The biology of *Onchocerca ochengi*, a filarial nematode from African cattle, and the implications on the epidemiology of the causative agent of river blindness, *Onchocerca volvulus*

Dissertation

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My sincere gratitude goes to all of you. Thank you so much!

Abbreviations

ABR	annual biting rate
ALT	abundant larval transcript
APOC	African Programme for Onchocerciasis Control
ATP	annual transmission potential
bp	base pair
CDTI	community-directed treatment with ivermectin
ELISA	enzyme-linked immunosorbent assay
ESP	excretory-secretory product
GC/MS	gas chromatography/mass spectrometry
IgG	immune globuline subclass G
IgM	immune globuline subclass M
IRAD	Institut de Recherche Agricole pour le Développement
L3	third-stage larva
mf	microfilaria
mff	microfilariae
mtDNA	mitochondrial DNA
MTP	monthly transmission potential
NLT	novel larval transcript
OCP	Onchocerciasis Control Programme
*Ov*103	*Onchocerca volvulus* microfilariae surface-associated antigen
Ov7	*Onchocerca volvulus* onchocystatin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pe	*post exposure*
REMO	rapid epidemiological mapping of onchocerciasis
rRNA	ribosomal RNA
s.l.	*sensu lato*
SNP	single nucleotide polymorphism
s. str.	*sensu stricto*

Summary in English

Onchocerca ochengi is a common filarial parasite in African zebu cattle, which is non-pathogenic, in contrast to its closest known relative *Onchocerca volvulus*, which causes onchocerciasis (river blindness) in humans. In Cameroon it serves as an animal model to study the vector-borne disease, transmitted by the same black fly species of the *Simulium damnosum* complex. The present study focuses on the biology of *O. ochengi*, including its population dynamics in a longitudinal follow-up study of a cattle herd exposed to natural transmission since their birth. Another research focus lies on the decline in biting rates and transmission potentials of the local vector in formerly meso- and hyperendemic onchocerciasis foci in North Cameroon, in order to determine the actual risk for the population and prospects for elimination of river blindness.

In the dry savannah focus at the Vina du Nord river basin the annual transmission potential dropped to 3.5 % of pre-control levels after 25 years of annual treatment of the human population with the microfilaricide ivermectin, whereas at the Vina du Sud focus on a highland plateau no transmission could be confirmed by molecular detection after 15 years of consecutive treatment intervention. The high cattle stock density on the Adamawa highlands has contributed to the regional interruption of transmission, and demonstrates that annual mass drug administration alone may not be sufficient to prevent onchocerciasis in hyperendemic foci.

Moreover, a high genetic diversity of *O. ochengi* was shown in cattle and vector, including the discovery of a new mitochondrial haplotype with phylogenetic similarities to *O. volvulus*, and a putative hybrid of *O. ochengi* with *O. dukei*. Other results in studying the bovine onchocercosis model were insights into the reproductive biology of *O. ochengi*, time-scaled dynamics of immunological reactivity to recombinant *O. volvulus* antigens in zebu cattle naturally exposed to *O. ochengi*, the production and biochemical analysis of excretory-secretory products of *O. ochengi* adults, including their putative function, and the optimization of separating viable microfilariae of *O. ochengi* and *O. gutturosa* from adult uteri and cattle skin for subsequent culturing.

Zusammenfassung auf Deutsch

Onchocerca ochengi ist ein häufiger Parasit in afrikanischen Zebu Rindern, und ist im Gegensatz zu seinem nächstbekannten Verwandten *Onchocerca volvulus*, der die Tropenkrankheit Flussblindheit im Menschen auslöst, weitgehend apathogen. In Kamerun ist es als Tiermodell etabliert zur Erforschung dieser vektorübertragenen Krankheit, die von den gleichen Kriebelmücken des *Simulium damnosum* Artenkomplexes übertragen werden. Diese Arbeit befasst sich mit der Biologie von *O. ochengi*, einschließlich der Populationsdynamik der Filarie in einer über 3 Jahre untersuchten Rinderherde, deren Tiere schon kurz nach der Geburt der natürlichen Übertragung ausgesetzt sind.

Eine hohe genetische Diversität von *O. ochengi* ließ sich im Rind und Vektor nachweisen, einschließlich der Entdeckung eines neuen mitochondriellen Haplotyps mit phylogenetischer Ähnlichkeit zu *O. volvulus*, und eines putativen Hybrids zwischen *O. ochengi* und *O. dukei*. Weitere Resultate in der Erforschung des Rindermodells der Onchozerkose beinhalten Einblicke in die Reproduktionsbiologie von *O. ochengi*, zeitliche Dynamiken von immunologischen Reaktionen auf rekombinant produzierte *O. volvulus* Antigene in *O. ochengi* exponierten Zebu Rindern, die Produktion und biochemische Analyse von exkretorisch-sekretorischen Proteinen von erwachsenen *O. ochengi*, mitsamt ihrer putativen Funktion, und die Optimierung der Auftrennung lebender Mikrofilarien von *O. ochengi* und *O. gutturosa* von den Uteri adulter Weibchen und Rinderhaut für die darauffolgende Kultivierung.

Ein weiterer Fokus liegt auf der Abnahme des Übertragungspotentials des lokalen Vektors in früher meso- und hyperendemischen Onchozerkosegebieten in Nordkamerun. Es gilt, das noch bestehende Risiko für die Bevölkerung und die Aussicht auf die Ausrottung der Flussblindheit zu bestimmen. In dem untersuchten Trockensavannen Fokus im Vina du Nord Flusstal hat das jährliche Übertragungs-potential nach 25 Jahren jährlicher Behandlung der menschlichen Bevölkerung mit dem Mikrofilarizid Ivermektin auf 3,5 % des Ausgangswerts vor der Behandlung abgenommen. Im Gegensatz dazu konnte im Vina du Sud Tal auf dem Hochlandplateau nach 15 Jahren Behandlung keine Übertragung mit molekularen Methoden bestätigt werden. Die hohe Rinderbestandsdichte auf dem Adamawa Hochland hat zur regionalen Unterbrechung der Transmission beigetragen, und es zeigt sich, dass jährliche Massenbehandlungen mit verfügbaren Medikamenten

alleine nicht ausreichen dürften, um die Onchozerkose in hyperendemischen Gebieten zu eliminieren.

List of publications in the cumulative thesis

a. published

1) Hildebrandt, J.C., **A. Eisenbarth**, A. Renz, and A. Streit. 2012. Single worm genotyping demonstrates that *Onchocerca ochengi* females simultaneously produce progeny sired by different males. *Parasitology Research.* 111:2217-21.
2) **Eisenbarth, A.**, D. Ekale, J. Hildebrandt, M.D. Achukwi, A. Streit, and A. Renz. 2013. Molecular evidence of 'Siisa form', a new genotype related to *Onchocerca ochengi* in cattle from North Cameroon. *Acta Tropica*. 127:261-65.
3) Hildebrandt, J.C., **A. Eisenbarth**, A. Renz, and A. Streit. 2014. Reproductive biology of *Onchocerca ochengi*, a nodule forming filarial nematode in zebu cattle. *Veterinary Parasitology*. 205:318-29.
4) Manchang, T.K., I. Ajonina-Ekoti, D. Ndjonka, **A. Eisenbarth**, M.D. Achukwi, A. Renz, N.W. Brattig, E. Liebau, and M. Breloer. 2015. Immune recognition of *Onchocerca volvulus* proteins in the human host and animal models of onchocerciasis. *Journal of Helminthology*. 89:375-389.
5) Eberle, R., N.W. Brattig, M. Trusch, H. Schluter, M.D. Achukwi, **A. Eisenbarth**, A. Renz, E. Liebau, M. Perbandt, and C. Betzel. 2015. Isolation, identification and functional profile of excretory-secretory peptides from *Onchocerca ochengi*. *Acta Tropica*. 142:156-66.

b. submitted

6) **Eisenbarth, A.**, M.D. Achukwi, and A. Renz. Ongoing transmission of *Onchocerca volvulus* after 25 years of annual ivermectin mass treatments in the Vina du Nord river valley in North Cameroon. *PLOS Neglected Tropical Diseases*.

Personal contribution

All my contributions were done under the framework of DFG-funded German-African research projects in infectiology. The first project aimed at the analysis of the secretome of *O. ochengi* (Liebau, LI 793/5-0 & RE 1536/1-1) and the succeeding project (COBE, Renz, RE 1536/2-1) deals with the question, whether onchocerciasis can be eradicated in Africa.

Two publications are co-authored with partner PD Dr. Adrian Streit and Dr. Julia Hildebrandt from the Max Planck institute of Developmental Biology, Tübingen, on the reproductive biology of *O. ochengi* in cattle (1,3). My task was to select the suitable cattle skins from the abattoir, to collect, map and isolate *Onchocerca* nodules and to organize the transport to Germany. In Cameroon, I identified the 'Siisa' strain of *O. ochengi*, first in the *Simulium* vector, then in cattle by using mitochondrial markers (2,3,6). With our Cameroonian partners Dr. Kingsley Manchang and Prof. Daniel Achukwi from the IRAD Wakwa Centre I participated in a longitudinal survey of an experimental cattle herd over the whole study period (2010 till present), including parasitological examination and data analysis, *Simulium* vector catches on cattle and their analysis, and a major revision on the co-authored publication on the immunologic responses after parasite acquisition (4) together with Prof. Minka Breloer. With Dr. Daniel Achukwi from the University of Ngaoundéré I collaborated with the supervision of several former Master, now PhD students trained in the Tübingen Programme Onchocercoses laboratory in Ngaoundéré (B. Abanda, N. Ngwasiri, A. Paguem), logistical support, and major revision on two jointly submitted manuscripts (4,7). Moreover, I assisted partner PD Dr. Norbert Brattig and Silke van Hoorn from the Bernhard Nocht institute, Hamburg, and Dr. Ralf Eberle and Dr. Markus Perbandt from the University of Münster on the ESP analysis of *O. ochengi*, such as the organization of the ESP production in Cameroon, the transport to Germany and drafting of the publication (5).

General introduction

Onchocerciasis, or river blindness, is a neglected tropical disease with still over 86 million people living in zones with a high risk of infection, predominantly in Africa (Noma *et al.*, 2014), and listed as second leading infectious cause of blindness (WHO, 2013). Other symptoms include skin and eye lesion, skin rashes, depigmentation and severe itching. The disease is caused by a parasitic nematode species of the Filarioidea suborder: *Onchocerca volvulus*. It is a vector-borne illness, transmitted by black flies of the *Simulium damnosum* complex, which need to deposit their eggs and developing larvae in fast flowing water (Fig. 1).

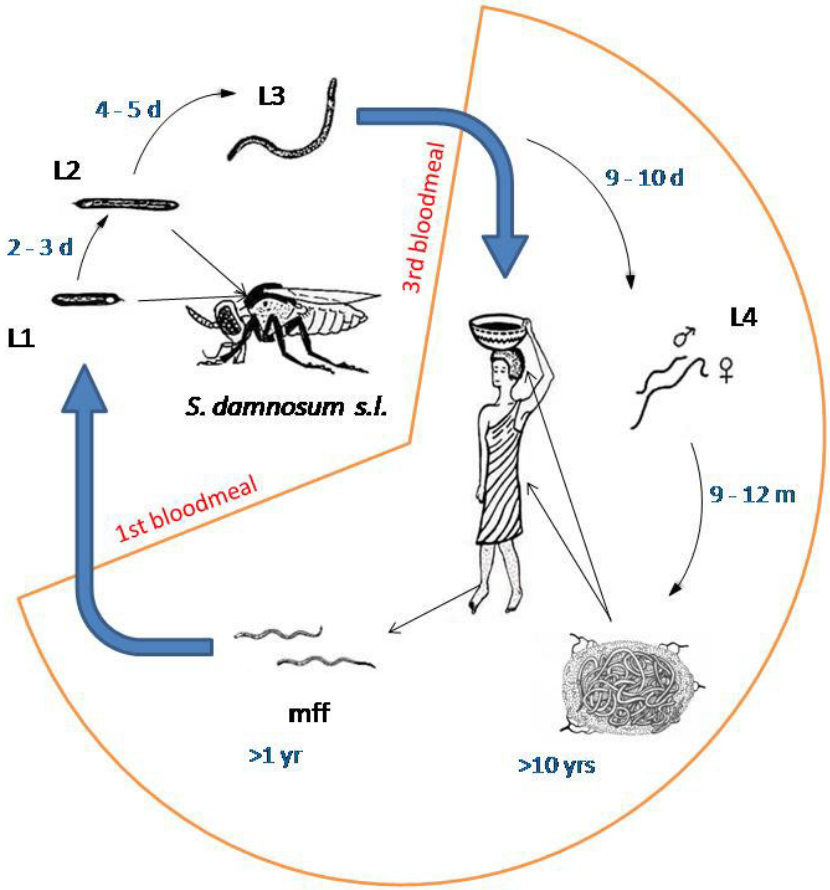

Fig. 1. Life cycle of *Onchocerca volvulus*. Female and male worms (on average 1:1) lie surrounded by host connective tissue (onchocercoma or nodule) under the skin of an infected human, normally around the hips or temples. One productive female gives birth to approx. 1,500 offspring per day. These microfilariae (mff) migrate to the skin predominantly in the leg region (lifetime > 1 year), where they get ingested by the vector (*S. damnosum s.l.*) in search of a bloodmeal. In the black fly, they penetrate the peritrophic membrane and encyst themselves in the flight muscle tissue to the so-called sausage stage (L1). After 2 to 3 days they molt to the encysted L2 stage, and 4 to 5 days later to the infective larva (L3). During another bloodmeal they enter a new host through the mouthpart of a sucking vector, and develop via another molting stage (L4) to male and female adults after 9 to 12 months. Their life expectancy exceeds 10 years. Graphs taken with copyright permission of A. Renz.

From 1974 to 2002 the United Nations have launched a disease control program called Onchocerciasis Control Programme (OCP) in 11 endemic West African countries. Initially control measures were large-scale aerial campaigns with insecticides directed against the larvae of *Simulium damnosum s.l.* in the fast-flowing rivers and streams. In 1988, the method began to phase out and was subsequently replaced by mass distribution of a drug targeted against the microfilariae in the skin: ivermectin. A pharmaceutical company has donated the compound under the name Mectizan free of charge until onchocerciasis will cease to be a threat to public health. Since then, and after the onset of mass distribution in the remaining 13 affected countries in Africa by the African Programme for Onchocerciasis Control (APOC) in 2003, prevalences of infection and clinical symptoms have reduced significantly (www.who.int/apoc/about/en/). However, given that the drug used does not kill the adult stage, treatments have to be repeated at least as long as the female worm lives, *i.e.* 10 years and longer. Nonetheless, if regional and therapeutic coverage is high enough, the transmission and hence the incidence of onchocerciasis can be suppressed, eventually eliminating the disease, as it was the case in Senegal and Mali (Diawara *et al.*, 2009), endemic foci in Uganda (Katabarwa *et al.*, 2014; Lakwo *et al.*, 2013) and Sudan (Higazi *et al.*, 2013). In other cases, however, a low but persisting incidence of *O. volvulus* remains in human and/or vector populations, *i.e.* Nigeria (Akinboye *et al.*, 2010; Evans *et al.*, 2014; Eyo *et al.*, 2013), Democratic Republic of Congo (Makenga Bof *et al.*, 2015), Cameroon (Katabarwa *et al.*, 2013; Katabarwa *et al.*, 2011), Ethiopia (Legesse *et al.*, 2010), Central African Republic (Yaya *et al.*, 2014), Ghana (Osei-Atweneboana *et al.*, 2007), and other African states (see REMO data: www.who.int/apoc/countries/en/).

Since it is not possible to conduct scientific research on humans for ethical considerations, an appropriate animal model is needed. Even though great apes, like chimpanzee or gorilla, are suitable surrogate hosts, they can no longer be used for infection experiments due to ecological and ethical reasons. Today the best model to investigate the biology of *O. volvulus* is the zebu cattle and its filarial parasite *O. ochengi* Bwangamoi, 1969 (Trees *et al.*, 1998). Like its human counterpart, the adult worms lie encapsulated in nodules under the skin and release thousands of microfilariae daily migrating into the epidermis (Bwangamoi, 1969), which conversely do not cause pathology in cattle. They also develop to infective larvae (L3) in the same vector species of the *S. damnosum* complex (Denke and Bain, 1978), which

complicates entomologic predictions of infection rates with the respective *Onchocerca* species (Renz *et al.*, 1994). Thus, for more than 20 years the bovine model has been used in Cameroon to study different aspects of onchocerciasis research, such as macrofilaricide drug screening (Bronsvoort *et al.*, 2008; Langworthy *et al.*, 2000; Renz *et al.*, 1995), epizootiology (Achukwi *et al.*, 2000; Wahl *et al.*, 1998), immunology (Achukwi *et al.*, 2007; Graham *et al.*, 1999; Wildenburg *et al.*, 1997), biochemistry (Cho-Ngwa *et al.*, 2007), population genetics and phylogeny (Krueger *et al.*, 2007; Morales-Hojas *et al.*, 2006).

There are at least four other filarial species in the African zebu, which are frequently found sympatric. *O. dukei* Bussieras, 1974 only occurs in Africa, forms nodules like *O. ochengi*, but mainly in dermis of the breast region, and its microfilariae are found in the epidermis. Adult *O. gutturosa* Neumann, 1910 worms lie in the connective tissue of the nuchal ligament or other ligaments, and occur worldwide. *O. armillata* Ralliet & Henry, 1909 inhabits the aortic endothelium, and its microfilariae are only occasionally found in the skin (Wahl *et al.*, 1994a). S*etaria* spec., whose microfilariae reside in the blood stream, are also found regularly in the abdominal cavity. Furthermore, other filarial worms, some of them nodule-forming, have been found in wild-roaming ungulates, but most of them have not yet been taxonomically described. The vector of *O. ramachandrini*, a parasite of warthogs, and *O. dukei* of zebu cattle have been discovered, which are *S. damnosum s.l.* (Wahl, 1996) and *S. bovis* (Wahl and Renz, 1991), respectively.

Zooprophylaxis describes the protective traits of animals against the transmission of anthroponotic diseases to man (Garrett-Jones, 1964). For instance, zebu cattle divert blood seeking flies to bite them instead of humans, therefore reducing the vector population for humans and thus the risk of onchocerciasis transmission (Renz *et al.*, 1994). Furthermore, cross-reactive immune responses caused by non-human filarial parasites transmitted to man also diminish the risk for onchocerciasis in humans in the vicinity of cattle and perhaps also game animals (Wahl *et al.*, 1998). In an experiment, cross-protecting vaccination has been demonstrated by inoculating live *O. volvulus* infective larvae to naive Zebu cattle. These animals were better protected from *O. ochengi* infection as compared to a mock-treated control (Achukwi *et al.*, 2007). But for all that, the epidemiologic

dimension and impact of zooprophylaxis for onchocerciasis in endemic foci has not been fully evaluated.

Apart from mass-drug administrations and zooprophylaxis, other factors impact on the epidemiology of river blindness. Jacobi and colleagues (2010) showed that the individual exposure to fly bites differs according to behavior and clothing. In his Bachelor thesis, Bürgel investigated the impact of the socio-economic development to transmission in three different river basins endemic for onchocerciasis in Cameroon, and compared it with historical records (Buergel, 2014). He also identified considerable individual and regional differences of exposure risk to *Simulium* bites based on gender, age, cultural background and occupation. Moreover, a gradual emergence of resistance against ivermectin (Osei-Atweneboana *et al.*, 2011; 2007) could have dramatic consequences on the effectiveness of control and lead to a recrudescence of onchocerciasis in former endemic foci.

The population dynamics of *O. ochengi* under natural transmission have been observed in a longitudinal study of cattle, looking at the acquisition of palpable nodules, densities of microfilariae in the skin (Achukwi *et al.*, 2004), in addition to *S. damnosum s.l.* biting and infection rates with *Onchocerca* spp. (Achukwi *et al.*, 2000). The experiment showed that the susceptibility to parasite acquisition varied, ranging from putative resistant, early-susceptible and late-susceptible animals. However, the observation period was terminated after three years well before the estimated lifetime of filarial worms (Achukwi *et al.*, 2004), so no data of the dynamics after this time point have been available.

In this and other studies, it was evident that the parasite population is regulated and controlled by interaction with the host, the vector, and by communication with conspecifics and other co-habitant filarial worms. This cross-talk at different interfaces is governed by excretory-secretory products (ESPs) from the parasite. Some ESPs have been found to exhibit immune-stimulatory effects in the host, e.g. *O. volvulus* superoxide dismutase (Ajonina-Ekoti *et al.*, 2012) or macrophage migration inhibitory factors (Ajonina-Ekoti *et al.*, 2013). In the vector, most ingested mff die in the fly's midgut, whereas a few escape by penetrating the peritrophic membrane, evading the internal defense system by intracellular sequestering in syncytial cells of the flight muscle tissue. After the L3 enters a new vertebrate host during the vector's blood meal, its development to L4 and adult stage,

as well as the migration is controlled by a range of ESPs from itself and the already established population of filarial nematodes. This leads to population-limiting regulation presumably by induced apoptosis, and a relatively even distribution of *Onchocerca ochengi* nodules across the inguinal region (A. Renz, personal communication and own observation). Also for the finding and behavior of mating partners, and the control of mf birth and longevity, the ESPs play a decisive role in the parasite's reproductive cycles. ESP-driven crosstalk with the host include the angiogenesis and nodule formation, and the stimulation and/or suppression of the immune system, both to ensure the survival of the worm in its host and to ward off intruding L3 to prevent overcrowding.

Objectives and expected output

The objective of my dissertation is to get a better insight of the biology of parasitic nematodes of the *Onchocerca* genus, in particular of *O. ochengi* and its human counterpart *O. volvulus*. Of high interest is the development and transmission of these species in the *Simulium damnosum* vector and their cross-influences. Therefore, the fly biting and infection rate shall be determined with traditional and molecular biological tools and compared with results dating back before ivermectin control was set in place. Especially the impact of zooprophylaxis shall be elucidated and the question addressed, whether elimination of *O. volvulus* after prolonged ivermectin treatment in hyperendemic populations is attainable.

In the ongoing longitudinal survey of a cattle herd under natural exposure to *O. ochengi* the dynamics of nodule formation and microfilariae in the skin shall be depicted over time. Moreover, the history of the immune responses in different cohorts of the herd shall be quantified, as well as the vector biting rates and transmission potentials of *O. ochengi* during the study period. Altogether, the dataset shall give a detailed picture how and to which extend the parasite develops in the animal model.

Furthermore, the reproduction pattern of *O. ochengi* in the host shall be identified, including mating strategies and reproductive potential of inseminated females to the pool of circulating microfilariae in the skin. Also, the mass production of excretory-secretory products from adult worms and microfilariae shall permit biochemical analysis of these, including their biological activity.

By employing molecular techniques for the identification of filarial nematodes, new strains and possibly species shall be discovered, and their phylogenetic relationship disclosed. This shall enlarge our knowledge of the evolution of *Onchocerca* spp. parasites in Central Africa.

Results and discussion

1. Longitudinal survey of cattle herd naturally infected with *Onchocerca ochengi*

Over an observation period of up to 55 months (April 2010 till November 2014) a cattle herd of 27 zebu Gudali cattle (eighteen 4.5 years old, nine 3.5 years old) was exposed to natural transmission of *O. ochengi* and other bovine filarial parasites (*i.e. O. gutturosa*, *O. armillata*, *Setaria labiatopapillosa*, *Cercopithifilaria* spec.). Every two months these animals were examined and their parasitemia level determined. All mentioned parasites have been detected, in addition to others, *e.g. Trypanosoma* spp., *Fasciola* spec., gastrointestinal nematodes, *Eimeria* spp., *Dermatophilus congolensis* infection and the foot-and-mouth disease virus. In the present thesis I concentrated my analysis on filarial worms. Most of the *Simulium* flies took their bloodmeal in the morning and/or late afternoon from the inguinal region and venter. This is also where most nodules were found (Fig. 2). Unlike *O. volvulus*, where nodules occur mostly in clumped aggregates, the nodules of *O. ochengi* remain individual entities and disperse more evenly. Table 1 summarizes the preferred location of nodules in infected animals. Depending on how many nodules develop after the same time and intensity of exposure, the animals were grouped according to susceptibility of parasite acquisition. In the low-susceptibility group ('putative-immune'), most nodules occur on the hind legs/buttocks, the lateral flanks and on the

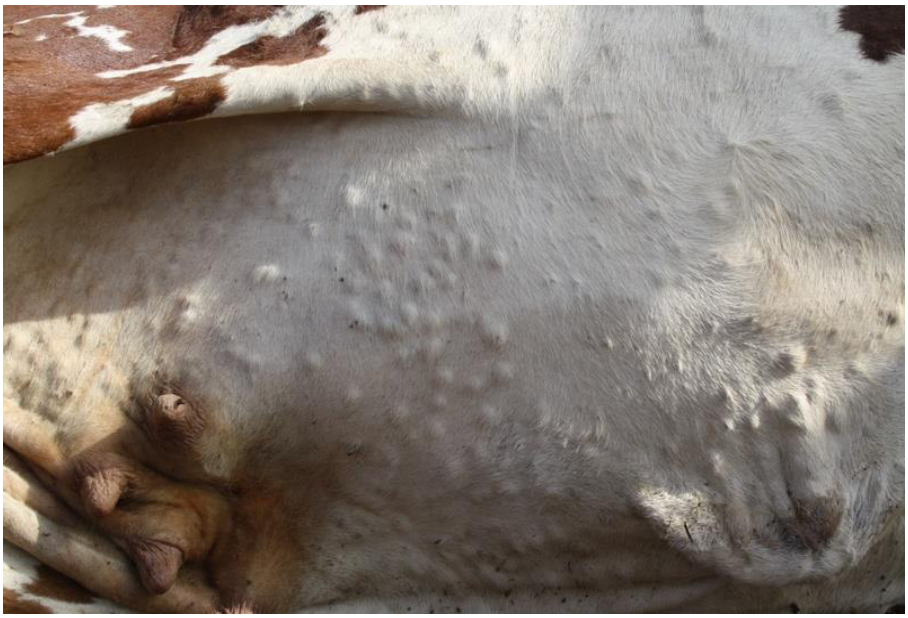

Fig. 2. Ventral skin of a highly susceptible zebu cow with many *Onchocerca ochengi* nodules in Cameroon. These animals, though, do not suffer from any clinical signs due to infection. Copyright permission by Programme Onchocercoses, A. Eisenbarth.

Table 1. Relative distribution of onchocercomata of *O. ochengi* in zebu cattle exposed to natural transmission. The data is based on two age cohorts of 53 months and 41 months pe, respectively, and is stratified according to susceptibility.

O. ochengi nodule load	n	buttocks, hind legs	udder, scrotum	belly	umbilicus	flanks	armpit, front legs	breast	neck	face, ears, eyes
low susceptible	9	++++	++	++	+	+++	+	(+)	+	+++
medium susceptible	9	++++	++	+++	(+)	+++	+	(+)	+	++
high susceptible	9	+++	+++	++++	++	+++	+	(+)	+	++
Total	27	++++	++	+++	+	+++	+	(+)	+	++

nodule frequency: (+) very low; + low; ++ medium; +++ high; ++++ very high

head predominantly at the base of the ears and surrounding the eyes. In the medium and high susceptible groups, more nodules also distribute around the belly, udder or scrotum, and partly the umbilicus. Occasionally, they are found in the front armpits, front legs and neck, whereas they are rare to very rare in the chest region and the back. The vast majority of microfilariae dwell in the whole inguinal and venter region - also of *O. gutturosa*, whose adult stages reside on the neck ligament.

At natural exposure in a zone of high biting intensity (mean effective ABR 2010 - 14: 54,180 bites/animal/year, approx. 148 bites/animal/day), only a small fraction of the incorporated L3 mature to adults and form nodules. Figure 3 illustrates that with a transmission burden of approx. 23,600 L3 per animal after 4.5 years, only every 60th (1.7 %, n = 18) developed to adult stage - if considered only L3 older than the patency period of 12 months (17,400), and that one nodule consists on average of 1 female, 1 male and 0.25 males migrating in search of a mating partner -, ranging from every 31st (3.2 %) in the early susceptible group, every 138th (0.7 %) in the late susceptible group, and every 858th (0.1%) in the low susceptible/resistant group. In contrast, inoculation experiments of a small quantity of *O. ochengi* L3 in unexposed zebu cattle (n = 9) exhibited approx. 6.5 -times higher mean development rates of 11 % (Achukwi *et al.*, 2007). This would render the L3 to adult development rate strongly density-dependent by an unknown control mechanism.

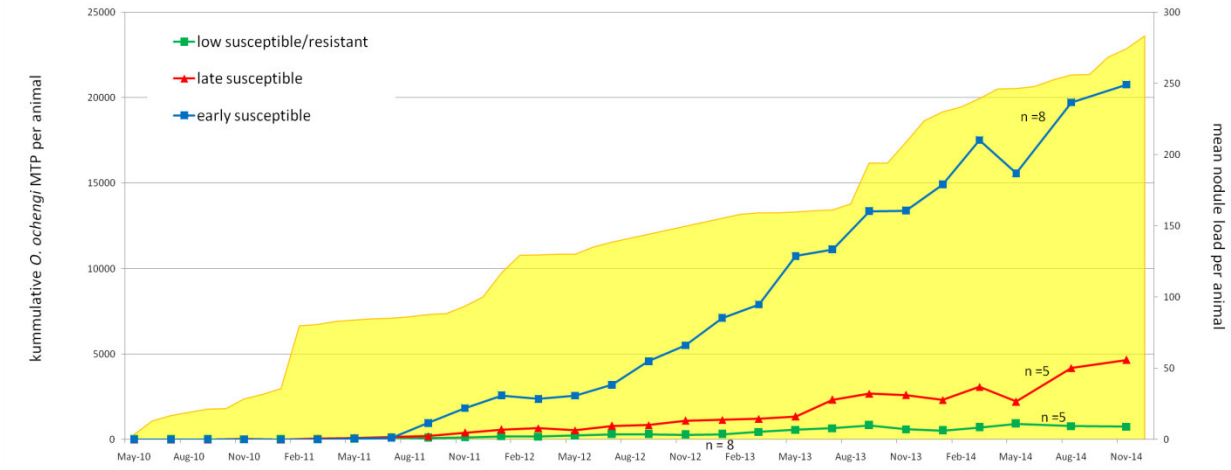

Fig. 3. *Onchocerca ochengi* nodule acquisition of zebu cattle (n = 27) after 4.5 years exposure, in comparison to the cumulative transmission potential (yellow line) of infective larvae from cattle biting *S. damnosum s.l.* The animals are grouped based on their level of susceptibility.

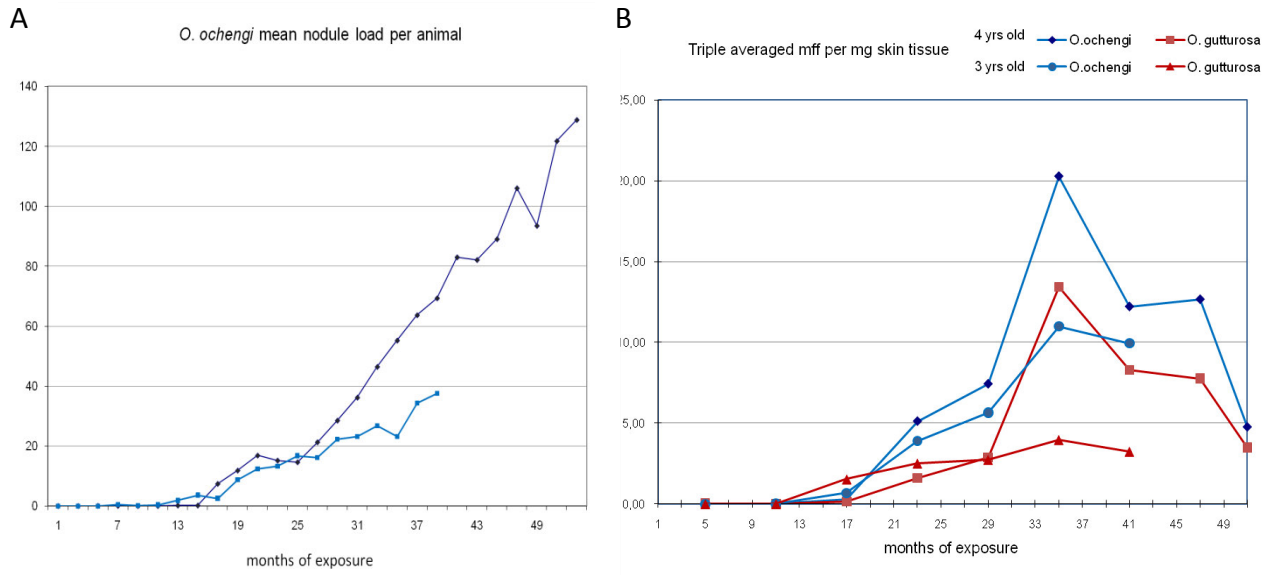

Fig. 4. Mean *Onchocerca ochengi* nodule loads **A** and 6 month averaged microfilarial densities **B** of *O. ochengi* (blue) and *O. gutturosa* (red) in zebu cattle after 43 (n = 9) and 52 months (n = 18) of natural exposure.

The first nodules are palpable after 11 to 13 months and continuously increase in number (Fig. 4A). The observed drop in nodule load 49 and 35 months pe, respectively, was with a very high chance a result of measuring errors. Patency of microfilariae in the cattle skin began at 15 months pe for both *O. ochengi* and *O. gutturosa*, reached its maximum between 31 and 33 months pe, and steadily decreased down to some 25 % of its highest value (Fig. 4B). Unlike in *O. volvulus*, which mff densities in the skin continue to rise under persistent exposure, the microfilarial *O. ochengi* population becomes at a certain time point largely independent of the numbers of adult worms present, indicating some form of

regulation. This may also explain why filarial worms are not detrimental to cattle, but to man. For instance, the fertility of females reached its peak after 32 months and constantly decreases thereafter due to biologic age or a control mechanism of the survival rate of microfilariae in the skin. Newly acquired younger females either cannot balance this loss of offspring productivity or are themselves suppressed by the established worm population. The reproductive biology of *O. ochengi* will be discussed in a later chapter.

2. Immunological and biochemical characteristics of *Onchocerca*-derived peptides

O. ochengi is the closest known relative to *O. volvulus*, but it does not inflict pathology in its natural host. Inflammatory responses against released molecules of dead and disintegrating mff are responsible for the symptoms in human onchocerciasis (Brattig, 2004). The immunogenic cross-reactivities between antigens of the two species, however, are extensive (Graham *et al.*, 2000). In the bovine model we compared the immunogenic reactivity by ELISA of four recombinantly produced *O. volvulus* proteins with sera from cattle (n = 10) at different time points between zero and 26 months pe (Manchang *et al.*, 2014). These ESPs, *Ov*ALT-2, *Ov*7, *Ov*103 and *Ov*NLT-1, are derived from different development stages, and have been previously used for immunologic studies. Figure 5 and 6 illustrate the kinetics of antibody titers to these ESPs for subclass M and G1, respectively. The lysate of homogenized *O. ochengi* worms and European cattle were the positive and negative controls, respectively. During the course of infection, antibody titers against all ESP tested reacted, as well as in the mother cows. Depending on the antibody class, time points of detection differed: for IgM 4 months pe, except for *Ov*NLT-1 after the first appearance of nodules and microfilariae in the skin (Fig. 5D-F), and for IgG1 before exposure to *Onchocerca* transmission for *O. ochengi* lysate and *Ov*NLT-1 (Fig. 6B). That indicates a maternal diaplacental and/or lactogenic transfer of IgG1 against NLT-1 and a whole array of other antigens not tested, also because significant IgG1 titers of *O. ochengi* lysate and NLT-1 reemerged only after 16 months pe onwards (Fig. 6E), which coincides with the emergence of skin microfilariae.

Sera of animals with a higher nodule and/or microfilarial load had stronger reactivity to OvNLT-1, both for IgM and IgG1. This low molecular weight ESP, which is detected first by IgM after 8 months pe (Fig. 5D), then by IgG1 at 16 months pe

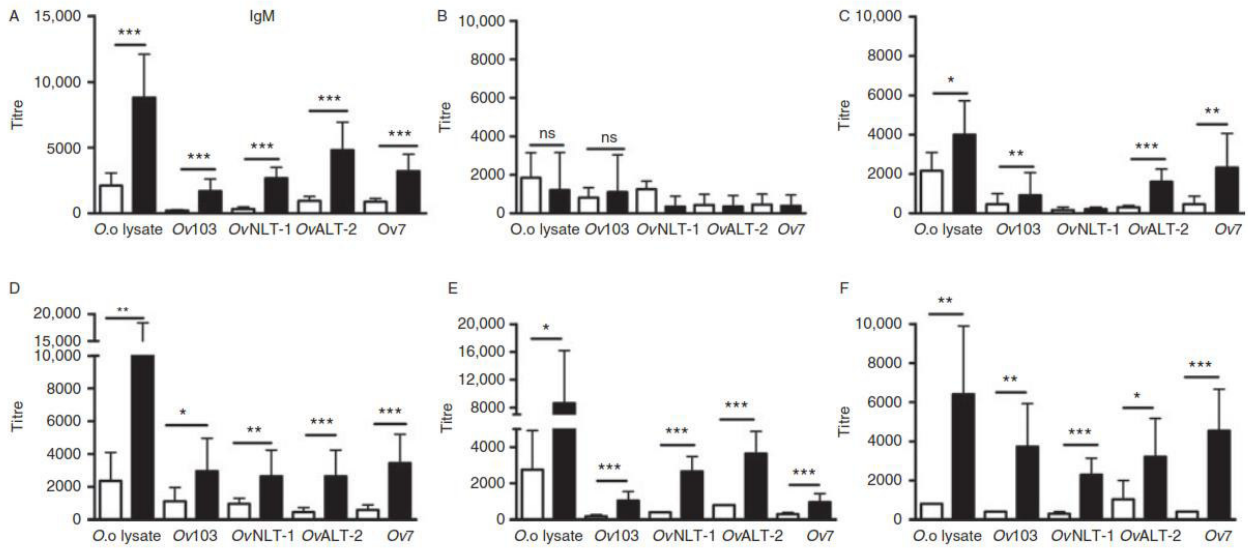

Fig. 5. Humoral IgM immune responses to*O. volvulus*-derived proteins and *O. ochengi* lysate in cattle sera. **A** mother cows, **B** calves pre-exposed to *O. ochengi* transmission, **C** 4 months post exposure, **D** 8 months post exposure, **E** 22 months post exposure and **F** 26 months post exposure. Exposed cattle sera (black bars, n = 10); unexposed European cattle control sera (white bars). From Manchang *et al.* (2014).

