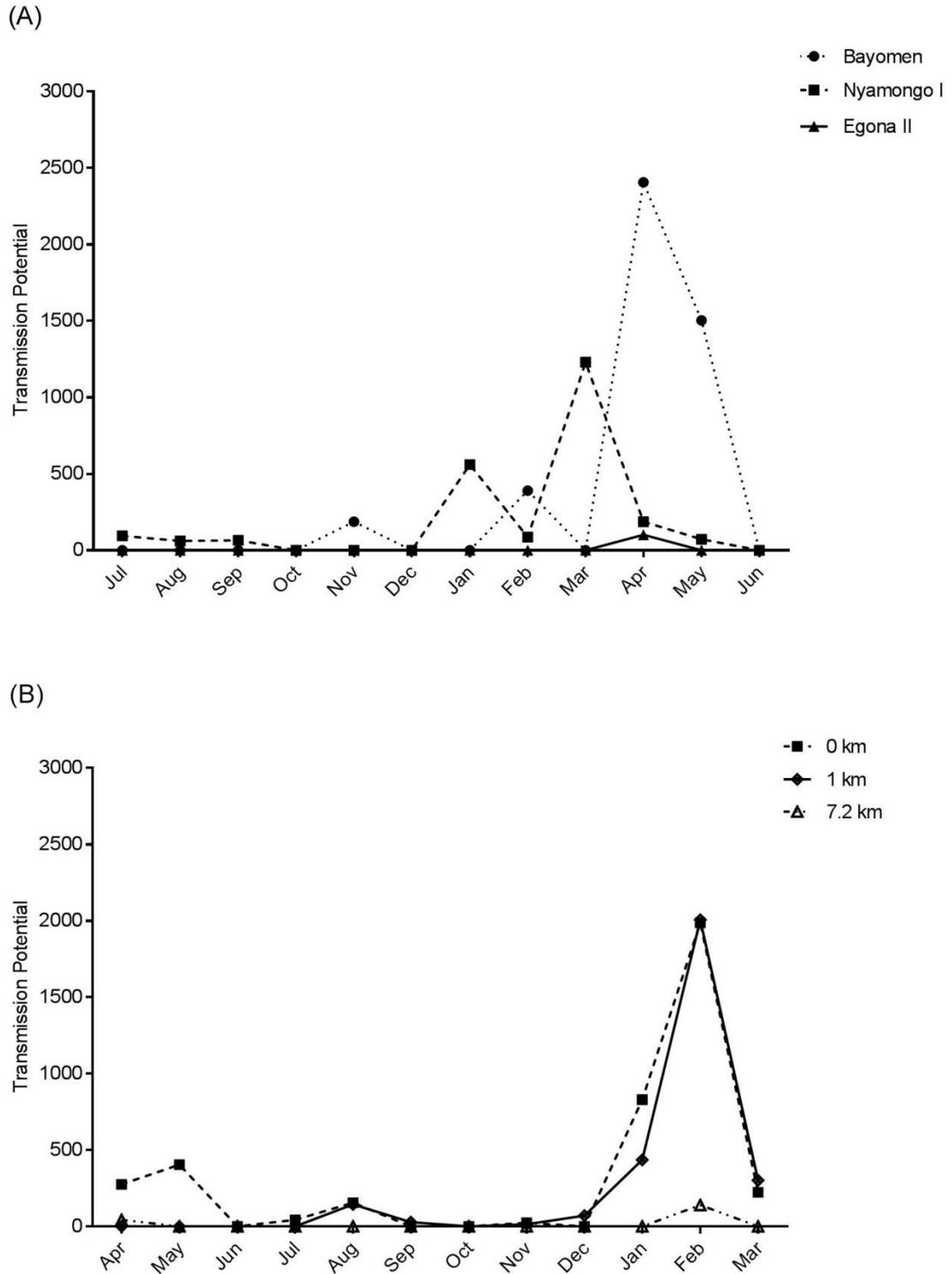


Fig 6. Transmission potentials in 2016/17 and 1993/94. (A) Monthly transmission potentials at Bayomen, Nyamongo I and Egona II (2016-17), estimated using dissection data only; data from Ondouano excluded since no larvae were found in dissected flies (n=496); (B) Monthly transmission potentials at Nyamongo I riverside site (0km), and sites 1km and 7.2km from the riverside (1993-94). Plotted using unpublished data from study by Barbazan *et al.* [1].

Ondouano, although blackfly activity decreased with increasing distance from the river (Fig 5A). Peaks in biting were recorded at both sites in October and March, but biting rates at Ondouano otherwise remained relatively low (DBR <85). The MBRs at Egona II and Ondouano were both significantly lower than at the Nyamongo I ($p < 0.001$) (Table 5).

Parity rates

The overall percentage of parous flies was higher at Bayomen (36.3% [95% CI 34.7%, 37.8%]), than at Nyamongo I (20.7% [95% CI 19.3%, 22%]) ($p < 0.001$) (Tables 1-2). Parity rates were lower at Egona II (11.1% [95% CI 9.6%, 12.6%], $p < 0.001$) and Ondouano (9.4% [95% CI 0.7%, 12.3%], $p < 0.001$) than at Nyamongo I, but there was no difference between Egona II and Ondouano ($p = 0.286$) (Tables 3-4). Parity rates were higher in the March – June rainy season than in the August – October rainy season at both riverside sites combined ($p < 0.001$) (Fig 5B). The odds ratio for being bitten by a parous fly was 1.7 times higher in the March to June rainy season when compared with the August to October rainy season. Parity rates were <10% at Egona II and <5% at Ondouano when biting rates peaked at these sites in October 2016 and March 2017.

Transmission

Onchocerca volvulus transmission occurred mainly between January and May during the long dry season and early rainy season. The ATP was higher, and peaks in transmission occurred slightly later, at Bayomen (ATP 4,488) than at Nyamongo I (2,360) (Table 6, Fig 6A). The ATP at Nyamongo I was slightly lower than that reported by Barbazan *et al.* (3,113) in the same area, although transmission appeared to follow a similar pattern (Fig 6B) [1]. The mean number of L3H per infective fly was higher at Bayomen (6.4) than Nyamongo I (2.4), and the highest estimated MTP (2,406) at Bayomen was calculated based on just two infective flies carrying 17 L3H parasites. When data were combined for the two riverside sites, there was no effect of rainy season on the proportion of flies carrying only L1 and L2 stage parasites ($p = 0.528$) (Fig 5B). However, the proportion of flies carrying L3H parasites was higher in the March – June rainy season than the August – October rainy season ($p = 0.043$) (Fig 5B). At Egona II, the ATP (102) was considerably lower than at the two riverside sites (Table 6, Fig 6A). Infective L3H parasites were only found at this site in two flies collected in April 2017, at the beginning of the rainy season and shortly after biting rates had peaked (Table 3). No parasites of any stage were found in the 489 flies dissected at Ondouano during the study (Table 4).

Preliminary results of real-time PCR pool screening (see method in Chapter 4) showed that ≈98% of infected pools containing blackflies collected between July 2016 and January 2017

were positive for *O. volvulus* (Table 7). As the pool screening work is ongoing, data are preliminary and no statistical analysis has been performed.

Table 7. Provisional pool screening results from blackfly collections made between July 2016 and January 2017, and analysed by real-time PCR. Data are preliminary and no statistical analysis has been performed.

Collection Site	Distance from River (km)	No. Pooled [†]	No. Pools [*]	<i>O. volvulus</i>		<i>O. ochengi</i>	
				Bodies	Heads	Bodies	Heads
Bayomen	0	33808	170	154	39	5	1
Nyamongo I	0	10707	56	55	31	0	0
Egona II	4.9	3345	20	19	8	0	0
Ondouano	7.9	580	8	6	2	0	0
Total		48440	254	234	80	5	1

[†]*S. damnosum* s.l.

^{*}= number of pools of heads and number of pools of bodies.

Discussion

Simulium damnosum complex

Collections of immature blackflies were limited to a single period in the dry season in January 2017 and consisted of *S. squamosum* s.l. and *S. mengense*, both of which are known from the area [1]. The involvement of 3C in sex determination and the presence of the newly described inversion 1L-57 indicate that this is a variant of *S. squamosum* E, and not the *S. squamosum* A previously reported. The E cytotype is known from western Côte d'Ivoire, Ghana, Guinea, Liberia and Sierra Leone [5, 31], whereas previous reports of its presence in Benin, Central African Republic and Togo were made in error [8, 31]. The collection of *S. squamosum* E2 from the Mbam River near Bafia is therefore thought to be the first record of this cytotype (or a variant of it) east of Lake Volta, more than 1,200km away. In addition, 5/54 larvae were identified as *S. mengense*. The composition is therefore similar to previous reports by Barbazan *et al.* (90% *S. squamosum*, 10% *S. mengense*) [1].

It appears likely that *S. squamosum* E2 is involved in human biting, at least during part of the year. It was the more abundant of the two cytoforms found at breeding sites, and there was a lack of morphological evidence to suggest that *S. mengense* was biting humans in the area. However, specimens used for identification were very limited in number, geographical distribution, and season of collection. The previous report that *S. squamosum* (A) was collected in the area is not unfounded, although there is no published chromosomal evidence to verify this [1]. It is possible that the species composition has changed in recent years. Reductions in rainfall and river discharge have occurred since the early 1970s, and dams were built upstream from Bafia on the Noun and Mbam rivers in 1974 and 1987 respectively [23]. Environmental changes such as these alter the physical properties of the river water and rates of discharge, and could affect the blackfly species present [23, 36]. However, these events pre-date Barbazan's collections in 1993/94, and another possible

explanation for the differences in respective studies is that the composition of cytotypes in the Mbam changes seasonally. Traoré-Lamizana and Lemasson [6] showed that *S. squamosum* s.l. spreads north from the forest-savannah transition zones and into the savannah rivers during the rainy season in Cameroon, but this was before the different cytotypes of *S. squamosum* had been described. The seasonal dynamics of *S. squamosum* cytotypic distribution are therefore not known.

The discovery of *S. squamosum* E2 at breeding sites shortly before peaks in *O. volvulus* transmission makes it of interest as a potential vector of *O. volvulus*. Investigations to determine its role and competence as a vector, and the extent and seasonality of its breeding range are therefore warranted in view of the severe clinical presentation of onchocerciasis in the Mbam Valley.

Biting rates

Blackfly biting occurred throughout the year at the riverside sites. The observed differences in biting rates between Bayomen and Nyamongo I were probably due to the relative proximity of collection sites to breeding sites (<300m and ≈1km, respectively). At Bayomen, biting rates were highest towards the end of the August – October rainy season. Mean DBRs remained >1,500 except in the dry months of July, December and February, when they decreased to ≈1,000 or less. Mean DBRs >2,000 were also recorded during the long dry season in January and there consequently appeared to be no clear seasonal trend other than for an intense blackfly nuisance that persisted throughout the year.

At Nyamongo I, similar seasonal trends to those observed in 1993/94 were recorded, although ABRs were higher during the current study [1]. This was partly due to the longer peak in biting that occurred during the long dry season compared with the same period in 1993/94 [1]. The dry season peak at Nyamongo I occurred at a time when the average river discharge is relatively low and stable (<500m³/s) (Fig 2) [23]. Under these conditions, more oviposition sites may become available and blackfly population instability caused by fluctuating water levels will be reduced [23, 37, 38]. Similar biting patterns were observed at Egona II and Ondouano, although biting peaked at these sites in October and March. The estimated ABR of 89,849 at Egona II was almost as high as the 98,208 estimated by Barbazan *et al.* at the riverside in 1993/94 [1]. However, the ABR declined markedly at Ondouano (20,540), 7.9km from the riverside, whereas it was still 43,790 at a similar distance (7.2km) in the 1993/94 study. In addition, Barbazan *et al.* had two more collection sites along the same transect at 13.5km and 23km, both of which had ABRs >43,000 [1]. Considering they reported no local breeding, it may be that blackflies dispersed greater

distances during the 1993/94 study than at present, although additional sampling would be required to confirm this.

Parity rates

The higher parity rates recorded at Bayomen compared to Nyamongo I may also be due to the relative proximity of collection sites to breeding sites. It was shown by Duke that parity rates can decrease rapidly with increasing distance from these habitats [39]. It is also possible that parity rates were higher at Bayomen because suitable non-human blood hosts such as cattle were present, or were available in greater numbers than at Nyamongo I. As a family, blackflies are quite catholic in their choice of host [40, 41]. Although blackfly blood meals were not analysed to investigate host preference, pool screening may yet yield information about the presence of alternative blood sources if non-human *Onchocerca* parasites are detected. It has been shown in northern Cameroon that *O. ochengi* appear in riverine blackflies at times that coincide with Bororo/cattle migrations [42].

Parity rates appeared to increase gradually throughout the sampling period (Fig 5B), although dissection data were very limited and should be interpreted cautiously. Nevertheless, combined parity rates at the two riverside sites were higher in the March – June rainy season than in the August – October rainy season, and this appears similar to previous findings that parity rates were lower at the end of the August – October rainy season and early dry season [1]. The parity rates recorded at Bayomen, Nyamongo I, Egon II and Ondouano were all consistently lower than the 78% average reported from the Mbam/Sanaga basin in 1993/94 [1]. Barbazan *et al.* also reported that there was little difference in parity between blackflies on the shoreline and at points further inland [1]. This contradicts current findings which show that parity rates at Egon II and Ondouano were significantly lower than at Nyamongo I. Parity rates decreased to <10% and <5% at Egon II and Ondouano respectively during months of peak biting. Considering the difference in ABRs between these three sites, there is likely to be relatively little exposure to parous flies at Ondouano in comparison to Nyamongo I.

Onchocerca volvulus transmission

The transmission of *Onchocerca* parasites morphologically indistinguishable from *O. volvulus* occurred mostly at the riverside sites (Fig 6A). Transmission appeared to peak slightly earlier at Nyamongo I (March) than at Bayomen (April), but generally occurred during the long dry season and early rainy season between January and May. At Nyamongo I, this was when biting rates were at their peak, whereas at Bayomen, biting rates were more or less continuously high. The higher ATP at Bayomen was due to the higher biting rates and the higher mean number of L3H per infective fly compared to Nyamongo I. However, the

collection site at Nyamongo I was adjacent to a ferry crossing where many people gather, and it is likely to be a point of significant human-vector contact and parasite transmission. In contrast, the Bayomen collection site was mainly accessed by farmers and fishermen. At the sites away from the riverside, transmission was clearly lower. Just two flies out of 1,626 dissected were found to be infective at Egona II (Table 3). However, the estimated ATP of 102 is still above the WHO threshold for interruption of transmission, and discontinuation of ivermectin treatment should not be considered given the intensity of transmission at the riverside [43]. No parasites were found at Ondouano where biting rates and parity rates were low (Table 4), although the ATP of 0 is unlikely to accurately represent transmission since only a small number of flies were dissected.