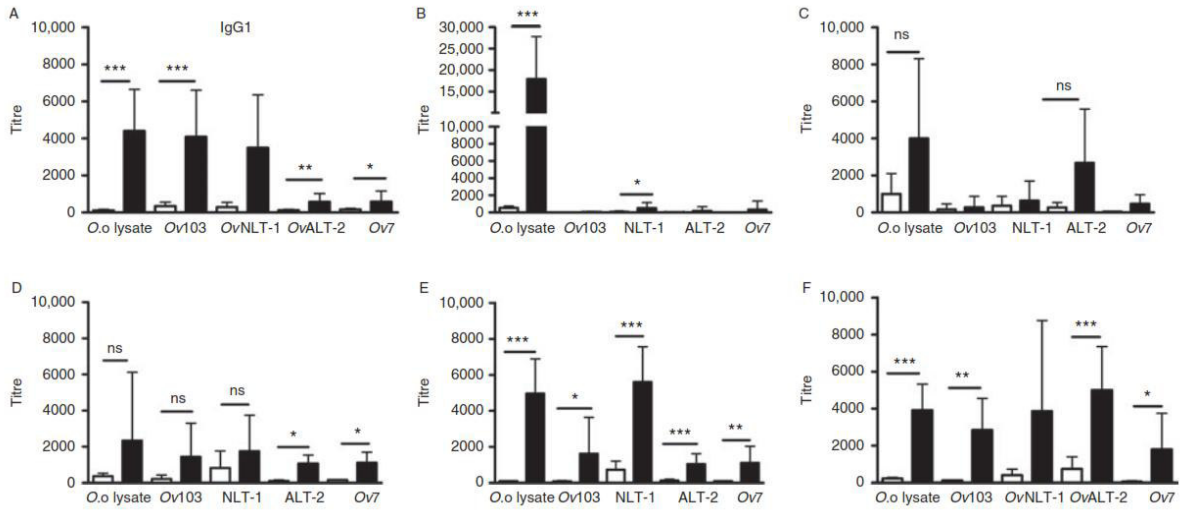

Fig. 6. Humoral IgG1 immune responses to *O. volvulus*-derived proteins and *O. ochengi* lysate in cattle sera. **A** mother cows, **B** calves pre-exposed to *O. ochengi* transmission, **C** 4 months post exposure, **D** 8 months post exposure, **E** 22 months post exposure and **F** 26 months post exposure. Exposed cattle sera (black bars, n = 10); unexposed European cattle control sera (white bars). From Manchang *et al.* (2014).

(Fig.6E), is believed to play a role in host immune evasion and immunopathology (Grieve, 1990). To a lower extend, *Ov*103 IgG1, but not IgM, correlates positively with microfilarial *O. ochengi* counts, starting at 22 months pe (Fig. 6E). The ESP is a

surface-associated antigen of microfilariae, and acts as a stimulant of immune responses (Graham *et al.*, 2000).

The question whether low susceptible/putative immune animals differ in their immune response to higher susceptible animals cannot be answered with this study. However, a negative correlation to certain ESP-directed antibodies was evident, but this could just reflect the lower exposure to antigens of adult or mff stages, since invading L3, L4 and young adult stages are neutralized over 27-times more efficient than in highly susceptible individuals (see calculations in chapter before).

We also analyzed the secretome of adult *O. ochengi* females to search for peptides of immune-modulatory and other functions (Eberle *et al.*, 2015). Therefore, several thousand worms were extracted from nodules of zebu cattle skin, which were obtained from a local abattoir in Ngaoundéré, North Cameroon, and subsequently cultivated *in vitro* in supplemental medium at 37°C. The ESP-enriched medium was collected and exchanged daily, and then subjected to biochemical purification and characterization using a GC/MS approach. Of the found 85 peptide fractions isolated, 21 % were classified as immunogenic proteins (Fig. 7). The majority was classified according to ESP from proteome databases of related species, such as *O. ochengi*, *O. volvulus*, *Brugia malayi* and *Wuchereria bancrofti*, 13 peptides were unmatched in the available databases, of which three showed antimicrobial activity. Like the homologue of host defense molecules, which are known to protect against bacterial infection, the function of these antimicrobial proteins could be to fend off potentially deleterious bacterial infections which harm the parasite and/or the host. Such helminth defense molecules have been discovered before (Robinson *et al.*, 2011), and may ensure the survival of its host, which is also essential for the helminth's survival.

In order to facilitate the analysis of the secretome of *Onchocerca* spp. microfilariae, an efficient protocol to isolate and purify highly viable *O. ochengi* mff and the clear separation from co-infecting *O. gutturosa* mff was developed (Paguem *et al.*, ready for submission). The different steps of this combined gradient density/gel filtration technique is demonstrated in Figure 8. Briefly, mff were either extracted under sterile conditions from chopped female *O. ochengi* uteri or small skin biopsies of parasitized zebu cow udders. After incubation in sterile PBS medium, the mff-containing supernatant is transferred and centrifuged. Next, the spun-down pellet

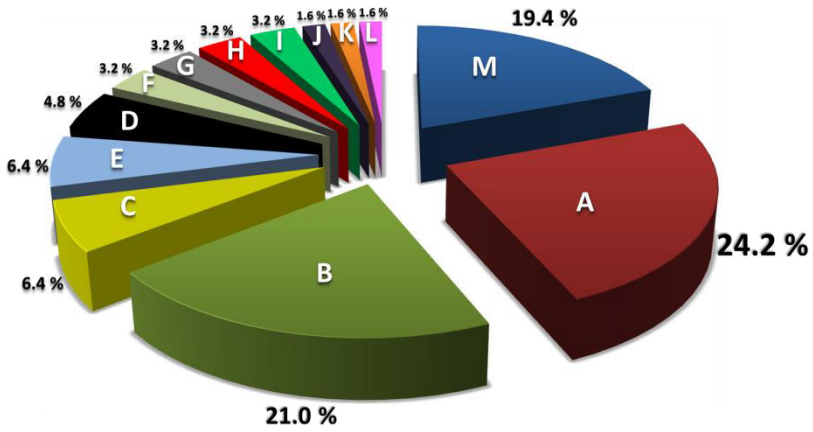

Fig 7. Functional profiles of the *O. ochengi* proteins releasing peptides by proteolytical processes. Pie-chart representing the percentage of proteins identified from protein families. **A**: metabolism, **B**: immunogenic proteins, **C**: antioxidants, **D**: protease, **E**: stress response proteins, **F**: glycoproteins, **G**: transport proteins, **H**: receptor proteins, **I**: construction, **J**: protein modification, **K**: signal transduction, **L**: ribosome, **M**: uncharacterized proteins. From Eberle *et al.* (2015).

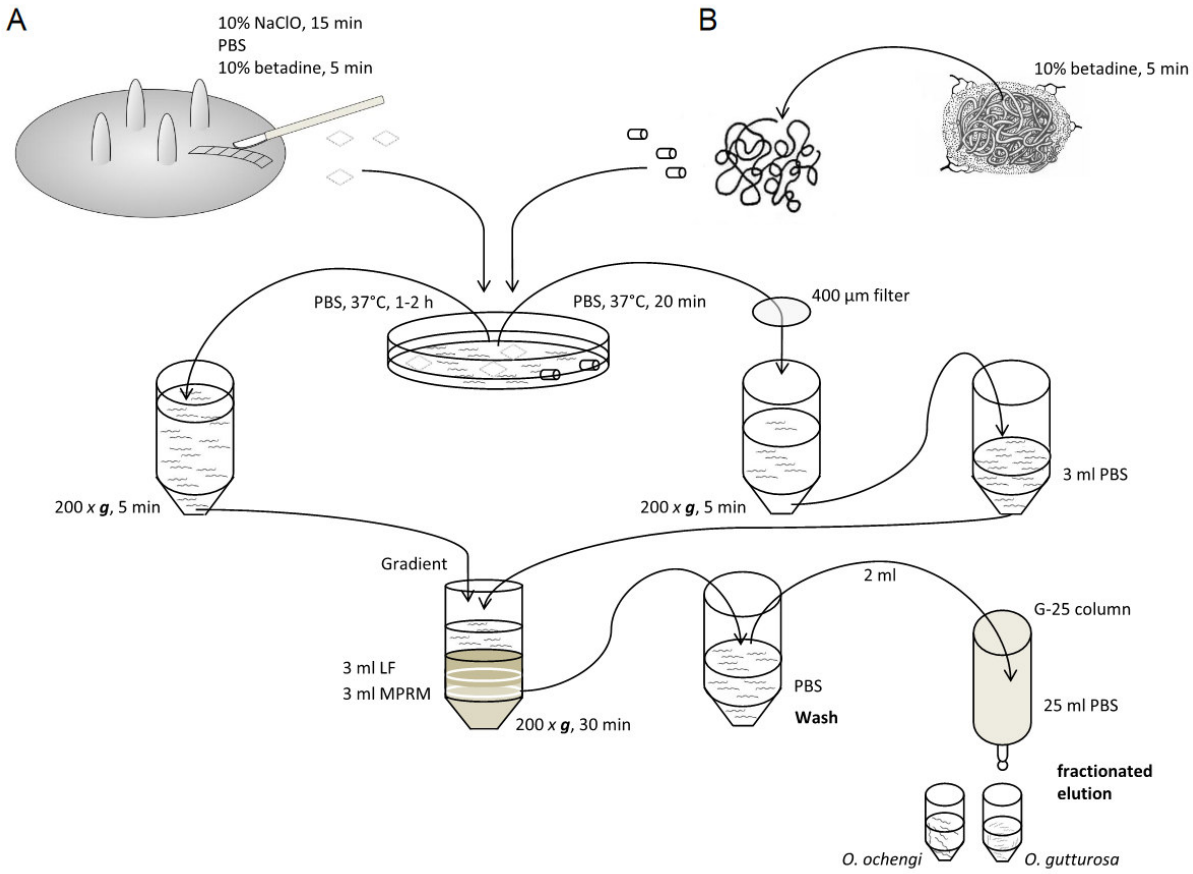

Fig. 8. Work flow of the isolation and separation of microfilariae of *O. ochengi* and *O. gutturosa* from cattle skin (**A**) and onchocercomas (**B**) of *O. ochengi*-infected cattle applying discontinuous gradient centrifugation with subsequent fractionated separation by Sephadex G-25 columns. LF: Lymphoflot; MPRM: Mono-Poly-Resolving Medium. From Paguem *et al.* (in preparation).

medium is loaded on top of a discontinuous gradient solution consisting of 3 ml Lymphoflot (Biorad, Germany) and Monopoly (MP Biomedical, USA), and then centrifuged. The viable microfilariae accumulate in a layer of the same density, forming a white ring in the gradient. When transferred into new PBS medium, they are highly motile and freed from debris and contaminants. By using Sepharose G25 columns (PD-10, GE Healthcare, Germany), the microfilarial populations of *O. ochengi* and *O. gutturosa* from cattle skin were separated in eluate fractions and used for *in vitro* culturing. From a total of 20 udder skins, more than 1.3 million mff were extracted by gradient density centrifugation, of which 80 % were *O. ochengi* and 20 % *O. gutturosa*. Following the Sephadex G-25 column filtration, 88.3 % and 92.5 % of the priorly extracted *O. ochengi* and *O. gutturosa*, respectively, were retrieved.

3. Implications of the reproductive biology of *O. ochengi*

Through the isolation, positional mapping and genetic analysis of the complete adult worm population in one host animal, including a fraction of the microfilariae from female uteri and skin biopsies of the cow udder, we were able to draw novel insights into the reproductive biology of *O. ochengi* (Hildebrandt *et al.*, 2014). With only a few exceptions, all of the 88 nodules found - mostly located around the udder and venter - consisted of one female together with an average of 1.7 males (range: 0 to 8) 64 % of the 87 *O. ochengi* females found carried progeny, of which one to four males were present in the same nodule (Fig. 9A).The spatial distribution of these reproductively active females was not different to the non-reproductive ones.

A reliable protocol for the DNA extraction of single mf was established and a number of molecular markers with at least one SNP locus used in order to screen for genetic variability and pedigree information (Hildebrandt *et al.*, 2012). In case of uterine mff, the number of fathers of the progeny was determined, and whether they match with the present males in the nodule. In many, but not all cases, some or all fathers were present in the nodule. Given that females simultaneously produced microfilariae sired by multiple males (Fig. 9B), reproduction is not exclusively monogamous. Most males tended to stay with their gravid females, hinting to a territorial strategy at least until their progeny have developed. A smaller proportion of males leave the nodules in search for other partners, aiming to maximize paring success by an opportunistic strategy. These findings match well with a positive

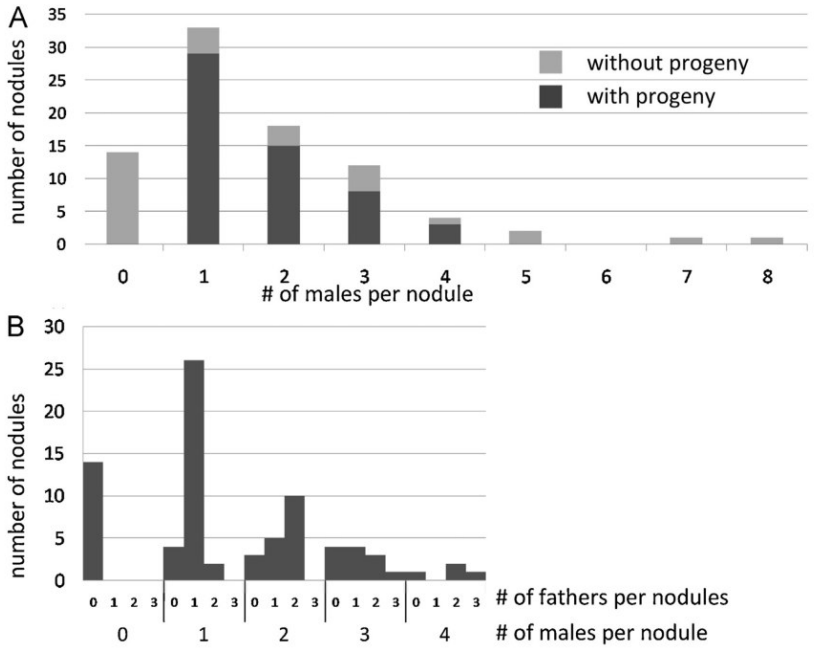

Fig. 9. Number of males per nodule. **A** Histogram showing the number of males per nodule (X-axis) and number of nodules with the corresponding number of males (Y-axis). The bars are subdivided; dark grey indicates nodules with progeny while light grey indicates nodules without progeny. **B** Minimum number of males required to explain the progeny found in a nodule. X-axis bottom label: number of males present in the nodule; X-axis upper label: minimal number of fathers; Y-axis: number of nodules with the corresponding number of males in the nodule and minimum number of fathers. From Hildebrandt *et al.* (2014).

correlation of the color values as age proxy of males and females inside *O. ochengi* nodules, but also with indications of migrating behavior of males (Maier, 2012). The proportion of fecundate females, however, declined with increasing number of present males (Fig. 9B) down to zero for 5 and more males (Fig. 9A). This observation may be a result of increased competition among males, which leads to reduced reproduction, or that females which start to become reproductively active - for the first time or repeatedly after phases of reproductive quiescence - are particularly attractive for males.

The molecular genetic analysis of skin microfilariae revealed that not all reproductive females contributed equally to the pool of circulating mff. Of the 121 skin mff screened, 74 % could be assigned to 11 mothers (13 % of total adult female population), of which 37 % came from a single female. However, since the temporal and spatial dimension was not covered in this study, it remains unclear whether these contributions are stable over place and time. Pheromones from other female worms and/or from newly arriving males may be responsible for an increased frequency of

dying progeny in gravid females and ultimately the temporal or permanent cessation of reproduction. The systematic analysis of embryograms, which quantify the proportion of different embryonic stages, including viable and degenerated mff (Schulz-Key, 1988) in a host's *O. ochengi* population could facilitate support or mitigation to this hypothesis (Trees *et al.*, 1992).

4. *Onchocerca* biodiversity in *Simulium damnosum* and zebu cattle

In order to study the genetic biodiversity of *Onchocerca* parasites in cattle and vector flies, mitochondrial (Eisenbarth *et al.*, 2013) and nuclear markers (Hildebrandt *et al.*, 2014) were used. This approach was also very useful to determine the species of morphologically similar L3 in *S. damnosum s.l.*, which is crucial for epidemiologic investigations (see chapter 5ff). Four different mitochondrial clades were found: *O. ramachandrini*, *O. ochengi*, *O sp.* 'Siisa' and *O. volvulus* (Fig. 10). The latter three taxons formed a group in the phylogenetic tree, with a slightly closer relation of *O. sp.* 'Siisa' with *O. volvulus*. In Cameroonian zebu cattle, the hitherto unknown definite host of 'Siisa' (Krueger *et al.*, 2007) was identified. Moreover, nuclear markers revealed that 'Siisa' is interbreeding with *O. ochengi*, thus belonging to the same species (Hildebrandt *et al.*, 2014).The most likely explanation is that the current population in Cameroon stems from previously separated but nowadays connected populations. The proportions of *O. ochengi* 'Siisa' haplotypes are almost identical in the examined zebu host (16.3 %, n = 233 adult worms) and the analyzed vector population (15.1 %, n = 119 *O. ochengi* L3), indicating a similar success rate of uptake and development in the *Simulium* vector. The only other confirmed finding of *O. ochengi* 'Siisa' was from a single *S. damnosum s.l.* vector fly at an onchocerciasis focus in Western Uganda along the Siisa river, where its name is actually derived from (Krueger *et al.*, 2007).

The isolated L3 from infected flies were also classified microscopically according to body length and shape of anterior and posterior ends. This method has been used to distinguish *O. volvulus* infective larvae from other L3 which have animals as definite host (Renz *et al.*, 1987; Wahl *et al.*, 1998). Figure 11 illustrates the variance in body lengths of the different species in comparison with microscopic and molecular-genetic classification (Eisenbarth *et al.*, submitted). The molecular data confirms the delimitation power of body length distribution for the three species,

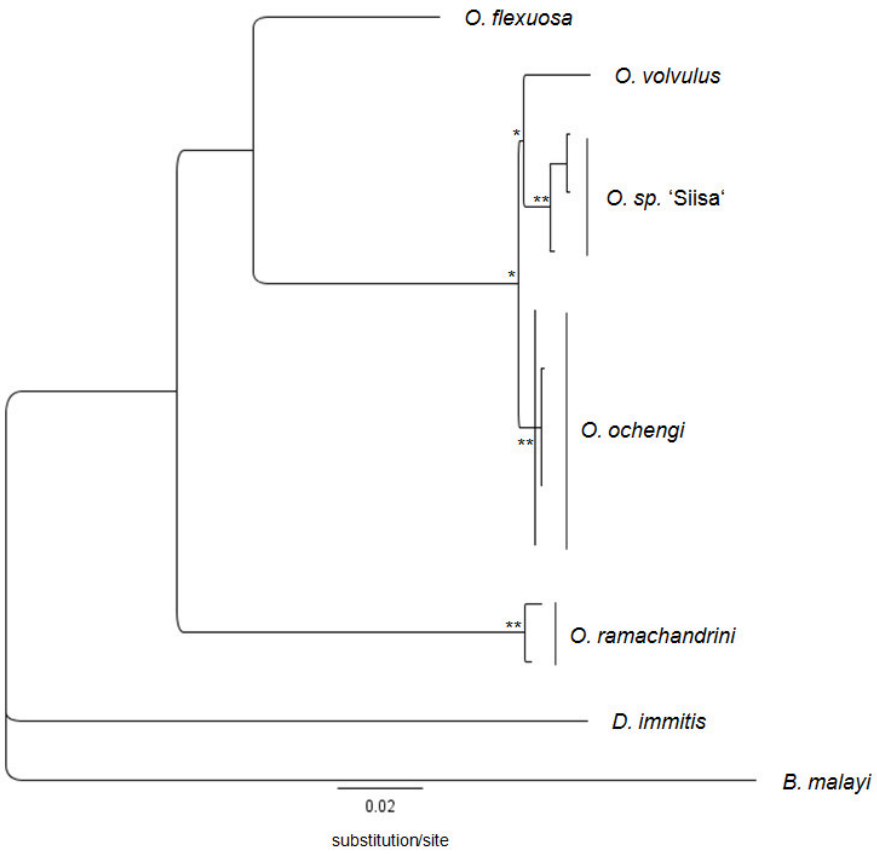

Fig. 10.Maximum likelihood tree showing the phylogeny of *Onchocerca* spp. on the concatenated analysis of three mtDNA sequences (12S and 16S rRNA, *coxI* mtDNA; 1545 bp). The asterisks indicate the pair-wise genetic distance (***$P < 0.001$; **$0.01 > P > 0.001$; *$P < 0.05$).Sequences of *O. flexuosa*, *Dirofilaria immitis* and *Brugia malayi* were taken from the Genbank database. The latter species was set as outgroup. From Eisenbarth *et al.* (2013).

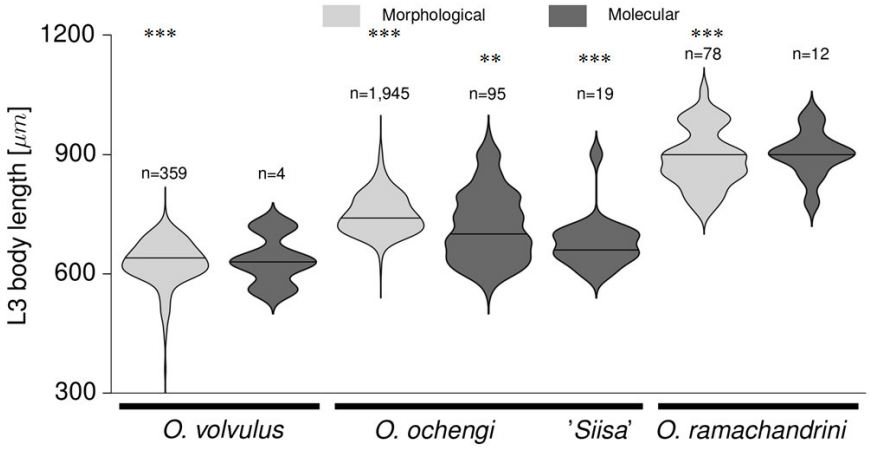

Fig. 11. Violin plot of the L3 body lengths of identified *Onchocerca* spp. in *S. damnosum s.l.* from North Cameroon. Species identification is based on morphological (light grey) and molecular-genetic (dark grey) characteristics. Two genotypes of *O. ochengi*, *O. ochengi s. str.* and *O. ochengi* 'Siisa', are shown based on their mitochondrial clades. ***$P < 0.001$; **$0.01 > P > 0.001$; *$P < 0.05$. From Eisenbarth *et al.* (submitted).

but it also indicates that apart from one outlier *O. ochengi* 'Siisa'L3 are shorter (n = 19) than those of *O. ochengi s. str.* (n = 93), and actually overlap largely with the size range of *O. volvulus*. This increases the risk of misidentification of animal-borne *Onchocerca* spec. L3 with *O. volvulus*, if only relying on morphological features, in particular in areas where onchocerciasis transmission rates have strongly reduced.

Consistent with our findings is the hypothesis that the human parasite *O. volvulus* originated from a spillover event of a common ancestor with *O. ochengi* from cattle (Bain, 1981). The most plausible time point would be during the domestication of cattle between 5,000 and 10,000 years ago. Whether the variant 'Siisa' is in more direct ancestry with *O. volvulus* remains speculative and requires further investigation of the genomes and geographic distribution of the species in question, both in taurine and zebu cattle breeds and also in ungulate game animals.

According to the variation and frequency of the nuclear alleles found in the *O. ochengi* population in one zebu cow (Hildebrandt *et al.*, 2014), as well as the mitochondrial alleles of *O. ochengi* found in caught vector flies (Eisenbarth *et al.*, 2013), the genetic diversity is high. This supports the hypothesis that only one or a few infective larvae get transferred to a new host during a blood meal (Renz, 1987), and therefore results in an unrelated heterogeneous parasite population per host with many independent transmission events.

Besides the above mentioned haplotypes, one solitary female, which was non-reproductive, and one male worm from another nodule had mitochondrial sequences very similar to *O. dukei* (Hildebrandt *et al.*, 2014). This bovine parasite is transmitted by *S. bovis*, a vector almost exclusively occurring in the Sudan savannah, but not on the Adamaoua highlands (Wahl and Renz, 1991). Nuclear markers were only functional for the male worm individual, and were in all cases heterozygous with a common allele and a very rare (one marker shared with another individual) or unique allele (all other markers tested). Most likely, it was a hybrid between *O. ochengi* and *O. dukei*. Given that another *O. ochengi* individual had a rare allele at one nuclear locus which almost resembles the corresponding unique allele of the hybrid, the findings suggest a limited gene flow between *O. dukei* and *O. ochengi*.

5. Vector dynamics and transmission of *Onchocerca* spp. in North Cameroon over the last 25 years

From April 2009 till March 2013 (48 months), the vector fly abundance, biting frequencies, *Onchocerca* spp. infection rate and L3 transmission potential in two onchocerciasis foci in North Cameroon have been monitored by conducting regular fly catches on human bait, and the ensuing dissection of flies under the microscope. This data was compared with available records previously taken and published from the same locations (Achukwi *et al.*, 2000; Renz, 1987; Seidenfaden *et al.*, 2001; Wahl *et al.*, 1998; 1994b), going back up to 36 years before community-directed treatment with ivermectin (CDTI) was set in place. One focus at the village Soramboum lies in the Sudan savannah in the Vina du Nord basin, where onchocerciasis was hyperendemic at baseline (Renz, 1987). The other focus at the

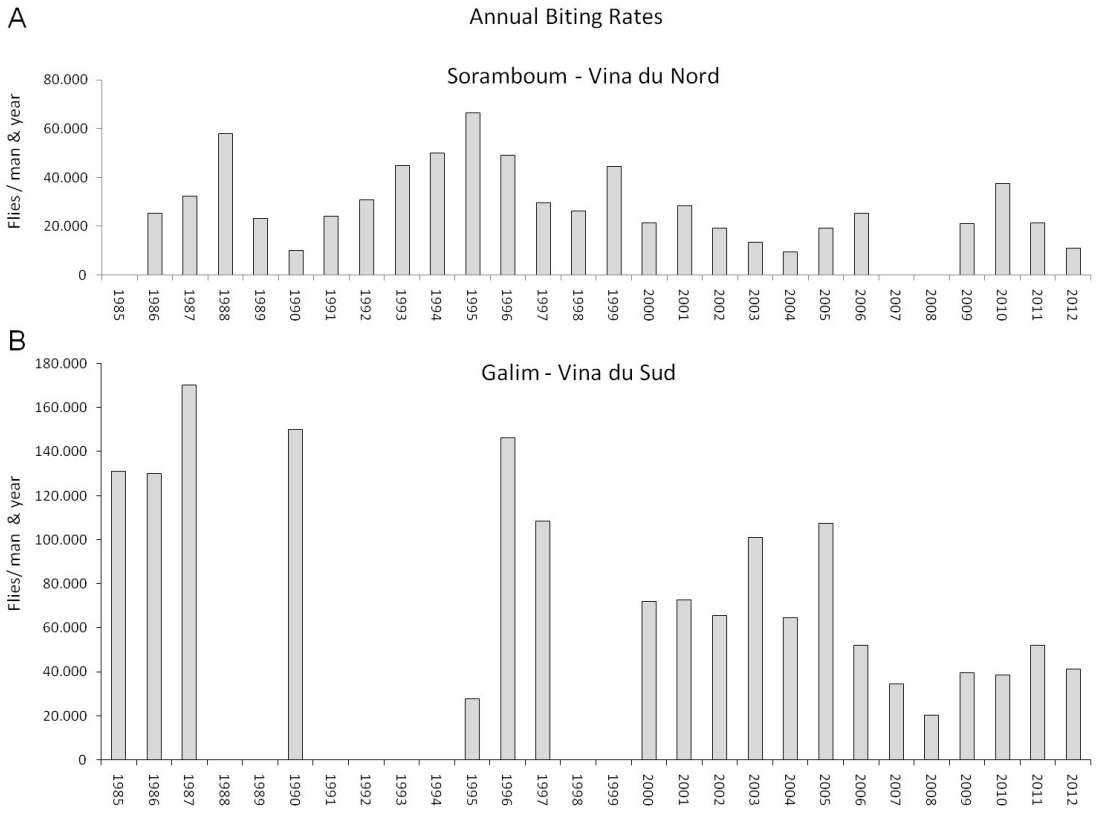

Fig. 12. Annual biting rates of *Onchocerca* spp. from two onchocerciasis foci in North Cameroon. Each data point starts at the beginning of the rainy season (April) till the end of the dry season (March of the following year). Years with no data are left blank. **A** Soramboum, Vina du Nord. Epidemiological data prior to 1998 was published before and modified to fit this graph. Data from 1976 was taken from Touboro, 30 km further downstream. **B** Galim, Vina du Sud. Epidemiological data prior to 1997 was published before and modified to fit this graph. From Eisenbarth *et al.* (submitted).

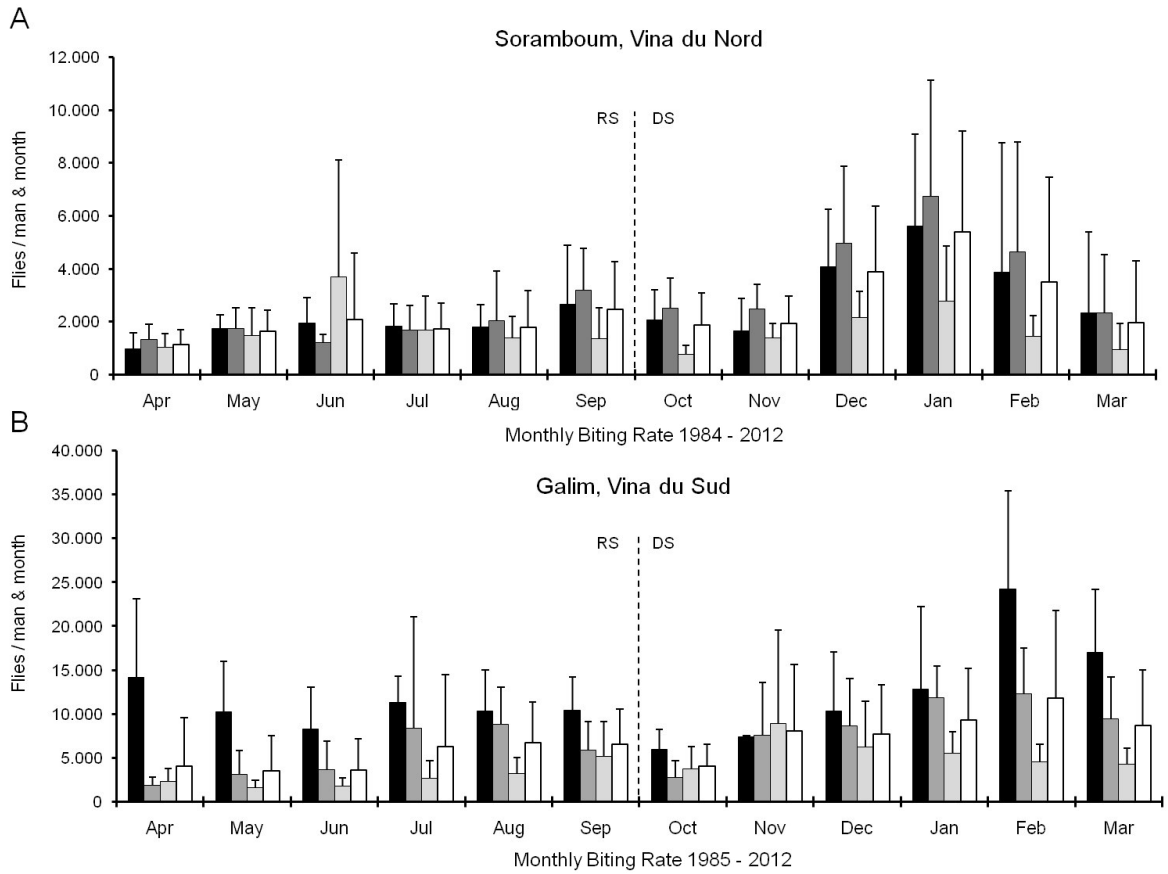

Fig. 13.Mean monthly biting rates of *Onchocerca* spp. from two onchocerciasis foci in North Cameroon. Whiskers show the standard deviation. Black bar: 1984 - 1993; dark grey bar: 1994 - 2003; light grey: 2004 - 2012; white bar: 1984 - 2012. RS: rainy season; DS: dry season. **A** Soramboum, Vina du Nord. **B** Galim, Vina du Sud. From Eisenbarth *et al.* (submitted).

village Galim in the Vina du Sud basin is approx. 170 km South-West of Soramboum in the Guinea grassland on the Adamaoua plateau, and was mesoendemic before treatment interventions have started. The Annual Biting Rates (ABR) have declined in recent years at both loci, in Soramboum since the early 2000's (Fig. 12A) and in Galim more severe since 2006 (Fig. 12B). The cause for this vector population drop is still under debate, but may be linked to decreased food availability for the filter-feeding aquatic *Simulium* larvae due to the local extermination of hippopotamuses, which fertilized the water columns with their excrement. Another conjecture would be a higher level of toxicants, like insecticides, acaricides, etc. in the rivers than before, decimating the number of larvae in the breeding sites. Also, the continuous rise of potential blood hosts in the course of population growth of both human and livestock could result in lower individual biting frequencies. Likewise, the degree of anthropophily in the vector fly population, meaning the attraction to human blood hosts, may have gradually shifted towards zoophily.

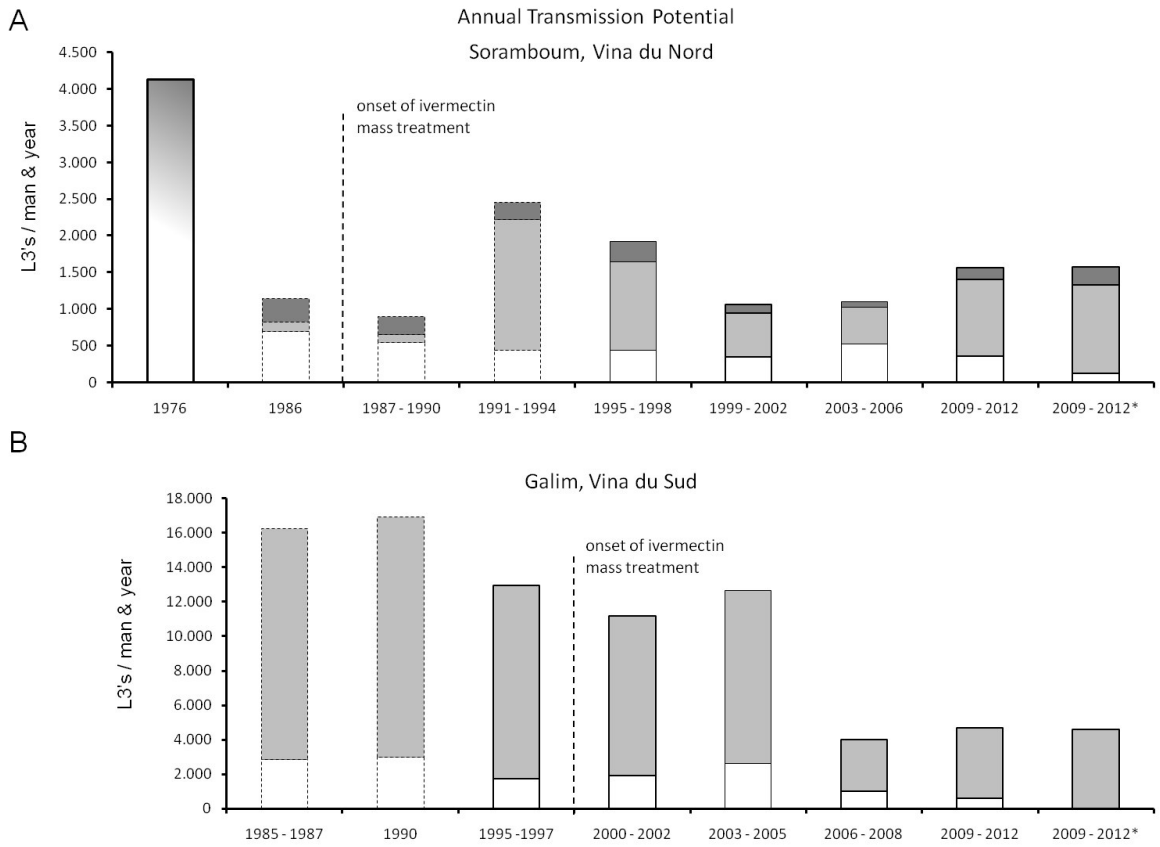

Fig. 14. Annual transmission potentials of *Onchocerca* spp. from two onchocerciasis foci in North Cameroon. Data is given at the beginning of the rainy season (April) till the end of the dry season (March of the following year). Dark grey: *O. ramachandrini*; light grey: *O. ochengi*; white: *O. volvulus*. In 1976 (color transition of white, light grey to dark grey) no species discrimination was done. ATP with available MTP data ≥ 80 % are bold framed, with data 80 % > x ≥ 30 % normal framed, and with data < 30 % broken line framed. The dotted line marks the beginning of ivermectin mass treatments. The asterisk indicates the introduction of PCR-based methods for species discrimination in the vector. Prior to 1990, the L3 species was determined according to the following body length criteria: *O. volvulus* ≤ 750 µm; 750 µm <*O. ochengi* < 900 µm; *O. ramachandrini* ≥ 900 µm. **A** Soramboum, Vina du Nord. Epidemiological data prior to 1998 was published before and modified to fit this graph. Data from 1976 was taken from Touboro, 30 km further downstream. **B** Galim, Vina du Sud. Epidemiological data prior to 1997 was published before and modified to fit this graph. From Eisenbarth *et al.* (submitted).

Climate change may also play a role, in particular dwindling rainfall and intensification of droughts. Figure 13 depicts the largest collapse in biting rates during the last 30 years in the dry season on the Sudan savannah (Fig.13A), and stretching to the peak of the rainy season on the Adamaoua plateau (Fig. 13B). The dry season is also the period where most *O. volvulus* and *O. ochengi* are transmitted, unlike *O. ramachandrini* which is reaching its peak in the rainy season.

When looking at the annual transmission potential (ATP), which estimates the number of infective larvae transmitted per exposed person in one year, we saw a decline mainly as a result of lower ABR since 2002 in Soramboum (Fig. 14A) and since 2006 in Galim (Fig. 14B). In Soramboum, conversely, total *Onchocerca spp.* transmission potential has risen moderately due to a higher infection rate of the vector (3.3 % *vs.* 2.3 % before 2002). The L3 have been differentiated into the three occurring species *O. ramachandrini* (only Soramboum), *O. ochengi* and *O. volvulus*. Before 2009, the body length and shape of head and tail were the chief discriminating parameters according to Renz (1987) and Wahl *et al.* (1994b), at which *O. ramachandrini* had a longer body length than *O. ochengi*, and *O. volvulus* the shortest mean body length (Fig. 11, white bars). Since 2009, additional molecular data from three rRNA markers of the mitochondrion were available and used for species classification (Eisenbarth *et al.*, 2013; submitted). Figure 15 shows the proportion of each identified *Onchocerca* species in the examined L3 population of the study sites. Accordingly to molecular investigation (Fig. 15B), 72 % of

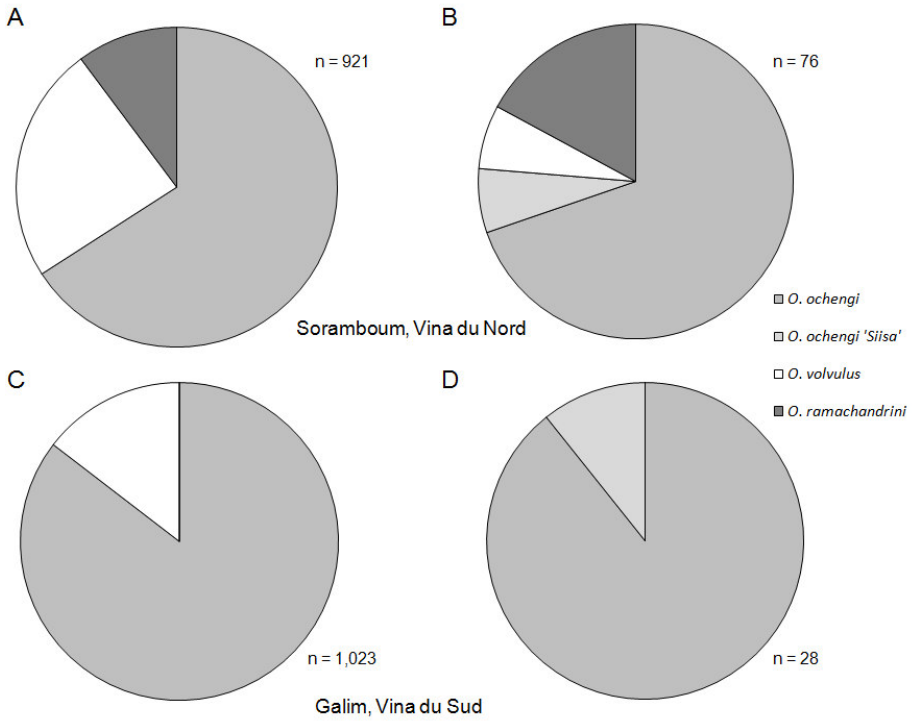

Fig. 15. Species composition of infective third-stage larvae from *S. damnosum s.l.* at two foci in North Cameroon. White: *O. volvulus*; light grey: *O. ochengi* 'Siisa' type; medium grey: *O. ochengi*; dark grey: *O. ramachandrini*. **A-B** Soramboum, Vina du Nord. **C-D** Galim, Vina du Sud. Left side (**A**, **C**): morphological identification. Right side (**B**, **D**): PCR-based identification. From Eisenbarth *et al.* (submitted).

microscopically classified *O. volvulus* from Soramboum (Fig. 15A) were filariae of animal origin. That would translate in an average ATP of 98 (range: 47 to 221) L3/man/year instead of 354 (range: 168 to 794) for the years 2009 to 2012. In Galim (Fig. 15C), no *O. volvulus* L3 could be confirmed by molecular investigation (Fig. 15D).

Taken together, the data shows that 25 years of annual CDTI measures have strongly reduced the transmission of onchocerciasis in the Sudan savannah, and in case of the Adamaoua highlands, our findings suggest even the interruption of transmission since at least 2009. However, it also raises concern whether yearly-given ivermectin to the exposed population is sufficient to eliminate river blindness from former hyperendemic loci. Furthermore, the low but stable rate of *O. volvulus* transmission in the Sudan savannah focus may hint to a lower threshold for maintaining endemicity than current mathematical models predict (Basanez *et al.*, 2002; Duerr *et al.*, 2011).

6. Protective impact of zooprophylaxis for the epidemiology of onchocerciasis

Remarkably, onchocerciasis has never reached such high endemicity in human and vector populations on the Adamaoua plateau than in the less than 200 km distant Sudan savannah, even though the biting rates have always been much higher (Fig. 14). Apart from different altitudes and climatic conditions, one striking contrast has been the much higher livestock to human ratio in the Adamaoua highlands, in particular of cattle (Fig. 16). This is both culturally inherited, *e.g.* migrating pastoralists of the North *vs.* sedentary cattle farmers of the South, and due to biologic conditions, *e.g.* water and food scarcity during the dry season in the Sudan savannah, and the absence of tsetse flies on the Adamaoua plateau, which transmit bovine trypanosomosis. It is thus very likely that zooprophylaxis, meaning the combined effects of diversion of the vector pool to animal blood hosts and immunological cross-reactivity with animal-borne *Onchocerca* spp., has substantially contributed to the local elimination of river blindness in the Vina du Sud basin (see also Renz et al., 1994; Wahl et al., 1998). Even more so, since no *O. volvulus* transmission was detected as early as 12 years of annually repeated CDTI.

Not before the 1980's were nomadic herdsmen and their cattle herds allowed to enter the Vina du Nord river basin. Not long after, a highly increased transmission

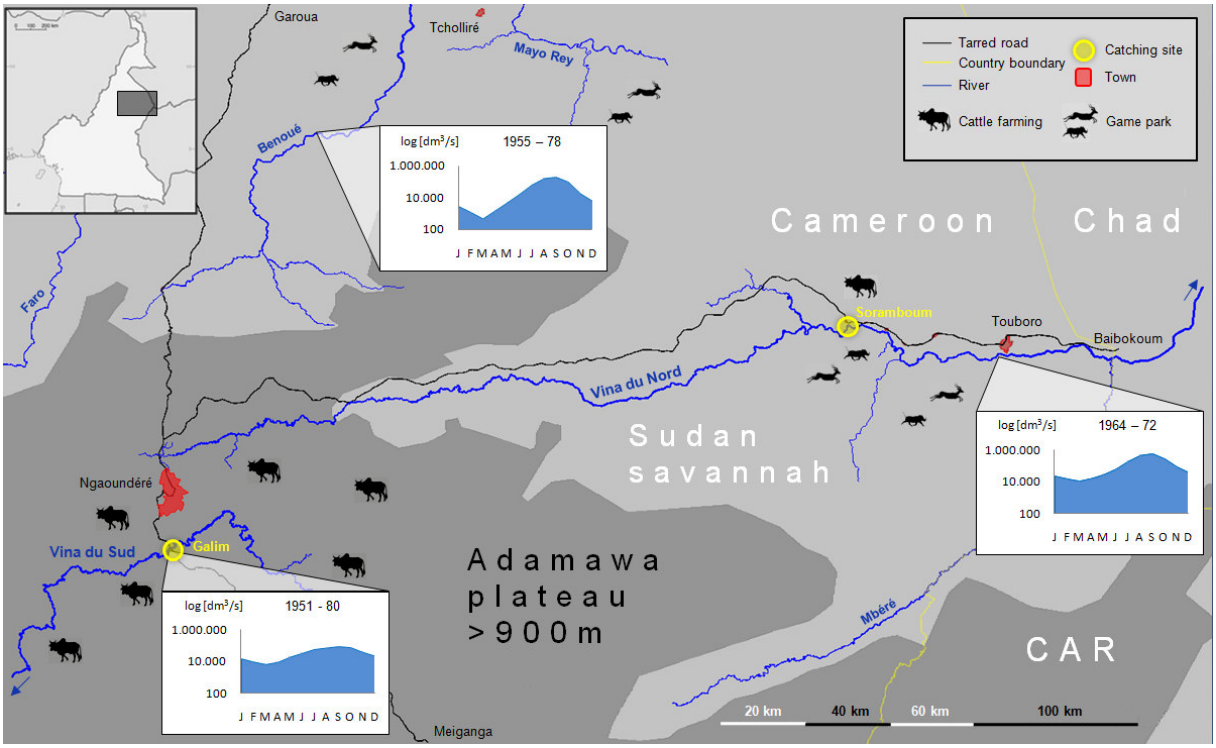

Fig. 16. Overview map of the study sites (yellow circle) from two river basins in Northern Cameroon. The village Galim at the Vina du Sud river near Ngaoundéré is on the Adamawa highland plateau, an area above 900 m altitude (dark grey area) with intense cattle husbandry. The village Soramboum at the Vina du Nord river towards Touboro is located in the Sudan savannah (light grey area), a region only restrictedly frequented by cattle herds and high wildlife density. Diagrams of hydrological data showing the average monthly water discharge from three river basins is taken from Olivry (1986). CAR: Central African Republic. From Eisenbarth *et al.* (submitted).

of animal-borne filariae was recorded, in particular of *O. ochengi* (Fig. 14A). This sudden jump of animal-filariae in the vector population implies the diversion of large quantities of local *S. damnosum s.l.* to take their blood meal from cattle, thereby reducing the vector pool and hence the risk of transmission for humans. In contrast to the Vina du Sud valley, the effect of zooprophylaxis in conjunction with ivermectin mass treatment and possibly socio-economical changes has not (yet) resulted in a breakdown of onchocerciasis transmission. Whether this may change in the future due to a recent increase of cattle herds in the region (unpublished observation) shall be seen in the coming years of vector monitoring.

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Appendix

1. accepted
    a. Hildebrandt *et al.* 2012
    b. Eisenbarth *et al.* 2013
    c. Hildebrandt *et al.* 2014
    d. Manchang *et al.* 2015
    e. Eberle *et al.* 2015

2. submitted
    a. Eisenbarth *et al.*

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SHORT COMMUNICATION

Single worm genotyping demonstrates that *Onchocerca ochengi* females simultaneously produce progeny sired by different males

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Abstract *Onchocerca ochengi* is a filarial nematode parasite of African cattle and most closely related to *Onchocerca volvulus*, the causing agent of river blindness. *O. ochengi* females induce the formation of a nodule in the dermis of the host, in which they remain sedentary in very close association with the host's tissue. Males, which do not adhere to the host's tissue, are also found within the nodules at an average number of about one male per nodule. Young *O. ochengi* females tend to avoid the immediate proximity of existing nodules. Therefore, *O. ochengi* nodules are dispersed in the ventral inguinal skin at considerable distances from each other. It has been speculated that males avoid the risk of leaving a female once they have found one and remain in the nodule as territorial males rendering the reproductive strategy of *O. ochengi* essentially monogamous. We developed a protocol that allows reliable PCR amplification of single copy loci from different developmental stages of *O. ochengi* including embryos and microfilariae. From 32 *O. ochengi* nodules, we genotyped the female worms and the 67 adult male worms, found in these nodules, together with a fraction of the progeny from within the uteri of females. In 18 of 32 gravid females progeny derived from multiple males were found. In five nodules, the males isolated from the same nodule as the female were not sufficient to explain the genotypes of the entire progeny. We conclude that frequently *O. ochengi* females simultaneously produce progeny sired by different males and that most but not all males are still present in the nodule when their offspring is ready to hatch.

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Introduction

The filarial nematode *Onchocerca ochengi* is a parasite of cattle in tropical and subtropical regions of Africa. It is most closely related to *Onchocerca volvulus*, the causative agent of human onchocerciasis. *O. ochengi* and *O. volvulus* share the black fly *Simulium damnosum s.l.* as a vector (Renz et al. 1994; Wahl et al. 1994). Due to the close phylogenetic relationship and the many parallels in the biology of these two worms, *O. ochengi* serves as an animal model for *O. volvulus* (Renz et al. 1995). In spite of ongoing efforts to combat onchocerciasis, i.e., by pan-African mass-treatments of endemic areas (APOC, WHO, http://www.who.int/blindness/partnerships/APOC/en/), *O. volvulus* continues to be a threat to the health of millions of people, and new therapies and control measures are required (Hoerauf et al. 2011). Development of resistance against ivermectin, the only one drug presently used in mass treatment, is likely to occur, and the spread of resistance will depend on the population biology dynamics and mating behavior of the *Onchocerca* worms.

O. ochengi females induce the formation of nodules in the dermis of the host, in which they remain sedentary in very close association with the host's tissue. They remain reproductive for many years, presumably as long as their hosts live (5 to 10 years; Determann et al. 1997; Wahl et al. 1994).

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Young *O. ochengi* females tend to avoid the immediate proximity of existing nodules. Therefore, *O. ochengi* nodules are dispersed in the ventral inguinal skin at considerable distances from each other. Sometimes, in very heavily infested cattle (>100 nodules), groups of 5 to 15 nodules can be found close to each other in the udder and teats. Nevertheless, each nodule remains separated from other nodules, like the grapes of wine. This is in contrast to females of *O. volvulus*, which tend to group together and form clumps of nodules, consisting of female worms of different ages (Wahl et al. 1994; Schulz-Key 1988).

Upon mating, the embryos develop and finally hatch in the uteri of the female. The microfilariae (first-stage larvae) migrate to the peripheral skin and wait to be taken up by a black fly during its blood meal. In the vector, the larvae develop to the third stage and during a later blood meal the fly transfers the infective third stage larvae to a new host. After reaching adulthood, females induce the formation of a nodule and males search for a mate.

The spacing of *O. ochengi* nodules, sometimes more than 10 to 50 cm, poses a certain challenge for males to find multiple mates. Males are much smaller than females and do not adhere to the host's tissue. They are found within the nodules at an average number of one male per nodule (Renz et al. 1994), and the situation with exactly two males is less frequent than expected by chance, indicating a territorial defense of single males (AR, unpublished observations). Further, it has been observed that the males and the females in a particular nodule often are of similar age. Based on these observations, it has been speculated that *O. ochengi* males become territorial once they have found a female and avoid the risk of leaving the nodule to search for additional mates. This would make the reproductive strategy of *O. ochengi* essentially monogamous (Renz et al. 2010).

O. ochengi microfilariae are notoriously difficult for DNA preparation for molecular genotyping. The so far most reliable protocol described involves cutting the microfilariae with a laser dissecting microscope (Post et al. 2009) but this procedure is very cost- and labor-intensive. Starting from the protocol we routinely use for *Strongyloides* spp. (Eberhardt et al. 2007; Nemetschke et al. 2010), we systematically varied all parameters and devised a protocol that allows reliable PCR amplification of single locus genomic sequences from individual *O. ochengi* microfilariae (Protocol 1).

Protocol 1. Preparation of single *O. ochengi* microfilariae for PCR amplification of single locus genomic DNA

Equipment and reagents

- *O. ochengi* worms in PBS
- 2x lysis buffer (20 mMTris-HCl pH 8.3, 100 mMKCl, 5 mM $MgCl_2$, 0,9 % NP-40, 0,9 % Tween 20, 0,02 % Gelatine, 240 µg/ml Proteinase K [add just before use])
- Mouth pipette
- PCR machine
- PCR grade water

Method

1. Transfer a single worm into a PCR tube with 20 µl H_2O
2. Close the tube and freeze, thaw and vortex vigorously. Repeat three times.
3. Add 20 µl of 2x lysis buffer and mix by finger tapping.
4. Incubate at 65°C for 8 hours in a PCR machine.
5. Incubate at 95°C for 15 minutes to inactivate the proteinase K.
6. Add 10 µl of water and use up to 5 µl as template for PCR amplification.

We isolated five molecular markers (*ytP159, ytP161, ytP62*, *ytP164*, *ytP169*, Table 1, Suppl. Table 1) based on Expressed Sequence Tags available from the National Center for Biotechnology Information following the strategy described earlier by our laboratory for *Strongyloides* sp. (Eberhardt et al. 2007; Nemetschke et al. 2010). "Molecular markers" is the term we use for fragments of genomic DNA that can be PCR-amplified with defined primers and contain one to several single nucleotide polymorphisms (SNPs).

We dissected 48 individual *O. ochengi* nodules from the skins of naturally infected cows that had been collected post mortem from Zebu cattle at the abattoir in Ngaoundéré, Cameroon. Of those nodules, eight contained no males and the females did not have progeny in their uteri. One nodule contained a gravid female, but no male. In seven nodules, the females were without progeny although males were found. Thirty-two nodules contained females with developing embryos in the uterus and at least one male. Of the last category, we genotyped all adults we found and a fraction of the progeny for multiple markers (Tables 2 and 3). In order to avoid selecting microfilariae that might have migrated to the nodule, we analyzed only embryonic progeny that were still in the eggshell or microfilariae directly from the uteri.

First, we asked if all progeny isolated from a particular female stem from the same partner, present or not in the nodule. In 18 of the 32 gravid females, progeny of multiple males were identified, indicating repeated inseminations by two or more males.

Next, we asked if the genotypes of the microfilariae were consistent with the hypothesis that their fathers were present in the nodule. In 27 of the 32 nodules, this was the case. In 3 of the 12 nodules containing one male, the present male was not the father of all progeny. Also in two of the nine nodules, containing three males, there was at least one father per nodule missing. As mentioned above, we also found a nodule with a gravid female but no male.

From our data, we conclude that reproduction in *O. ochengi* is not predominantly monogamous, though most fathers tend to stay with their gravid females at least for as long as it takes for their progeny to reach the microfilarial stage. Nevertheless, at least some males appear to leave the

Table 1 Molecular markers used

Marker	Primers[a]	Length in base pairs[b]	Number of different alleles found[c]
ytP159	fw: TGCGTTTTCTGATCGTATTT rev: CCCTTTTGAATCAATGATGA seq: TGCGTTTTCTGATCGTATTT	446	8
ytP161	fw: TATCTCCTCTTTCGGTGTCA rev: ATTCTGCTGAAGCTTTCCTT seq: TATCTCCTCTTTCGGTGTCA	405	14
ytP162	fw: AGGCACATGTTTTGGTAGTGG rev: AGTTTGCCGGTCATTGATTC seq1: CCTATAGAACTTCTCTTGAG seq2: CTCAAGAGAAGTTCTATAGG	629	25
ytP164	fw: GCATCTTCGCTATCCTTTGC rev: CGAATGGAAACAGCAGCAG seq: AGACTTATCCGTGGTT	448	7
ytP169	fw: CGACATTTGCTATGGGAAGC rev: CACCATCGCAGCTGTGTACT seq: CGACATTTGCTATGGGAA	372	15

[a] *fw* forward primer; *rev* reverse primer; *seq* primer used for sequencing. For *ytP162* two sequencing primers pointing from the same position into opposite directions were used

[b] Overall length of the PCR product including the primers

[c] Each marker contains multiple SNPs. One particular combination of bases at the variable positions within a marker is referred to as an allele. Details are given in Supplementary Table 1. PCR reactions were done with 5 μl of worm lysate (see Protocol 1) in a total volume of 25 μl of ThermoPol Buffer (New England Biolabs) supplemented with 0.2 mg/ml bovine serum albumin, 0.5 mM $MgCl_2$, 0.2 μM primer (each), 120 μM dNTPs (each) and 1.25 U of Taq DNA polymerase (New England Biolabs). An initial denaturation step of 95 °C for 3′ was followed by 35 cycles of 95 °C for 30″, 58 °C for 30″, 72 °C for 1′ and a final extension step of 72 °C for 7′. 0.3 μl of the resulting product were used for sequencing using the BDTv3.1 kit (Applied Biosystems) following the manufacturer's instructions. The samples were submitted to the in house sequencing facility for analysis

Table 2 Results for the individual nodules with males and progeny

Nodule number[a]	Number of males found	Number of progeny genotyped	Minimal number of fathers	Males in nodule sufficient to explain progeny	Minimal number of fathers not found
AI	1	14	1	Yes	0
AH	2	15	2	Yes	0
A19	3	8	2	No	1
A22	4	4	2	Yes	0
A35	2	11	2	Yes	0
B1	1	9	2	No	1
B3	1	11	1	Yes	0
B8	3	7	2	Yes	0
B9	3	6	2	Yes	0
B10	1	12	1	Yes	0
B11	1	3	2	No	1
B13	4	12	3	Yes	0
B15	3	7	1	Yes	0
B16	1	13	2	No	1
B20	3	6	1	Yes	0
B21	3	21	2	Yes	0
B23	2	27	2	Yes	0
B24	4	11	2	Yes	0
B25	3	14	2	No	1
B26	3	15	1	Yes	0
B30	1	22	1	Yes	0
B31	2	15	2	Yes	0
B32	2	16	1	Yes	0
B33	1	19	1	Yes	0
B34	2	18	2	Yes	0
B35	1	13	1	Yes	0
B36	3	15	3	Yes	0
B37	1	17	1	Yes	0
B38	1	20	1	Yes	0
B39	2	21	2	Yes	0
B40	1	10	1	Yes	0
B44	2	26	1	Yes	0

[a]Nodules A19, A22, A35 were isolated on 19.01.2011 from one animal, nodules B1-44 were isolated on 13.01.2011 from one animal; Nodules AI, AH were isolated in the context of an earlier study and recovered from the freezer. All nodules contained only a single female worm

nodule after siring progeny. It is possible that different males follow different strategies. Some males may be territorial and by remaining in the nodule they may father a large portion of the progeny of the corresponding female. Others may be roamers and try to mate with multiple females thereby "stealing" a portion of the progeny from the territorial males. Mixed strategies like this have been described for various organisms from different phyla (Gross 1996).

Table 3 Results from Table 2 summarized

Nodules (females) with	Number	Number of nodules consistent with one father	Number of nodules consistent with all fathers in nodule	Minimal number of males not found
1 male	12	9	9	3
2 males	8	2	8	0
3 males	9	3	7	2
4 males	3	0	3	0
Total	32	14	27	5

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Conflict of interest The authors declare that they have no conflict of interest.

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Molecular evidence of 'Siisa form', a new genotype related to *Onchocerca ochengi* in cattle from North Cameroon☆

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ABSTRACT

Onchocerca ochengi, a filarial nematode parasite from African Zebu cattle is considered to be the closest relative of *Onchocerca volvulus*, the causative agent of river blindness. Both *Onchocerca* species share the vector, black flies of the *Simulium damnosum* complex. Correct identification of their infective third-stage larvae in man-biting vectors is crucial to distinguish the transmission of human or animal parasites. In order to identify different closely related *Onchocerca* species we surveyed the sequences from the three mitochondrial loci 12S rRNA, 16S rRNA and *coxI* in both adult worms isolated from *Onchocerca*-induced nodules in cattle and infective third stage larvae isolated from vector flies from North Cameroon. Two distinct groups of mitochondrial haplotypes were found in cattle as well as in flies. One of them has been formerly mentioned in the literature as *Onchocerca* sp. 'Siisa', a filaria isolated from the vector *S. damnosum sensu lato* in Uganda with hitherto unknown host. Both variants are found sympatric, also in the same nodule of the animal host and in the vector. In the flies we also found the mitochondrial haplotype that had been described for *O. volvulus* which is about equally different from the two previously mentioned ones as they are from each other. These results suggest a higher genetic diversification of *Onchocerca ochengi* than previously reported.

1. Introduction

In areas where cattle-biting *Simulium damnosum* flies and Zebu cattle are present in tropical regions in Africa, infections with the filarial nematode *Onchocerca ochengi* (Bwangamoi, 1969) are prevalent. This parasite is closely related to the causative agent of human onchocerciasis *Onchocerca volvulus*, both in respect of phylogenetic distance (Morales-Hojas et al., 2006) and biology (Wahl et al., 1994). Therefore, it serves as an excellent animal model for exploring the biology, chemotherapy and immunology of *Onchocerca* parasites (Achukwi et al., 2007; Renz et al., 1995; Trees et al., 1998). Both species are amongst the filarial worms transmitted by the black fly vector *S. damnosum sensu lato* also including *O. ramachandrini*, a filaria from warthogs (Bain et al., 1993; Wahl, 1996), *Onchocerca* sp. 'Siisa' which definite host is unknown (Krueger et al., 2007), and other yet undefined filarial species (Duke, 1967; Garms and Voelker, 1969). Correct identification of infective third-stage larvae (L3) of *O. volvulus* and differentiation from other filarial species is paramount for the realistic calculation of Annual Transmission Potentials (Duke, 1968), an important epidemiologic parameter to determine the infection risk of a population in endemic areas (Renz et al., 1987; Wahl et al., 1998).

L3s can be classified morphologically according to their shape and length (Duke, 1967; Eichner and Renz, 1990; Franz and Renz, 1980; McCall and Trees, 1989; Wahl and Schibel, 1998), however not unequivocally due to overlaps in their body length distribution. Moreover, different populations within a species and morphologically highly similar sibling species may remain undetected, in particular when no adult specimens are examined (Denke and Bain, 1978) or no supplementary information is available, e.g. differences in pathology (Duke et al., 1966). More recently, DNA-based techniques have been introduced for *O. volvulus* detection, namely dot blot hybridization assays with specific DNA probes (Fischer et al.,

Abbreviations: BI, Bayesian inference; bp, nucleotide base pair; CI, confidence interval; L3, third stage larva; ML, maximum likelihood; MP, maximum parsimony; RPMI, Roswell Park Memorial Institute medium; rRNA, ribosomal RNA; *s.l.*, *sensu lato*.

☆ *Note*: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers: KC167330–KC167358.

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1997; Meredith et al., 1991; Wahl and Schibel, 1998). However, due to qualitative and/or quantitative limitations, e.g. background hybridization and hybridization signal failure, respectively, the practicability of this method is constrained. Furthermore, the routinely used 150 bp long diagnostic marker for *O. volvulus* O-150 clusters with another *Onchocerca* species, namely *Onchocerca* sp. 'Siisa' (Krueger et al., 2007), thereby hampering species discrimination. Modern molecular-genetic tools enable us to overcome these drawbacks by comparison of the genetic sequences of defined conservative regions across specimens (Ferri et al., 2009). We used three primer pairs to amplify portions of the mitochondrial DNA, for which sufficient data entries of the *Onchocerca* genus are publicly available from GenBank™, namely the 12S and 16S rRNA regions, and parts of the cytochrome oxidase subunit 1 gene *coxI*.

2. Materials and methods

2.1. Infective stage larvae from the vector S. damnosum s.l.

From December 2009 to March 2012, samples were collected at two locations in Northern Cameroon adjacent to *S. damnosum s.l.* breeding sites, namely at Soramboum near the Vina du Nord river in the Sudan savannah: 7°47′14″ N; 15°0′22″ E, where *S. damnosum sensu stricto* and *S. sirbanum* are most prevalent (Renz and Wenk, 1987 and own unpublished data), and at Galim near the Vina du Sud river on the Guinea-grassland of the Adamaoua plateau: 7°12′2″ N; 13°34′56″ E, where *S. squamosum* is the common vector (own unpublished data). The village population from both areas has been treated annually with the antifilarial drug ivermectin for about 15 (Galim) and 25 years (Soramboum). Fly catchers attracted female *Simulium* flies by exposing their legs and trapped the flies with a sucking tube. Daily catches were brought to the Programme Onchocercoses Field Research Station in Ngaoundéré (www.riverblindness.eu), stored at −15 °C and subsequently dissected for filarial infections. The length of intact L3 stages was measured at 50× magnification by an eye-micrometer attached to the stereomicroscope (Wild M5, Switzerland).

2.2. Adult O. ochengi worms extracted from nodules in cattle

To investigate the genetic heterogeneity of *O. ochengi* in cattle, skin samples with palpable worm-nodules in the dermis were obtained from the local abattoir in Ngaoundéré. Worm-nodules containing male and female adults were excised and stored at −15 °C for later analysis. Adult worms were isolated from the nodule tissue by collagenase digestion modified from Schulz-Key et al. (1977). Briefly, nodules were incubated at 37 °C overnight in 0.125% collagenase in RPMI or PBS solution and transferred in fresh medium afterwards.

2.3. DNA preparation, PCR and sequencing

Isolated L3s' and fragments of adult stages were lysed in 75 μl DirectPCR™ lysis reagent (Viagen Biotech, USA) or reaction buffer (30 mM Tris–HCl, 10 mM EDTA, 1% SDS, pH 8.0), supplemented with 1–2 μl proteinase K (20 μg/μl stock, Genaxxon, Germany). Digestion conditions were 5 h or overnight at 55 °C, followed by an enzyme denaturation step (85 °C, 45 min). For lysis of microfilariae and embryonic stages, the protocol according to Hildebrandt et al. (2012) was used. Two microliters of each extract was added in a total volume of 25 μl PCR reaction containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol forward and reverse primer (see below) and 1 U Taq polymerase (Qiagen, Germany). The thermocycler model GeneAmp PCR System (Perkin Elmer, USA) was used with the following program: an initial denaturation step of 4 min at 94 °C was followed by 40 cycles of denaturation (94 °C, 40 s), annealing (conditions amplicon specific, see below), and elongation (72 °C, 90 s) and followed by a final elongation period of 7 min at 72 °C. The primers and annealing conditions were: for 12S as described by Casiraghi et al. (2004) (fw-primer: 5′-GTTCCAGAATAATCGGCTA-3′, rev-primer: 5′-ATTGACGGATGRTTTGTACC-3′; 62 °C, 30 s; for 16S: fw-primer: 5′-TGGCAGCCTTAGCGTGATG-3′, rev-primer: 5′-CAAGATAAACCGCTCTGTCTCAC-3′, 55 °C, 30 s; and for *coxI*: fw-primer: 5′-TGATYGGYGGTTTTGGWAA-3′, rev-primer: 5′-ATAMGTACGAGTATCAATATC-3′, 52 °C, 45 s) (modified from Casiraghi et al., 2001). The PCR products were purified using the PCR Purification Kit (Qiagen, Germany) and sequenced from both ends with the respective PCR primers using the BigDye™ v3.1 Ready Reaction Terminator Kit (Applied Biosystems, USA) according the manufacturer's protocol. Next, excess fluorescent nucleotides were removed by Sephadex G50 (GE Healthcare, UK) column purification (Tabak and Flavell, 1978) or isopropanol precipitation, and the reactions were analyzed on an ABI3100 automated sequencer (Applied Biosystems, USA) according to the manufacturers' instructions.

2.4. Statistic and phylogenetic analysis

Non-parametric multiple comparison between the different species' L3 lengths was done using the Steel Dwass' test by the statistical software program JMP 10.0 (SAS, USA). For the creation of sequence alignments and neighbor-joining consensus trees, the bioinformatics program Geneious version 5.6.5 (Drummond et al., 2012) was used. Extracts from published mitochondrial sequences from the following taxa were added to the phylogenetic analysis: *Brugia malayi* (Genbank: AF538716, Ghedin et al., 2007), *Dirofilaria immitis* (Genbank: NC_005305, Hu et al., 2003) and *Onchocerca flexuosa* (Genbank: HQ214004, McNulty et al., 2012), where *B. malayi* was set as outgroup, as well as own records of *O. ramachandrini* from Soramboum (GenBank: [12S] KC167340–KC167341, [16S] KC167348–KC167349, [*coxI*] KC167356–KC167357). For maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) trees, PAUP*4.0b10 (Swofford, 2002) and MrBayes 2.0.5 (Huelsenbeck and Ronquist, 2001) were used, respectively, and implemented evolution model analyses were estimated by jModeltest 0.1.1 (Posada, 2008) according to Akaike and Bayesian information criteria, as well as decision theory performance-based selection. For the MP and ML trees, heuristic searches with tree bisection-reconnection (TBR) as the branch-swapping algorithm were used. The starting trees were obtained via stepwise addition with random addition of sequences. Additionally, 100 replicates were performed for MP analysis, and all characters were treated as unordered, given equal weights, with gaps treated as missing data. For the BI tree, posterior probabilities were calculated using 1,100,000 generations, employing four simultaneous tree-building chains, with every 200th tree being saved. A 50% majority rule consensus tree was constructed based on the final 75% of generated trees.

3. Results and discussion

3.1. Genotypes of L3s' found in the S. damnosum s.l. vector

From 2872 L3s isolated of 27,425 dissected flies, we determined the 12S rRNA, 16S rRNA and *coxI* sequences of 78 L3 and two L2 isolated from 43 *S. damnosum s.l.* caught at the two different sites in North Cameroon and compared them with GenBank entries. The larvae fell into three groups. 62 (77.5%) grouped together with entries for *O. ochengi* from Mali and Cameroon (12S-GenBank: AY462914; 16S-GenBank: AY462897 (Morales-Hojas et al., 2006);

Table 1
Phylogenetic sequences of L3, adults and microfilariae of closely related *Onchocerca* spp.

Marker	Genotype	GenBank accession number	Consensus sequence[a]	Single Nucleotide Polymorphisms[b]
12S	*O. ochengi* (n=190)	KC167330 – KC167335	CCTTATTTATTAAATTCATTAARACATT AARAAAAAATTACTTTCTTTTCCAATTT CAAAAAAAAATAAAAAACTAATCCAAA AAAAATTCATAATAGTAACACATGAAA CATAAATTCATAAGCCAAATATATATCT GTTTTTAATGCCAAAACAAVAAAGCATT ACAAAATAAAATAAAAACTAAACAATC	1 AACGCC* * AC* T* CC 2 AACGCC* * C* T* CC 3 GACGCC* * AC* T* CC 4 AACGCC* AAC* T* CC 5 AACGCCAAAC* T* CC 6 AACGTC* * AC* T* CC
	O. sp. 'Siisa' (n=22)	KC167336 – KC167338	ATACATGTGCCAACAAAATTCACCAAA AAAGAGGGCTCTCCAGCAAATCACATR TTCCAAAGAAAAATCTAAAGTCAGTCA ATATTTTTTCGGTTTAAAVAAAACTTTA CTCCCGAATTATTAAATTTTGATTACCT GGGTACTAATCCAGTTCAAAAAACAAA	1 AGCACC* * * T* C* CA 2 AGCACC* * * C* C* CA 3 AGCACC* * ACTCACA
	O. volvulus (n=4)	KC167339	TTTTTGATACCAVAAGAAACT***AAAA AAAATVAAA*AVATTTTAC*AAAAAACA AVATAAAAACAAAMTAAAAACTCATAG CACCACAACAAATAGAGTCAAAAATTA AAACGCA	1 AGTATT* * AT* T* TC
16S	*O. ochengi* (n=84)	KC167342 – KC167343	TGGYRTAAAAGTAGCGTAAGTGATTTGT TTTTTTAATGGTTTCARGTATGAATGAA GTTTTTAGCAGTTTTTTTATTTACTTTTT RTTTGAATTATTTTTTTRATTAAAAATTA TTAGTTAAGGTATTACAAAGATAAGTCT TCGGAAATTTTGTTTTGAATTTTGAAATT	1 CGATAGGGAA 2 CGGTAGGGAA
	O sp. 'Siisa' (n=11)	KC167344 – KC167345	TTTRTTTTTAATTTTTTCTTGGGGATGGA TTTTAAGAAAGTTTTATACTATTRTTATT ATTAAAAATTACTCCGGAGTTAACAGGG TTGTAGACATATAAATAGRTTTTTATAT	1 CGACAGAAGA 2 CGACAGGAGA
	O. volvulus (n=3)	KC167346 – KC167347	TAGTGTGCTGCGCTACATCGATGTTGTA TATTTTTTTTGATAATGGAGAGGTTTTTT TTRTTTTGAGACTGTTCTTCTTGTATAAA AAATTGACTTGATATTAGTTTAGTTCGT CG	1 CAACGAAGGG 2 TAAT GAAGGG
coxI	*O. ochengi* (n=6)	KC167350 – KC167351, KC167358	TTGGATGTTGCCTTTRATRTTRGGGGCT CCTGAGATRGCGTTTCCTCGGRTAAATG CTTTRTCTTTTTGRTTTACTTTTGTGGCT TTRTTGATAGTTTATCAGTCTTTTTTTAT YGGRGGTGGYCCTGGTAGRAGTTGGAC TTTTTATCCTCCTCTTAGGGTTGAAGGY CAACCAGAATTGTCTTTRGATACTATGA TTTTAGGTTTACATACTGTRGGAATTGG	1 AAAGGAAGTGCAATAA 2 AAAGGAAGTGCAATAG 3 AAAAGAAGTGCAATAG 1ff. TGGTAGAGTGAT 2ff. TGGTAGAGTGAT 3ff. TGGTAGAGTGAT
	O. sp 'Siisa' (n=3)	KC167352 – KC167354	TTCTTTGTTGGGTGCTATTAATTTTATGG TAACTACTCAGAATATACGGTCVACTGC TGTGACTTTGGATCAAATTAGTATGTTT GTTTGGACTTCTTATTTGACTTCTTTTTT RTTAGTTTTRTCTGTGCCTGTTTTGGCTG GTTCTTTATTGTTTTTGTTGTTGGATCGT AATTTTAATACTTCTTTTTATGATACTAA GAAGGGGGGTAATCCTTTGTTGTATCAG CATTTGTTTTGATTTTTTGGTCATCCTGA	1 GGAGGGAGCATACGA 2 GGAGGGAGCATACGA 3 GGAGAGAGCATACGA 1ff. CAGCAGGACGAT 2ff. CAGTAGGACGAT 3ff. CAGTAGGACGAT
	O. volvulus (n=2)	KC167355	GGTGTATGTTATTATTTTACCTGTTTTYG GTATTATTAGGGARGCGGTTTTATTTTT GACTGATAAGGATCGTTTRTTTGGTCAG ACTAGGATRACTTTTGCTTCTATTTGRA TTGCTGTTTTAGGYACTTCTGTRTGRGG YCATCATATGTATACGGCTGGTTTG	1 GGGGGAGATGTGTAA 1ff. TAATGAGGTAGC

[a] Ambiguity codes at variable positions are in bold and color coded as follows: A or G – yellow; C or T – pink; A or C – red; insertion or deletion (*) – turquoise.
[b] The bases present at the variable positions are listed in the order of occurrence. The numbers indicate different alleles. ff. – continuance of the allele sequence.

coxI-GenBank: AJ271618 (Casiraghi et al., 2001)). The sequences of 13 (16.3%) worms grouped with entries for *Onchocerca* sp. 'Siisa', a phylogenetically closely related filaria from Uganda which has been speculated to originate from wild animals (12S-GenBank: DQ523738; 16S-GenBank: DQ523749, (Krueger et al., 2007); *coxI*-no entry). Finally, the sequences of five samples (6.3%) grouped together with sequences from *O. volvulus* from Mali, Uganda and Cameroon (12S-GenBank: AY462920; 16S-GenBank: AY462902 (Morales-Hojas et al., 2006); *coxI*-GenBank: AM749285 (Ferri et al., 2009)). Mixed infections of *O. ochengi* and *Onchocerca* sp. 'Siisa' occurred in six flies, whereas only two flies carried *O. ochengi* and *O. volvulus* together. No flies had *O. volvulus* and *Onchocerca* sp. 'Siisa' at the same time.