Despite 16 years of CDTI, the high transmission potentials encountered at riverside sites were perhaps not surprising when considering the results of recent epidemiological surveys [3, 20]. CDTI currently takes place around July each year [3, 16], and the drug is most effective at suppressing microfilariae during the first six months after treatment [44]. This would at first appear to be the reason for the lower rates of transmission observed between July and December during the current study. However, similar patterns of *O. volvulus* transmission were also documented by Barbazan *et al.* at Nyamongo I in 1993/94, and at additional sites along the Sanaga River where *S. squamosum* B breeds and bites perennially [1, 13]. These entomological studies were conducted at a time that pre-dates ivermectin mass treatment [16, 45]. Even the earliest mass treatments (1994 – 1997) only covered approximately 10% of the population, and high (>65%) therapeutic ivermectin coverage was not achieved until several years after CDTI commenced [3, 16]. Ivermectin would therefore not have affected the results of this earlier study, and unless other significant intruding factors were involved, the patterns of *O. volvulus* transmission documented by Barbazan *et al.* probably reflect true seasonal cycles [1]. Similar transmission cycles have been reported for *S. squamosum* in Togo [46], where breeding and biting occurred perennially, but at higher rates during the dry season. At this time, parity rates were also higher and coincided with a peak in *O. volvulus* transmission [46]. Despite another biting peak occurring during the rainy season, parity rates were lower and parasite transmission was consequently lower [46]. In support of this, Millest *et al.* also demonstrated that *S. squamosum* in Togo lived longer in the dry season than the rainy season [47]. It is also thought that favourable weather conditions increase the probability of blackfly survival and human-vector contact, resulting in dry season peaks in *O. volvulus* transmission by the forest cytoform, *S. yahense* [46, 48].

At present, it appears that seasonal variation in fly survival (as indicated by higher parity rates and proportions of L3 infected flies in the late dry-season/early rainy season) provides the strongest argument for the observed transmission cycle. However, other possibilities should not be excluded. Differences in vector competence, which might be influenced by the existence of *Onchocerca-Simulium* complexes, could potentially affect transmission in areas where the cytoform composition is not stable [41, 49]. The annual arrival of the Bororo with their cattle may also contribute to the peak in transmission. The bovine parasite *O. ochengi* is difficult to distinguish morphologically from *O. volvulus* and could potentially distort transmission indices if estimated from dissection data alone.

Conclusion

It is clear from recent epidemiological, parasitological and social studies, that onchocerciasis remains a public health problem among communities along the lower Mbam River [3, 20, 45]. High pre-CDTI parasite burdens and poor adherence to ivermectin, particularly among younger people, have been cited as probable reasons for disease prevalence being higher than expected following >15 years of CDTI [3]. The frequency and timing of treatment are important factors in determining how quickly a focus progresses towards elimination, although it is not clear how the latter may affect progress where *O. volvulus* transmission occurs seasonally [50, 51]. The current programme may therefore benefit from treating communities early in the dry season (ca. December), before blackfly parity rates and parasite transmission increase.

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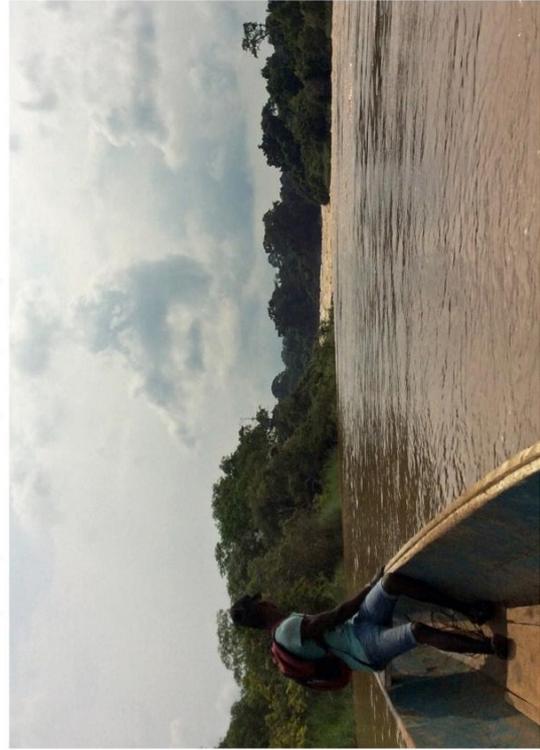
Ferry crossing the Mbam River at Nyamongo I.



Business at ferry crossing, selling socks to protect from biting blackflies.



Boat ride to rapids upstream from Nyamongo I, July 2016.



Rapids approximately 1 km upstream from Nyamongo I, July 2016.





Looking downstream towards rapids from fishing site near Bayomen, January 2017.



Demonstrating use of aspirators for human landing collections at Ondouano.



Training in human landing collection methods at Nyamongo I.



Field laboratory at dissection point near Nyamongo I.

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Chapter 6

Discussion

Summary

Infection with the blackfly-borne filarial parasite, *Onchocerca volvulus*, was one of the leading causes of preventable blindness worldwide prior to the commencement of the Onchocerciasis Control Programme in West Africa (OCP) in 1974 [1, 2]. Clinical pathologies were particularly severe in savannah bioclimatic zones where the blackfly species *Simulium damnosum* s.str. and *Simulium sirbanum* were responsible for the majority of transmission [1]. Despite the undeniable success of the OCP and the African Programme for Onchocerciasis Control (APOC) in reducing disease burden, it is not known whether current interventions are sufficient to achieve widespread elimination [3]. That said, interruption or elimination has been achieved in some isolated foci of former APOC countries [3-8]. However, these are mostly areas where ivermectin has been distributed biannually, or where ivermectin treatment has been supplemented with vector control. Despite these successes, the current strategy of onchocerciasis control in most former APOC countries remains through annual community directed treatment with ivermectin (CDTI) [9]. While this is proving to be effective in many areas, Tekle *et al.* recently identified eight foci that were underperforming in their progress towards elimination [5, 10]. Reasons for this are thought to include poor ivermectin coverage and poor adherence to the drug [5, 10, 11]. The data of Tekle *et al.* are certainly important, but assessments were based on human skin snip (parasitological) surveys [5]. This method, while acceptable during intermediate phases of control programmes, is not recommended for evaluating the interruption or elimination of transmission [12, 13]. The World Health Organization (WHO) has set the ambitious target of achieving elimination (defined in Chapter 1) of onchocerciasis by 2025 [9]. Since many of the CDTI projects established during the lifetime of APOC have now been treating communities >15 years, there is a need to evaluate the impact of chemotherapeutic and vector-based interventions on parasite transmission in blackflies.

This study aimed to provide a detailed investigation of the ecology of anthropophilic blackflies and the status of *O. volvulus* transmission in three formerly hyperendemic disease foci under long-term control with either annual CDTI, or vector control in combination with biannual CDTI.

The collection of blackflies for entomological evaluation

The need to develop new tools for blackfly collections as CDTI programmes approach phases of interruption, elimination and post-elimination surveillance was discussed in Chapter 2, and an evaluation of the efficacy of Esperanza Window Traps (EWTs) was performed for this purpose. The decision to conduct the study was also influenced by difficulties in obtaining ethical approval to collect blackflies using human bait in Uganda in 2014. The delays were unfortunate as unpublished Uganda Ministry of Health (MOH) records showed that *S. damnosum* s.l. biting rates were >100/day at Awere Bridge in Pader district at the time. The species was also abundant at Beyogoya village in Lamwo district in 2014 (personal observation). However, *S. damnosum* s.l. populations had declined dramatically by the time ethical approval was obtained, and only 130 flies of this species were collected on human bait in Kitgum, Lamwo and Pader districts in 2015/16 (Chapter 3). If a well-functioning and easy-to-deploy trap had been available, the outcome of the Uganda investigations might have been different. At present, the EWT appears to work well for the collection of *Simulium damnosum* s.str. when the species is abundant, and a recent study at the same Ugandan site (Ayago Bridge) has shown that traps can be effectively operated by community members [14-16]. However, it does not appear to be equally attractive to all African human biting blackflies [15]. Further research to improve upon the understanding of blackfly behavioural responses to traps is needed, particularly in East African foci where vectors other than *S. damnosum* s.str. are responsible for the majority of transmission [17]. In addition, improving the traps ergonomically would increase their appeal. Building multiple traps was time consuming and building them poorly or using inappropriate materials led to problems in the field [15].