The sequence divergence between the different groups (Table 1) was 2.11–2.75% (12S), 0.75–2.00% (16S) and 2.00–2.93% (*coxI*) while the intra-group variability of the sequences was between 0.15–0.31% (*coxI*) and 0.21–0.64% (12S) for *O. ochengi*, between 0.15–0.31% (*coxI*) and 0.21–0.85% (12S) for *Onchocerca* sp. 'Siisa', and between 0% (12S, *coxI*) and 0.50% (16S) for *O. volvulus*.

From a total of 86 sequenced L3s the body lengths of 87.2% ($n = 75$) could have been determined. Because it had been reported that the L3 length distributions of *O. ochengi* and *O. volvulus* overlap mainly in the area below 700 μm (Wahl and Schibel, 1998) we biased our molecular analysis toward such larvae (61.3% of all measured L3). As expected, our results confirmed the difficulty to correctly identify a few *O. volvulus* L3 larvae among a larger number of L3s of animal origin. Although there was no misidentification of *O. ramachandrini* (mean length: 888 ± 61, $n = 8$) many of the larvae classified as *O. volvulus* proved to be short *O. ochengi* (mean length: 695 ± 77.5, $n = 52$) and *Onchocerca* sp. 'Siisa' (mean: 665 ± 36.2, $n = 11$). The *O. volvulus* L3s confirmed by sequencing measured 635 ± 66.1 μm ($n = 4$).

3.2. Genotypes found in the Zebu cattle host

To investigate the genetic heterogeneity of *O. ochengi* in Zebu cattle, 33 nodules extracted from three cow skins purchased at the local abattoir in Ngaoundéré were dissected, and the adult

Table 2
Allele frequencies of 12S rRNA from different life cycle stages of *Onchocerca ochengi*, *Onchocerca* sp. 'Siisa' and *O. volvulus* from Northern Cameroon.

Species	Developmental stage[a]	Isolated from	*n*	Allele frequencies[b] [%]					
				1	2	3	4	5	6
O. ochengi	Adult	Zebu cattle	48	46	25	19	8	–	2
	– Female		23	39	35	22	–	–	4
	– Male		19	58	21	16	5	–	–
	L3	*S. damnosum s.l.*	62	52	24	16	3	3	2
	– Galim		41	44	27	17	5	5	2
	– Soramboum		21	67	19	14	–	–	–
	L2	*S. damnosum s.l.*	2	100	–	–	–	–	–
	– Galim		1	100	–	–	–	–	–
	– Soramboum		1	100	–	–	–	–	–
	Microfilaria	Zebu cattle	52	71	2	27	–	–	–
	'brezel' stage[c]	Zebu cattle	26	35	42	23	–	–	–
	Total		**190**	**53.7**	**20.5**	**20.5**	**3.2**	**1.1**	**1.1**
Onchocerca sp. 'Siisa'	Adult	Zebu cattle	9	56	44	–			
	– Female		6	67	33	–			
	– Male		2	50	50	–			
	L3	*S. damnosum s.l.*	13	54	31	15			
	– Galim		11	64	27	9			
	– Soramboum		2	–	50	50			
	Total		**22**	**54.5**	**36.4**	**9.1**			
O. volvulus	L3	*S. damnosum s.l.*	4	100					
	– Soramboum		4	100					

[a] For isolated larvae from the vector the respective fly catching sites are given.
[b] The allele numbers correspond to the sequences listed in Table 1 and represent the order of occurrence when excluding data of first-stage larvae.
[c] The 'brezel' stage is an embryonic stage from the female's uterus.

worms (21 males, 29 females and 7 worms of unidentified gender) were subjected to 12S and 16S rRNA analysis. Again, we could clearly identify two distinct groups of sequences. These groups were identical with the *O. ochengi* and the *Onchocerca* sp. 'Siisa' groups respectively (Table 1), identified in the larval stages we found in *S. damnosum s.l.* from both fly-catching sites (see Section 3.1). Details about the 12S rRNA allele frequencies of each genotype are given in Table 2. In five nodules originating from two of the three skins we found adult specimens of both types. It is noteworthy that the proportion of 'Siisa' in the definite host's adult worm population (15.8%, $n = 57$) is similar to the proportion of 'Siisa' L3 of all *O. ochengi* found in the vector (17.3%, $n = 75$) indicating a comparable transmission success between both genotypes. Furthermore, some microfilariae ($n = 52$) and embryonic stages ($n = 26$) from five nodules of a cow's whole skin could be successfully sequenced, showing only alleles of the *O. ochengi* genotype (Table 2). The majority of the worm nodules examined ($n = 31$), however, had no (77.4%) or only decayed microfilariae with disintegrated DNA (6.5%). The reason for this is unclear but may be attributed to a recent anti-helminthic treatment regimen or to density-dependent self regulation of the parasite.

Taken together, we found proof that *Onchocerca* sp. 'Siisa' occurs in cattle, forms nodules like *O. ochengi* and is transmitted by the same black fly vector, namely female *S. damnosum s.l.* At the moment it is not clear whether *Onchocerca* sp. 'Siisa' is a variant of *O. ochengi* with sympatric distribution or if this variant should be considered a sister species. According to the maternal heredity of mitochondrial DNA, our data cannot conclude whether fertile mating between *Onchocerca* sp. 'Siisa' and *O. ochengi* occurs. Of six female 'Siisa' worms found, three were alone in the nodule, two with at least one adult *O. ochengi* male, and one with only microfilarial stages of *O. ochengi*. As the latter contradicts the maternal transfer of mitochondria to the next generation, this phenomenon can be best explained by infiltration of microfilariae from a worm nodule in close proximity. Whereas one of the two identified male 'Siisa' worms was located in a nodule with a female worm and microfilariae of unknown genotype, the other was found together with one female, two males and microfilariae of *O. ochengi*. The gender of one 'Siisa' genotype worm which was associated with adult *O. ochengi* female and males could not be determined.

The phylogenetic relationship between 'Siisa', *O. ochengi* and *O. volvulus* remains puzzling. In all phylogenetic trees (Fig. 1) they form a monophyletic group showing their close evolutionary association, but only the ML-tree groups 'Siisa' as a sister taxon to *O. volvulus* (Fig. 1b). The MP-tree (Fig. 1a) groups 'Siisa' together with *O. ochengi* with a bootstrap support below the 50% threshold, and the BI-tree (Fig. 1c) does not resolve the event either. Nonetheless, the employed substitution model TIM3 + G in the ML-tree ranked first on all varied selection criteria during the model test. It is tempting to speculate that *Onchocerca* sp. 'Siisa' is in a more direct lineage with *O. volvulus*, although comparison of nuclear DNA would be necessary to corroborate this idea. Should this be the case, two scenarios exist, namely either *Onchocerca* sp. 'Siisa', as a variant of *O. ochengi*, is in direct ancestry to *O. volvulus*, or *O. volvulus* re-switched from its former human host back to the bovine host, hence showing the reversal of a host switch event which possibly occurred some 10,000 years ago during the domestication of cattle by man (Bain, 1981). Krueger et al. (2007) already postulated a higher volatility of host switch events in *Onchocerca* species which our study would support. The 'Siisa' variant could even stem from a hybridization event between *O. ochengi* and *O. volvulus*, although this seems unlikely given the difference in chromosome pair numbers (5 vs. 4, respectively, Post et al., 1989). Investigation of the haplotype of *Onchocerca* sp. 'Siisa' could shed more light to this aspect. In any regard, this is a prime example for the co-evolution of *Onchocerca* – *Simulium* complexes, as already observed with the discovery of

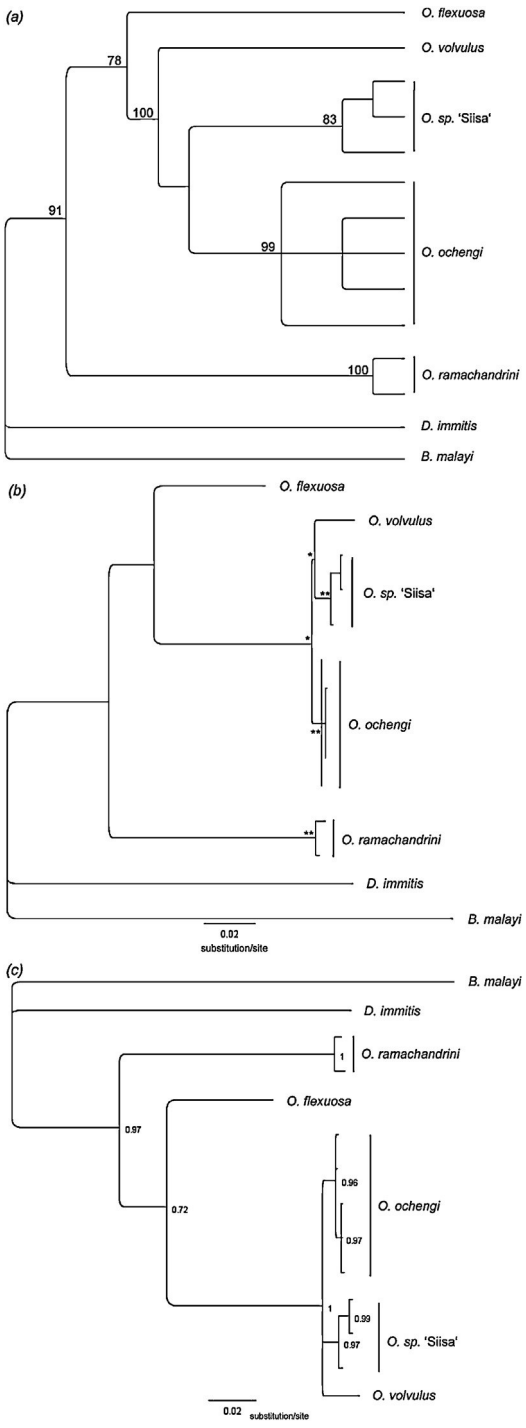

Fig. 1. Phylogeny of *Onchocerca* spp. on the concatenated analysis of three mtDNA sequences (12S and 16S rRNA, *coxI* mtDNA; 1545 bp). (a) Maximum parsimony bootstrap 50% majority-rule consensus tree. (b) Maximum likelihood tree (−ln likelihood = 4179.4), estimated under the TIM3 + G evolution model (nucleotide frequencies: A 0.2407, C 0.0883, G 0.1868, T 0.4842; substitution rate matrix: [AC] 0.2029, [AG] 8.9414, [AT] 1.0000, [CG] 0.2029, [CT] 2.7256, [GT] 1.0000; gamma distribution shape parameter 0.1470). (c) Bayesian inference tree (−ln likelihood = 4178.4), estimated under the GTR + G evolution model (nucleotide frequencies: A 0.2380, C 0.0879, G 0.1893, T 0.4847; substitution rate matrix: [AC] 0.1461, [AG] 11.4273, [AT] 1.5213, [CG] 0.3971, [CT] 3.5493, [GT] 1.0000; gamma distribution shape parameter 0.1440). Analyses have been run on PAUP* 4.0b10 (a, b), and MrBayes 2.0.5 (c). The implemented evolution models have been estimated by jModeltest 0.1.1. Numbers on the branches display values of bootstrap support (a) and Bayesian posterior probabilities (c), respectively, and the asterisks (b) indicate the pair-wise genetic distance (***$P < 0.001$; **$0.01 > P > 0.001$; *$P < 0.05$).

rainforest and savannah strains of *O. volvulus* in West Africa (Duke et al., 1966).

4. Conclusion

This study has identified cattle as at least one of the definitive hosts of *Onchocerca sp.* 'Siisa', a filarial nematode previously only isolated from the vector *S. damnosum s.l.* The mitochondrial genotypes of what is generally considered to be *O. ochengi* form two distinct clades.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Reproductive biology of *Onchocerca ochengi*, a nodule forming filarial nematode in zebu cattle

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ABSTRACT

Onchocerca ochengi is a nodule-forming filarial nematode parasite of cattle in tropical Africa and closely related to the human pathogen *Onchocerca volvulus*. The adult worms reside in intradermal nodules. While females are sedentary, males may move between nodules. The first stage larvae (microfilariae) disperse in the skin of the host waiting to be taken up by the intermediate host. The density of microfilariae in the skin is largely independent of the number of adult worms present indicating some form of density dependent control. Recently, *Onchocerca* sp. Siisa, a form of *Onchocerca* distinguishable from *O. ochengi* by mitochondrial DNA sequences but not by morphology, was described to occur in cattle. This raised the question if *Onchocerca* sp. Siisa represents a different mitochondrial clade of *O. ochengi* or a new species. In order to study the reproductive biology and to understand this self-control of the off-spring population we systematically analyzed all *Onchocerca* nodules from the skin of one zebu cow and we examined a sample of microfilariae from a skin biopsy. We identified 87 *O. ochengi* females and 146 males. 56 (64.4%) of the females contained developing embryos. In order to assign the progeny to their respective parents we determined the genotypes at six nuclear and two mitochondrial molecular genetic markers in the adult worms, in a fraction of the progeny present in the uteri of the females and in the skin microfilariae. The 121 skin microfilariae we analyzed originated from at least 17 different mothers, which contributed rather differently to the total. Forty-five larvae (37.2%) were the progeny of a single female. Of the adult worms 16.7% were of the type *Onchocerca* sp. Siisa. These worms appeared to interbreed freely with the rest of the *O. ochengi* population and therefore belong to the same species.

1. Introduction

Most representatives of the filarial nematode genus *Onchocerca* are species-specific parasites of various ungulates. Many elicit the formation of nodules (Anderson, 2000). One of the very few non-ungulate hosts with *Onchocerca* parasites is man. *Onchocerca volvulus* is the causing agent of river blindness and, in spite of the success of *Onchocerca* control programs, continues to be a threat to millions of people (Hoerauf et al., 2011).

Onchocerca ochengi is a parasite of cattle in tropical Africa, best characterized in zebu (*Bos primigenius indicus*). With an estimated time of evolutionary separation of as

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little as 10,000 years, it is very closely related to the human pathogen *O. volvulus*, with which it shares the vector, the black fly *Simulium damnosum s.l.* (Krueger et al., 2007; Renz et al., 1994; Wahl et al., 1994). *O. ochengi* is well accessible because it lives in intradermal nodules in the inguinal region of the bovine host that are identifiable by palpation and can be easily removed for examination. This makes *O. ochengi* an attractive model case for studying the biology of nodule forming *Onchocerca* sp. (Renz et al., 1995; Trees et al., 1998).

O. ochengi females induce the collagenous nodules (Fig. 1), in which they grow up entangled and in very close contact with the host's tissue. They can reach up to 30 cm in length and they can reproduce for many years—presumably as long as their hosts live (5 to 10 years) (Determann et al., 1997; Wahl et al., 1994). Young *O. ochengi* females appear to avoid the immediate proximity of existing nodules. Thus *O. ochengi* nodules are rather dispersed. Mainly in heavily infested cattle, nodules can sometimes be found close to each other in the udder, teats and umbilicus, but still the individual nodules remain separate. This behavior is different from females of *O. volvulus*, which tend to form clumps of nodules consisting of female worms of different ages (Schulz-Key, 1988; Wahl et al., 1994). Males are much smaller than females (2 to 4 cm). They migrate to the females and are found within the nodules at various numbers, in average about one male per female (Renz et al., 1994). *O. ochengi* embryos develop and hatch in the uteri of their mothers (Fig. 1). The first stage larvae (called microfilariae) are released and disperse in the peripheral skin around the nodules where they accumulate and wait to be taken up by a black fly during a blood meal. This accumulation of skin-microfilariae is not strictly linked to the number of reproducing female worms. Rather in adult cattle, the density of skin microfilariae becomes largely independent of the number of adult worms present indicating some form of regulation (Trees et al., 1992). In principle there are three non-mutually exclusive ways of achieving this. First, many (in the extreme case all) females may produce fewer progeny when the density of circulating microfilariae is high. Second, a few (in the extreme case one) dominant females may reproduce at high rates while suppressing the reproduction of other females. Third, microfilariae may have reduced survival in a density-dependent manner.

Other than *O. ochengi*, a second nodule-forming species of *Onchocerca*, *O. dukei*, has been described in cattle. Because of the restricted occurrence of its vector, *Simulium bovis*, in Cameroon this species is believed to be limited to the Sudan-savanna, which lies about 150 km to the North of our sampling site (Renz et al., 1994; Wahl et al., 1994; Wahl and Renz, 1991). Further, based on a single observation of two larvae in a *Simulium damnosum s.l.* vector in Uganda, a form of *Onchocerca* morphologically and with respect to its mitochondrial DNA similar to *O. ochengi* was described as *Onchocerca* sp. variant Siisa (Krueger et al., 2007).

In two recent studies we reported (A) that *O. ochengi* females frequently produce progeny sired by different males simultaneously and that these males most of the time but not always were present in the nodule along with the female (Hildebrandt et al., 2012) and (B) that *Onchocerca* sp. variant Siisa occurs in black flies and as nodule forming adults in cattle in Cameroon demonstrating that this variant exists also in West Africa and that the zebu is at least one of its definite hosts (Eisenbarth et al., 2013). In these two studies we analyzed worms isolated in different places and at different times from multiple host individuals but from each host individual only a rather small fraction of the worms present. Further the two studies were limited to the characterization of either nuclear (Hildebrandt et al., 2012) or mitochondrial (Eisenbarth et al., 2013) genetic markers. Therefore, these investigations did not provide any information about what fraction of the adult worms were actually reproductively active and to what extent the different adults contribute to the pool of microfilariae present. Also we could not address the question if the different mitochondrial clades interbreed and therefore belong to one species or if *O. ochengi* and *Onchocerca*. sp. variant Siisa are reproductively isolated from each other and represent different species.

Therefore we undertook a detailed analysis of the population of nodule forming *Onchocerca* in one particular host individual with an intermediate parasite load. We isolated all *Onchocerca* nodules we could find in this zebu and we genotyped at multiple nuclear and mitochondrial loci (A) the adult worms, (B) a fraction of the progeny in the uteri of their mothers (if present), and (C) microfilariae from a skin sample. We show that a significant fraction of the adults contribute variably to the pool of circulating microfilariae and we present evidence strongly suggesting that the members of the two mitochondrial clades interbreed freely and therefore belong to the same species.

2. Materials and methods

2.1. Parasite material

The skin of a freshly slaughtered 3.5 years old female Zebu cattle was purchased on January 13th 2011 at the abattoir in Ngaoundéré, Cameroon. The skin was spread on the floor and all 88 nodules found were excised. After every nodule excision the position was marked and a picture of the skin taken. Based on these pictures the positions of the individual nodules were marked on the map shown in Fig. 2. The nodules were cut open and transferred individually into 1.8 ml Nunc cryofreezing tubes filled with 95% ethanol for storage and transportation at ambient temperature. Later, the nodules were individually transferred into PBS and dissected immediately.

In addition, skin samples (about 25 g each) were taken from the belly, the udder, and the back of the cow and preserved in ethanol. To obtain skin microfilariae, several small chunks (about 1 mm^3 each) were cut out and washed in 500 μl PBS for 5 to 6 h at 700 rpm in a Thermomixer at room temperature. The samples were digested in 500 μl collagenase of type II 2,5 mg/ml in PBS supplemented with 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 37 °C for 18 to 24 h at 700 rpm in a Thermomixer. Individual microfilariae were isolated and lysed immediately. Microfilariae were only found in the sample from the udder. For comparison, a second skin (containing 33 nodules) was purchased and processed in the same way on January 19th 2011. Partial

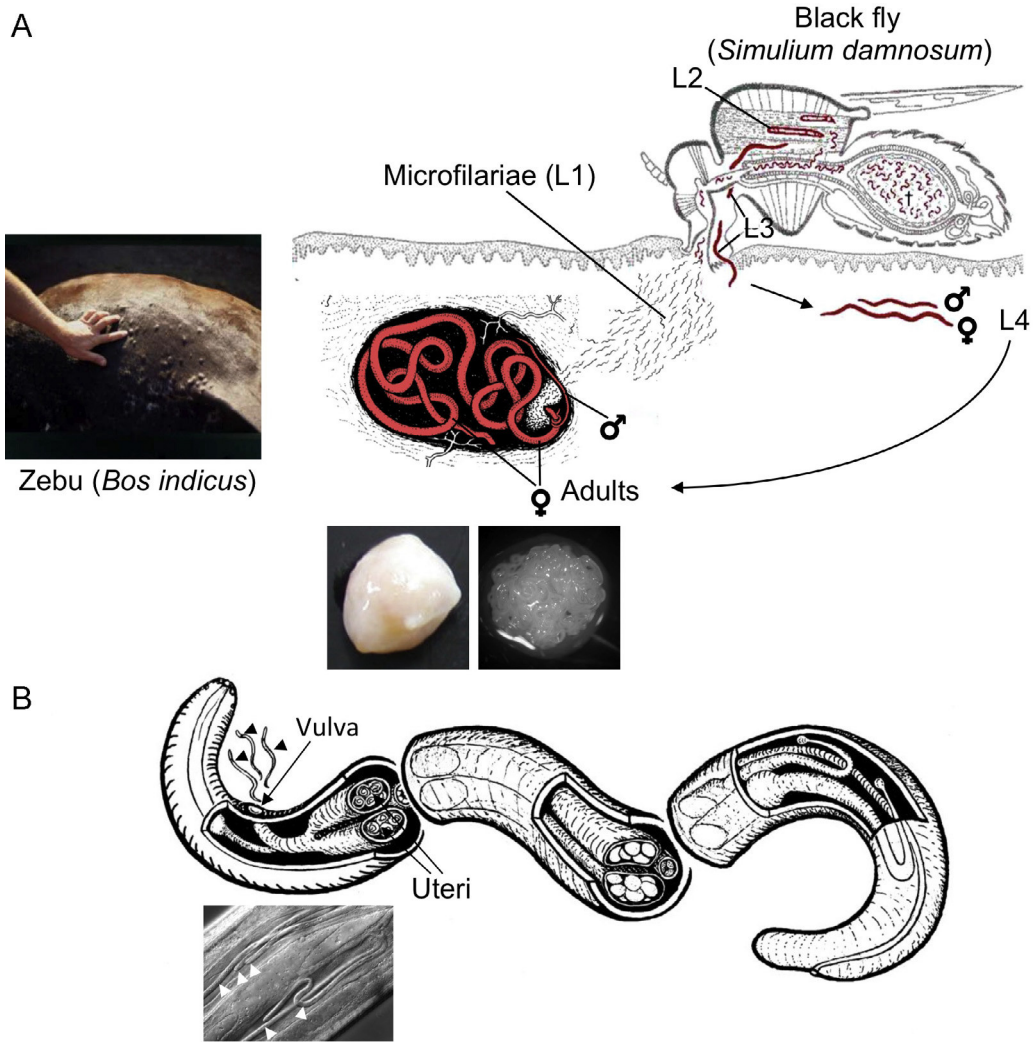

Fig. 1. Anatomy and life cycle of *O. ochengi*.
(A) The life cycle of *O. ochengi*. For explanation see text. The photographs show (from left) the belly of a zebu with numerous *O. ochengi* nodules, an indidvidual nodule (diameter about 1 cm) dissected from the skin of a zebu and an adult *O. ochengi* female after the digestion of the nodule with collagenase. (B) Schematic representation of an adult *O. ochengi* female. Anterior is to the left. The total length of the worm is about 20–30 cm. The embryos develop in the two-armed gonad. The first stage larvae (microfilariae, arrowheads) hatch within the uterus (differential interference contrast image) and leave their mother through the anteriorely located vulva. The figure was compiled using copyright protected drawings and photographs. Permission was granted by the copy right holder A. Renz.

analysis of 27 of the nodules from the first skin and of three nodules from the second skin was reported in our previous study (Hildebrandt et al., 2012).

All steps up to the conservation of the material in ethanol were carried out in the Programme Onchocercoses laboratory of the University of Tübingen in Ngaoundéré (www.riverblindness.eu). All subsequent analyses were done at the Max Planck Institute for Developmental Biology in Tübingen, Germany.

2.2. Genotyping

The worms were lysed and prepared for genotyping as previously described (Hildebrandt et al., 2012). Worms were genotyped at six nuclear and two mitochondrial markers. For marker details see Supplementary Table 1. Nuclear marker sequences were PCR amplified and sequenced as described in Hildebrandt et al. (2012) and mitochondrial marker sequences were PCR amplified and sequenced as described in Eisenbarth et al. (2013).

Except for *ytP160* the markers used were the same as in Hildebrandt et al. (2012) (nuclear markers) and in Eisenbarth et al. (2013) (mitochondrial markers). Each marker contains several polymorphic positions. For nuclear markers each combination of nucleotides at polymorphic positions within one copy of the marker is referred to as an allele. Because certain combinations of heterozygous and homozygous positions at polymorphic sites can result from more than one combination of alleles, it is not always possible to determine an individual's genotype solely based on the sequencing result. For reproductively active individuals, alleles were determined based on the genotypes

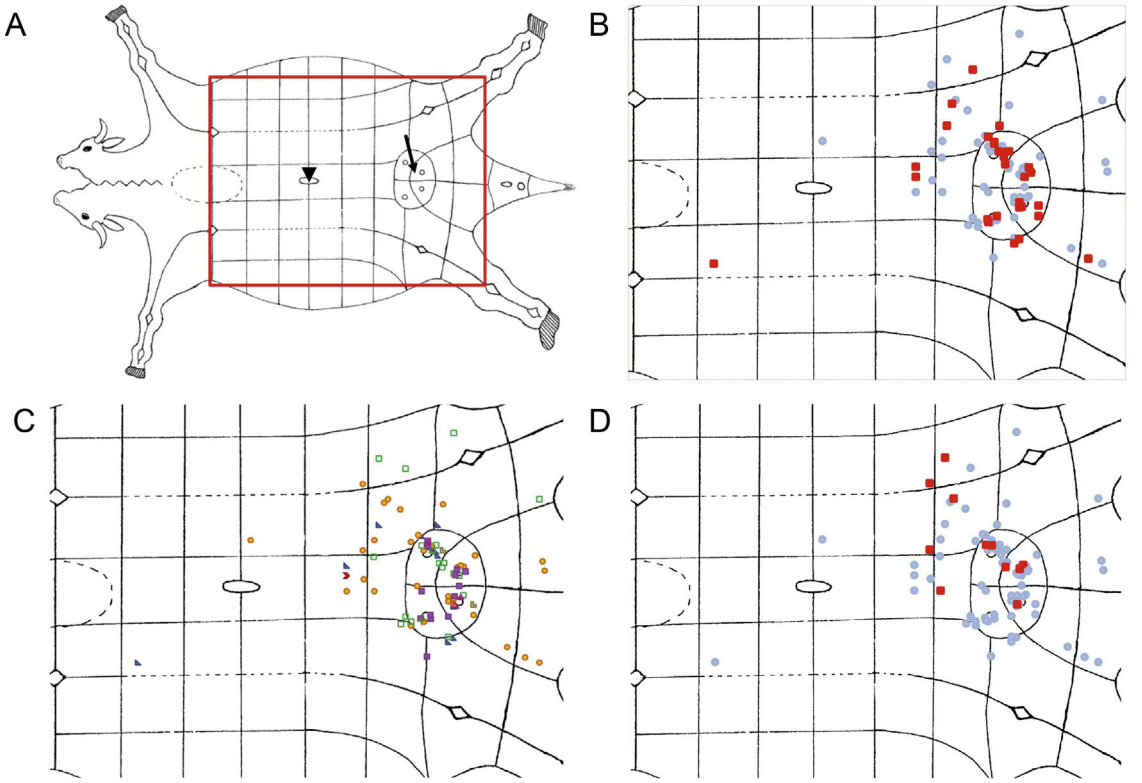

Fig. 2. Location of the *O. ochengi* nodules.
(A) Schematic representation of the skin of a zebu. Anterior is to the left, the skin is opened at the dorsal side. The arrow head and the arrow point to the umbilicus and the udder, respectively. The section shown in B–D is framed. (B) Distribution of nodules containing reproductive and non-reproductive females. Blue (in the bw version light) circles: nodules with offspring, red (dark) squares: nodules without offspring. (C) Distribution of the males. The following symbols indicate nodules with the number of males indicated: 0 males 1 male 2 males 3 males 4 males 5 males 7 males 8 males. (D) Distribution of nodules (mothers), to which microfilariae isolated from the skin could be assigned. Red (filled) squares: nodules with assigned microfilariae, blue (open) circles: other nodules. At least six more females must have contributed to the pool of sampled microfilariae. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of their progeny. Nucleotides present in the same copy of a locus are inherited in a strictly coupled manner. For non-reproducing animals, alleles were determined by cloning the PCR products and the sequencing of multiple clones; however, this was not done for all individuals.

2.3. Assigning parents to progeny

For embryos isolated from the uteri of their mothers a male present in the nodule was accepted as the likely father, if the genotypes at all loci successfully determined were consistent with the embryo being the offspring of this particular male and the mother. For skin microfilariae assignment to a mother was made, if based on nuclear and mitochondrial markers, all females but one could be excluded as mothers.

2.4. Phylogenetic analysis of the mitochondrial sequences

For each animal, the 12S and the 16S sequences were concatenated prior to analysis. This resulted in 25 different sequences (haplotypes 1 to 25). The following sequences were also included in our phylogenetic analysis (GenBank accession numbers): DQ523738, DQ523749 (*O.* sp. Siisa); DQ523740, DQ523751 (*O. dukei*); AY462920, AY462902, KC167339, KC167346, KC167347 (*O. volvulus*); AJ537512 (*Dirofilaria immitis*); AY462914, AY462897 (*O. ochengi*). Alignment and phylogenetic estimation were carried out using MEGA 5 version 5.2 (Tamura et al., 2011) choosing the MUSCLE algorithm for sequence alignment and maximum likelihood using the Tamura–Nei model for estimating the phylogeny. Gaps were included. Node support was estimated by bootstrapping (1000 bootstrap pseudoreplicates). For comparison, other trees, using different models and including or excluding gaps, were also calculated (see legend to Fig. 5).

2.5. Comparison of the allele distributions in the different mitochondrial clades

To visualize the relationships of the different alleles at a given locus we calculated a haplotype network using the program TCS ver. 1.21 (Clement et al., 2000). The analysis was performed separately for each nuclear marker using NEXUS files as input and allowing a maximum number of

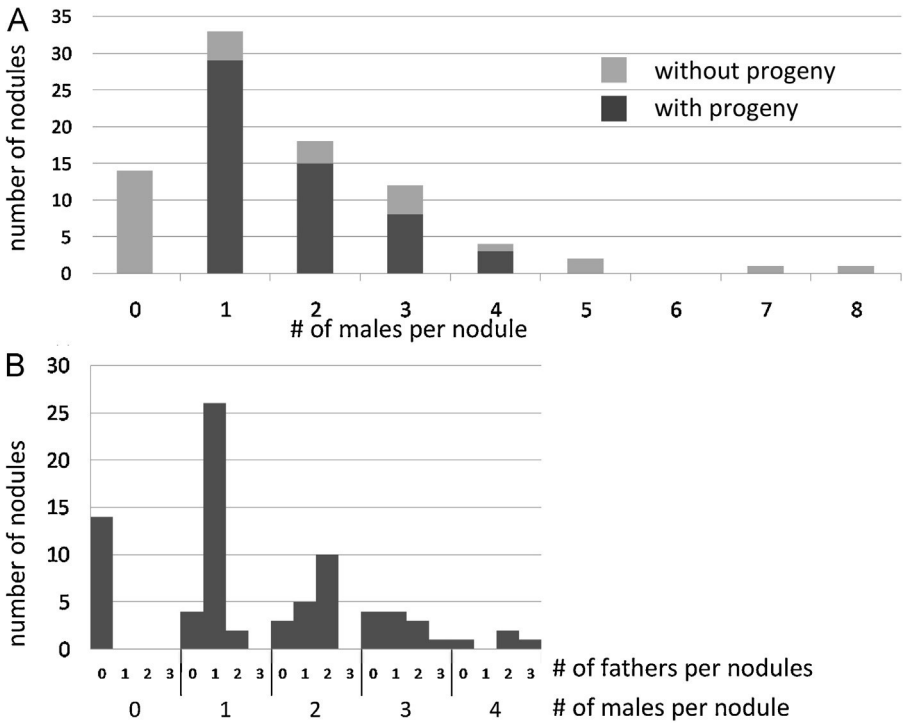

Fig. 3. Number of males per nodule.(A) Histogramm showing the number of males per nodule (*X*-axis) and number of nodules with the corresponding number of males (*Y*-axis). The bars are subdivided; dark grey indicates nodules with progeny while light grey indicates nodules without progeny. (B) Minimum number of males required to explain the progeny found in a nodule. *X*-axis bottom label: number of males present in the nodule; *X*-axis upper label: minimal number of fathers; *Y*-axis: number of nodules with the corresponding number of males in the nodule and minimum number of fathers. Notice that one of the reproductively active females found in a nodule with a single male carried only a very small number of very young embryos; determination of fatherhood of these embryos failed, and this nodule is included in A but not in B.

mutational steps of 100. The different mitochondrial clades (see Fig. 5) were mapped manually onto the TCS output.

2.6. Animal experimentation and ethics statement

Both cows were slaughtered in the context of the normal operation of the abattoir and were processed for human consumption. No special animal experimentation and ethical clearance was required.

3. Results

3.1. Distribution of the adult worms

A total of 88 nodules were found and dissected from the skin of an individual female zebu. The nodules were located on the ventral side of the host animal and strongly clustered at and around the udder (Fig. 2). Two nodules contained decaying females, indicating that either the worms that had induced the nodules were not anymore alive at the time of sampling or that the nodules were insufficiently preserved and had perished during transportation. Two nodules contained two females along with one male, whereas in all other nodules we found a single female and zero to eight males (Figs. 2C and Fig. 3). The total number of males isolated was 146 (in average 1.7 per nodule).

One non-reproductive female that was found in a nodule without males showed mitochondrial sequences very similar to what had been published for *O. dukei* (see below "interesting observations"). This individual was not included in any subsequent analyses. Of the remaining 87 females, 56 contained developing embryos, indicating that they were reproductively active (Fig. 2B and Fig. 3). Consistent with our earlier findings (Hildebrandt et al., 2012), the progeny within a particular female was sometimes derived from multiple males (Fig. 3B). Note that the datasets of this publication and of Hildebrandt et al. (2012) are overlapping but not identical, as described in Section 2.

3.2. High diversity in nuclear markers

Earlier studies had suggested that a single bite by the vector transfers, in most cases, only one or a few *O. volvulus* larvae (Renz, 1987). There is indication that this is also the case for *O. ochengi* (AR unpublished observation). As a consequence, one would expect that the different parasite individuals in a particular host would have been acquired independently, and therefore be unrelated. Consistent with this, we found high genetic diversity (up to 24 different alleles per locus) among the *Onchocerca* worms within this one host animal (Fig. 4). The notion that the worms present in this one host individual represent a large fraction of the genetic variation present in the entire population is also supported by data derived from a second Zebu slaughtered one week later. In this second animal we found a total of 33 nodules containing 33 females and 20 males. From the

Fig. 4. High genetic diversity.Apparent allele distributions in females, males, and skin microfilariae (columns) are given for the six markers tested (rows). Colors (shades of grey) indicate the allele numbers. Between four (*ytP160*) and 24 (*ytP162*) different alleles per locus were present in this particular host individual. Notice: not all alleles we know of were found in the host individual described here; therefore the allele numbers go up to 27. The allele distributions shown are approximations but not accurate measures of the allele frequencies (see Section 4). Only unambiguously identified alleles are included in the figure (cf. Section 2). Therefore, uneven numbers are possible. We were not able to genotype all individuals at all six markers. Therefore, the numbers differ between markers.

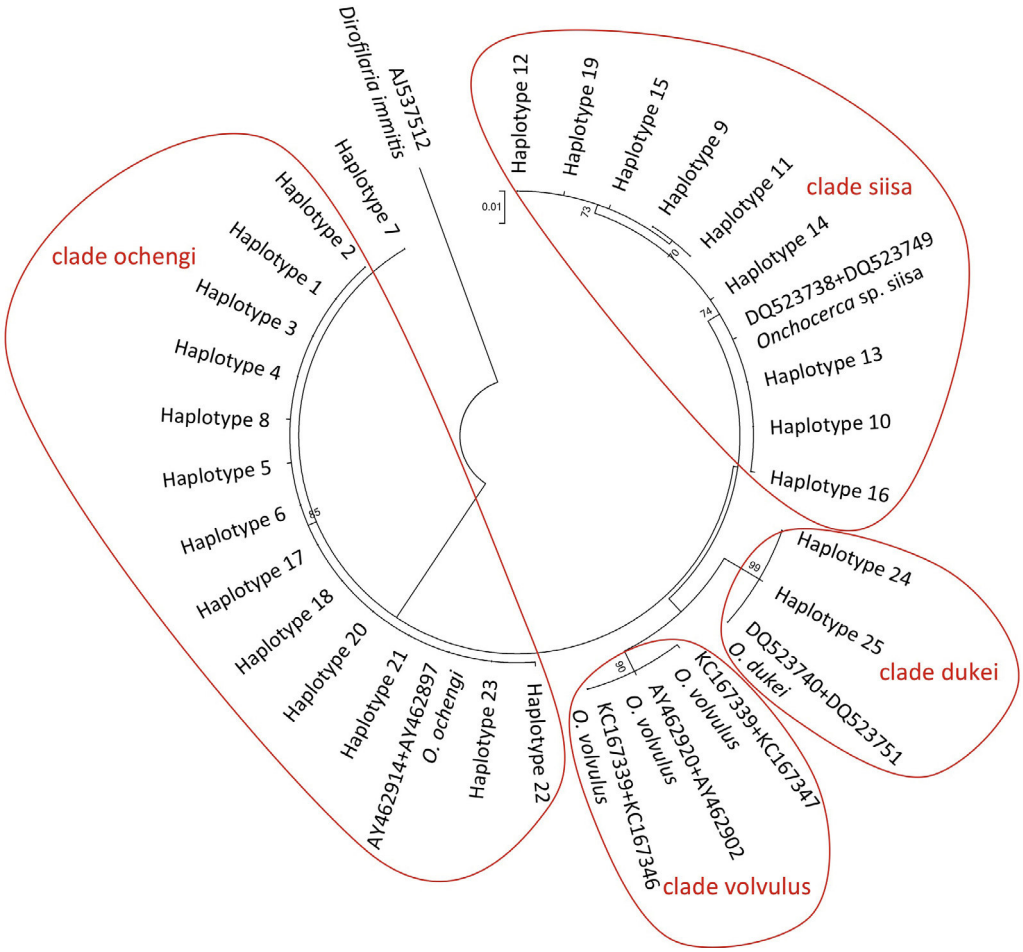

Fig. 5. Mitochondrial haplotypes found.Unrooted maximum likelihood tree reconstructed from concatenated portions of the 12S and the 16S mitochondrial rDNA genes. "Haplotype . . ." indicates sequences observed in this study; sequences taken from Genbank are labeled by accession number and the species name they belong to. Only bootstrap values of 70 and above are shown. Similar trees were also calculated using different models (neighbor joining, maximum parsimony) and including or excluding gaps. The separation into the major groups, also referred to as "clades" (framed), was very robust. The resulting relationships of the major clades and of haplotype 7 changed with the exact parameter settings and the model used. Therefore, the phylogenetic relationships of these major groups cannot be resolved with the available data.