While there is clearly still work needed to improve blackfly trapping methods, the recent flurry of relevant publications should encourage blackfly researchers [14-16, 18-20]. The example of utilising traps to control tsetse flies shows just how successful methodical approaches to development can be [21-23]. The distribution and prevalence of onchocerciasis is far greater than of tsetse-borne sleeping-sickness (trypanosomiasis) [24, 25], yet there has been rigorous and systematic development of tools to control the tsetse vector [21, 22, 26], and this has been critical to the success of trypanosomiasis control programmes. Significant biological differences exist between tsetse and blackfly vectors which make the former more appealing to control using traps. Importantly, female tsetse are larviparous and each fly gives birth to just a single live larva every 9-10 days [27, 28]. As a result, they have very low reproductive rates. In contrast, each female blackfly can lay hundreds of eggs every 3-4 days [29]. While traps may not have such an obvious role in onchocerciasis control as they do for trypanosomiasis (and control with EWTs might be

interesting to attempt using impregnated materials in isolated foci), there is both ethical and operational value in funding future research and development as critical phases of onchocerciasis control programmes approach.

Approaches to disease control

Uganda and Tanzania are among three former APOC countries (the other being Equatorial Guinea) that have supplemented ivermectin treatment with vector control to control onchocerciasis [30]. However, Uganda is unique among these, currently being the only country in sub-Saharan Africa to regularly and extensively integrate both methods [31]. In 2007 the Uganda MOH decided to switch focus from control to elimination based on biannual ivermectin treatment where necessary, and vector control by ground larviciding where appropriate [4]. The decision was partly based on the observed greater impact of integrated control on onchocerciasis in Itwara focus, western Uganda, when compared with annual CDTI alone [32], but also due to concerns about donor fatigue and political commitment to sustaining projects in the long-term [3, 33]. The use of integrated control in Uganda has contributed to the interruption of transmission in 10/17 formerly endemic foci, and interruption is suspected in several more [4]. Remaining problem areas are those with cross-border transmission, including the Madi-Mid North focus where the current study took place [4, 34]. Vector control in Tanzania has been less widespread in recent years, and has only been implemented in the Tukuyu focus where there was thought to be little risk of reinvasion [35, 36]. Elsewhere in Tanzania, and throughout Cameroon, control has relied almost exclusively upon annual CDTI.

*Blackfly vectors and status of *O. volvulus* transmission*

It is clear from the work in northern Uganda that *O. volvulus* transmission was suppressed in Kitgum, Lamwo and Pader districts at the time of adult blackfly collections in 2015/16. The abundance and distribution of *S. damnosum* s.str. reported by Post in 2012 [37] makes it the most likely vector in the Mid North, although problems locating productive *S. damnosum* s.l. breeding sites in 2015/16 made it difficult to verify this. Reasons for the scarcity of breeding sites and low biting rates are unclear, but are likely to include vector control and possibly a natural decline in *S. damnosum* s.l. populations caused by hot and dry conditions at the time of collections [38]. It is important to note that *Simulium bovis*, a species that breeds sympatrically with *S. damnosum* s.l., was breeding (Achwa River, Te Lute) and biting in reasonable numbers in the south west of Lamwo district in 2015/16 (Chapter 3: Table 3, Fig 5). It therefore appears that low *S. damnosum* s.l. biting rates are not exclusively due to vector control, but this may only become clear when the MOH publish details of ongoing blackfly surveys and vector control methods in the Mid North. The *S. damnosum* s.l.

collected during the current study were insufficient in number and duration of collection to permit a satisfactory evaluation according to WHO guidelines [12]. In addition, it is not known whether *S. bovis* contributes to *O. volvulus* transmission. Regardless, the integrated approach to control only commenced in 2012 and it is too soon to consider withdrawing ivermectin treatment. Despite current data only providing limited evidence for suppression of *O. volvulus* transmission, the outcome of blackfly collections which took place at times of peak biting and the results of *O. volvulus* screening should be seen as encouraging.

In contrast to northern Uganda, *O. volvulus* transmission was clearly ongoing throughout the Mahenge Mountains in Tanzania, and also in areas surrounding the lower Mbam River near Bafia in Cameroon. These are both foci where onchocerciasis control relies entirely upon annual CDTI, and results in both areas should provide cause for concern [39, 40]. While it was not possible to determine the relative vectorial roles of *Simulium kilibanum* and 'Nkusi J' in Mahenge, the latter appeared to be the predominant cytoform. This agrees with Häusermann's observations from the 1960s [41]. It is known that 'Nkusi J' is a vector elsewhere in Tanzania, while *S. kilibanum* has not been incriminated other than in western Uganda [17, 42, 43]. Differences in the analysis of blackfly infection rates by Häusermann [41] (based on dissections) and those presented here (based on pool screening), make direct comparisons difficult. A combined dissection/pool screening approach would have provided more insightful data. Nevertheless, evidence of ongoing transmission in blackflies supports new Ov-16 serological data that demonstrates exposure to *O. volvulus* among children aged 6-10 years living in rural villages (Mdindo and Msogezi) near Mahenge [44]. It has been shown elsewhere, that even in areas with moderate ivermectin coverage (>60%) or in those experiencing problems with drug adherence, community microfilarial loads (CMFLs) can still be reduced to subclinical levels after 3+ years of annual treatment [5, 10]. Consequently, onchocerciasis may no longer be perceived to be problematic within communities, particularly among younger people less familiar with clinical complications of the disease [10]. However, transmission and new infections will continue to occur, and while clinical cases are likely to disappear, the risk of developing ivermectin resistance increases [45, 46]. The Mahenge focus may benefit from a detailed treatment coverage survey to identify possible causes of ongoing transmission, or it may just be that annual ivermectin treatment is insufficient to interrupt transmission at this stage [47, 48].

The entomological study conducted near Bafia in Cameroon was by far the most comprehensive of the three. The discovery of a chromosomal variant of *Simulium squamosum* E merits further investigation into its distribution and vector competence. Explanations for the seasonality and intensity of *O. volvulus* transmission remain

hypothetical, but may be clarified once the pool screening work has concluded. The combined dissection/pool screening approach will eventually yield a more detailed data set than was compiled in Mahenge. It will allow for a more precise estimation of transmission potentials, while clarifying the relative abundance of *O. volvulus*, *Onchocerca ochengi*, or possibly *Onchocerca* species 'Siisa' which is known from northern Cameroon [49]. Combining methods should therefore be considered for future studies (cost-permitting) in which detailed transmission data are required, or if conducting longitudinal follow-ups of dissection-based studies. However, in areas where there is enough reason to expect interruption of transmission, pool screening alone should suffice.

Again, rates of *O. volvulus* transmission near Bafia are worrying considering that ivermectin mass drug administration began in 2000, and that therapeutic coverage has been >65% since 2002 [10]. Transmission potentials were exacerbated by the very high biting rates encountered at riverside sites. Barbazan *et al.* [50] suggested that vector control at times of peak transmission might be an option considering the localised nature of breeding sites along the lower Mbam and Sanaga rivers. The authors proposed that two to three dosage points might be sufficient for this purpose [50, 51], although breeding along the Noun River would also need to be taken into consideration. Improving infrastructure at the ferry crossing near Nyamongo I might also reduce biting at a site of high human-vector contact. At present, annual ivermectin treatment alone seems highly unlikely to interrupt transmission along the lower Mbam River. However, optimising the timing of mass treatment to occur before the seasonal peak in transmission (rather than just the peak in biting as suggested by Coffeng *et al.* [52]), may improve the programme outcome without requiring significant new investments.