6 nuclear markers combined, we identified a total of 48 different alleles, only 2 of which were not present in the cow that is the principle subject of this study. The high number of possible allele combinations allowed us to determine likely parents for a large fraction of embryos and larvae.

3.3. Multiple interbreeding clades of mitochondrial haplotypes

We determined portions of the mitochondrial 12S and 16S rDNA sequences, in addition to the nuclear sequences described above. Most of the sequences grouped with one of the mitochondrial clades described by Eisenbarth et al. (2013) (Figs. Fig. 5 and 6A). One clade contains the sequences previously published for *O. ochengi* (referred to as clade or type "Ochengi"), whereas the other one includes the sequence derived from *Onchocerca* sp. variant Siisa (referred to as clade or type "Siisa"). However, three sequences did not fit this pattern. The sequences for two worms (one male, one female) grouped with sequences published for *O. dukei* (Krueger et al., 2007) (haplotypes 24 and 25, Fig. 5, see "additional interesting observations"), The sequence for another male (haplotype 7, Fig. 5), depending on the model used to reconstruct the phylogenetic relationship and the exact parameter settings, sometimes grouped with the clade "Ochengi" and sometimes appeared to represent its own additional clade.

In the following we address the question of the species status of the two mitochondrial clades described. The three haplotypes 7, 24 and 25 were not included in this analysis. First, we asked if there is evidence for assorted pairing. We considered each combination of a female and a male present in the same nodule a pair and asked if individuals were more likely to form pairs with partners from the same clade. 82.5% of the available females were of the Ochengi and 17.5% of the Siisa type (Fig. 6A). If they had no preference, for males of both clades, one would expect 82.5% of the pairs to be with females of the Ochengi and 17.5% of the Siisa type. We observed (Fig. 6B) 19 (expected 19) pairs of

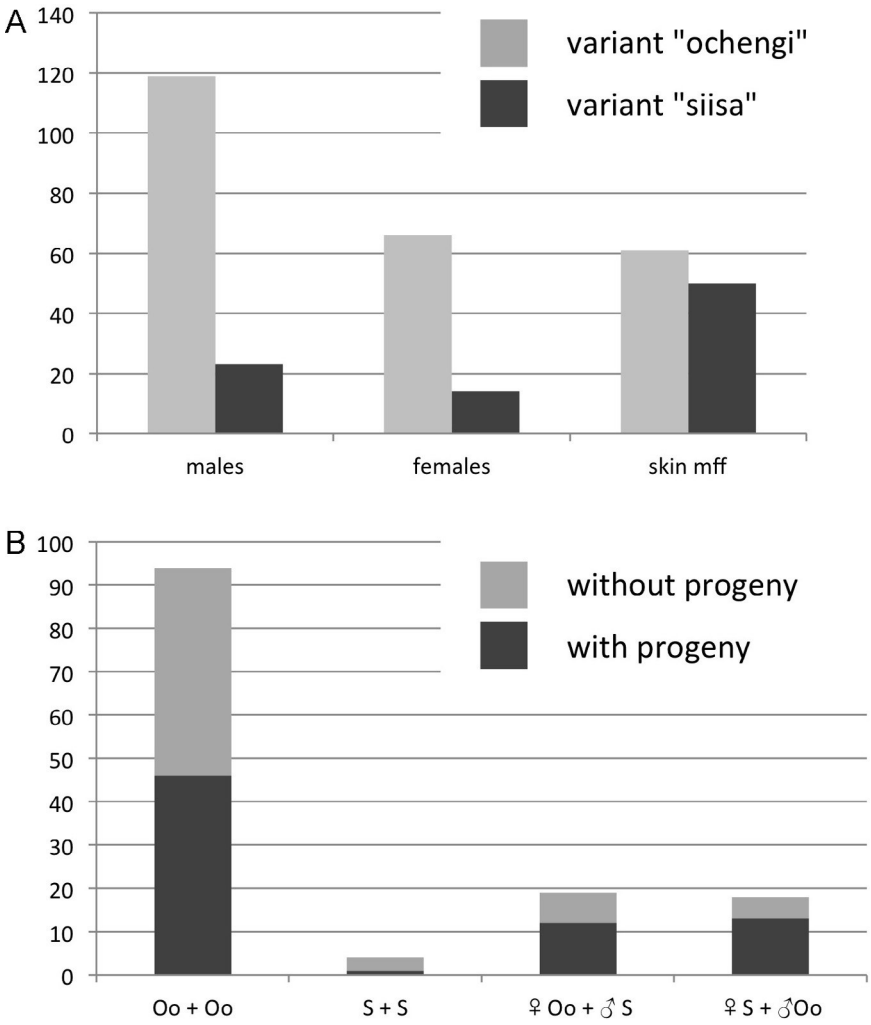

Fig. 6. Interbreeding of the two mitochondrial clades.(A) Number of males, females, and skin microfilariae with a mitochondrial haploptype variant "ochengi" (light grey) and variant "Siisa" (dark grey). (B) Number of male/female pairs with a particular combination of mitochondrial haplotypes. Every male and female found in the same nodule were considered a pair; as such, individuals can contribute to more than one pair when more than one male or female is present. Pairs were considered to have produced progeny together if we found offspring of the male in the uterus of the female (cf. Section 2.3). Oo variant "ochengi"; S: variant "Siisa". Pairs with progeny are in dark grey, pairs without progeny are in light grey. Note that we were not able to establish the mitochondrial haplotypes for 2 males, 7 females, and 10 microfilariae.

Siisa males with Ochengi females, 4 (expected 4) pairs of Siisa males with Siisa females, 18 (expected 19.6) pairs of Ochengi males with Siisa females, and 94 (expected 92.4) pairs of Ochengi males with Ochengi females. All observed values are very close to the expected ones and there is no indication of assorted pairing.

Second, we asked if the inter clade pairs did mate successfully. For 25 (67.6%) out of 37 inter clade pairs we could confirm successful mating because we found the resulting progeny in the uteri of the females (Fig. 6B). Of 98 intra clade pairs we could confirm successful mating for 47 (48.0%). From this we conclude that inter-clade pairs are not less likely to produce progeny than intra clade pairs. Among the skin microfilariae we genotyped (see Section 3.4) we found 46 that, based on their nuclear genotypes, most likely had parents of different mitochondrial type. This shows that the progeny derived from inter clade mating events are viable at least up to the skin microfilarial stage.

Third we analyzed the nuclear allele distribution among the two mitochondrial clades. We calculated the relationships of the different alleles using the program TCS ver. 1.21 (Clement et al., 2000) (see Section 2 for details) and mapped the mitochondrial clades onto the resulting network (Fig. 7). Also this analysis did not provide any indication for two separate genetically isolated populations.

Although no formal proof, these results very strongly suggest that the two mitochondrial clades, with respect to their nuclear genomes, form one population. There is no reason to postulate that *Onchocerca* sp. Siisa is a new species different from *O. ochengi*. The presence of separable mitochondrial clades probably indicates that the *O. ochengi* population currently found in Cameroon is the product of previously separated but currently connected populations.

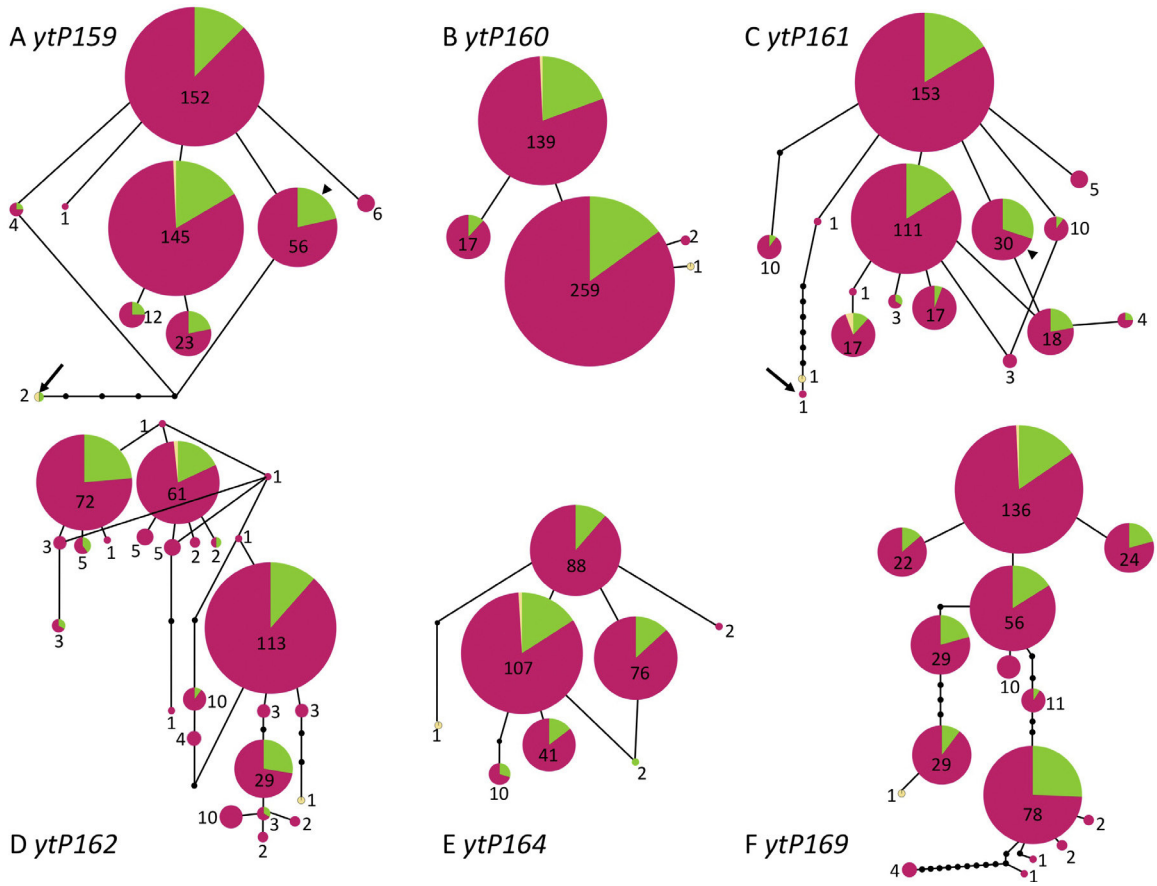

Fig. 7. Nuclear allele distribution in the different mitochondrial clades.Relationship of the different alleles at the six nuclear loci analyzed as determined by the program TCS (Clement et al., 2000, see Section 2). Every edge represents one difference (one different nucleotide or one nucleotide inserted/deleted). The length of an edge is not informative. Colored disks represent alleles (haplotypes) present in our samples. The size of the disk roughly represents the allele frequencies (see also caption to Fig. 4). The absolute number of occurrences is indicated in or next to the disks. The mitochondrial haplotype clades (Fig. 5) were mapped onto the allele networks. For each allele the fraction present in animals of a particular mitochondrial clade is represented by color/shade of grey (type ochengi—red/dark, type Siisa—green/intermediate, type *dukei*—yellow/light). The only animal with the mitochondrial haplotype 7 is not included in this figure. In A and C the possible *O. dukei* derived alleles in animals with non-*dukei* mitochondrial genotypes (arrows) and the common alleles they were heterozygous with (arrowheads) are indicated (see Section 3.5). (A) marker *ytP159*. (B) marker *ytP160*. (C) marker *ytP161*. (D) marker *ytP162*. (E) marker *ytP164*. (F) marker *ytP169*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Multiple females contribute variably to the pool of circulating microfilariae

As outlined above, 56 females contained embryos and larvae in their uteri indicating that they were reproductively active (Fig. 2B). The pool of microfilariae isolated from the skin biopsy from the udder (see Section 2) was genetically diverse (Fig. 4). Assuming that we found and genotyped all the parents of the microfilariae we analyzed (see Section 4) and taking into consideration the bi-parentally inherited nuclear markers and the maternally inherited (Sato and Sato, 2011 and references therein) mitochondrial markers, we were able to assign 89 of the 121 skin microfilariae to 11 different mothers (Fig. 2D). The mothers of the remaining 32 microfilariae could not be unambiguously determined but the larvae must have originated from at least six different additional mothers. The minimal number of mothers required to explain the genotypes of the microfilariae isolated from this one skin biopsy is therefore 17. However, 45 microfilariae (37.2% of the total) were the progeny of a single female. The genotypes of all 45 microfilariae were consistent with the assumption that they were sired by either of the two males present in the mother's nodule. This female was located in the udder but was not the closest reproductively active female to the place where the microfilariae were collected (Fig. 2D). The other 10 females contributed 12, 11, 5, 4, 3 (3 animals), and 1 (3 animals) microfilariae, respectively. Interestingly, the females that contributed 12 and 11 larvae were located outside of the udder and rather distant from the sampling site. Together, these findings argue against the hypothesis that all reproductive females contribute more or less equally to the population of skin microfilariae. However, based on this study, we cannot know if these contributions remain more or less stable over space and time.

3.5. Additional interesting observations

Below, we describe a few additional observations we consider interesting. These are single observations and they cannot be used to draw firm conclusions about *Onchocerca* biology. However, we believe that making them public may be worthwhile for others studying *Onchocerca* parasites.

First, for one non-reproductive female in a nodule without males, the mitochondrial 12S and 16S sequences were close to what had been published for *O. dukei* (Krueger et al., 2007) (haplotype 25, Fig. 5), a species so far only found in the Sudan savanna of Northern Cameroon, approximately 150 km to the North-East from our study area (Wahl and Renz, 1991). Genotyping of nuclear markers failed. We do not know if this animal was truly *O. dukei* or may be a hybrid between the two species (see also below). This female is not included in any of the analyses.

Second, one male was heterozygous at all markers analyzed and always contained one common allele and one allele found in only one (*ytP159*, see below) or no (all other markers) other individuals in our study (Fig. 7 yellow (light grey in print version) label). With respect to its mitochondrial sequences (haplotype 24, Fig. 5), it grouped very closely with sequences published for *O. dukei*. Most likely, this individual was a hybrid between an *O. ochengi* father and an *O. dukei* mother. This male did father progeny, which developed at least to the stage we genotyped (late embryo) and for each nuclear marker locus both alleles were represented in the progeny.

Interestingly, two males shared rare alleles with this putative hybrid at a single locus each (Fig. 7). One was of the mitochondrial type Siisa and contained, at locus *ytP159*, the same allele as the putative hybrid male (arrow in Fig. 7A) along with a common allele (arrowhead in Fig. 7A). At all other loci it had alleles found multiple times in our sample. The other one was of the mitochondrial type Ochengi and contained at locus *ytP161* an allele very similar to the unique allele of the putative hybrid (arrow in Fig. 7C) along with a common allele (arrowhead in Fig. 7C). At all other loci it had alleles found multiple times in our sample. These findings suggest that there might be limited gene flow between *O. ochengi* and *O. dukei*.

4. Discussion

Here, we systematically analyzed the *O. ochengi* population in one particular host animal. With 88 nodules, the worm burden of this cow was in the usual range for *Onchocerca*-susceptible animals of this age and exposed to natural transmission on the Adamaoua plateau near Ngaoundéré (Achukwi et al., 2004; Renz et al., 1995; Trees et al., 1992; Wahl et al., 1994). The number of males we found in each nodule (average of 1.7 per nodule) was somewhat higher than the previously described (Renz et al., 1994). Of the 87 females, 56 (64%) contained developing embryos, indicating that they were reproductively active at the time of sampling. These numbers are in agreement with earlier findings for *O. ochengi* and *O. volvulus*, which had suggested that *Onchocerca* females undergo phases of reproduction interspersed with times of reproductive quiescence (Duke, 1993; Duke et al., 1990; Schulz-Key, 1990).

We found the population of *O. ochengi* in this one host animal to be highly genetically diverse. This suggests that the *O. ochengi* present in one host animal do not tend to be closely related. This was expected based on earlier studies that had demonstrated that the number of infective larvae transmitted in a single bite of *Simulium damnosun s.l.* is very small (Renz, 1987) and that the number of nodules in a particular host grows gradually (Achukwi et al., 2004). While the data presented in Fig. 4 are fully suitable to support the claim that many different alleles exist at very different frequencies, they should not be taken as accurate measurements of the allele frequencies. It is likely that some alleles, for which our primers do not work, exist. Also, if the marker sequencing results of non-reproductive individuals could be explained with known alleles, in many cases we did not clone the PCR products and sequence individual clones to confirm these alleles. If only two alleles were possible, they were accepted and included in the analysis, but if multiple allele combinations were possible then the animal was not included in Figs. 4 and 7.

In agreement with earlier observations (Hildebrandt et al., 2012), we found that reproductive activity is almost always associated with the physical presence of at least one male in the nodule. It is, however, striking that all four females in nodules with more than four males were not reproducing. Given that 77.5% (55/71) of females in nodules with males did contain progeny (Fig. 3A) it is very unlikely (0.26% by simple probability calculation) that the four nodules with the highest number of males did not contain progeny just by chance. There are two possible explanations for this. First, it may be that these females were just becoming reproductively active, either for the first time or after a period of reproductive quiescence, and they were therefore particularly attractive for males. Alternatively, too many males may actually be detrimental for the reproduction of females, as has been observed in the model nematode *Caenorhabditis elegans* (Wegewitz et al., 2008).

The fact that a female contains developing embryos does not necessarily mean that it also contributes substantially to the pool of circulating microfilariae. For example, it has been observed that gravid females frequently contain dying progeny (Renz et al., 1995). This may indicate that some factor, for example signals by other females or the actions of a newly arrived male, may at least temporarily prevent certain females from successfully reproducing.

To our knowledge, this is the first study that determined parentage of circulating microfilariae. Our analysis demonstrated that different females contributed differently to the pool of circulating microfilariae at a particular location and time. Our study is a snap shot, looking at one location (the udder) at one particular time point and we cannot conclude anything about the dynamics of this population of worms waiting for a vector.

It must be noted that our assignment of circulating microfilariae to particular parents assumes that we did indeed find and successfully genotype all reproductive adults, but it is likely that some adults were missed. First, we cannot be absolutely sure that we found all nodules in the first place. Furthermore, one would expect that a few males were in the process of migrating between nodules

at the time of sampling; such males were certainly missed. We were unable to obtain any nuclear genetic information for eight females; none of these eight females were reproducing at the time of sampling, but this does not exclude the possibility that they had earlier produced microfilariae, and that these microfilariae were still present in the periphery. The reason for the relative high failure rate among non-reproducing females is that for females we used only the most anterior portion of the body, which is devoid of any part of the gonad, which might contain genetically distinct progeny or sperm (see Hildebrandt et al., 2012). As a consequence, only very little DNA was available for females. For reproductive females the genotypes of the mothers could also be derived/confirmed from the genotype of the progeny, which, of course, were not available for non-reproductive females. In spite of these caveats, we are confident that the vast majority of our parental assignments were generally correct for the following reasons: (1) All of the alleles found in the microfilariae (51 nuclear alleles, 13 mitochrondrial haplotypes) were also found in adults. (2) The microfilariae were assigned to mothers solely based on their own and on the mother's genotypes; nevertheless, in the vast majority of cases (86 out of 89), the genotypes of the males found in the nodule with the putative mother were compatible with the genotypes of the progeny. (3) There were only four microfilariae (likely siblings) for which we found no possible mother in our sample. This indicates that though we are indeed missing some mothers, their number is presumably small.

Based on morphology and on mitochondrial sequences, *O.* sp. Siisa had been described as a variant of *Onchocerca* very closely related to *O. ochengi* and *O. volvulus* (Krueger et al., 2007). While it was originally described based on two individuals found in one black fly in East Africa (Krueger et al., 2007), it was later also found in Cameroon (Eisenbarth et al., 2013). This study also demonstrated that *O.* sp. Siisa is a nodule forming parasite of cattle, which, by morphological criteria, would have been classified as *O. ochengi*. Our data strongly suggest that *O. ochengi* and *O.* sp. Siisa interbreed freely and therefore belong to the same species. Since, for technical reasons we could not demonstrate directly that the inter clade progeny formed is indeed fertile we cannot formally exclude an extremely recent genetic isolation of the two clades. However, we consider this most unlikely, mainly for two reasons. First, we could not detect any assorted mating. Should the two mitochondrial clades indeed belong to different species one would have to postulate that the worms themselves cannot tell their own species apart from the sister species. Second, both species resulting from this very recent speciation event would have retained essentially the entire ancestral genetic diversity, indicating that in the process none went through a genetic bottleneck. This is very unlikely.

We identified one individual that likely was a hybrid between *O. ochengi* and *O. dukei* and two more individuals that at one locus each carried an allele that might have been of *O. dukei* origin. These findings indicate that there might be occasional gene flow between these two species. Additional studies, preferentially of whole genome sequences of *O. ochengi* and *O. dukei* individuals, will be required to confirm or reject this hypothesis. Although not yet formally published, a reference genome sequence for *O. ochengi* is already publically available (see http://www.nematodes.org/genomes/onchocerca_ochengi/). Given the rapid development of sequencing technologies single worm genome sequencing should be technically possible very soon, at least for adult males, which provide much more genomic DNA than microfilariae and other than adult females do not carry progeny with different genotypes in their bodies.

To end on a very speculative note: it is interesting that, based on mitochondrial sequences, *O. volvulus* is phylogenetically as closely (Fig. 5) or even slightly more closely (Krueger et al., 2007) related to *O. ochengi* than is *O. dukei*. This opens the possibility that there might also be limited gene flow between *O. volvulus* and *O. ochengi*. Should this be the case, this would create the possibility for genetic features, for example resistance against ivermectin, that arise in *O. ochengi* in cattle to spread into the human pathogen *O. volvulus*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2014.06.006.

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Immune recognition of *Onchocerca volvulus* proteins in the human host and animal models of onchocerciasis

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Abstract

Onchocerca volvulus is a tissue-dwelling, vector-borne nematode parasite of humans and is the causative agent of onchocerciasis or river blindness. Natural infections of BALB/c mice with *Litomosoides sigmodontis* and of cattle with *Onchocerca ochengi* were used as models to study the immune responses to *O. volvulus*-derived recombinant proteins (*Ov*ALT-2, *Ov*NLT-1, *Ov*103 and *Ov*7). The humoral immune response of *O. volvulus*-infected humans against *Ov*ALT-2, *Ov*NLT-1 and *Ov*7 revealed pronounced immunoglobulin G (IgG) titres which were, however, significantly lower than against the lysate of *O. volvulus* adult female worms. Sera derived from patients displaying the hyperreactive form of onchocerciasis showed a uniform trend of higher IgG reactivity both to the single proteins and the *O. volvulus* lysate. Sera derived from *L. sigmodontis*-infected mice and from calves exposed to *O. ochengi* transmission in a hyperendemic area also contained IgM and IgG1 specific for *O. volvulus*-derived recombinant proteins. These results strongly suggest that *L. sigmodontis*-specific and *O. ochengi*-specific immunoglobulins elicited during natural infection of mice and cattle cross-reacted with *O. volvulus*-derived recombinant antigens. Monitoring *O. ochengi*-infected calves over a 26-month period, provided a comprehensive kinetic of the humoral response to infection that was strictly correlated with parasite load and occurrence of microfilariae.

Introduction

Onchocerca volvulus is a tissue-dwelling, vector-borne nematode parasite of humans and is the causative agent of onchocerciasis or river blindness (http://www.who.int/tdr/diseases-topics/onchocerciasis/en/). Onchocerciasis is the second leading cause of infectious blindness worldwide. It affects about 18 million people worldwide – 99% living in Africa – 270,000 of whom are blind and 500,000 visually impaired. *Onchocerca volvulus* is transmitted during blood meals of black flies of the *Simulium damnosum* complex and can persist in the host for 10 years or longer (Karam *et al.*, 1987;

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Plaiser *et al.*, 1991). The adult worms dwell in subcutaneous nodules. The pathology associated with onchocerciasis is mediated mainly by migrating first-stage larvae called microfilariae (mf) in the skin and by immune responses against the mf which are continuously released by fertilized adult female worms (Kazura *et al.*, 1993; Allen *et al.*, 2008). The migrating mf can survive more than 1 year and thus accumulate in the skin. They may invade the eyes, and their presence in both anatomical positions can lead to tissue destruction (Anderson *et al.*, 1974). The inflammatory response against excretory/secretory products (ESP) of the living and internal components of the dead and disintegrating mf results in dermatitis, of which there are two types: hyperreactive onchodermatitis ('sowda') and hyporeactive onchodermatitis (Brattig, 2004; Korten *et al.*, 2011).

Studies on the biology of *O. volvulus* have been limited due to lack of appropriate animal models (Lok & Abraham, 1992). In the recent past, investigations on the immunology of onchocerciasis have been focused on two animal models, using mice and cattle (Allen *et al.*, 2008). The mouse model employs the nematode *Litomosoides sigmodontis*, which is a natural parasite of the cotton rat, *Sigmodon hispidus*. This filaria was found to undergo complete development in BALB/c mice and can produce patent infections with blood-circulating, sheathed mf within 55–60 days post infection (Petit *et al.*, 1992). Furthermore, *L. sigmodontis* belongs to the family of onchocercidae, like the human filariae, and has several common features, such as patterns of larval migrations (Bain *et al.*, 1994), and genomic and biochemical structures (Xie *et al.*, 1994; Zahner *et al.*, 1995). *Litomosoides sigmodontis* also shows extensive immunological cross-reactivities with *O. volvulus* (Bain *et al.*, 1994), *Brugia* species, *Loa loa*, *Wuchereria bancrofti* and other *Onchocerca* species (Harnett *et al.*, 1989; Maréchal *et al.*, 1994).

Another adequate approach is to study the interaction of the nematode *Onchocerca ochengi*, with its natural bovine host, the Zebu cattle in Africa. The skin-dwelling mf are very similar to those of *O. volvulus* and are transmitted by the same black fly vector *S. damnosum sensu lato* (Wahl *et al.*, 1998). It is the closest known relative of *O. volvulus* and therefore regarded as an excellent model for onchocerciasis research (Renz *et al.*, 1995; Achukwi *et al.*, 2007). There is extensive antigen cross-reactivity between *O. ochengi* and *O. volvulus* antigens (Hock *et al.*, 1992; Graham *et al.*, 2000). The immunological cross-protection suggested (Wahl *et al.*, 1998) was demonstrated following the vaccination of cattle with live *O. volvulus* L3 (Achukwi *et al.*, 2007). Infection of cattle with *O. ochengi* leads to the formation of nodules, which resemble those induced by *O. volvulus* and can be quantified and enumerated by palpation of the skin (Trees *et al.*, 1992; Wahl *et al.*, 1994; Achukwi *et al.*, 2004). In general, four *Onchocerca* species are common parasites of cattle in Africa: *O. ochengi* (Bwangamoi, 1969) and *O. dukei* (Bain *et al.*, 1974), which form nodules in the ventral skin or on the fasciae of the thoracic muscles, respectively. The adult worms of *O. gutturosa* (Neumann, 1910) normally reside in the loose connective tissues of the nuchal ligament and other tendons, whereas *O. armillata* (Raillet & Henri, 1909) inhibits the intima of the aorta. All these species generally exhibit low pathogenicity in cattle. Microfilariae of the four species can be detected by examination of infected skin biopsies (Wahl *et al.*, 1994).

In these two models, the interaction of filarial stages with their hosts can be investigated (Allen *et al.*, 2008).

Four excretory/secretory proteins which are expressed at various stages of the life cycle of *O. volvulus* were selected for the study. These are: abundant larval transcript-2 (*Ov*ALT-2, 18 kDa) which is secreted in the late L2 and L3 larvae during growth in the vector, and also via the pseudocoelom and cuticle in the definitive host. This protein plays an important role in parasite entry into the host, and immunization experiments yielded 74–76% protection in animal models (Gregory *et al.*, 2000; Yang *et al.*, 2004; Madhumathi *et al.*, 2010). Previous studies have suggested ALT-2 as a promising vaccine candidate antigen (Munirathinam *et al.*, 2004; Ramachandran *et al.*, 2004). BALB/c mice immunized with DNA as well as recombinant ALT-2 provided partial (DNA) to 70% (protein) protection against challenge with *Brugia malayi* (Ramachandran *et al.*, 2004). Similar immunization experiments with ALT-2 in jirds gave the same level of protection (Munirathinam *et al.*, 2004). Antibodies to antigens of low molecular mass are considered to be the most reliable serological indicators of infection in humans (Weiss & Karam, 1989), and molecules of 16, 17 and 20 kDa have been cloned and applied for immunodiagnosis of onchocerciasis (Ramachandran, 1993).

Onchocystatin (*Ov*7, 17 kDa) is expressed in all developmental stages of the parasite except the mf. It is a cysteine protease inhibitor (Lustigman *et al.*, 1992a) which inhibits human T-cell proliferative responses and induces interleukin (IL)-10 release by macrophages (Schonemeyer *et al.*, 2001).

Microfilariae surface-associated antigen (*Ov*103, 15 kDa), is expressed in mf and also found in the cuticle and hypodermis of female adult worms (Lustigman *et al.*, 1991). This protein stimulates immune responses in chimpanzees and cattle (Johnson *et al.*, 1995; Graham *et al.*, 2000).

Novel larval transcript 1 (*Ov*NLT-1, 24 kDa) is expressed by the larvae and adult worms (Frank *et al.*, 1999) and plays a role in evasion of host immune response and immunopathology (Grieve, 1990).

The kinetics of specific and cross-reactive immune responses during the natural course of infection was studied. The proteins were produced and mice experimentally infected with *L. sigmodontis* were examined to see if they produce antibodies that cross-react with these *O. volvulus* antigens. Likewise, studies were conducted to see whether calves naturally exposed to the transmission of *O. ochengi, O. gutturosa* and other filarial species will produce antibody responses against these heterologous proteins, and hence whether there is a relationship between the *O. ochengi* nodule loads, the microfilaria levels in the skin and the protein-specific immune responses over a 26-month period of natural exposure.

Materials and methods

Preparation of DNA and candidate antigens

Total RNA was extracted from adult *O. volvulus* using Trizol reagent (Invitrogen, Carlsbad, California, USA)

according to the manufacturer's protocol. First strand c′-DNA was synthesized using 1.5 μg of total RNA as template following the manufacturer's recommendations.

The accession numbers of proteins supplied the information needed for primer design: *Ov*NLT-1 (AAD11969.1), *Ov*ALT-2 (AAD27588), Ov103 (AAA63412) and *Ov*7 (P22085). Following amplification, the polymerase chain reaction (PCR) products and the expression vector pJC40 (Clos & Brandau, 1994) were digested using appropriate FastDigest® restriction enzymes (Thermo Fisher Scientific, Lafayette, Colorado, USA) and ligated using T4 DNA ligase (Invitrogen). Five microlitres (μl) of each ligation were transformed into XL10-gold ultra competent cells according to the supplier's protocol (Stratagene, California, USA). Positive clones were identified by test digestion and sequencing. Following transformation of the respective expression plasmids into *Escherichia coli* Rossetta gami DE3 cells (*Ov*NLT-1) or *E. coli* BL21DE3 Star cells (*Ov*ALT-2, *Ov*103, *Ov*7) (Stratagene), expression of the tagged proteins was initiated by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) (0.05 mM IPTG for *Ov*NLT-1, 0.5 mM IPTG for *Ov*ALT-2 and 1 mM IPTG for *Ov*7) once the cultures had reached $A_{600} = 0.5$ (*Ov*-ALT-2, *Ov*7) or $A_{600} = 0.2$ (*Ov*NLT-1). Following induction, cells were left to grow for additional 3 h at 37°C (*Ov*7) or overnight (*Ov*NLT-1, *Ov*ALT-2). Expression of *Ov*103was carried out by auto-induction (Studier, 2005).

Cells were harvested by centrifugation and the resulting bacterial pellets were stored at −20°C until further use. Pellets were resuspended in 50 mM Tris buffer (pH 8.0) containing 500 mM NaCl, 10% glycerol, 0.1% (v/v) Triton X-100, 10 mM imidazole and 1 mM phenylmethylsulphonyl fluoride (*Ov*103, *Ov*7), with the addition of 5 mM dithiothreitol (DTT) to the lysis buffer (*Ov*NLT-1 and *Ov*ALT-2), and sonicated using a digital sonifier set to 30 W and 30% amplitude (Branson Ultrasonic Corp., Chicago, Illinois, USA). Following sonication, the resulting lysate was clarified by centrifugation at 10,000 × *g* for 30 min.

The resulting supernatant was purified by affinity column chromatography with profinity™ IMAC Ni^{2+}-nitrilotriacetic acid resin (Bio-Rad Laboratories, Germany). Incubation of the resin–lysate mixture was increased to 2 h at 4°C and washing was carried out using 25 bed volumes of washing buffer (lysis buffer containing 20 mM imidazole and 5 mM DTT). The recombinant proteins were eluted with lysis buffer containing 300 mM imidazole (*Ov*NLT-1, *Ov*ALT-2, *Ov*7) or 250 mM imidazole (*Ov*103) and dialysed in 20 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 0.2 mM DTT.

The purity of the dialysed proteins was checked by resolution on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining or Western blot analysis using anti-His antibody (Invitrogen). The purified and dialysed proteins were concentrated using Millipore 10,000 MWCO (Amicon Ultra, EMD Millipore Corporation, Billerica, Massachusetts, USA) (6000–8000 MWCO for *Ov*ALT-2) and the concentration was determined by Bradford protein assay (Bradford, 1976). To reduce the bacterial lipopolysaccharide (LPS) in the recombinant protein preparations, 60 μg/ml of polymyxin B was added to all stages of purification and the purified proteins were dialysed in buffers prepared with LPS-free water (Aqua B, Braun, Melsungen AG, Germany). The recombinant proteins were tested for contamination with LPS using a commercially available LAL Chromogenic Kit (*Limulus* amoebocytes lysate; QCL-1000, Lonza, Walkersville, Maryland, USA). Endotoxin contamination was: *Ov*ALT-2, 0.03 EU/mg; O*v*103, 2.4 EU/mg; *Ov*NLT-1, 1.2 EU/mg; and *Ov*7, 0.95 EU/mg.

Infection of mice

Female BALB/c mice and cotton rats (*S. hispidus*) were purchased from Charles River (Sulzfeld, Germany). The animals were kept in individually ventilated cages. The life cycle of *L. sigmodontis* was maintained in cotton rats, and natural infections of 6- to 8-week-old mice were performed by exposure to infected mites, as described by Al-Qaoud *et al.* (1997). *Litomosoides sigmodontis*-infected mice were sacrificed at days 30 and 64 post infection (pi) to collect sera and spleen tissues for analysis. Successful infection was confirmed by counting *L. sigmodontis* adults in the pleural cavity.

Litomosoides sigmodontis antigen was prepared by homogenization of vital worms isolated from infected BALB/c mice, followed by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant was passed through a 0.22 μm sterile filter and the protein concentration was determined by Bradford protein assay (Bradford, 1976) with subsequent storage at −80°C until use.

Detection of proteins by immunoglobulins and proliferation of immune cells of mice

Litomosoides sigmodontis-specific immunoglobulins (Ig) in the serum were quantified by enzyme-linked immunosorbent assay (ELISA) as described by Maréchal *et al.* (1997). Briefly, *L. sigmodontis* lysate or *Ov*NLT-1, *Ov*103, *Ov*ALT-2, *Ov*7 (2.5 μg/ml in phosphate-buffered saline (PBS), pH 7.2) was coated overnight at 4°C on Microlon ELISA plates (Greiner Bio-one, Frickenhausen, Germany). Plates were washed four times with PBS 0.5% Tween 20 and blocked by incubation with PBS 1% bovine serum albumin (BSA) for 2 h at room temperature (RT). Serial dilutions of sera derived from naïve or *L. sigmodontis*-infected mice in PBS 0.1% BSA were incubated in duplicate, adding 50 μl/well overnight at 4°C. Plates were washed five times and antigen-specific Ig was detected by incubation with 50 μl/well of horseradish peroxidase-conjugated anti-mouse IgM or IgG1, respectively (Invitrogen, Karlsruhe, Germany) for 1 h at RT. Plates were washed five times and developed by incubation with 100 μl/well tetramethylbenzidine (TMB) 0.1 mg/ml, 30% H_2O_2 in 100 mM NaH_2PO_4 (pH 5.5) for 2.5 min. The reaction was stopped by addition of 25 μl/well of 2 M H_2SO_4 and the optical density at 450 nm (OD 450) was measured. The titre was defined as the highest dilution of serum that led to an OD 450 above doubled background. Non-specific background detection was always below an OD 450 of 0.15.

In vitro *splenocyte stimulation and Western blotting*

A total of 2×10^5 splenocytes derived from *L. sigmodontis*-infected or age-matched naïve BALB/c mice were cultured in 96-well round-bottomed plates in

RPMI 1640 medium supplemented with 10% fetal calf serum, 20 mM HEPES, L-glutamine (2 mM) and gentamycin (50 μg/ml) at 37°C and 5% CO_2. Cells were stimulated in triplicate with *Ov*NLT-1, *Ov*103, *Ov*ALT-2, *Ov*7 (10 μg/ml), *L. sigmodontis* lysate (10 μg/ml), anti-CD3 (1 μg/ml) or left unstimulated for 72 h. Cell proliferation was measured by the uptake of [H^3]thymidine during an additional 18 h.