Methods of parasite identification

The methods of identifying parasites in blackfly vectors should be reviewed before evaluating CDTI programmes on a large scale. At present, identification relies upon the use of the conventional O-150 PCR [53]. Cross reactivity of the primers with *O. ochengi* and *O.* sp. 'Siisa' was discussed in Chapter 3, and could contribute to false-positive results in areas where these *Onchocerca* species are present in anthropophilic blackflies [49]. So far, *O.* sp. 'Siisa' has been identified from western [54] and northern Uganda (Chapter 3), and also northern Cameroon [49]. Domesticated Zebu cattle are definitive hosts, and the distribution of *O.* sp. 'Siisa' is therefore likely to be more widespread than is currently known. Real-time PCR assays, which differentiate all three 'species', may provide the best option going forward.

Original contribution to knowledge:**Overall**

- Demonstrated ongoing *O. volvulus* transmission at unacceptable levels after >15 years annual CDTI in two formerly hyperendemic onchocerciasis foci in Tanzania and Cameroon.
- Provided a systematic evaluation of Esperanza Window Traps for the collection of anthropophilic blackflies in two onchocerciasis foci, demonstrating different responses to traps in each area.

Uganda

- Mapped recent distribution (2012 – 2016) of breeding and biting anthropophilic blackflies in Madi-Mid North onchocerciasis focus of northern Uganda.
- Demonstrated wider distribution of anthropophilic *S. bovis* in the Mid North than was previously known (present in Kitgum, Lamwo and Pader districts) using morphological and molecular (ITS1) methods.
- Identified *O. sp.* 'Siisa' in Mid North and showed possible development to infective (L3) stages in anthropophilic blackflies, and also cross reactivity with O-150 primers used in control programmes to identify *O. volvulus*.
- Illustrated chromosomal forms of *S. damnosum* s.str. and *S. sirbanum* present in the Mid North.

Tanzania

- Provided an updated inventory of *S. damnosum* complex cytoforms present in Mahenge, using chromosomal and molecular (ITS1) methods.
- Demonstrated the presence of both 'Turiani' and *S. kilibanum* cytoforms in Mahenge for the first time.
- Showed that both 'Nkusi J' and *S. kilibanum* are anthropophilic in the focus.

Cameroon

- Documented seasonality and spatial patterns of blackfly biting and *O. volvulus* transmission at sites along the lower Mbam River in *Région du Centre*, Cameroon.
- Highlighted possible seasonal transmission of the parasite as a reason for the control programme underperforming, and proposed adjusting the timing of annual treatment.
- Described and illustrated a new chromosomal variant of *S. squamosum* E, the first time this cytoform has been collected East of Lake Volta (Ghana).

Operational elimination by 2025

It seems improbable based on current data that the WHO target of achieving operational elimination of onchocerciasis by 2025 can be met through annual mass drug administration with ivermectin alone [9]. Where interruption or elimination of transmission has been achieved, more intensive control efforts have been required [4, 55]. This has been through either biannual ivermectin treatment alone [8] or integrated chemotherapeutic and vector-based approaches [3, 7, 31, 56]. The Uganda National Onchocerciasis Control Programme (NOCP) provides an example of the success that can be achieved when a country is motivated to intervene. The strength of personnel and NOCP infrastructure, along with a rich history of onchocerciasis control, provides a strong foundation for combating the disease [3, 56]. For other countries to build and sustain equally strong programmes with limited resources will be challenging. In addition, even in Uganda, there are likely to be persisting problems with cross border transmission that will need to be resolved in close cooperation with neighbouring countries [4].

Conclusion

The OCP in West Africa was a pioneering vector-based public health intervention that had remarkable success in reducing the prevalence of the most severe ocular complications of onchocerciasis. APOC built on the success of the OCP, and through a strategy of annual mass drug administration with ivermectin, has largely succeeded in controlling onchocerciasis as a public health problem. The public health achievements of both programmes are undeniable, but many affected countries are in the midst of a lengthy battle against a resilient parasite, and one that could easily recrudescence in previously controlled areas. Donor fatigue or ivermectin resistance are two possible weaknesses in the sustainability of current control programmes, the longevity of which should not be taken for granted. This study showed that *O. volvulus* transmission is continuing at unacceptable levels despite >15 years of annual ivermectin treatment in two formerly hyperendemic onchocerciasis foci. Kazura [57] recently said that control programmes should not be “static or inflexible with respect to changes in MDA policy”, so rather than hoping the problem will eventually fade, countries should be proactive in evaluating underperforming programmes and addressing issues using appropriate interventions.

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Supplementary information

Supplementary figure (S1)

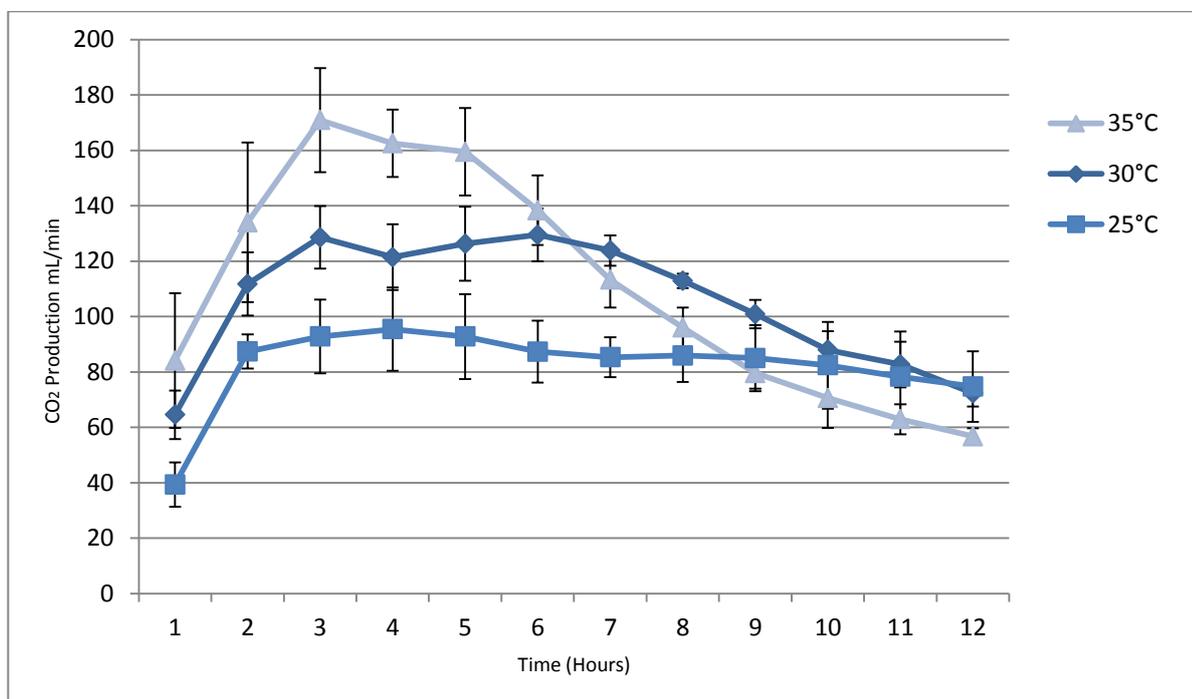


Fig S1. Laboratory production of CO₂. Mean values and 95% CIs of CO₂ (mL/min) produced by mixing 500g white sugar (Delhaize 365 Fine granulated sugar, Delhaize, Belgium), 50g baker's yeast (Saf-instant Red, Lesaffre, France), and 2.5L water, in a 10L container. Mixtures were incubated at 25°C, 30°C and 35°C. Measurements were made hourly for 12 hours and experiments were repeated four times at each temperature. Experiments were carried out at the Institute of Tropical Medicine, Antwerp, Belgium.