Proteins (1–10 μg/ml), were separated on 12% SDS–PAGE, transferred on to a 0.45 μm nitrocellulose membrane (Hybond ECL) at a constant amperage of 60 mA, 120 V at RT for 1 h 30 min. After blocking with PBS in 4% (w/v) skimmed milk overnight, the membranes were washed three times with PBS with 0.05% Tween 20. Subsequently, they were exposed to sera (derived from naïve or *L. sigmodontis*-infected mice) diluted at 1:200 in PBS in 1% w/v skimmed milk at RT for 1 h 30 min. After washing with PBS and 0.05% Tween 20, the membranes were incubated with polyclonal goat anti-mouse immunoglobulin (1:2000 diluted) conjugated with horseradish peroxidase (Dako, Hamburg, Germany) for 1 h at RT. After repeated washings, the immobilized monoclonal antibodies were detected using the ECL-Western blotting detection system (GE Healthcare, Germany) according to the manufacturer's instructions.

Selection of calves for natural transmission

A total of 30 calves (Zebu Goudali breed), less than 1 month old, were purchased locally and kept on the project-owned fenced meadow along the 'Vina du Sud' river in a high-grass savanna area situated about 20 km south of Ngaoundere (Adamawa plateau, Northern Cameroon). Together with their dams, calves were introduced on to the large project site (approximately 15 ha) and exposed to natural transmission of the bovine filarial, *O. ochengi*, transmitted by the local population of *S. damnosum s.l.* In this hyperendemic area, the biting density of *S. damnosum* on cattle is high. The annual transmission potential (ATP) for *O. ochengi* has been measured by regular fly catches and dissections at a monitoring point located on the paddock at the 'Vina du Sud' close to the fly biting sites. Regular fly catches on cattle and human bait were carried out close to the *S. damnosum* breeding sites at the river bank and upland near the paddock, where the calves spent the majority of the day. Flies were caught between 07.00 and 18.00 hours and were screened for filarial infections, identified to species by morphological identification and verification by a PCR-based approach (Eisenbarth *et al.*, 2013). During their 26 months of exposure, calves received an estimated total of 127,000 fly-bites per calf and 19,300 infective L3 larvae of *O. ochengi*. Calves were monitored at 2-monthly intervals (for 26 months), during which skin biopsies were taken for mf counts. Sera from blood were collected from the jugular vein, heat inactivated at 56°C and frozen at −20°C. Calves were weaned after 1 year and their dams removed.

Nodule mapping and examination of microfilariae

At every time point, nodule and mf counts were made according to the method described by Renz *et al.* (1995). Briefly, each animal was restrained in a lateral recumbent position and the nodules counted by palpation of both lateral sites of the animal. The position of all known and newly acquired worm nodules were identified and marked on a sketch map ('nodule map').

After shaving, three superficial skin biopsies were collected with a sterile scalpel blade along the linea alba: one posterior to the umbilicus, one mid-way between the umbilicus and udder and the last one just anterior to the udder/scrotum. Skin biopsies (ranging from 15 to 40 mg for the total of three skin snips) were processed (Achukwi *et al.*, 1994) and mf were identified morphologically and distinguished following the method described by Wahl *et al.* (1994).

Recognition of Onchocerca *proteins by immunoglobulins in sera from exposed cattle*

Serum levels of antigen-specific IgM, IgG1 and IgG2 were measured by ELISA as described by Achukwi *et al.* (2004) with some modifications. Briefly, 50 μl of solution containing 2.5 μg/ml of either *Ov*NLT-1, *Ov*103, *Ov*ALT-2, *Ov*7 or *O. ochengi* lysate (positive control) in PBS (pH 7.2) were coated in 96-well high-binding plates (Microlon, Greiner bio-one, Germany) overnight at 4°C. After washing with PBS/0.05% Tween 20, 100 μl of blocking buffer, containing 5% milk powder (Roth, Karlsruhe, Germany) in PBS, 0.05% Tween 20 (PBS/T), was added to each well and incubated for 2 h. Individual cattle sera (at initial dilutions of 1:100) in 0.2% milk in PBS/T were serially diluted, and incubated overnight at 4°C. Freshly collected sera from non-infected European cows (control) were pooled and diluted side by side with the test sera. After washing, rabbit anti-bovine IgM or sheep anti-bovine IgG1 or IgG2 (Bethyl laboratories Inc., Texas, USA) conjugated with horseradish peroxidase diluted at 1:4000 in 0.2% milk in PBS/T was added and incubated for at least 1 h. After 5–6 washings, the plates were incubated with 100 μl substrate buffer (0.1 M NaH_2PO_4, pH 5.5 in TMB, 30% H_2O_2) for 2.5 min and the reaction afterwards stopped by adding 25 μl 2 M H_2SO_4. Optical densities were read at 450 nm in a Dynatech MRX ELISA reader (MTX Lab. Systems Inc., Vienna, Virginia, USA). Titres were defined as the highest serum dilution that resulted in an OD 450 above twice the OD 450 level of the background for *L. sigmodontis* lysate-specific IgM, *L. sigmodontis* lysate-specific IgG1, protein-specific IgM, respectively, and the highest dilution that gave an OD 450 value above the mean of the background for protein-specific IgG1 and IgG2.

Withdrawal of nodules/sera from human hosts

The collection of sera and removal of nodules were performed as previously documented by Liebau *et al.* (2008). Onchocercomas were derived from studies conducted in Liberia and Ghana (Liebau *et al.*, 2008). The storage of adult worms was accomplished by freezing in liquid nitrogen. Production of total worm extract was carried out by appropriate homogenization and subsequent centrifugation steps. Cell-free protein supernatant was then adjusted to 1 mg/ml protein and stored at −20°C until further use. Blood was collected from 24 individuals with onchocerciasis residing in Dungwa (Ghana), an area

highly endemic for *O. volvulus* infection (Brattig *et al.*, 2002), as well as from eight healthy Europeans as controls. The diagnosis was confirmed by the detection of skin microfilariae and/or onchocercomas. Sixteen patients expressed a generalized form of onchocerciasis, while eight of the onchocerciasis patients showed the rare hyperreactive form (Mpagi *et al.*, 2000; Brattig, 2004).

Recognition of onchocercal proteins in human sera

A semi-quantitative analysis of serum IgG antibody levels was performed by ELISA as described previously (Liebau *et al.*, 2008), with modifications. In brief, polystyrene microtitre plates (Maxi-Sorb, Nunc, Roskilde, Denmark) were coated with recombinant *Ov*ALT-2, *Ov*NLT-1 and *Ov*7, as well as *O. volvulus* lysate, at a concentration of 200 ng per well in carbonate buffer (pH 9.6). After sealing with saran wrap, the plates were incubated overnight at 4°C. After removal of unbound protein by washing three times with PBS/T, the plates were blocked with 5% (w/v) BSA in PBS for 1 h at 37°C. Human sera were diluted 1:300, 1:600 and 1:1200 in PBS/0.5% BSA, and were then incubated at 37°C for 1 h. Unspecifically bound proteins were removed by three washing steps. For detection of bound IgG anti-human peroxidase-conjugated IgG (Jackson ImmunoResearch, Dianova, Hamburg, Germany) was applied at a final concentration of 1:5000 and TMB (Sigma, Deisenhofen, Germany) was used as the substrate. Data are expressed as endpoint titres derived from titration curves and presented as boxplots and whiskers (Mpagi *et al.*, 2000).

Data analysis

Data analysis was performed with Graphpad prism software (San Diego, California, USA) using Student's unpaired *t*-test for the tested groups of mice and cattle. The data are represented as arithmetic means ± SD. A value of $P < 0.05$ was considered statistically significant. In addition, Mann–Whitney *U*-test was used for comparison of the titres obtained for the tested group of humans. Analysis by bivariate Pearson correlation coefficients for nodule loads, mf counts and protein-specific IgM and IgG1 responses were performed using SSPS 15.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Cross-reactivity to recombinant proteins in mice

Using Western blots, three of the four recombinant proteins, namely *Ov*ALT-2, *Ov*NLT-1 and *Ov*7, were specifically detected by immunoglobulin present in sera

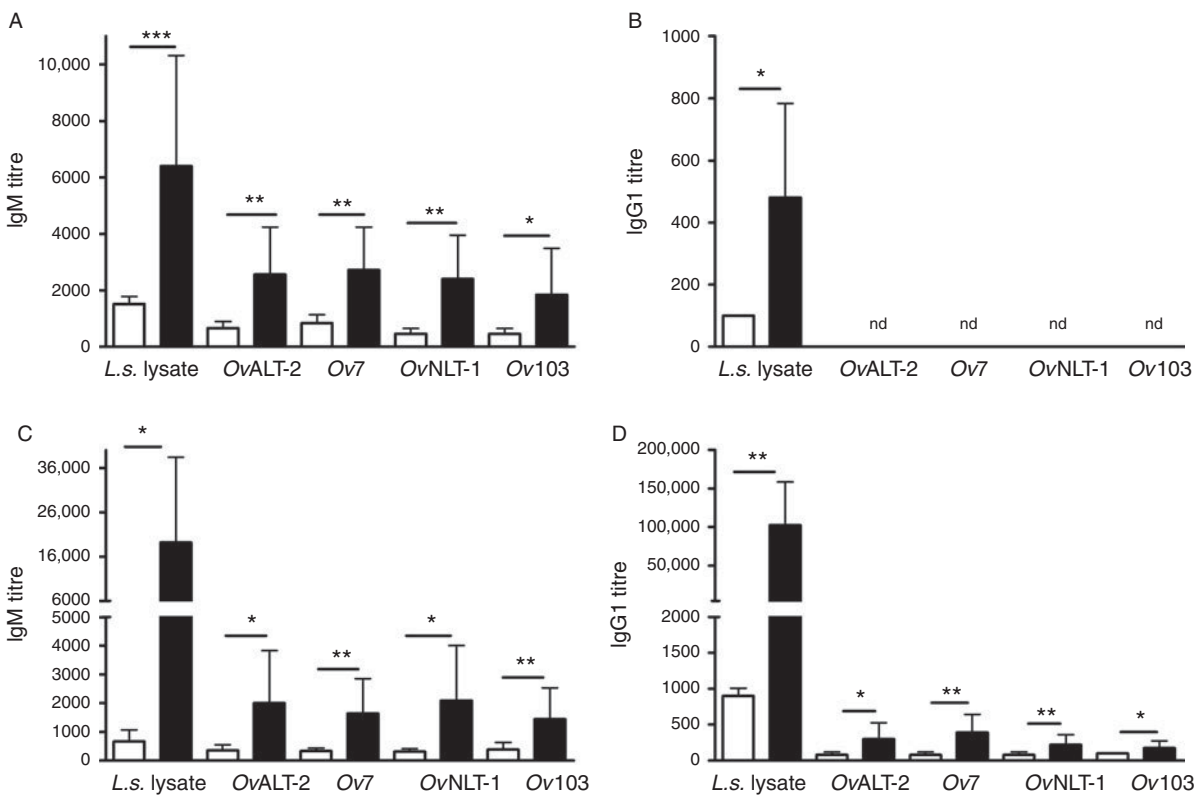

Fig. 1. Mean titres (+ SD) of *O. volvulus*-derived proteins and *L. sigmodontis* lysate-reactive (A) IgM day 30, (B) IgG1 day 30, (C) IgM day 64 and (D) IgG1 day 64 post infection, derived from infected mice (black bars) or left naïve (white bars), with levels of significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; nd, not detected.

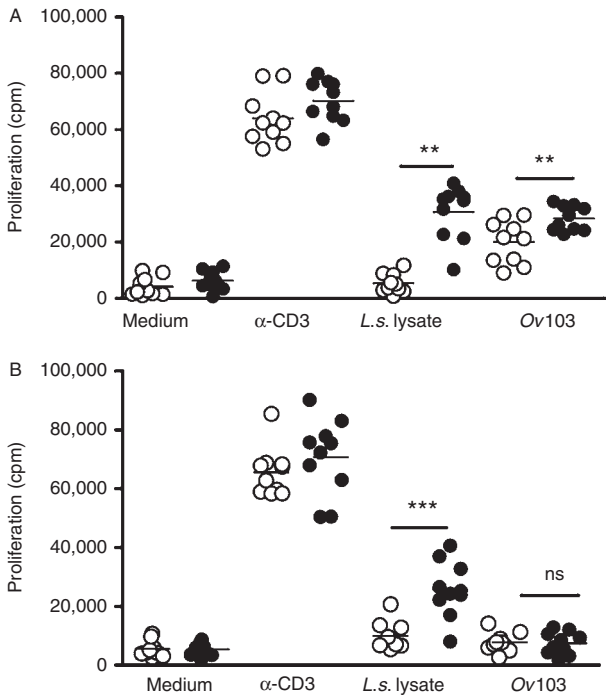

Fig. 2. Cellular immune responses to recombinant proteins to show the proliferation of splenocyte cells from naïve (○) and infected (●) mice on (A) day 30 and (B) day 64 post infection, in the presence of medium, *L. sigmodontis* lysate, *Ov*103 or anti-CD3, with levels of significance $^{**}P < 0.01$ or $^{***}P < 0.001$.

derived from *L. sigmodontis*-infected BALB/c mice but not by sera derived from naïve mice (figure not shown). *Ov*103 was neither detected by infected nor naïve animals. To identify the isotype of the antigen-specific immunoglobulins and to quantify the immunoglobulin responses, further ELISA studies were performed. Binding of sera derived from ten naïve and ten *L. sigmodontis*-infected BALB/c mice to the recombinant proteins and to complete *L. sigmodontis* antigen-lysate as a positive control were tested (fig. 1A–D). Sera derived from infected mice contained *L. sigmodontis* lysate-specific IgM and IgG1 at day 30 and day 64 post infection (pi). Furthermore, in infected mice, all recombinant proteins were specifically recognized by IgM (fig. 1A and C). Sera derived from naïve mice did not bind to the recombinant *O. volvulus* proteins, thus demonstrating antigen-specificity of this IgM. However, a higher immunoglobulin titre for complete *L. sigmodontis*-lysate was recorded compared to the individual recombinant proteins. This quantitative difference is most likely due to the high number of potential antigens in the lysate compared to single antigens in the purified proteins. Titres for protein-specific IgG1 at day 30 pi were not detected, although *L. sigmodontis* lysate-specific IgG1 was produced (fig. 1B). At a later time point, i.e. day 64 pi, IgG1 specific for the investigated *O. volvulus* proteins *Ov*ALT-2, *Ov*NLT-1, *Ov*7 and *Ov*103 was detected in sera derived from *L. sigmodontis*-infected mice (fig. 1D). No antigen-specific IgG2a and IgG2b against the recombinant proteins or complete *L. sigmodontis* antigen lysate were detected (data not shown).

To test whether the recombinant *O. volvulus* proteins are targeted by cellular immune responses, splenocytes derived from naïve and *L. sigmodontis*-infected BALB/c mice were stimulated with *Ov*ALT-2, *Ov*NLT-1, *Ov*7, *Ov*103, *L. sigmodontis* lysate or anti-CD3, respectively. Anti-CD3 stimulation readily induces proliferation of splenocytes, hence proving viability of T cells within the splenocyte culture. *Litomosoides sigmodontis* lysate antigens specifically induced proliferation of splenocytes derived from infected mice, but not from naïve mice, demonstrating antigen-specific proliferation (fig. 2A and B). Also, the purified recombinant protein *Ov*103 selectively induced a higher proliferation in splenocytes derived from infected mice at an early time point (day 30 pi, fig. 2A), but this proliferation was not recorded at a later time point (i.e. day 64 pi, fig. 2B). *Ov*ALT-2, *Ov*NLT-1 and *Ov*7 did not induce any proliferation in splenocytes, neither from naïve nor from infected mice. These results strongly suggest that *L. sigmodontis*-specific IgM and IgG1 elicited during *L. sigmodontis* infection of BALB/c mice cross-reacted with the *O. volvulus* recombinant proteins. In addition, *Ov*103 was recognized by *L. sigmodontis*-specific T cells at an early time point of infection.

Immune responses to bovine filarial infections

To study the recognition of *O. volvulus* recombinant antigens in the bovine model of onchocerciasis, sera were analysed for antigen-specific humoral responses, i.e. IgM, IgG1 and IgG2. To monitor parasite burden, the cumulative nodule loads during the first 26 months of exposure were counted (fig. 3A). Intradermal nodules

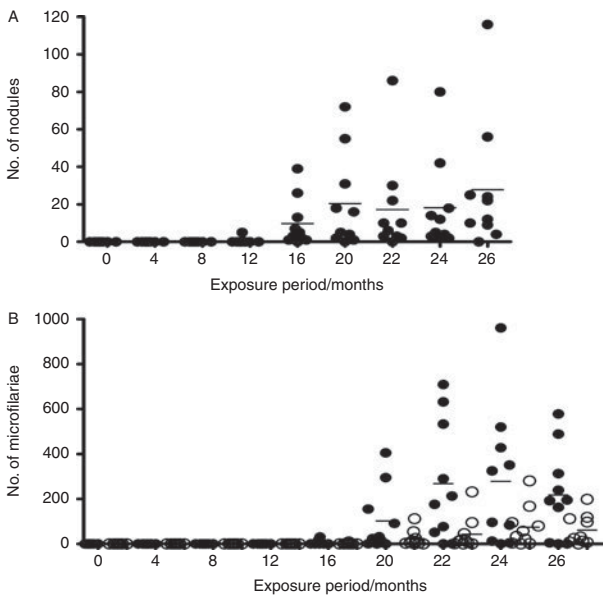

Fig. 3. Infection levels of calves naturally exposed to *Onchocerca* infections, to show (A) the number of nodules and (B) levels of microfilariae of *O. ochengi* (●) and *O. gutturosa* (○) in skin snips ($n = 10$).

were first detected after 12 months post exposure (pe) and subsequently the nodule loads increased steadily over time. Meanwhile, detection of mf in skin snips occurred from 16 months pe onwards, and mf counts increased steadily thereafter (fig. 3B). *Onchocerca ochengi* and *O. gutturosa* were the *Onchocerca* species detected from skin snips. The present results show that calves naturally exposed to *Onchocerca* transmission, develop patency after 12 months, with the appearance of palpable intradermal nodules and mf in the skin after 16 months pe.

Nodule and total microfilaria counts (*O. ochengi* and *O. gutturosa*) correlated positively with individual protein-specific immune responses (table 1). Significant positive correlations ($P < 0.01$) were found between nodule and total microfilaria loads, between total mf and *Ov*NLT-1-specific IgM ($P < 0.01$), *Ov*NLT-1-specific IgG1 and *Ov*103-specific IgG1 ($P < 0.05$) (table 1).

Next, sera were analysed for humoral immune responses during this period of natural infection. Figure 4A shows that all the recombinant proteins *Ov*ALT-2, *Ov*NLT-1, *Ov7* and *Ov*103 and adult worm lysate were specifically recognized by IgM in sera derived from cows (mothers), while sera derived from the control (uninfected European cattle) did not react with the recombinant proteins or with the *O. ochengi* lysate. The cows (mothers) were pre-exposed to the *Onchocerca* infections before they entered the study site. Similarly, analysis of the sera derived from calves before exposure (<1 month after birth) revealed the absence of IgM reactive with the recombinant proteins or *O. ochengi* lysate (fig. 4B). This was also observed after 4 months pe (figure not shown). After 8 months of exposure, the first antibodies of the IgM class were seen, reacting against both the *O. ochengi* lysate and the recombinant *O. volvulus* antigens (fig. 4C). After 12 months pe the sera from exposed calves showed IgM detection with all the proteins tested (fig. 4D). This finding is concomitant with the first appearance of palpable intradermal worm nodules (patency) in the calves (fig. 3A). We did not detect *Ov*NLT-1-specific IgM in sera derived from calves at early time points but *Ov*NLT-1-specific IgM was detected at the same time point when mf occurred (fig. 4D–F).

Cows introduced into the study site contained IgG1 specific for *Ov*-ALT-2, *Ov*NLT-1, *Ov7* and *Ov*103 in their sera at an early time point (fig. 5A). Likewise, analysis of sera of the calves at 1 month after birth for IgG1 isotype show *O. ochengi* lysate- and *Ov*NLT-1-specific responses (fig. 5B), which most likely reflects a maternal diaplacenta and/or lactogene transfer of IgG1. At 4 months pe, a decline in *O. ochengi* lysate-specific titres for IgG1 (figure not shown) was observed, which in part recovered at 12 months pe (fig. 5D). There was no *Ov*103 and *Ov*NLT-1 protein-specific recognition in exposed calves up to 12 months pe (fig. 5B–E). After 12 months exposure, a re-emergence of *Ov*ALT-2 and *Ov7* protein-specific IgG1 responses was detected (fig. 5E), which was maintained throughout the rest of the exposure period (fig. 5E–F). In addition, this was evident for the other proteins tested (*O. ochengi* lysate, *Ov*103 and *Ov*NLT-1; fig. 5E–F). This consistent protein-specific recognition of sera at the onset of 16 months exposure throughout the rest of the exposure period (fig. 5E–F) coincided with the appearance of worm nodules and mf in the skin. There was no

Table 1. Pearson correlation coefficients between the number (N) of nodule loads, total microfilarial counts, including *O. ochengi* and *O. gutturosa*, and protein-specific immune titres; levels of significance $^{**}P < 0.01$ and $^{*}P < 0.05$.

	Nodule load	Microfilariae	*O. ochengi*	*O. gutturosa*	IgM *Ov*ALT-2	IgM *Ov*NLT-1	IgM *Ov7*	IgM *Ov*103	IgG1 *Ov*ALT-2	IgG1 *Ov*NLT-1	IgG1 *Ov7*	IgG1 *Ov*103
N	90	90	90	90	82	76	81	84	72	78	78	85
Nodule load	1	0.591(**)	0.651(**)	0.007	0.160	0.209	0.032	0.052	0.126	0.297(**)	0.084	0.169
Microfilariae	0.591(**)	1			0.214	0.341(**)	0.143	−0.025	0.076	0.509(**)	0.135	0.220(*)
O.ochengi	0.651(**)		1	0.345(**)	0.202	0.352(**)	0.151	−0.015	0.063	0.477(**)	0.105	0.215(*)
O.gutturosa	0.007		0.345(**)	1	0.127	0.155	0.120	0.005	0.166	0.429(**)	0.192	0.156

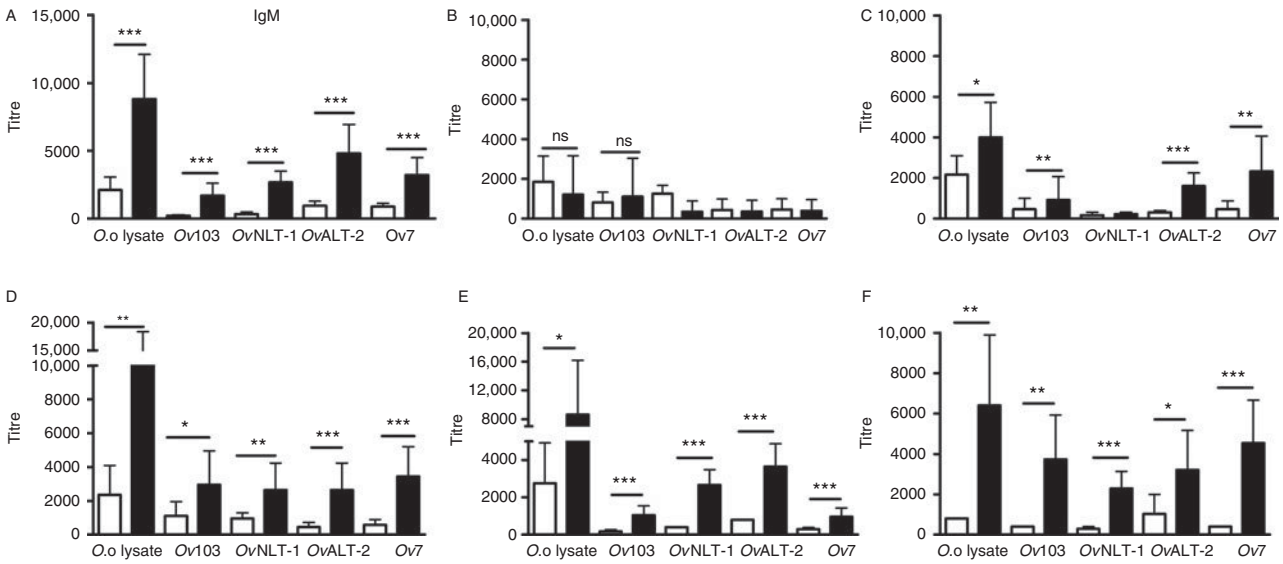

Fig. 4. Mean titres (+SD) of the *O. volvulus*-derived proteins and *O. ochengi* lysate-reactive IgM: (A) mother cows, (B) pre-exposure calves, (C) 4 months pe, (D) 8 months pe, (E) 22 months pe and (F) 26 months pe cattle (black bars) or control sera (white bars), with levels of significance $*P < 0.05$; $**P < 0.01$ or $***P < 0.001$ ($n = 10$).

observed protein or *O. ochengi* lysate-reactive recognition of IgG2 by sera in all the time points tested.

The present results show that there is no maternal transfer of antigen-specific IgM from cow to calf, as expected. Protein-specific recognition of IgM was first detected after 8 months pe and antigens of *O. ochengi* lysate, *Ov*ALT-2, *Ov*NLT-1, *Ov*7 and *Ov*103 were gradually recognized following prolonged exposure to the parasite. Moreover, antigen-specific IgG1 is maternally transferred, as expected, and declines after 8 months pe. A second re-emergence of antigen-specific IgG1 starts as from 12 months pe, thereby most likely reflecting the humoral immune response elicited by prepatent and early patent infections.

Immune recognition of onchocercal proteins in human sera

In order to examine the immunogenicity of three of the investigated onchocercal proteins, a possible IgG reactivity of sera from 24 onchocerciasis patients, eight of those expressing the rare hyperreactive form of the infection, towards *Ov*ALT-2, *Ov*NLT-1 and *Ov*7, applying total *O. volvulus* lysate as the positive control, was investigated. The IgG reactivities of these sera were compared to the reaction found in sera from eight healthy European controls. IgG in sera from patients with generalized onchocerciasis compared to the control persons reacted strongly with the total protein extract of *O. volvulus* (median titre for patients versus controls: 10,380 versus 35; $P < 0.001$) and showed lower reactivities towards the single onchocercal proteins: *Ov*ALT-2 (median titre: 3913 versus 145; $P < 0.001$), *Ov*NLT-1 (median titre: 4850 versus 195; $P < 0.01$) and *Ov*7 (median titre: 3770 versus 70; $P < 0.01$) (fig. 6). Interestingly, the eight sera from the rare hyperreactive form uniformly showed a trend of higher IgG reactivities for onchocercal extract (median titre: 10,550), *Ov*ALT-2, *Ov*NLT-1 and *Ov*7 (median titres: 4175, 4975 and 4060, respectively).

Discussion

Using murine and bovine models for human *O. volvulus* infection, we show that recombinant *O. volvulus* antigens (*Ov*ALT-2, *Ov*NLT-1, *Ov*103 and *Ov*7) are targets of the immune responses. In mice, specific IgM and, at a later stage of infection, also IgG1 exclusively in *L. sigmodontis*-infected but not in naïve animals, were detected. These experiments indicate that antigens generally function as targets for B-cell response during nematode infection. The detection of IgG1 at a later time point indicates a T-helper (Th)-dependent class-switch, thus suggesting that a preceding priming of specific T cells occurs. Also, the very early *Ov*103-specific proliferation at day 30 pi indicates the presence of *Ov*103-specific T cells. There were no observed *Ov*ALT-2-, *Ov*NLT-1-, *Ov*103- and *Ov*7- specific IgG2a and IgG2b titres in mice in these studies. This is in line with the association of IgG2a or IgG2b with Th1-associated immune responses, whereas *L. sigmodontis* infection predominantly provokes Th2-associated responses in BALB/c mice (Torrero *et al.*, 2010).

Proliferation of spleen cells was recorded in response to *Ov*103 at 30 days, but not at 64 days post *L. sigmodontis* infection. Previous studies suggested a possible correlation between levels of mf and antibody response to *Ov*103 antigen (Lustigman *et al.*, 1992b). They found that a significantly greater proportion of individuals with low levels of skin mf recognized the protein encoded by clone *Ov*103 than did individuals with higher levels of skin mf. Dynamics of response mirrors that observed in the current study, in which *Ov*103-protein-specific cellular response at an early time point (day 30) was recorded, but was absent

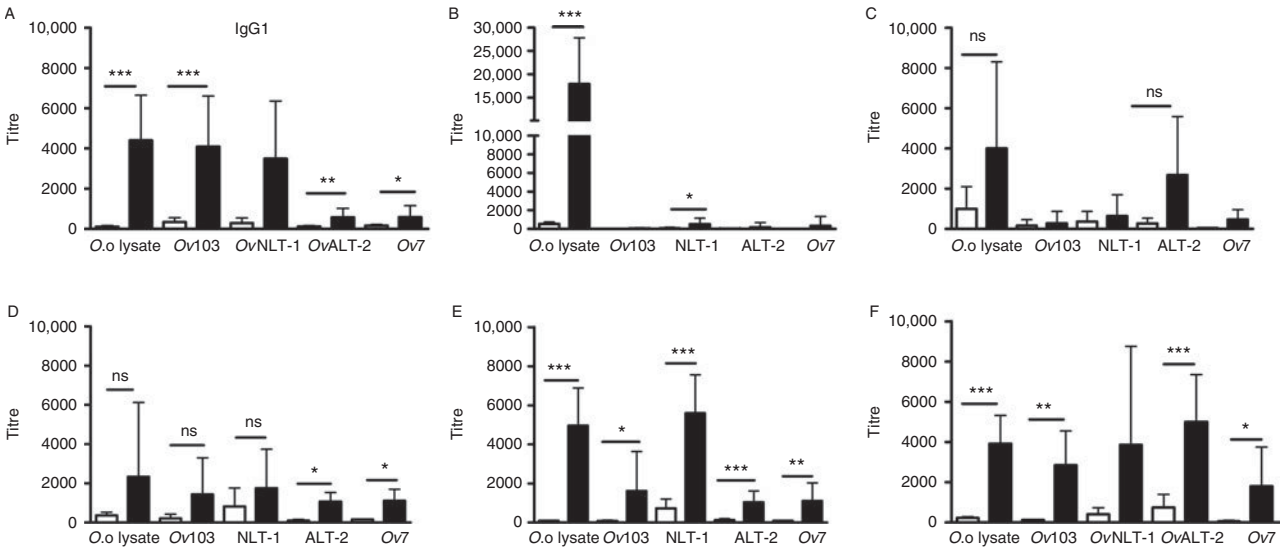

Fig. 5. Mean titres (+SD) of *O. volvulus*-derived proteins and *O. ochengi* lysate-reactive IgG1: (A) mother cows, (B) pre-exposure calves, (C) 4 months pe, (D) 8 months pe, (E) 22 months pe and (F) 26 months pe cattle (black bars) or control sera (white bars), with levels of significance $*P < 0.05$; $**P < 0.01$ or $***P < 0.001$ ($n = 10$).

at a later time point (day 64) in the *L. sigmodontis* model. In human onchocerciasis, Lustigman *et al.* (1992b) therefore proposed that the presence of specific anti-*Ov*103 antibodies before patency may reflect the existence of *Ov*103 epitopes in early developmental stages. A probable reason for a proliferation in response to *Ov*103 at the early time point of day 30, but not on day 64, may be that the protein is only transiently released on to the surface (or in solution) at an early course of infection and not continuously. Further studies, such as a longitudinal study of the cellular immune response to *Ov*103 in *L. sigmodontis* infection, could be used to test this hypothesis.

Calves (Goudali breed, *Bos indicus*), which were naturally exposed to the *Simulium* and other arthropod vectors at our study site, developed intradermal nodules of *O. ochengi* and harboured *O. ochengi* and *O. gutturosa* microfilariae (and other filariae) in the skin, as has already been shown in previous studies (Wahl *et al.*, 1994; Achukwi *et al.*, 2000). By introducing very young Goudali calves into an area with high parasite transmission, dynamics of nodule acquisition, microfilaria loads and protein-specific immune responses for more than 2 years have been studied. This time-frame correlates to a series of biological events occurring during the life cycle of bovine *Onchocerca* parasites.

First, the serological profiles (IgM and IgG1) of the cows (mothers) were established. Their protein-specific responses were an indication of ongoing infection with filarial parasites (figs 4A and 5A). The significant protein-specific IgM and IgG1 responses imply that the cows were already exposed to *Onchocerca* infection. In addition, intradermal palpation of the mother cows indicated that 7 out of 10 were already positive for *Onchocerca* nodules and skin mf of *O. ochengi* and *O. gutturosa*.

*Ov*NLT-1- and *O. ochengi* lysate-reactivity of IgG1 of pre-exposed calves (fig. 5B) indicated a maternal transfer of specific antibodies through the colostrum. In cattle, IgG1 is the only (predominant) isotype transferred maternally in large amounts (Butler, 1999) and this transfer gradually declines after the calves start to build their own protective immunity. Hence, there was no protein-specific IgM and a reduced *O. ochengi* lysate-reactive IgG1 response in calves after 4 months pe. The predominant IgG1 responses

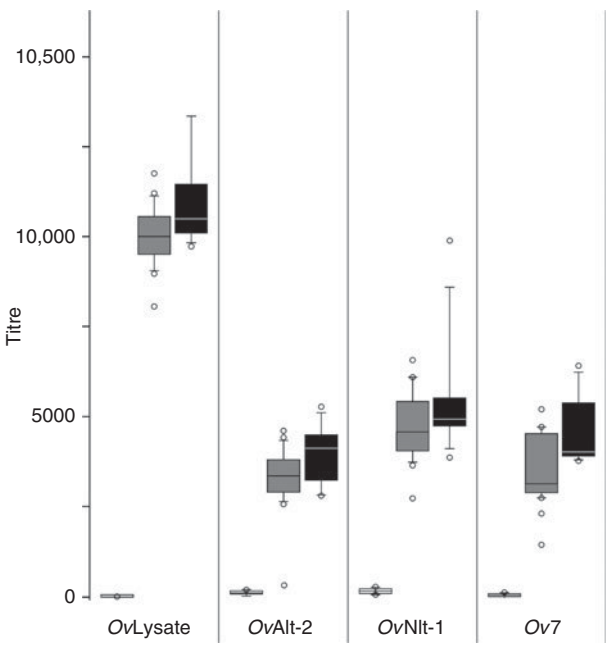

Fig. 6. Humoral immune responses to the mean titres of *O. volvulus* proteins compared to *O. volvulus* lysate-reactive IgG in human patients with a generalized common form (generalized onchocerciasis) (grey bars) or hyperreactive form (black bars) of onchocerciasis, compared with those in healthy Europeans (white bars).

observed in this study are in line with earlier reports of cattle experimentally infested with *O. lienalis* (Kuo & Bianco, 1995) and cattle naturally exposed to *O. ochengi* (Achukwi *et al.*, 2004). The high anti-*Onchocerca* IgG1 levels at an early stage of a calf's life could have an inhibitory effect on the establishment of L3s and therefore delay the acquisition of nodules (Achukwi *et al.*, 2004).

In general, infections with helminths typically lead to a strong Th2-immune response (Allen & Maizels, 2011), more probably because generic invertebrates produce signals through host receptors in a way that amplifies Th2 responses and is antigen specific. Bovine IgG consists of two isotypes, IgG1 and IgG2 (with allotypes 2a and 2b), neither of which is a homologue of human or murine IgG isotypes. Production of IgG2 responses were stimulated by interferon (IFN)-γ (a Th1-associated cytokine) from bovine cells *in vitro* (Estes, 1996) and *in vivo* (Graham *et al.*, 2001). Results show that protein-specific IgG1 antibody responses could be an indicator of infection with bovine onchocerciasis. However, in the current study, a Th2-associated IgG1 response in mice was observed, while in cattle there was no such correlation.

Previous work conducted on a ranch along the Vina du Sud river in Ngaoundere, Cameroon (Achukwi *et al.*, 2004) identified morphologically other species (*O. gutturosa*, *O. armillata* and *Setaria* species) with yet largely unknown transmission cycles. In the present study, *O. ochengi* and *O. gutturosa* were identified morphologically in the investigated cattle. Even though *O. ochengi* was the predominant filarial parasite identified in this study, the infection with *O. gutturosa* was closely related to that of *O. ochengi* (fig. 3) which is similar to earlier observations (Voung *et al.*, 1994; Wahl *et al.*, 1994; Achukwi *et al.*, 2004). Also, previous studies (Bradley *et al.*, 1989) have already indicated that surface antigens of *O. gutturosa* from bovines and *O. volvulus* from humans are similar in size and share antigenic cross-reactivities. Thus, it is very likely that the E/S protein-specific immune responses observed in this study are also a result of *O. gutturosa* infection, or of undetected filarial species, and not only of *O. ochengi*.

The immunogenicity and the exposure of the investigated proteins from *O. volvulus* to its natural host was examined in 24 individuals infected by *O. volvulus*. By ELISA analysis high IgG titres were demonstrated in the sera of the onchocerciasis patients for *Ov*ALT-2, *Ov*NLT-1, *Ov*7 and much higher for *O. volvulus* lysate. The reaction of the sera of the more rare chronic hyperreactive form was uniformly more pronounced with all tested proteins and the total extract, representing the characteristic immune response pattern as demonstrated earlier (Mpagi *et al.*, 2000; Brattig, 2004).

This study confirms cross-reactions to proteins expressed by the different developmental stages of *Onchocerca* and *Litomosoides* in filaria-infected cattle and mice. Mice display antigen-specific immunoglobulin levels in the sera 30 days pi, thus before patency is achieved, and a marked increase after production of mf 64 days pi. Calves naturally exposed to *O. ochengi* develop patency after 12 months of exposure. Protein-specific immune responses are proportional to the parasite loads and may function as an indicator of infection. This study suggests that the proteins are highly immunogenic and qualifies them as potential vaccine candidates for human onchocerciasis.

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Conflict of interest

None.

Ethical standards

The *in vivo* experiments were carried out in the animal facility of the Bernhard Nocht Institute for Tropical Medicine, Hamburg, with permission of the federal health authorities of the state of Hamburg, Germany. The collection of human sera and removal of nodules was approved by the Ethics Commission of the Medical Board in Hamburg (Germany) and by ethic committees and medical authorities in the respective countries, in accordance with the principles of the Helsinki declaration of 1975, revised 2000.

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Isolation, identification and functional profile of excretory–secretory peptides from *Onchocerca ochengi*

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ABSTRACT

Parasitic helminths excrete or secrete a variety of functional molecules into the internal milieu of their mammalian hosts and arthropod vectors which reveal distinct immunomodulatory and other biological activities. We identified and initially characterized the low molecular weight peptide composition of the secretome from the filarial parasite *Onchocerca ochengi*. A total of 85 peptides were purified by liquid chromatography and further characterized by mass spectrometry. 72 of these peptides were derived from already described *Onchocerca* proteins and 13 peptide sequences are included in the sequence of uncharacterized proteins. Three peptides, similar to host defense peptides, revealed antibacterial activity. The present analysis confirms the putative involvement of low molecular weight compounds in the parasite-host cross-talk.

1. Introduction

Human onchocerciasis or river blindness, endemic in parts of Africa, Latin America and Yemen is caused by the nodule-dwelling filarial nematode *Onchocerca volvulus* (WHO, 1995). The cattle parasite *Onchocerca ochengi* represents today the most feasible *onchocercid* model parasite (Renz et al., 1995). Based on morphological and biological criteria, *O. ochengi* is the phylogenetically most closely related species to *O. volvulus*, and the two species share the same vector (Achukwi et al., 2004). These facts imply a high degree of similarity at the protein and genome levels (Trees, 1992).

In the present report, the investigation of low molecular weight peptides released by adult *O. ochengi* provides important information contributing to the understanding of the *Onchocerca*-host cross-talk. Furthermore, the helminths excretory–secretory products (ESPs) obviously represent a substantial reservoir of biologically active molecules with potential pharmacological applications. The capacity of parasites to modulate the host immune system is a relevant factor supporting their long-term survival in the mammalian host (Behnke et al., 1992; Maizels and Yazdanbakhsh, 2003). Hence, there is a particular interest to analyse, identify and characterize mediators released by parasites and to investigate how these products, in total and as individual components, influence and interact with the host system (Hewitson et al., 2009). These mediators can ligate, degrade or interact with host molecules or immune cells (Lightowlers and Rickard, 1988). In summary, they are composed of a mixture of different proteins, peptides, lipid mediators and polysaccharides and are termed ESPs (Hewitson et al., 2009; Dzik, 2006). However, the definition fails to distinguish between components actively secreted and

Abbreviations: ESPs, excretory/secretory products; HDM, helminth defense molecules; HDP, host defense peptides; LPS, lipopolysaccharides; RPC, reversed phase chromatography.

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compounds produced as a consequence of other physiological processes (Lightowlers and Rickard, 1988).

Parasites ESP and peptides homologous to host defence peptides (HDP) have been reported to modulate the immune response via molecular mimicry of host defence peptides thus providing anti-inflammatory activities commonly observed in helminth infections (Cotton et al., 2012; Robinson et al., 2011). HDP have distinct activities against bacteria, fungi, eukaryotic parasites and viruses (Mookherjee and Hancock, 2007). The potential of HDP to influence an infection is mediated by direct antimicrobial properties as well as by a modulation of the immune response (Bommarius et al., 2010). Thus, these peptides are known to neutralize LPS-mediated responses and prevent lethal endotoxemia (Giuliani et al., 2010). They also participate in inflammatory responses e.g. by acting as chemotaxins for immune cells (Chertov et al., 1996). HDP can promote phagocytosis while inhibiting oxidant responses of neutrophils or monocytes (Miles et al., 2009; Tecle et al., 2010), they stimulate wound healing as well as angiogenesis (Aarbiou et al., 2002; Murphy et al., 1993). Furthermore, HDP exhibit a modulatory effect on pathways regulating cell survival and apoptosis and they can induce the production of chemokines or other immune mediators (Mookherjee and Hancock, 2007). HDP are low molecular weight short peptides, composed of 12–50 amino acids, with an overall positive charge of +2 to +9 at neutral pH 7.0. Due to the predominance of basic amino acids e.g. arginine, lysine and histidine the interaction with negatively charged bacterial cell wall surfaces is supported (Hancock and Chapple, 1999). These peptides can be divided into subgroups on the basis of their amino acid composition and secondary structure (Hancock and Chapple, 1999; Gennaro and Zanetti, 2000) including cationic peptides rich in proline, arginine, lysine, histidine, phenylalanine or tryptophan, anionic compounds and fragments of larger proteins that arise through metabolic events (Otvos, 2002). The secretion of molecules mimicking HDP-type peptides with antibacterial activities was described before for helminths (Robinson et al., 2011). The objective of the present study was to identify peptides in ESPs of the filaria *O. ochengi* and to analyse these peptides for potential biological activity. Because of the limited amount of available experimental material obtained from the *O. ochengi*-endemic area in Cameroon, we could only focus towards analyzing anti-bacterial activities.

2. Material and methods

2.1. Peptide preparation

In the context of the Cameroonian-German Cooperation Project "Analysis of host-parasite cross-talk based on the bovine model for human onchocerciasis, *O. ochengi*" funded by the German Research Foundation (DFG, via project PAK296) adult female *O. ochengi* filariae were isolated from nodules extirpated from the skin of Gudali Zebu cattle slaughtered in the municipal abattoir in Ngaoundéré, Adamawa region, Cameroon. Pieces of the ventral skin from the inguinal region, where most of the nodules are found (Wahl et al., 1994) were shaved, thoroughly cleaned and antiseptically treated. Then the worm nodules were removed and individually incubated at 35 °C in 0.125% collagenase RPMI solution to digest the nodule capsule. Male and female worms were carefully cleaned from any debris and host tissue with PBS.

Only adult females of *O. ochengi* were used to reduce the gender influence in the peptide composition. Isolated intact and motile female worms were multiple times washed, pre-incubated for 1 h, then the medium changed and subsequently incubated individually at 37 °C for 96 h in RPMI media (Sigma-Aldrich, Taufkirchen, Germany), supplemented with 10 mM Hepes, 200 U/ml penicillin, streptomycin 200 μg/ml, 50 μg/ml gentamycin (pH 7.0–7.2). Every 24 h the supernatant was harvested and the medium was changed. After the incubation period, vitality of the nematode and sterility of the culture supernatants were checked microscopically on blood agar plates (Soblik et al., 2011). Only sterile cultures were used for further processing. Analyzed were supernatants of TCA-precipitated culture fluids from three experiments each performed using five adult female *O. ochengi*.

Proteins in the supernatant were precipitated by trichloroacetic acid (TCA)-treatment (10%) and removed by centrifugation. Only the unprecipitated low molecular fractions were used for further investigations. The peptides were separated from lipids, carbohydrates and salts using an Oasis HLB Plus cartridge (Waters 186000132, Milford, USA). The matrix was rinsed with 2 ml methanol, and equilibrated with 3 ml 0.2% formic acid. A 5 ml sample was loaded, and afterwards washed with 5 ml 0.2% formic acid. The flow through wash step was collected and stored at −20 °C for further investigation. Bound peptides were eluted with 1 ml 30% acetonitrile, 0.2% formic acid followed by 1 ml 60% acetonitrile, 0.2% formic acid. The desalted elution fractions were lyophilized and further separated via reversed phase chromatography (RPC). The lyophilized desalted samples were solved in 0.2% formic acid. Separation was performed using a chromolith® RP-18e 100 × 4.6 mm column (Merck Millipore). Buffer A (sample and washing buffer) consisted of 0.2% formic acid. Buffer B (elution buffer) consisted of 0.2% formic acid and 60% acetonitrile. In total 45 sample fractions were collected, lyophilized and stored at −80 °C until used for mass spectroscopic analysis. The peptide concentration of each fraction was determined by measuring the absorbance of the peptide bond at 205 nm according to the method of Scopes (1974) a standard curve of the peptide with the sequence SAVLQSGFRK (Genescript, USA) to calculate the concentrations of the peptide samples.

2.2. Bactericidal agar plate assay

E. coli BL 21 Star™ (DE3) (Invitrogen, Darmstadt, Germany) were used for the agar plate assay. The cells were cultured in LB medium until OD_{600} reached a value of 0.4. Aliquots were placed on antibiotic-free LB agar plates. Subsequently the peptides were poured into a 60 mm-diameter wells of the LB plates and incubated for 12 h at 37 °C. Desalted peptide mixtures were used to analyse antibacterial activity and efficiency following a procedure described by Lin et al. (2010). Identified peptides with antibacterial activity were compared with so far known antibacterial peptides deposited within the LAMP (Zhao et al., 2013) and CAMP (Thomas et al., 2010) databases.

2.3. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Three replicates of MALDI-TOF and TOF/-TOF analyses were performed applying fresh purified *O. ochengi* secretome samples without proteinase inhibitors but processing the samples consequently at 4 °C to exclude proteolysis. Further, the samples for the MALDI-TOF and TOF/-TOF analyses were not tryptically digested.

These samples were lyophilized after reversed phase chromatography. Resolved in 30% acetonitrile, 0.1% trifluoroacetic acid (TFA) in H_2O and 0.75 μl and spotted on a MALDI target plate (MTP AnchorChip 384, Bruker Daltonik). After drying 0.75 μl MALDI matrix (0.7 mg/ml cyano-4-hydroxy-cinnamic acid (Bruker Daltonik)), dissolved in 85% acetonitrile, 1 mM $NH_4H_2PO_4$ and 0.1% TFA in H_2O were spotted on the sample dots. Data acquisition was performed in positive ion mode under the control of the FlexControl software 3.3. The instrumental parameters of the UltrafleXtreme MALDI-TOF-TOF-MS (Bruker Daltonik) were set as follows: ion source 1: 25 kV, ion source 2: 23.6 kV, lens: 7.5 kV. MS data were collected automatically using autoXecute. Parameters were set as

follows: laser power: 47%; laser shots: 1000; movement, random walk with 100 shots per raster spot. MS spectra were processed in flexAnalysis (version 3.3, Bruker Daltonik) mode. Further data analysis was performed using BioTools (version 3.2, Bruker Daltonik). Mascot version 2.1.03 was used for a computational search within the subset "other metazoa" of the NCBI database. Precursor ion mass tolerance was set at 0.1%; fragment ion mass tolerance was set at 0.5 Da. Furthermore the identified peptide sequences were compared with proteome data deposited so far in terms of the *O. ochengi* genome project (http://www.nematodes.org/genomes/onchocerca_ochengi/), the *O. volvulus* genome project (http://www.sanger.ac.uk/resources/downloads/helminths/onchocerca-volvulus.html) and the *O. flexuosa* genome project (http://www.nematodes.org/nembase4/,species_info.php?species=OFC). Presently, the NCBI or Uniprot database does not provide entire genome information of *O. ochengi*. Identified peptide sequences were cross-checked performing a BLASTP search against the non-redundant protein database, to identify potential contaminations of the experimental material with human, cattle or *Wolbachia spec.*-derived proteins.

2.4. Characterization of the net charge, hydrophobicity and secondary structure from the identified peptides

To calculate and predict net charge, hydrophobicity and secondary structure of the identified peptides bioinformatics tools were applied.

The program PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/index.html) was used for the calculation of the net charges at neutral pH 7.0 and the hydrophobicity whilst the prediction of the secondary structure was undertaken applying the program NetSurfP (Petersen et al., 2009) in combination with the program NetTurnP (Petersen et al., 2010) = 30.

3. Results

3.1. Identification of peptides released from O. ochengi by MALDI-TOF-TOF-MS

Proteins, carbohydrates and lipids were separated from the sterile supernatants of the TCA-precipitated culture fluids from three experiments, each with five adult female *O. ochengi* to identify and characterize functional peptides. The separated peptides were further purified by reversed phase chromatography (RPC). During the separation overall 45 fractions were collected and 13 peaks were observed in the chromatogram (Fig. 1).

All fractions were analyzed by MALDI-TOF-TOF-MS and in total 85 peptides with molecular weights between 528 and 2409 Da were detected (Tables A1 and A2 in the appendix).

13 of the 85 peptides arise from so far uncharacterized proteins of *O. ochengi* and have not been described before. The 62 proteins that release 85 peptides through proteolytic processes belong all to different protein families (appendix, Table A2), like enzymes (e.g. GAPDH), immunogenic proteins (e.g. major antigen), antioxidant proteins (peroxidoxin-2), transport proteins (fatty acid and retinol-binding protein-1), proteases (cathepsin L-like cysteine proteinase), stress response (small heat shock protein), protein modifications (cyclophilin-4) and signal transduction proteins (ankyrin homologues). For all 85 peptides the theoretical net charge was calculated, 26 peptides revealed a neutral net charge, 30 peptides revealed an anionic net charge and 29 peptides revealed a cationic net charge, respectively. Especially the peptide with the sequence DGGDEEGGNDENEDVPRGSF showed a relatively high net charge of −7 and coincidently also a relatively high hydrophobicity of +44.7 kcal/mol. The calculated hydrophobicity for the 41 peptides is ranging from +0.4 kcal/mol to +44.7 kcal/mol. Information about the secondary structure prediction for all peptides is summarized in the appendix, Table A1.

3.2. Influence on bacterial growth

The antibacterial activity of all *O. ochengi* peptides was investigated applying a bactericidal agar plate assay. The desalted peptide sample (containing all peptides) concentration was 36 μg/ml and the agar plate assay of the desalted peptide mixture resulted in an inhibition zone with a diameter of 20 mm (Fig. 2A). Thus the antibacterial activity of the desalted *O. ochengi* peptide mixture with a concentration of 36 μg/ml corresponds to an ampicillin concentration of 15 μg/ml (Fig. 2A and B). Antibiotics added in the worm cultivating culture, were removed by the desalting process of the fluid and during the RPC runs.

Subsequently all 45 fractions derived from the reversed phase chromatography were investigated by the agar plate assay to identify those fractions with antibacterial activity. 10 fractions showed antibacterial activity with corresponding ampicillin concentrations ranging from 5.5 to 12 μg/ml (Table 1).

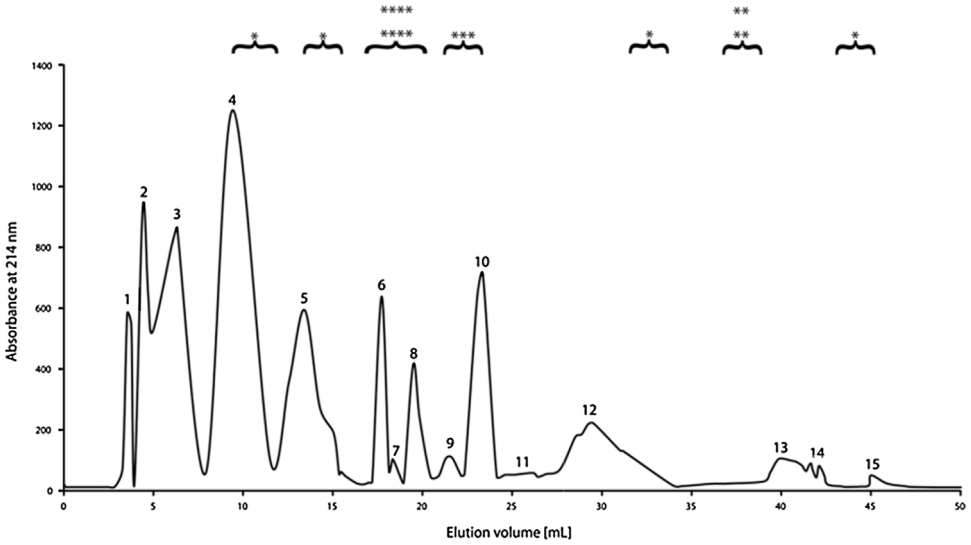

Fig. 1. Reversed phase chromatogram of *O. ochengi* enriched peptides. Fractions marked with asterisks include peptides with antibacterial activity.

Table 1
Peptide composition of RPC fractions with antibacterial activity.

Ranking	RPC fraction	Peptide sequences	Antibacterial activity equivalent to Amp (μg/ml) 100% = 20 μg/ml Amp	Peptide concentration fraction (μg/ml)	Relative effect*
1	37	GNRLIQLTNK, GKHTIFGRVVG, GTIFNSERPTEQIE, RILTNYGAINLELF, VVWTRETLFEYLLDPK, VENSPDTLLLIVSNPVDVLT,	12 (60%)	0.4	30
2	16	TARIVP, SQDAFIN, TVATKLAIL, NKGVITVKD, IKPEVKESKEYFGKH	9.5 (47.5%)	0.5	19
3	18	EEIARATVTA, FGCLFFFDAVRNVNEQIGPA, MEKQENFIEIAKSKDVEVLS	8.5 (42.5%)	0.1	85
4	34	SELMRPQIRN, LEIRGQPFLD	11 (55%)	0.9	12
5	14	QKEVKEYEHR	6.5 (33%)	0.7	9
6	44	DGGDEEGGNDENEDVPRGSF	8.5 (42.5%)	0.6	14
7	23	LDQAPSHCL, EERAPLF LAKVIHDKFG, PYRDVTPQTWK, QPIESNPAKPNAM, QRNVLNGLNLAAYAG, GTHQELVEFDGKYA	11 (55%)	1.2	9
8	19	RQKSQQIQAIM, VLYSFGKISAEN, SCDDAEFSLNINS	7 (35%)	0.4	18
9	11	VCPANWQPGSET	6 (30%)	0.6	10
10	17	MVFSGFCA, QRDEREIPP, TSFQRS, GPGSSWTFYPP, RWLYDQLTPIT, IRHFAGSVCYQTSLFL	5.5 (27.5%)	0.4	14

The absolute and relative effects to the peptide corresponding concentration are summarized.

* The relative effect is a factor, without dimensions, characterize the relationship between the observed "antibacterial activity" and the corresponding "peptide concentration per RPC fraction".

Three of these ten fractions contained only one peptide. Thus antibacterial activity could be attributed unambiguously to the peptides QKEVKEYEHR**,** DGGDEEGGNDENEDVPRGSF and VCPANWQPGSET. The strongest absolute antibacterial activity was shown by DGGDEEGGNDENEDVPRGSF and indicates also the highest relative antibacterial activity in context to the peptide concentration (Table 1). The calculated theoretical net charge, hydrophobicity and secondary structure prediction for all peptides is summarized in the appendix, Table A1. The remaining seven fractions with antibacterial activity contained overall 33 peptides with potential antibacterial activity. In summary, ten RPC fractions that display antibacterial activity contained 36 peptides with potential antibacterial activity. The peptide concentrations, composition, as well as the absolute and relative antibacterial activity of each fraction are summarized in Table 1.

4. Discussion

85 different peptides in the secretion of *O. ochengi* adult females were identified by mass spectrometric analysis and data base searches. The analysis revealed that 72 peptides are proteolytic products originating from *O. ochengi* proteins, which can be classified according to their putative function (Fig. 3).

The remaining identified 13 peptides originate from so far uncharacterized *O. ochengi* proteins with unknown function (appendix, Table A1). The sequence analyses of the 72 peptides released from known proteins showed sequence homologies up to 100% for most of the peptides towards *Onchocerca* species and helminths molecules, as summarized in the appendix, Table A2. The majority of these proteins has been described before for other *Onchocerca* species and helminths, like *Brugia malayi*, e.g. GAPDH, enolase, cytochrome c oxidase, cyclophilin, ALT, peroxidoxin (Bennuru et al., 2009; Hewitson et al., 2008).

The peptide QKEVKEYEHR (galectin) is conserved in *O. ochengi*, *O. volvulus*, *O. flexuosa*, *B. malayi* and *W. bancrofti*. With exception of *O. flexuosa*, the peptide VCPANWQPGSET (peroxidoxin-2) is conserved within all analyzed species. In contrast, the peptide DGGDEEGGNDENEDVPRGSF (ALT-1) shows substantial sequence differences, compared to the corresponding sequences found in other nematodes (appendix Table A2).

However, it has not been described before that galectin, peroxidoxin-2 or ALT-1 contains peptide sequences with antibacterial activity, which can be released through the action of proteases. It is known that several antimicrobial peptides described so far are derived from proteins by proteolysis, for instance buforin II from histone 2A (Kim et al., 2000) or lactoferricin from lactoferrin (Kuwata et al., 1998). However, it remains unclear whether the peptides reported here, which are part of a precursor protein, are generated by specific proteolysis from pre-proteins or by random proteolysis. An analysis of the 62 protein sequences applying the ExPASy PeptideCutter tool (Gasteiger et al., 2005) and the MEROPS database (Rawlings et al., 2012) revealed mainly cleavage sites for serine proteases and for metallo-proteases, flanking the identified peptides. Further, ten proteins contain a signal peptide (ALT-1, Ov33-3, rainforest immunodominant hypodermal antigen, cathepsin L-like cysteine proteinase, fatty acid and retinol binding protein-1, peptidyl-prolyl *cis–trans* isomerase, activation-associated secreted protein-1, extracellular Cu/Zn superoxide dismutase, Ov16 antigen and Ov39 antigen). However, the identified peptides are not derived from a signal peptide cleavage process. In summary we can speculate that these peptides arose from random proteolysis – e.g. naturally in the onchocercoma habitat of the females – proteolysis of ESP proteases, e.g. blisterase (Poole et al., 2003) or through host protease cleavage, as in the activation process of the *O. volvulus* TGF-beta homodimer (Korten et al., 2009). Thus, in some selected cases the respective corresponding proteins were also found by mass spectrometric analysis of the proteins which had been precipitated from the culture supernatants of the females by TCA (Brattig et al., unpublished). Nevertheless, the peptides could be naturally generated by host's or by parasite's proteases from distinct proteins released from the onchocercoma-dwelling females. The peptides can result from proteolytic cleavage by parasite's exported proteinases like astacin (Borchert et al., 2007), aspartic protease (Jolodar et al., 2004a,b), serine proteinase (Haffner et al., 1998; Lackey et al., 1989; Jolodar et al., 2004a,b; Ford et al., 2005) as well as secreted host-originated proteinases like plasmin and meprin metalloproteinase.

The functional activity of released *O. ochengi* molecules certainly depends on the developmental stage of the worm and the state of the host immune system. For example, it has been shown for *B. malayi* microfilariae that the composition of the secreted material depends on the local environment of the microfilariae in the human host (Moreno and Geary, 2008). Our results provide molecular details to support the understanding of the molecular

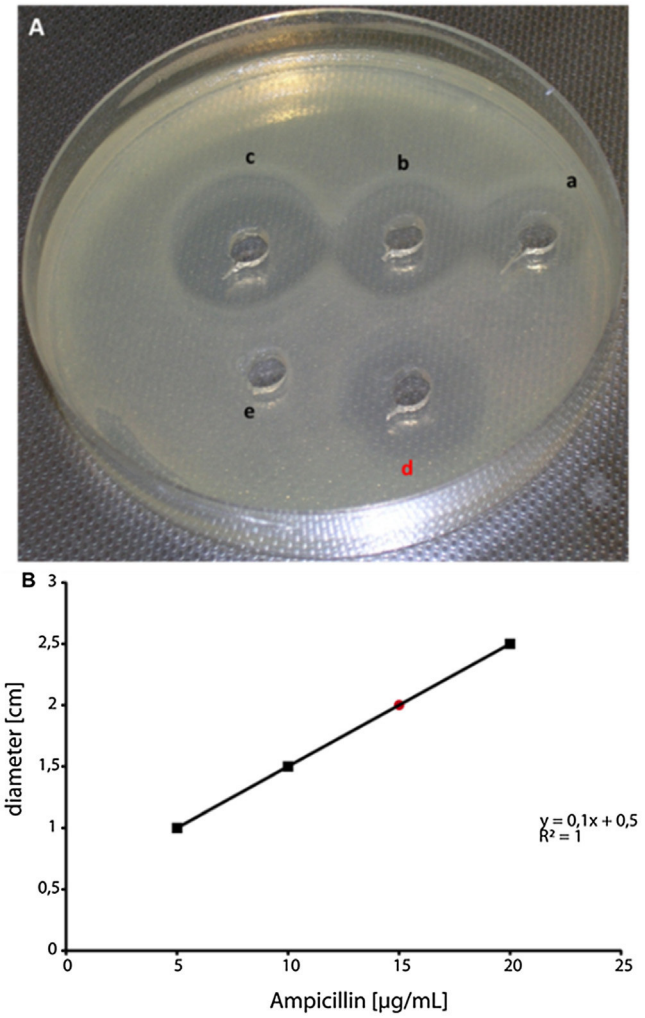

Fig. 2. Representative results of the agar plate assay applying the peptide mixture after the desalting process. The bactericidal activity was tested against BL21 Star^{Tm} (DE3) *E. coli* cells. The antibacterial efficiency of the peptides was calculated on the basis of the diameters of inhibition zones obtained from three different ampicillin concentrations on the same plate (a: 5 µM, b: 10 µM and c: 20 µM; d: sample; e: control). (A) Agar plate assay of the *O. ochengi* peptide mixture with sample d showing an inhibition zone for bacterial growth. (B) Determination of antibacterial efficiency of the desalted peptide sample based on the diameters of the inhibition zones obtained from 5, 10, and 20 µg/ml of ampicillin with the same plate. A standard equation was used to calculate the antibacterial potency of the peptide sample. The determined antibacterial potency of the desalted peptide sample corresponds to 15 µM ampicillin.

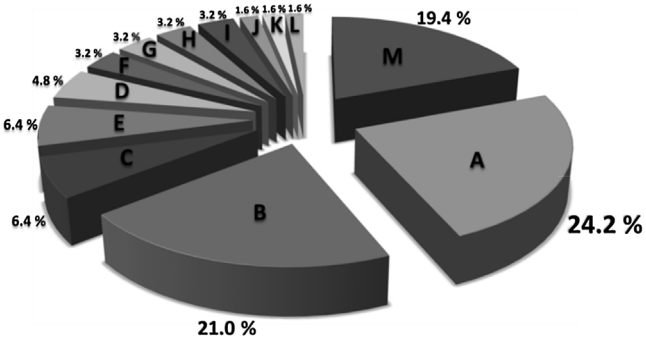

Fig. 3. Functional profiles of the *O. ochengi* proteins releasing peptides by proteolytical processes. Pie-chart representing the percentage of proteins identified from protein families. (A) metabolism, (B) immunogenic proteins, (C) antioxidants, (D) proteases, (E) stress response proteins, (F) glycoproteins, (G) transport proteins, (H) receptor proteins, (I) construction, (J) protein modification, (K) signal transduction, (L) ribosome, (M) uncharacterized proteins.

mechanisms and of the cross-talk between parasites and hosts (Thomas et al., 2005). Preceding investigations demonstrated that not only secreted proteins and peptides are involved, but also carbohydrates (Jenkins et al., 2005; Thomas et al., 2003) and lipid mediators play an important role in the modulation of the host immune system (Brattig et al., 2009; van der Kleij et al., 2002). For the first time we have been able to unambiguously show that three secreted *O. ochengi* peptides have antibacterial activity against the gram-negative *E. coli*. Furthermore, six RPC fractions containing more than one peptide, also revealed antibacterial effects. Since antibacterial effects are so far unaccounted for filarial infections, this functional activity of the peptides may stand for a further biochemical activity beyond the effect on bacteria, virtually as a representative activity on distinct host cells or structures which has not yet been identified.

Robinson et al. (2011) described peptides secreted by the helminth *Fasciola hepatica* with functional and biochemical characteristics similar to host defense peptides: defensins and cathelicidins. These peptides – designated as helminth defense molecules (HDM) – exhibits similar structural features to LL-37, an antimicrobial peptide proteolytically released from the human cathelicidin precursor. By inhibiting the interaction between bacterial LPS and macrophages, *F. hepatica* HDM-1 prevents the activation of an inflammatory immune response (Robinson et al., 2011).

However, analyses of the primary sequence of the identified *O. ochengi* peptides with fragments of the above described helminth HDM-1 (Robinson et al., 2011) showed only a very low amino acid sequence similarity (<5%). In contrast, DGGDEEGGNDENEDVPRGSF showed a sequence similarity of around 30% to the human antibacterial peptide dermcidin (Schittek et al., 2001) and QKEVKEYEHR showed a sequence similarity of approximately 30% to ixosin from *Ixodes sinensis* (Yu et al., 2006). Furthermore the *O. ochengi* galectin showed a sequence similarity of 33% to the human galectin-3, which induces a proven antibacterial and antifungal function (Kohatsu et al., 2006).

Moreover, it is already well known that helminths cause also tissue damages allowing a systemic movement of bacteria (Ferraz et al., 2005; Ogunrinade and Adegoke, 1982). In this context it is of interest that in areas endemic for helminth parasites, a co-infection with gram-negative bacteria, most commonly by *Salmonella sp*, is frequently observed (Melhem and LoVerde, 1984). Robinson et al. (2011) reported, that the active secretion of host defense molecules by parasites during their lifespan in the mammalian host can ensure that pathogenic or even potentially lethal LPS, derived from microbial co-infections, is neutralized and that LPS-mediated activation of macrophages is controlled (Robinson et al., 2011). Thus, by preventing the activation of innate immune responses normally induced by LPS, the helminths may enhance the survival of their hosts and in consequence increase their own longevity (Robinson et al., 2012).

The possible pharmaceutical application of antimicrobial peptides has a strong impact today (Zasloff, 2002). So far, more than 800 antimicrobial peptides have been identified in different tissues and cell types of a variety of invertebrate, plant and animal species. They comprise peptide cytokines and chemokines, selected neuropeptides and peptide hormones or proteolysis processing products of proteins. In general, peptides with antibacterial activity share common characteristics comprising size, sequence, charge, conformation and structure, hydrophobicity and amphipathicity (Brogden, 2005). The antimicrobial peptides share these common characteristics like the strong positive or negative charge, the high hydrophobicity and a high content of secondary structure. In particular DGGDEEGGNDENEDVPRGSF shows the highest net charge of −7 and coincidently also a relatively high hydrophobicity with +44.7 kcal/mol within the identified four antibacterial peptides.

The observed antibacterial activity of this peptide is equivalent to the antibacterial potency of 8.5 μg/ml ampicillin, considering that the corresponding peptide concentration was calculated to be 0.6 μg/ml (Table 1).

It has also been reported that antimicrobial peptides released by helminth parasites interfere with the vitality of protozoan parasites like *Plasmodium spec.* or *Leishmania spec*. (Afacan et al., 2012; Torrent et al., 2012). Indeed, in initial experiments applying the desalted peptide fraction, an inhibitory effect against *Plasmodium falciparum* strain 3D-7 was observed (data not shown).

The dual function of HDP/HDM regarding activity against human pathogens and a modulation of the human immune system are key features of these molecules and corresponds to their central task and activity in the innate immune response. The capability of *O. ochengi* female worms to release peptides mimicking the human HDP confirm their potential to modulate the immune system (Hartmann et al., 2013). In summary, well-adapted helminths may even enhance the survival of the host to support their own survival. Therefore, ESPs of helminths are a rich source of pharmaceutically interesting molecules, for example displaying activities against bacteria, fungi, virus and eukaryotic parasites.

5. Conclusions

85 different peptides have been identified in the ESPs of female *O. ochengi* by mass spectrometry, mainly deriving from proteins with various metabolic functions, including stress response or representing immunogenic proteins. Within these peptides we identified three peptides with substantial antibacterial activity against *E. coli* (VCPANWQPGSET, QKEVKEYEHR and DGGDEEGGNDENEDVPRGSF).

DGGDEEGGNDENEDVPRGSF shows common features known for anionic antibacterial peptides, like high negative charge, high hydrophobicity and an abundance of secondary structural elements.

Interestingly the desalted peptide mixture presented antibacterial and antiplasmodial activity. In summary the ESPs of helminths like *O. ochengi* present an interesting reservoir of bioactive molecules, and further characterization of these molecules will provide new important insights into the parasite host molecular cross-talk. In this context also lipids, possible lipid mediators and carbohydrates within the *O. ochengi* ESP have to be considered and analyzed accordingly.

Conflict of interest statement

There is no conflict of interest for all authors.

Acknowledgements

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Appendix A.

Tables A1–A2

Table A1
Peptides identified in *O. ochengi* secretome.

Fraction No.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
24	528	GGVALL	30	Heat shock protein 60 (genome: 9027) *O. volvulus*	0	+7.7	Coiled coil regions
16	656	TARIVP	33	Uncharacterized protein[1] (genome: 3717) *O. ochengi*	+1	+9.0	Coiled coil regions
24	667	LLLVLV	30	Peptidyl-prolyl *cis–trans* isomerase (O96336) *O. volvulus*	0	+2.0	Coiled coil regions
6	692	FLEVAGG	28	Protein disulfide isomerase (genome: 168) *O. volvulus*	−1	+10.9	Coiled coil regions
17	724	TSFQRS	30	OV71 protein (genome: 7873) *O. volvulus*	+1	+9.9	Coiled coil regions
15	732	QVPEIF	26	Uncharacterized protein[2] (genome: 6633) *O. ochengi*	−1	+9.2	Coiled coil regions
6	770	SKPNARV	25	Putative heparan sulfate (genome: 11235) *O. volvulus*	+2	+14.0	Coiled coil regions, beta-turn
7	773	QQREGVG	29	Activation-associated secreted protein-1 (genome: 11358) *O. volvulus*	0	+16.7	Coiled coil regions, beta-turn
21	780	EMNRTE	41	Ankyrin homolog (O96411) *O. ochengi*	−1	+17.4	Coiled coil regions
7	786	ATPAALRS	28	Ankyrin homolog (O96411) *O. ochengi*	+1	+10.8	Coiled coil regions
7	790	PNFLLTS	30	Putative ivermectin receptor (O61641) *O. ochengi*	0	+5.4	Coiled coil regions
Fraction no.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
16	794	SQDAFIN	36	Uncharacterized protein[3] (genome: 2707) *O. ochengi*	−1	+11.3	Coiled coil regions, beta-turn
6	844	EIPPFLE	30	Rainforest immunodominant hypodermal antigen (B2D1W6) *O. ochengi*	−2	+11.4	Coiled coil regions, beta-turn
13	851	AKLHPTKG	27	Chitinase (genome: 13332) *O. volvulus*	+2	+16.6	Coiled coil regions
17	861	MVFSGFCA	41	NADH dehydrogenase subunit 5 (Q5QIQ2) *O. ochengi*	0	+5.4	Coiled coil regions, beta-turn

Table A1 (*Continued*)

Fraction No.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
23	862	EERAPLF	32	Uncharacterized protein[4] (genome: 8359) *O. ochengi*	−1	+14.7	Coiled coil regions
29	877	AVSATPFIA	30	Uncharacterized protein[5] (genome: 140) *O. ochengi*	0	+6.9	Coiled coil regions
20	882	SITPSYKS	38	B20 protein (B2D1X0) *O. ochengi*	+1	+10.6	Coiled coil regions
25	902	ADYERIH	31	Rainforest immunodominant hypodermal antigen (B2D1W6) *O. ochengi*	−1	+18.0	Coiled coil regions
22	907	LFGEKSNL	30	Immunodominant antigen Ov33-3 (genome: 11807) *O. volvulus*	0	+12.6	Coiled coil regions
15	917	DRRTLAAD	27	Uncharacterized protein[6] (genome: 990) *O. ochengi*	0	+18.8	Coiled coil regions
15	921	SQITEQES	29	Novel immunogenic protein (genome:6296) *O. volvulus*	−2	+16.8	Coiled coil regions
Fraction no.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
16	928	TVATKLAIL	41	B8 protein (B2D1W7) *O. ochengi*	+1	+8.1	Coiled coil regions
43	932	VPDIVLYN	35	Acetylcholine receptor non-alpha chain (P54247) *O. volvulus*	−1	+8.5	β-Strand, coiled coil regions
26	964	YLKLGSRE	32	Uncharacterized protein[7] (genome: 3568) *O. ochengi*	+1	+14.5	Coiled coil regions, beta-turn
16	973	NKGVITVKD	39	Chaperonine protein (genome: 11357) *O. volvulus*	+1	+17.4	Coiled coil regions, beta-turn
23	982	LDQAPSHCL	26	Uncharacterized protein[8] (genome: 8867) *O. ochengi*	−1	+13.2	Coiled coil regions
18	995	FGCLFFFD	30	NADH-ubiquinone oxidoreductase (O47572) *O. volvulus*	−1	+4.6	β-Strand, coiled coil regions
26	1010	EYYGIHDD	31	Ankyrin homolog (O96411) *O. ochengi*	−3	+19.8	Coiled coil regions
13	1014	AKVIHDKFG	29	Glyceraldehyde 3-phosphate-dehydrogenase (genome: 13534) *O. ochengi*	+1	+16.7	β-Strand, coiled coil regions
7	1021	RSGKPQEYG	27	Protein disulfide isomerase (genome: 168) *O. volvulus*	+1	+19.1	Coiled coil regions
26	1039	DYLGLNGMAS	32	Cytochrome c oxidase subunit 3 (genome: 13917) *O. volvulus*	−1	+11.8	Coiled coil regions, beta-turn
15	1042	NNGLQIRDI	34	Uncharacterized protein[9] (genome: 2815) *O. ochengi*	0	+13.5	Coiled coil regions
Fraction no.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
12	1050	PPTSVPAPPTS	31	Pyrrolidone-rich antigen Ov42 (genome: 3939) *O. volvulus*	0	+10.1	Coiled coil regions
18	1060	EEIARATVTA	34	Fructose-1,6-bisphosphate aldolase (B2D1W9) *O. ochengi*	−1	+17.4	Coiled coil regions
20	1105	GANGVAEVYIN	30	Extracellular Cu/Zn superoxide dismutase (genome: 8918) *O. volvulus*	−1	+13.8	β-Strand, coiled coil regions
24	1125	QYYFDGCMV	32	Immunodominant antigen Ov33-3 (genome: 11807) *O. volvulus*	−1	+9.2	Coiled coil regions, beta-turn
23	1127	LAKVIHDKFG	44	Glyceraldehyde 3-phosphate-dehydrogenase (genome: 13534) *O. ochengi*	+1	+16.6	α-Helical regions, coiled coil regions
17	1139	QRDEREIPP	33	Rainforest immunodominant hypodermal antigen (B2D1W6) *O. ochengi*	−1	+22.4	Coiled coil regions
37	1156	GNRLIQLTNK	37	Major antigen (genome: 7406) *O. volvulus*	+2	+12.8	Coiled coil regions, beta-turn
25	1158	VLAGSLLFLLL	30	Cytochrome c oxidase subunit 1 (Q9GC21) *O. ochengi*	0	+0.4	β-Strand, coiled coil regions
45	1166	HILIAVGGYPK	36	Glutathione reductase (B5MAH7) *O. ochengi*	+1	+11.3	β-Strand, coiled coil regions
37	1169	GKHTIFGRVVG	77	Cyclophilin 4 (genome: 7109) *O. volvulus*	+2	+14.8	β-Strand, coiled coil regions
34	1187	LEIRGQPFLD	30	Blisterase precursor (genome: 6241) *O. volvulus*	−1	+13.7	Coiled coil regions, beta-turn