Supplementary figure (S2)

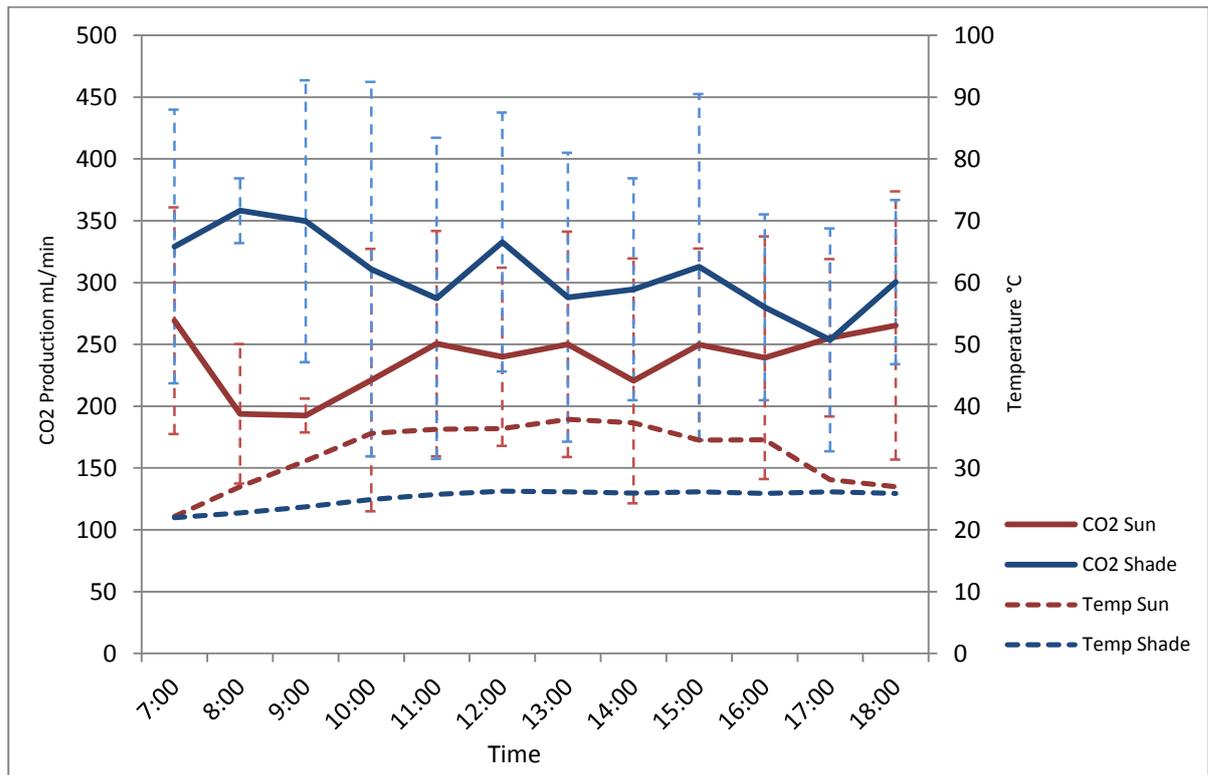
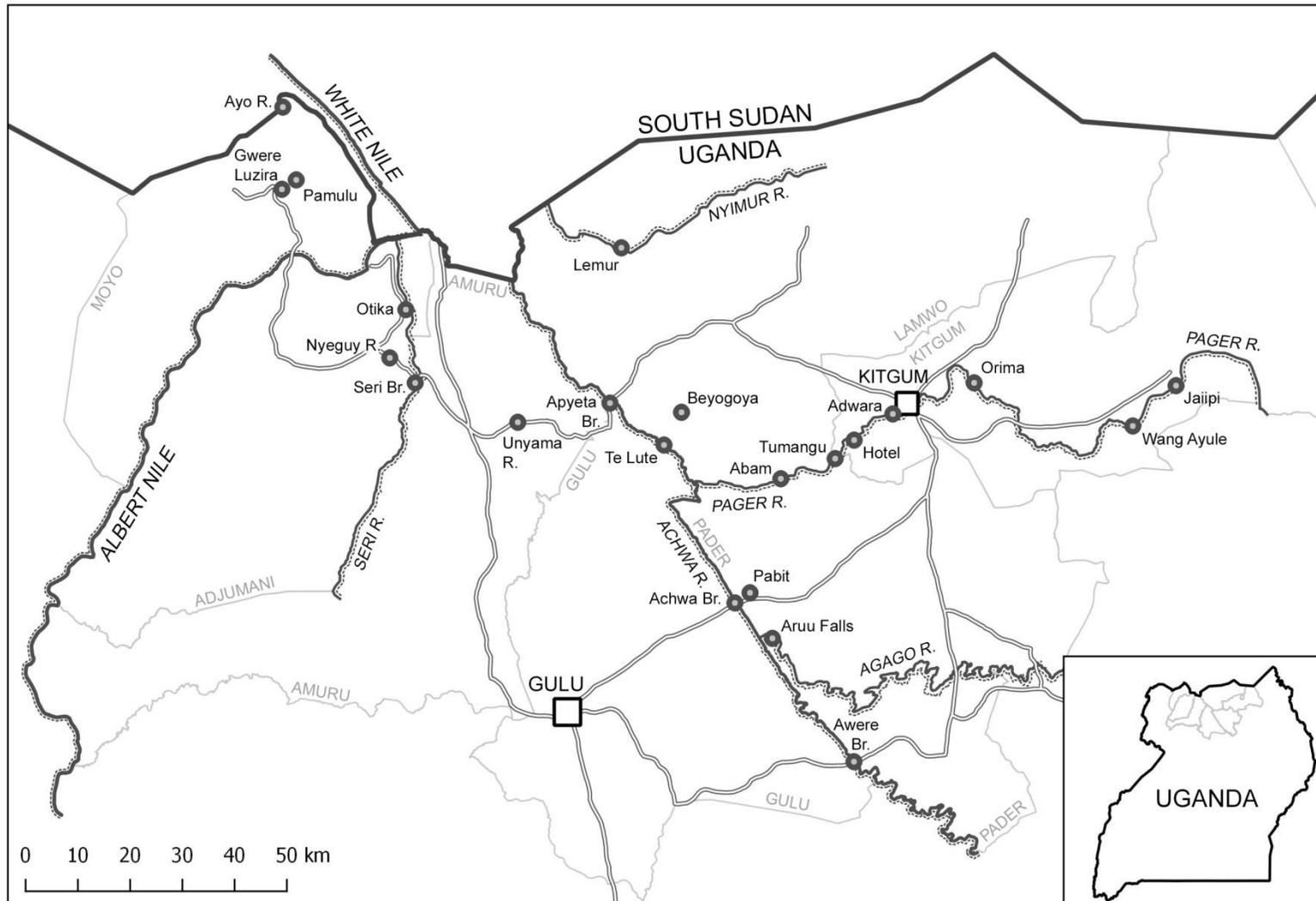


Fig S2. Semi-field production of CO₂. Mean values and 95% CIs of CO₂ (mL/min) produced by mixing 500g brown sugar (locally purchased, Gulu market, Uganda), 50g baker's yeast (Saf-instant Red, Lesaffre, France), and 2.5L water, in 10L containers placed in either in the sun or shade at Gulu University, Uganda. Measurements were made hourly for 11 hours (07:00 – 18:00) and were repeated for four consecutive days.

Supplementary figure (S3)

Fig S3. Map of northern Uganda showing key locations in the Madi-Mid North districts.



Supplementary table (S4)

Year	Month	District	Location	Latitude	Longitude	S.d	S.b	Source
2012	October	Amuru	Pajaa nr Pwomu village	3.18028	32.21556	+	-	RJP
2012	October	Kitgum	Hotel nr Laraba village	3.23667	32.78750	+	-	RJP
2012	October	Kitgum	Adwara nr Liba village	3.28056	32.85333	+	+	RJP
2012	October	Kitgum	Gozi nr Tumangu village	3.20417	32.75333	+	-	RJP
2012	October	Kitgum	Pachua Bridge	3.36417	32.97694	+	-	RJP
2012	October	Lamwo	Apyeta Bridge	3.29944	32.36250	+	+	RJP
2012	October	Lamwo	Waligo Bridge	3.56361	32.38611	+	+	RJP
2012	October	Pader	Awere Bridge	2.68778	32.78639	+	-	RJP
2012	October	Pader	Aruu Falls	2.89806	32.64639	+	-	RJP
2013	September	Adjumani	Otika	3.45833	32.01500	+	+	RJP
2013	September	Adjumani	Eyiguru, nr Madulu South	3.47000	32.01167	+	+	RJP
2013	September	Adjumani	Adjumani-Atiak Bridge	3.37667	31.99333	+	-	RJP
2013	September	Adjumani	Adjumani-Atiak Bridge	3.34833	32.04000	+	+	RJP
2013	September	Adjumani	Seri Bridge	3.21000	32.00000	+	+	RJP
2013	September	Amuru	Near Pogo	3.11000	32.08000	+	-	RJP
2013	September	Moyo	Gwere Luzira	3.66333	31.80167	-	-	RJP
2013	September	Moyo	Moyo-Ferry Bridge	3.64000	31.79000	-	-	RJP
2013	September	Moyo	nr Oyo village	3.76333	31.82000	-	-	RJP
2013	September	Moyo	Gbari	3.78667	31.81000	-	-	RJP
2013	September	Moyo	NFA House	3.79333	31.81000	-	-	RJP
2013	September	Moyo	Waterfall	3.62833	31.89000	-	-	RJP
2013	September	Moyo	Pakaruhwe upstream	3.62000	31.91167	-	-	RJP
2013	September	Moyo	nr Ramogi north	3.64500	31.92167	-	-	RJP
2013	September	Moyo	Pakaruhwe downstream	3.59167	31.92500	-	-	RJP
2013	September	Moyo	nr Gwere West	3.61000	31.53500	-	-	RJP
2013	September	Moyo	nr Cefo village	3.64500	31.92167	-	-	RJP
2013	September	Moyo	North Eria village	3.64333	31.64833	-	-	RJP
2013	September	Moyo	nr Lauro Samba	3.75000	31.75167	-	-	RJP
2013	September	Moyo	Lea River confluence	3.80333	31.80333	+	-	RJP
2014	September	Adjumani	Ayugi (Tete) River	3.45807	32.01608	-	-	AJH
2014	September	Adjumani	Seri Bridge	3.33337	32.03108	-	-	AJH
2014	September	Amuru	Unyama River	3.26637	32.20777	-	-	AJH
2014	September	Gulu/Pader	Awere Bridge	2.68828	32.78653	-	-	AJH
2014	September	Kitgum	Lanyadyang	3.17122	32.80355	-	-	AJH
2014	September	Kitgum	Tumangu/Gozi	3.20443	32.75405	+	-	AJH
2014	September	Lamwo	Beyogoya village	3.29174	32.50573	-	-	AJH
2014	September	Lamwo	Beyogoya village	3.29138	32.50610	-	-	AJH
2014	September	Lamwo	Burukung River	3.43013	32.52392	-	-	AJH
2014	September	Lamwo	Lemur (Nyimur)	3.56363	32.38607	-	-	AJH
2014	September	Lamwo	Apyeta Bridge	3.29903	32.36643	-	-	AJH
2014	September	Lamwo	Pabo River	3.29898	32.36652	-	-	AJH
2014	September	Lamwo	Pager River	3.18577	32.69273	-	-	AJH
2014	September	Lamwo	Unknown	3.34058	32.62590	-	-	AJH
2014	September	Pader	Agago River	2.80307	32.78755	-	-	AJH
2014	September	Pader	Ajani River	3.03068	32.76719	-	-	AJH

Year	Month	District	Location	Latitude	Longitude	<i>S.d</i>	<i>S.b</i>	Source
2014	September	Pader	Aruu Falls	2.89803	32.64605	+	-	AJH
2014	October	Adjumani	Irei	3.37608	31.98735	-	-	AJH
2014	October	Adjumani	Seri Bridge	3.33337	32.03108	-	-	AJH
2014	October	Adjumani	Irei River	3.37608	31.98735	-	-	AJH
2014	October	Adjumani	Irei River	3.37608	31.98735	-	-	AJH
2014	October	Adjumani	Seri Bridge	3.33337	32.03108	-	-	AJH
2014	October	Adjumani	Irei	3.37693	31.98887	+	-	AJH
2014	October	Kitgum	Tumangu/Gozi	3.20443	32.75405	-	-	AJH
2014	October	Kitgum	Adwara	3.28056	32.85333	-	-	AJH
2014	October	Kitgum	Unknown	3.24272	32.76017	-	-	AJH
2014	October	Kitgum	Abam Village	3.17012	32.66050	-	-	AJH
2014	October	Kitgum	Orima	3.33267	32.99462	-	-	AJH
2014	October	Kitgum	Lanyadyang	3.17112	32.80355	-	-	AJH
2014	October	Kitgum	Hotel	3.23595	32.78675	-	-	AJH
2014	October	Kitgum	Orima	3.33355	32.99327	+	-	AJH
2014	October	Lamwo	Lagura River	3.30052	32.65262	-	-	AJH
2014	October	Lamwo	Odwere River	3.29450	32.87625	-	-	AJH
2014	October	Lamwo	Aruu Falls (Lamwo)	3.17012	32.66050	-	-	AJH
2014	October	Lamwo	Aruu Falls (Lamwo)	3.17077	32.66378	-	-	AJH
2014	October	Lamwo	Laroya River	3.46992	32.35975	-	-	AJH
2014	October	Lamwo	Pamoo (nr Pager)	3.24267	32.76018	-	-	AJH
2014	October	Lamwo	Lagura River	3.30052	32.65262	-	-	AJH
2015	June	Kitgum	Adwara	3.28108	32.85367	-	-	AJH/TT
2015	June	Kitgum	Tumangu/Gozi	3.20443	32.75405	-	-	AJH/TT
2015	June	Nwoya	Ayago Bridge	2.43197	32.00710	-	-	AJH/TT
2015	July	Adjumani	Adjumani-Atiak Bridge	3.37802	31.99342	-	-	AJH/TT
2015	July	Adjumani	Adjumani-Atiak Bridge	3.34822	32.04068	-	-	AJH/TT
2015	July	Amuru/Lamwo	Apyeta Bridge	3.29903	32.36643	+	+	AJH/TT
2015	July	Gulu/Pader	Awere Bridge	2.69011	32.78603	-	-	AJH/TT
2015	July	Gulu/Pader	Achwa Bridge	2.95848	32.58142	+	-	AJH/TT
2015	July	Kitgum	Wang Ayule	3.26003	33.26640	-	-	AJH/TT
2015	July	Kitgum	Jaipii	3.32877	33.34087	-	-	AJH/TT
2015	July	Lamwo	Beyogoya village	3.28337	32.48953	-	-	AJH/TT
2015	July	Lamwo	Te Lute (Achwa River)	3.22757	32.45945	+	+	AJH/TT
2015	July	Moyo	Pamulu	3.67930	31.82637	-	-	AJH/TT
2015	July	Moyo	Meria (Lea River)	3.69972	31.88245	-	-	AJH/TT
2015	July	Moyo	Amua River	3.66378	31.80093	-	-	AJH/TT
2015	July	Moyo	River nr Moyo	3.64035	31.78940	-	-	AJH/TT
2015	August	Nwoya	Ayago/Nile confluence	2.43197	32.00710	+	-	AJH/TT

Table S4. Sites of larval and pupal blackfly surveys at major rivers and tributaries, mainly in the Madi-Mid North focus made between 2012 and 2015. *S.d* = *Simulium damnosum*, *S.b* = *Simulium bovis*; Source = RJP (Rory Post), AJH (Adam Hendy), TT (Taylor Tushar).

Supplementary table (S5)

Collection Dates	Nearest Village	River	Latitude	Longitude	Altitude	<i>S. damnosum</i>	Other Larvae	<i>S. adersi</i>	<i>S. hargreavesi</i>	<i>S. hirsutum</i>	<i>S. mcmahoni</i>	<i>S. rotundum</i>	<i>S. vorax</i>
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						

Table S5. Sites of blackfly breeding, indicating presence/absence of *S. damnosum* s.l., and other species identified by the morphology of the pupal respiratory organ.

Curriculum vitae

ADAM HENDY

Date of birth: 7th January 1982
Place of birth: Leicester, United Kingdom
Nationality: British

Education

Nov 2013 – Feb 2018 PhD Biomedical Sciences
Thesis: “Blackfly ecology and *Onchocerca volvulus* transmission in three formerly hyperendemic foci in Uganda, Tanzania and Cameroon.”
Unit of Medical Entomology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium.

Sep 2011 – Sep 2012 MSc Biology and Control of Disease Vectors
Thesis: “Effect of permethrin-impregnated school uniforms on *Aedes (Stegomyia) aegypti* mosquitoes in Thailand: an investigation of susceptibility, efficacy and residual effects.”
London School of Hygiene and Tropical Medicine, London, United Kingdom.

Sep 2001 – Sep 2004 BSc Biological Sciences with Specialisation in Parasitology
Thesis: “Comparing the infectivity and behaviour of two strains of *Schistosoma mansoni*.”
King’s College London, London, United Kingdom.

Other Research Experience

Jan – Oct 2013	Curatorial and Research Assistant	Life Sciences Department; Angela Marmont Centre for UK Biodiversity, Natural History Museum, London, UK.
Feb 2010 – Sep 2011	Volunteer; Project Support Assistant; Research Assistant	Life Sciences Department, Natural History Museum, London, UK

Publications

Hendy, A., Krüger, A., Pfarr, K., De Witte, J., Kibweja, A., Mwingira, U., Dujardin, J.C., Post, R., Colebunders, R., O'Neill, S. & Kalinga, A., 2017. The blackfly vectors and transmission of *Onchocerca volvulus* in Mahenge, south eastern Tanzania. Submitted (*Acta Tropica*) July 2017.

Hendy, A., Sluydts, V., Tushar, T., De Witte, J., Odonga, P., Loum, D., Nyaraga, M., Lakwo, T., Dujardin, J.C., Post R.J., Kalinga, A. & Echodu, R., 2017. Esperanza Window Traps for the collection of anthropophilic blackflies (Diptera: Simuliidae) in Uganda and Tanzania. *PLOS Neglected Tropical Diseases*, 11(6): e0005688. <https://doi.org/10.1371/journal.pntd.0005688>

Colebunders, R., **Hendy, A.** & Van Oijen, M., 2014. Nodding syndrome in onchocerciasis endemic areas. *Trends in Parasitology*, doi: 10.1016/j.pt.2016.05.013.

Orsborne, J., DeRaedt Banks, S., **Hendy, A.**, Gezan, S.A., Kaur, H., Wilder-Smith, A., Lindsay, S.W. & Logan, J.G., 2016. Personal protection of permethrin-treated clothing against *Aedes aegypti*, the vector of dengue and Zika virus, in the laboratory. *PLOS ONE*, 11(5): e0152805. <https://doi.org/10.1371/journal.pone.0152805>

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Deblauwe, I., Demeulemeester, J., De Witte, J., **Hendy, A.**, Sohier, C. & Madder, M., 2015. Increased detection of *Aedes albopictus* in Belgium: no overwintering yet, but an intervention strategy is still lacking. *Parasitology Research*, 114, pp.3469–3477.

Colebunders, R., Post, R., O'Neill, S., Haesaert, G., Opar, B., Lakwo, T., Laudisoit, A. & **Hendy, A.**, 2015. Nodding syndrome since 2012: recent progress, challenges and recommendations for future research. *Tropical Medicine & International Health*, 20(2), pp.194-200.

Colebunders, R., **Hendy, A.**, Nanyunja, M., Wamala, J.F. & van Oijen, M., 2014. Nodding syndrome—a new hypothesis and new direction for research. *International Journal of Infectious Diseases*, 27, pp.74-77. <http://dx.doi.org/10.1016/j.ijid.2014.08.001>

Demeulemeester, J., Deblauwe, I., De Witte, J., Jansen, F., **Hendy, A.** & Madder, M., 2014. First interception of *Aedes (Stegomyia) albopictus* in Lucky bamboo shipments in Belgium. *Journal of the European Mosquito Control Association*, 32, pp.14-16.

Funding and Awards

FWO Travel Grant, 2015. *Applicant*. Awarded funding for a one-month research stay to learn techniques in blackfly cytotoxicology at Clemson University, South Carolina, USA.

Institute of Tropical Medicine, Antwerp, and the Flemish Interuniversity Council South Initiative (VLIR-UOS) "Structural Research Funding" (SOFI) grant, 2014. *Co-applicant*. Awarded

€665,038 for the project entitled “An interdisciplinary study contributing to the identification of the cause of nodding syndrome in four countries.”

John Spedan Lewis Foundation, 2013. *Applicant*. Awarded £5,000 to study the taxonomy and bionomics of *Albuginosus* spp. tree-hole breeding mosquitoes at the Natural History Museum, London, UK.

Mansfield Aders Scholarship, 2011. *Applicant*. Awarded full tuition fees to study MSc Biology and Control of Disease Vectors at the London School of Hygiene and Tropical Medicine.

Conferences and Presentations

10th European Congress on Tropical Medicine and International Health (ECTMIH), 2017. Antwerp, Belgium. “The blackfly vectors and transmission of *Onchocerca volvulus* in Mahenge, south eastern Tanzania.” *Oral presentation*.

10th European Congress on Tropical Medicine and International Health (ECTMIH), 2017. Antwerp, Belgium. “Esperanza Window Traps for the collection of anthropophilic blackflies (Diptera: Simuliidae) in Uganda and Tanzania.” *Poster presentation*.

1st International Workshop on Onchocerciasis Associated Epilepsy (OAE), 2017. Antwerp, Belgium. “Blackfly ecology and *O. volvulus* transmission in Uganda, Tanzania and Cameroon.” *Oral presentation*.

1st International Workshop on Onchocerciasis Associated Epilepsy (OAE), 2017. Antwerp, Belgium. “The blackfly vectors and transmission of *Onchocerca volvulus* in Mahenge, south eastern Tanzania.” *Poster presentation*.

2nd International Conference on Nodding Syndrome, 2015. Gulu, Uganda. “Blackflies (Diptera: Simuliidae) and *Onchocerca* infection in Nodding Syndrome-affected districts of northern Uganda.” *Oral Presentation*.

13th Annual North American Black Fly Association (NABFA) Meeting, 2015, Athens, Georgia, USA. “Investigating a relationship between blackflies (Diptera: Simuliidae), onchocerciasis and Nodding Syndrome: An entomological perspective.” *Oral Presentation*.

Student Mentorship

Taylor Tushar

May – Sep 2015

MSc

Biology and Control of Disease Vectors

Thesis: “Analysis of *Simulium bovis* distribution, anthropophily, and infection rates in Northern Uganda, a focus of Nodding Syndrome.” London School of Hygiene and Tropical Medicine, London, United Kingdom.

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