Table A1 (*Continued*)

Fraction no.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
17	1195	GPGSSWTFYPP	31	Cytochrome c oxidase subunit 1 (Q9GC21) *O. ochengi*	0	+7.3	Coiled coil regions, beta-turn
13	1201	NLGAPEMAFPR	32	Cytochrome c oxidase subunit 1 (Q9GC21) *O. ochengi*	0	+13.0	Coiled coil regions
7	1208	RSYITQLTLN	31	Mitochondrial solute carrier (genome: 5398) *O. volvulus*	+1	+8.0	Coiled coil regions
24	1227	PKRPNVPGAEY	36	Glutathione reductase (B5MAH7) *O. ochengi*	+1	+17.9	Coiled coil regions
34	1244	SELMRPQIRN	40	Uncharacterized protein[10] (genome: 582) *O. ochengi*	+1	+14.3	Coiled coil regions
25	1246	SSANMNQPAIIT	35	Blisterase precursor (genome: 6241) *O. volvulus*	0	+9.8	β-Strand, coiled coil regions
13	1251	HTVGIGSLLGAIN	40	Cytochrome c oxidase subunit 1 (Q9GC21) *O. ochengi*	0	+10.5	α-Helical regions, coiled coil regions
24	1258	SEKKLPYPIIA	31	Putative peroxiredoxin (genome: 340) *O. volvulus*	+1	+14.2	Coiled coil regions
18	1267	AVRNVNEQIGPA	34	Enolase (genome: 7559) *O. volvulus*	0	+16.1	β-Strand, coiled coil regions
11	1288	VCPANWQPGSET	31	Peroxidoxin-2 (O76452) *O. ochengi*	−1	+13.2	Coiled coil regions
13	1315	RTAEDGRVNAVQ	40	Major antigen (genome: 7406) *O. volvulus*	0	+21.9	β-Strand, coiled coil regions

Fraction no.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
25	1322	TACERAKRTLSS	32	Heat shock 70 kDa protein (genome: 2756) *O. volvulus*	+2	+19.1	Coiled coil regions, beta-turn
19	1327	VLYSFGKISAEN	35	Ov16 antigen precursor (genome: 13238) *O. volvulus*	0	+12.5	α-Helical regions, coiled coil regions
19	1329	RQKSQQIQAIM	41	Rainforest immunodominant hypodermal antigen (B2D1W6) *O. ochengi*	+2	+13.6	α-Helical regions, coiled coil regions
26	1335	YVSGRKEPDPGM	38	Reprolysin (genome: 1930) *W. bancrofti*	0	+21.0	Coiled coil regions, beta-turn
14	1344	QKEVKEYEHR	29	Galectin OvGalBP (genome: 8682) *O. volvulus*	0	+28.1	β-Strand, coiled coil regions
26	1358	SNQRRETQQVI	38	Ov39 antigen (genome: 8307) *O. volvulus*	+1	+17.4	α-Helical regions, coiled coil regions
21	1362	LGEVINEKDKFA	50	Small heat shock protein (genome: 13179) *O. volvulus*	−1	+22.4	Coiled coil regions
23	1391	PYRDVTPQTWK	30	39S ribosomal protein L45 (genome: 365) *W. bancrofti*	+1	+14.4	Coiled coil regions
25	1394	LETIEGMKFDRG	32	Chaperonine protein (genome: 11357) *O. volvulus*	−1	+21.2	β-Strand, coiled coil regions
23	1396	QPIESNPAKPNAM	35	Gln-rich protein (genome: 12833) *O. volvulus*	0	+17.0	Coiled coil regions, beta-turn
17	1405	RWLYDQLTPIT	34	Glutamate-cysteine ligase (genome: 11266) *O. volvulus*	0	+8.3	β-Strand, coiled coil regions

Fraction no.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
19	1414	SCDDAEFSLNINS	34	Uncharacterized protein[11] (genome: 9013) *O. ochengi*	−3	+18.3	Coiled coil regions, beta-turn
12	1446	MMNGLRFPNETH	34	Cathepsin L-like cysteine proteinase (genome: 1047) *O. volvulus*	0	+14.6	Coiled coil regions, beta-turn
7	1493	SNRRNGSSSWRW	33	Uncharacterized protein[12] (genome: 12974) *O. ochengi*	+3	+13.8	Coiled coil regions, beta-turn
30	1572	AAHWCPQCRRFTP	28	Thioredoxin-2 (genome: 8110) *O. volvulus*	+2	+12.3	Coiled coil regions, beta-turn
23	1573	QRNVLNGLNLAAYAG	32	P-glycoprotein (genome: 5563) *O. volvulus*	+1	+11.9	β-Strand, coiled coil regions
23	1593	GTHQELVEFDGKYA	30	P-glycoprotein (genome: 5563) *O. volvulus*	−2	+23.6	α-Helical regions, coiled coil regions
29	1595	IPEEVKNFYKNLT	30	Fatty-acid and retinol-binding protein 1 (Q8WT57) *O. ochengi*	0	+17.6	α-Helical regions, coiled coil regions
37	1621	GTIFNSERPTEQIE	37	Uncharacterized protein[13] (genome: 8555) *O. ochengi*	−2	+20.5	Coiled coil regions, beta-turn
25	1633	IAVHNSKDPAEIPWG	45	Glyceraldehyde 3-phosphate-dehydrogenase (genome: 13534) *O. Ochengi*	−1	+19.3	Coiled coil regions

Table A1 (*Continued*)

Fraction No.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
37	1636	RILTNYGAINLELF	31	Cyclophilin-4 (genome: 7109) *O. volvulus*	0	+8.5	β-Strand, coiled coil regions
16	1818	IKPEVKESKEYFGKS	45	Peroxidoxin-2 (O76452) *O. ochengi*	+1	+30.0	α-Helical regions, coiled coil regions

Fraction no.	Observed *m/z*	Sequence determined	Peptide-score Mascot	Precursor	Net charge	Hydrophobicity (kcal/mol)	Secondary structure prediction
17	1842	IRHFAGSVCYQTSLFL	32	Body wall myosin (genome: 191) *O. volvulus*	+1	+7.4	β-Strand, coiled coil regions
37	2009	VVWTRETLFEYLLDPK	42	Cytochrome c type-1 (genome: 2616) *O. volvulus*	−1	+14.9	α-Helical regions, coiled coil regions
20	2093	KDSRGVIQTDEEGDKCESP	34	B8 protein (B2D1W7) *O. ochengi*	−3	+39.9	β-Strand, coiled coil regions
44	2095	DGGDEEGGNDENEDVPRGSR	32	Abundant larval transcript 1 (B2D1W8) *O. ochengi*	−7	+44.7	Coiled coil regions
37	2138	VENSPDTLLLIVSNPVDVLT	62	L-lactate dehydrogenase (genome: 2770) *O. volvulus*	−3	+14.3	β-Strand, coiled coil regions
18	2337	MEKQENFIEIAKSKDVEVLS	36	Ankyrin homolog (O96411) *O. ochengi*	−2	+30.7	α-Helical regions, coiled coil regions
27	2394	VYQSFFIGGGPGSSWTFYPPLS	40	Cytochrome c oxidase subunit 1 (Q9GC21) *O. ochengi*	0	+4.3	β-Strand, coiled coil regions
29	2409	WLPKAMAAPTPVSCLVHSSTDVT	37	NADH dehydrogenase subunit 5 (Q5QIQ6) *O. ochengi*	+1	+9.2	β-Strand, coiled coil regions

Net charge and hydrophobicity was determined by PepDraw, the secondary structure prediction was calculated by NetSurfP and NetTurnP. The antibacterial activity was analysed by a bactericidal agar plate assay.

Table A2
Proteins releasing identified peptides, arranged regarding their function.

	Protein class	Precursor protein	*O. ochengi*	*O. volvulus*	*O. flexuosa*	*B. malayi*	*W. bancrofti*
A	Metabolism	Glyceraldehyde 3-phosphate-dehydrogenase	√ (100%)	√ (100%)	N/S	√ (100%)	√ (100%)
		Cytochrome c type-1	√ (100%)	√ (100%)	N/S	√ (100%)	√ (100%)
		Cytochrome c oxidase subunit 1	√ (100%)	√ (99%)	√ (99%)	√ (94%)	√ (100%)
		Cytochrome c oxidase subunit 3	√ (100%)	√ (100%)	√ (100%)	√ (90%)	√ (90%)
		NADH dehydrogenase subunit 5	√ (100%)	√ (100%)	√ (100%)	√ (100%)	√ (100%)
		NADH-ubiquinone oxidoreductase	√ (100%)	√ (100%)	√ (75%)	√ (75%)	√ (75%)
		Enolase	√ (100%)	√ (92%)	N/S	√ (100%)	√ (100%)
		L-lactate dehydrogenase	√ (100%)	√ (100%)	N/S	√ (93%)	√ (98%)
		Fructose-1,6-bisphosphate aldolase	√ (100%)	√ (100%)	N/S	√ (90%)	√ (90%)
		Chitinase	√ (100%)	√ (100%)	N/S	√ (87%)	√ (75%)
		Glutathione reductase	√ (100%)	√ (100%)	N/S	√ (100%)	√ (100%)
		Glutamate-cysteine ligase	√ (100%)	√ (100%)	√ (100%)	√ (100%)	√ (100%)
		Putative heparan sulfate	√ (100%)	√ (100%)	N/S	√ (33%)	N/S
		Protein disulfide isomerase	√ (100%)	√ (100%)	√ (40%)	√ (93%)	√ (93%)
		Peptidyl-prolyl cis-trans isomerase	√ (100%)	√ (100%)	√ (20%)	√ (83%)	√ (30%)
B	Immunogenic	Major antigen	√ (100%)	√ (98%)	√ (98%)	√ (50%)	√ (0%)
		B8 protein	√ (100%)	√ (100%)	N/S	√ (70%)	√ (20%)
		B20 protein	√ (100%)	√ (100%)	√ (50%)	√ (88%)	√ (100%)
		Rainforest immunodominant hypodermal antigen	√ (100%)	√ (100%)	√ (15%)	√ (70%)	√ (65%)
		Ov39 antigen	√ (100%)	√ (100%)	N/S	√ (60%)	√ (60%)
		Abundant larval transcript 1	√ (100%)	√ (100%)	N/S	√ (20%)	√ (45%)
		Immunodominant antigen Ov33-3	√ (100%)	√ (100%)	N/S	√ (80%)	√ (75%)
		Ov16 antigen precursor	√ (100%)	√ (100%)	N/S	√ (17%)	√ (17%)
		Gln-rich protein	√ (100%)	√ (100%)	N/S	N/S	N/S
		Pyrrolidone-rich antigen Ov42	√ (100%)	√ (100%)	N/S	√ (80%)	√ (73%)
		Novel immunogenic protein	√ (100%)	√ (100%)	N/S	N/S	N/S
		Activation-associated secreted protein-1	√ (100%)	√ (100%)	N/S	√ (70%)	√ (70%)
C	Antioxidant	Putative peroxiredoxin	√ (100%)	√ (100%)	N/S	√ (73%)	√ (73%)
		Peroxidoxin-2	√ (100%)	√ (100%)	N/S	√ (99%)	√ (99%)
		Thioredoxin-2	√ (100%)	√ (100%)	N/S	√ (92%)	√ (50%)
		Extracellular Cu/Zn superoxide dismutase	√ (100%)	√ (100%)	√ (30%)	√ (73%)	√ (55%)

	Protein class	Homologue protein	*O. ochengi*	*O. volvulus*	*O. flexuosa*	*B. malayi*	*W. bancrofti*
D	Protease	Cathepsin L-like cysteine proteinase	√ (100%)	√ (100%)	N/S	√ (45%)	√ (70%)
		Blisterase precursor	√ (100%)	√ (100%)	N/S	√ (86%)	√ (86%)
		Reprolysin	√ (100%)	√ (100%)	N/S	√ (83%)	√ (100)
E	Stress response	Small heat shock protein	√ (100%)	√ (100%)	√ (30%)	√ (100%)	√ (95%)
		Heat shock protein 60	√ (100%)	√ (100%)	N/S	√ (100%)	√ (100%)
		Chaperonine protein	√ (100%)	√ (100%)	N/S	√ (100%)	√ (100%)
		Heat shock 70 kDa protein	√ (100%)	√ (100%)	N/S	√ (100%)	√ (100%)
F	Glycoprotein	Galectin OvGalBP	√ (100%)	√ (100%)	√ (50%)	√ (100%)	√ (100%)
		P-glycoprotein	√ (100%)	√ (100%)	√ (0%)	√ (86%)	√ (53%)
G	Transport	Fatty acid and retinol binding protein-1	√ (100%)	√ (100%)	√ (0%)	√ (92%)	√ (92%)
		Mitochondrial solute carrier	√ (100%)	√ (100%)	N/S	√ (100%)	√ (100%)

Table A2 (*Continued*)

	Protein class	Homologue protein	*O. ochengi*	*O. volvulus*	*O. flexuosa*	*B. malayi*	*W. bancrofti*
H	Receptor	Putative ivermectin receptor	√(100%)	√(60%)	N/S	√(100%)	√(100%)
		Acetylcholine receptor non-alpha chain	√(100%)	√(100%)	N/S	√(100%)	√(100%)
I	Construction	Body wall myosin	√(100%)	√(100%)	√(30%)	√(50%)	√(80%)
		Ov71 protein	√(100%)	√(100%)	N/S	√(100%)	√(83%)
J	Protein modification	Cyclophilin-4	√(100%)	√(100%)	N/S	√(100%)	√(90%)
K	Signal transduction	Ankyrin homologue	√(100%)	√(100%)	N/S	√(0%)	√(66%)
L	Ribosome	39S ribosomal protein L45	√(100%)	√(50%)	N/S	√(100%)	√(100%)
M	Uncharacterized	Uncharacterized protein[1]	√(100%)	√(100%)	N/S	√(100%)	√(100%)
		Uncharacterized protein[2]	√(100%)	√(100%)	N/S	√(100%)	√(100%)
		Uncharacterized protein[3]	√(100%)	√(100%)	N/S	√(86%)	√(100%)
		Uncharacterized protein[4]	√(100%)	√(100%)	N/S	√(100%)	√(100%)
		Uncharacterized protein[5]	√(100%)	√(100%)	N/S	√(56%)	√(0%)
		Uncharacterized protein[6]	√(100%)	√(50%)	N/S	N/S	N/S
		Uncharacterized protein[7]	√(100%)	√(100%)	N/S	√(88%)	√(88%)
		Uncharacterized protein[8]	√(100%)	√(100%)	N/S	√(0%)	√(0%)
		Uncharacterized protein[9]	√(100%)	√(100%)	N/S	√(33%)	√(50%)
		Uncharacterized protein[10]	√(100%)	√(100%)	N/S	√(0%)	√(0%)
		Uncharacterized protein[11]	√(100%)	√(100%)	N/S	√(83%)	√(92%)
		Uncharacterized protein[12]	√(100%)	√(100%)	N/S	√(100%)	√(100%)
		Uncharacterized protein[13]	√(100%)	√(60%)	N/S	√(50%)	√(50%)

O. ochengi proteins are compared with sequences of *O. volvulus* and other filarial species like *Brugia malayi*. The conserved sequence identities of the identified *O. ochengi* peptides within the species are shown in %. N/S: not specified.

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Ongoing Transmission of *Onchocerca volvulus* after 25 Years of Annual Ivermectin Mass Treatments in the Vina du Nord River Valley, in North Cameroon

Short title: River Blindness Not Eliminated in North Cameroon

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Abstract

Background:

Recent reports of transmission interruption of *Onchocerca volvulus*, the causing agent of river blindness, in former endemic foci in the Americas and, more recently, in West and East Africa raise the question whether elimination of this debilitating disease is underway after long-term treatment of the population at risk with ivermectin. The situation in Central Africa has not yet been clearly assessed.

Methods and findings:

Entomologic data from two former endemic river basins in North Cameroon were generated over a period of 43 and 48 months to follow-up transmission levels in areas under prolonged ivermectin control. Moreover, epidemiologic parameters of animal-borne *Onchocerca* spp. transmitted by the same local black fly vectors of the *Simulium damnosum* complex were recorded and their impact on *O. volvulus* transmission success evaluated. With mitochondrial DNA markers we unambiguously confirmed the presence of infective *O. volvulus* larvae in vectors from the Sudan savannah region (mean Annual Transmission Potential 2009 - 2012: 98, range 47 - 221), but not from the Adamawa highland region. Transmission rates of *O. ochengi*, a parasite of Zebu cattle, were high in both foci.

Conclusions/significance:

The high cattle livestock density in conjunction with the high transmission rates of the bovine filaria *O. ochengi* prevents the transmission of *O. volvulus* on the Adamawa plateau, whereas transmission in a former hyperendemic focus was markedly reduced, but not completely interrupted after 25 years of ivermectin control. This study may be helpful to gauge the impact of the presence of animal-filariae for *O. volvulus* transmission in terms of the growing human and livestock populations in sub-Saharan countries.

Author Summary

Over the past decades the fight against river blindness, a tropical disease caused by a nematode worm, has been relatively successful, and a number of countries have been reported to be free of parasite transmission. In North Cameroon, we checked the occurrence of infective stages of *Onchocerca volvulus* in the transmitting black fly populations for more than three years and were able to confirm that the transmission there is low, but not yet interrupted. In a second location on a highland plateau, however, no infective stages of the human parasite were found. Instead, a closely-related parasite of cattle was present in both places. Given that the areas are not far away from each other and the biting frequencies of the black fly populations are similar, the historically earlier and higher density of cattle herds in one of the regions would explain why it is now free of the parasite due to the effects called zooprophylaxis and cross-reacting premunition. Changes in the socio-economic environment, especially the increase of human and cattle populations have a strong influence on the spread of river blindness in Africa.

Introduction

The interruption of transmission of *Onchocerca volvulus*, the causing agent of river blindness or onchocerciasis, has been confirmed for a growing number of endemic foci on the American continent [1,2,3] and in West [4] and East Africa [5,6,7]. The recent success in onchocerciasis control can be mainly attributed to the extensive and sustained mass treatment programs with the microfilaricide ivermectin, governed by institutions of the World Health Organization, like the African Programme for Onchocerciasis Control [8]. Long treatment rounds are necessary because the drug is only lethal to the larval stage and not the adult worm.

There are thus good prospects that elimination of onchocerciasis is well underway in the Americas [9] and may also have begun at different foci on the African continent [6,10,11]. However, currently there is a paucity of information on the actual situation in Central and Southern Africa, in particular with respect to vector transmission, albeit a significant proportion of these regions have been hyperendemic. Recent studies on the effects of ivermectin treatment on the epidemiology of *O. volvulus* in humans and the black fly vector *Simulium damnosum sensu lato* have been done in North and West Cameroon [12,13,14,15,16]. The caveat of the most recent studies is that the filarial species in the vector were not always correctly identified, and the prevalence of infective *O. volvulus* larvae and thus the transmission potential remains unknown. Local *S. damnosum s.l.* populations are vectors of at least two other species from the *Onchocerca* genus: *O. ochengi*, a common parasite of Zebu cattle *Bos indicus* [17] and *O. ramachandrini*, a filaria from the warthog *Phacochoerus africanus* [18]. The proportion of animal-filariae in the vector has direct and indirect consequences for parasite transmission to humans [19,20,21] rendering it an important factor to understand the epidemiology of river blindness. Furthermore, filariae closely-related to *O. volvulus* might repopulate the human host [22] posing a potential risk of infection, or they might transfer genes to *O. volvulus* which negatively affect the effectiveness

of ivermectin, presently the sole drug intervention in use [23]. For this reason we combine microscopic differentiation of infective larvae with a PCR-based molecular approach which allows the detection of yet unknown filarial species and strains in addition to already known *Onchocerca* spp. [22].

This study presents the latest entomologic data of a longitudinal study in the Vina du Nord valley, North Cameroon, dating back to 1976 when ivermectin mass treatment had not yet commenced [24,25]. The impact on *O. volvulus* transmission after 25 years of annual community-directed treatment with ivermectin (CDTI) is demonstrated here. Furthermore, a second site endemic for onchocerciasis in an economically important cattle livestock region has been monitored since 1985. The epidemiologic data is also complemented with *Onchocerca* spp. transmitted by the same local vectors of the *S. damnosum* complex and discussed in light of their impact on transmission success of *O. volvulus*. We have not studied onchocerciasis transmission in regions where ivermectin treatment is contraindicated, such as co-endemic foci of *Loa loa* in the Central African rainforest, although they remain potential source-areas for reinvasion.

Methods

Study sites

Two *S. damnosum* fly catching sites at two foci in Northern Cameroon were visited between two to four times per month (Fig. 1). One former hyperendemic onchocerciasis focus is the village Soramboum close to the Vina du Nord river in the Sudan savannah (500 m altitude): 7°47'14"N; 15°0'22"E where ivermectin mass treatments have been conducted since 1987. Here we present entomological data collected from September 2009 till March 2013. The village has approximately 1000 inhabitants today, and between 1000 and 2000 cattle are located in the vicinity (personal observation). The Vina du Nord river is flowing perennially with an average annual water discharge of 150 m^3 per second, with highest values between July and October [26]. The other formerly hypo- to mesoendemic focus monitored is the village Galim located 15 km south of Ngaoundéré (population: 500 inhabitants, approximately 5000 cattle in the vicinity, personal observation) at the Vina du Sud river (mean annual water discharge: 37 m^3/s, 1050 m altitude): 7°12'2"N; 13°34'56"E, where CDTI has started in 1997. The entomological data was collected from April 2009 till March 2013. The area belongs to the Guinea-grassland of the Adamawa plateau, located in an important area for cattle livestock production in Cameroon [27]. Baseline and follow-up data of *O. volvulus* transmission to man before ivermectin mass treatments started is available for both foci [15,16,19,25,28], and publicly available via the project website www.riverblindness.eu. Whereas *S. damnosum sensu stricto* and *S. sirbanum* are the predominant vector species at the Vina du Nord river, it is *S. squamosum* at the Vina du Sud river [29].

Fly catching was performed according to Duke *et al.* [30] with the following modifications. Blood meal-searching female *Simulium* flies were attracted on man by exposing the fly catcher's legs and trapped with sucking tubes as soon as they settled before starting to probe. Usually the catching period was from 7 am to 6 pm, interrupted by a one

hour break at noon. Afterwards, the catches were transported to the research station in Ngaoundéré and stored at -15°C until dissection.

Fly dissection and identification of filarial species

Flies were sorted, counted, and a maximum of 100 female *S. damnosum s.l.* flies per site and day were dissected with needles under a stereomicroscope (Wild M5, Switzerland). The parous rate was determined by examination of the ovaries in the abdomen [31]. From parous flies infested with filarial worms the location (head, thorax or abdomen), molting stage and quantity was noted. Following the identification key of Wahl *et al.* [15], infective third-stage larvae (L3) were classified to species according to body length, measured by an ocular eye-micrometer attached to the stereomicroscope at 50x magnification, and shape of the anterior and posterior ends. For a subdivision of the L3 taken between February 2010 and February 2012, a molecular investigation of their mitochondrial DNA was conducted according to Eisenbarth *et al.* [22]. Briefly, single L3's were digested with 1 to 2 µl proteinase K (20 µg/µl stock) in 75 µl DirectPCR^{TM} lysis reagent (Viagen Biotech, USA) at 55°C. Two µl of the crude extract was used for each 25 µl PCR reaction. Primer pairs of three mitochondrial loci (12S rRNA, 16S rRNA and *coxI)* that allow for the discrimination of filarial species were used. The amplified PCR products were sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, USA) following the manufacturer's protocol. For the comparison of the body lengths of L3's identified by molecular markers, a larger sample size was taken from flies caught in the same period at the Vina du Sud river about one kilometer downstream of the site near Galim. These flies were collected both from man and cattle.

Calculation of Annual Biting Rates and Transmission Potentials

The Annual Biting Rate (ABR) and Annual Transmission Potential (ATP) of *Simulium damnosum* flies were determined according to the literature [24,25,32]. First, the monthly biting rates (MBR) were calculated by the sum of the flies caught per month divided by the

number of catching days and multiplied by the number of days per month. No correction was made for the missing hours due to rain, sandstorm, or any other reasons. The ABR is the sum of 12 MBRs per hydrological year, measured from April (beginning of rainy season) till March next year (end of dry season). For months during which no catches were attempted, the mean MBR value for the corresponding month and site over the respective decade was estimated by interpolation. The monthly infection rate was the sum of the infective L3 of *O. volvulus*, *O. ochengi* and *O. ramachandrini* from the head, thorax and abdomen found in all parous flies, divided by the sum of dissected flies. By multiplying the monthly infection rate with the respective MBR, the Monthly Transmission Potential (MTP) was determined. The ATP is the sum of 12 MTPs for one year, starting from April till March next year. Missing data points were extrapolated by the sum of all MTP divided by the number of months with data, and multiplied by factor 12. If the MTP data per year was below 3, the mean annual infection rate of proximate years multiplied with the respective ABR was used instead for the ATP calculation.

Statistical analysis

The statistical program Python version 3.4.1 was used for statistical analysis employing student *t*-tests. Results were considered statistically significant when the p-value was below 0.05. P-values were corrected for multiple testing by multiplying with the number of tests done. The effect size was calculated according to Cohen [33]. For depicting the distribution of the L3 body length from a random sample, violin plots*, i.e.* box plots with a rotated kernel density plot on each side, were used.

Results

Entomological data

During the study period, a total of 39,082 flies were caught on human fly catchers, and 21,897 (55.6 %) of them were dissected: a total of 2096 L3 were found (Tab. 1). Depending on the catching site, a mean of 1.96 ±0.53 L3 (max. 20) were harvested per infective fly in Soramboum and a mean of 3.49 ±0.13 L3 (max. 23) in Galim. Near Soramboum at the Vina du Nord site the infection rate (flies carrying L1, L2 and L3) of parous flies in the rainy season was significantly higher than in the dry season (mean: 10.9 *vs.* 5.9 %, *t*-value = -4.91, $p < 0.001$), as well as the infection rate with infective L3 stages (mean: 7.8 *vs.* 3.3 %, *t*-value = -4.88, $p < 0.001$). The opposite was true at the Vina du Sud site near Galim (mean infection rate: 11.1 *vs.* 16.6 %, *t*-value = 2.36, $p > 0.05$; mean L3 infection rate: 4.3 *vs.* 7.1 %, *t*-value = 1.89, $p > 0.05$). Moreover, the parous rate, which is a parameter of age structure, was on average 9.6 % higher in the wet season than in the dry season in Soramboum (range: -18.3 - 19.3%, t-value = 5.31, $p < 0.001$), but in Galim on average 11.8 % lower in the wet season compared to the dry season (range: 6.7 - 24.3 %, t-value = 3.83, $p < 0.001$). The higher proportion of infective L3 to developing larvae during the rainy season in Soramboum (64.0 % *vs.* 53.5 %, t-value = -1.88, $p > 0.05$), but not in Galim (33.9 % *vs.* 35.3 %, t-value = 0.88, $p > 0.05$) is lacking statistical support. A generally higher proportion of L3 in Soramboum can be explained by a longer storage time of the caught flies at ambient temperature during the time (often days) until they were brought to the laboratory, 225 km away by public transport, so that more larval stages developed further.

Comparison of Simulium *biting rates and* Onchocerca *transmission potentials pre- and post-ivermectin distribution*

Figure 2 shows the ABR for the two study sites starting prior to the distribution of ivermectin. With the exception of 1995 the ABR in Galim was higher than in Soramboum, on

average by a factor of 4.26 (SD ±3.30; range: 0.42 - 14.67; n = 17). The yearly fluctuations were more pronounced in the Vina du Nord valley and followed a cyclical pattern (Fig. 2A). In contrast, the ABR at the Vina du Sud fluctuated only mildly apart from intermittent dips, which reached previous levels in the following year (Fig. 2B). An ongoing trend of lower biting frequencies was evident in Soramboum since 2002 (mean: 19,700 flies per person and year *vs.* 35,348 before) and in Galim since 2006 (mean: 39,628 flies per person and year *vs.* 103,564 before). In Soramboum the decline in biting rate occurred mainly in the dry season from October till March with only little changes during the rainy season (Fig. 3A), whereas in Galim the highest decline was within the peak of the dry season and the peak of the rainy season from February till August (Fig. 3B). In the same period of declining ABRs, the monthly infection rate of all L3-harboring flies of all *Onchocerca* spp. increased in Soramboum from 2.25 % (1987 - 2001: 95%-CI: 0.45; n = 90) to 3.26 % (2002 - 2012: 95%-CI: 0.53; n = 89), while it remained stable in Galim (1989 - 2005: 3.34 %, 95%-CI: 0.57, n = 100 *vs.* 2006 - 2012: 3.19 %, 95%-CI: 0.62, n = 65).

A historic summary of the Annual Transmission Potentials over the last 36 years in Soramboum (Fig. 4A) and 27 years in Galim (Fig. 4B) illustrates the alterations in the ratio of animal-filariae and the human filaria *O. volvulus* in the vector. In Galim, annual filarial transmission rates remained high till 2006 (mean: 13,525 L3 per person and year, SD ±5334), when it dropped to 32.5 % of previous levels (mean: 4395 L3 per person and year, SD ±2348; Fig 3B). In contrast, Soramboum experienced an increase of L3 transmission after the early years of ivermectin mass treatments, from an average ATP of 1045 ±438 L3 per person and year in 1987 - 88 to 2286 ±1338 in 1993 - 98, which later returned to former levels, *i.e.* an ATP of 1242 ±741 in 1999 - 2012 (Fig 4A), although the pre-ivermectin control ATP from the adjacent Touboro site was much higher (4140 L3 per person and year, Fig. 3A).

Species composition of the infective Onchocerca *larvae from* Simulium damnosum s.l.

According to morphological classification the species composition of the L3 population in Soramboum from 2009 till 2012 was 23.9 % *O. volvulus*, 65.9 % *O. ochengi* and 10.2 % *O. ramachandrini* (Fig. 5A). In previous years the species composition of *O. volvulus* - *O. ochengi* - *O. ramachandrini* fluctuated from 60.7 % - 12.3 % - 27.0 % (1987 - 88) to 22.3 % - 65.3 % - 10.2 % (1993 - 99) and 40.5 % - 50.8 % - 8.7 % (2000 - 06, Fig 4A). Correspondingly, the species were composed as follows in Galim: 11.3 % *O. volvulus*, 88.7 % *O. ochengi* (1995 - 96), 19.2 %, 80.8 % (2000 - 05) and 17.4 %, 82.6 % (2006 - 12, Fig. 4B). No *O. ramachandrini* L3 were not found at all.

At the Vina du Nord site 96 isolated L3 (10.3 % of all found) from 52 infected flies (7.2 % of all dissected) were subjected to molecular identification, of which 76 (79.2 %) PCR products were successfully sequenced. At Galim from the Vina du Sud site, 40 L3 (3.5 % of all found) from 22 infected flies (2.8 % of all dissected) provided 28 (70 %) amplicons of *Onchocerca* spec. which could be successfully sequenced. The species composition of these L3 from Soramboum was 6.6 % *O. volvulus*, 76.3 % *O. ochengi* and 17.1 % *O. ramachandrini* (Fig. 5B), whereas in Galim only *O. ochengi* was found (Fig. 5D). A recently discovered *O. ochengi* genotpye called 'Siisa' [22,23,16] contributed to 8.6 % and 10.7 %, respectively, of the local *O. ochengi* L3 population in Soramboum and Galim (Fig. 4B and D, respectively). In comparison with morphological classification (n = 71), 72.2 % (13/18) of so-called *O. volvulus* in Soramboum were in fact *O. ochengi*, and 2.4 % (1/41) of *O. ochengi* were *O. ramachandrini*. All examined *O. ramachandrini* (n = 12) were correctly identified. Hence, the respective effective ATP in Soramboum for the years 2009 to 2012 was 68, 221, 58 and 47 for *O. volvulus* (mean: 98): 773, 2503, 1388 and 475 for *O. ochengi* (mean: 1285) and 18, 392, 238 and 70 for *O. ramachandrini* (mean: 180). Accordingly, the adjusted species proportion of the L3 population for these years were on average 6.3 % *O. volvulus*, 82.2 % *O. ochengi* and 11.5 % *O. ramachandrini* (Fig. 4A, right side).

At the Vina du Sud site near Galim (n = 67) *O. volvulus* (0/46) and *O. ramachandrini* (0/0) have not been detected since the introduction of molecular methods for L3 species identification (Fig. 5D), although there were morphologically identified specimens of *O. volvulus* (n = 149, 14.6 % of total, Fig. 5C). All morphologically classed *O. ochengi* (n = 21) were correctly identified. Hence, the whole L3 population in the observation period 2009 - 2012 consisted of *O. ochengi* (Fig. 4B, right side) with an ATP of 5096, 4525, 6753 and 2046 (mean: 4605).

L3 body length as a parameter in species identification

In order to evaluate the reliability of body length as a characteristic trait that can be used for species discrimination of infective larvae, the body lengths of occurring *Onchocerca* spp. L3 in *S. damnosum s.l.* were compared with morphological and molecular identification methods (Fig. 6). Whereas the inter-specific differences according to morphological criteria are significant ($p < 0.001$), no within-species length difference has been detected between morphological and molecular identification of *O. volvulus* and *O. ramachandrini*. A significant ($p < 0.01$) within-species difference has been found in the common genotype of *O. ochengi sensu stricto*, but with a low effect size ($d = 0.598$, $n = 95$); a significant difference ($p < 0.001$, $d = 1.471$, $n = 19$) was also found for the genotype *O. ochengi* 'Siisa'. Interestingly, a more than 4-times higher proportion of morphologically misidentified *O. volvulus* were *O. ochengi* 'Siisa' (25.4 %; 15/59) than in the morphologically identified *O. ochengi* group (6.3 %; 4/64). For the genotpye *O. ochengi s.s.*, this was vice versa (74.6 %; 44/59 of misidentified *O. volvulus vs.* 92.2 %; 59/64 of *O. ochengi* group).

Discussion

This study represents a comprehensive 4-year dataset of transmission from sites in two onchocerciasis-endemic river basins and re-evaluates data collected up to 36 years ago. Whereas we can observe a break of the transmission cycle on the Adamawa plateau, the decline of parasite transmission seems to be halted in the focus of the Sudan savannah despite ongoing treatment intervention. The treatment intervention in this focus has passed the estimated life expectancy of the worm (10 to 15 years) almost by factor two. Conjected that the residual transmission does not stem from invading infected flies from other endemic foci by wind drift, the elimination of *O. volvulus* in previous hyperendemic foci in North Cameroon by yearly-given CDTI appears to be difficult, even though the actual risk of skin-lesions and blindness due to onchocerciasis is presumably very low.

Despite the fact that the Mectizan treatment campaigns on the mesoendemic Adamawa plateau have started about 10 years later, the transmission of the parasites there seems to be disrupted. One reason might be that the transmission rates in the past were partly overestimated due to misidentification of infective larval stages derived from animals. However, several studies including this one emphasize the adequate discriminative power of morphological characters for species delimitation, in particular the body length (Fig. 6) and shape of head and tail [15,19,35]. It is thus very likely that at least a fraction of the L3 found were correctly identified as *O. volvulus*. However, if the endemicity of a region reaches hypoendemic levels in an area of intensive transmission of filariae of non-human origin, such as in this case, the reliability of microscopic examination is limited. For confirmation of interruptions of *O. volvulus* transmission, molecular methods like PCR are necessary.

Decline of the Simulium damnosum *biting rates of humans*

Since 2006 there is a steady trend of lower ABRs, in particular at the Vina du Sud river, where biting rates before were with only one exception above 60,000 per man and year

(Fig. 2B). The vector transmission of filarial stages have also dropped during this time (Fig.4B), but to a lesser extent in the Sudan savannah (Fig. 4A) due to a concomitant gain of the infection rate by bovine filariae. The reason for this vector decline could be the result of decreased availability of breeding sites or food for the aquatic *Simulium* larvae, and hence a drop in population size. A distinct increase of endoparasitic mermithids in human-biting nulliparous flies was evident at the Vina du Sud breeding sites (supplemental data S1). It is, however, unlikely that these mermithids or other *Simulium* parasites are the main drivers for the massive decline in biting rates of recent years. A reduced longevity of adult flies was not observed, when comparing the current parous rate with those of flies at baseline [24]. Furthermore, a continuous rise in the pool of potential blood hosts, both human and livestock, may also contribute to lower individual biting frequencies. The regional impact of climate change cannot be excluded, either, although the water delivery of the investigated rivers have not changed drastically until 1980 [26].

Endemicity of Onchocerca volvulus *in North Cameroon*

The longitudinal monitoring in the Vina du Nord valley indicates that the average transmission of *O. volvulus* remained around 500 L3 per man and year for 20 years after the onset of ivermectin mass treatments (Fig. 4A). This seems to contradict the reduction of onchocerciasis-positive patients in the region as a result of control strategies with ivermectin [12]. One reason could be that there is a variable degree of misidentification of *O. volvulus* L3. In the most recent monitored years 2009 -2012, when molecular detection methods were used, the degree of morphological misidentification was 72 %. However, earlier epidemiological data from the Sudan savannah [25] showed ATP above 4000 L3 per man and year (Fig. 4A, left side). Even though no differentiation of the species had been undertaken at that time, the proportion of animal-filariae in *S. damnosum s.l.* were likely low due to the lack of cattle as potential blood hosts [for explanation see 36]. Hence, only filariae from the

warthog could have been co-transmitted, and the infection rate of the vector with *O. ramachandrini* has not changed considerably during the observation period. Another theory states that the regulation of parasite transmission may be density-dependent instead of linear. That means the effective reproductive ratio of filarial worms equals one even though the basic reproductive ratio is much higher. In the Vina du Nord river basin, Renz [25] compared the prevalence of onchocerciasis and burden of microfilariae with the L3 infection rate in the vector and found no linear correlation, but rather a dependency of fly infection rate with prevalence in the human population instead of the community's microfilarial load (mff/mg). A density-dependent mechanism has already been shown for the parasite acquisition in cattle when inoculated with infective larvae of *O. ochengi* [37,38].

The observed seasonal variations of the entomological parameters match well with baseline data from the Vina du Nord river [25], including the number of infective flies with L3. Nonetheless, the *O. volvulus* ATP has drastically reduced to 3.5 % of the baseline value meaning that the majority of infective flies now carry filariae of animal origin. Additionally, the number of L3 per infective fly decreased moderately (from 3.2 to 1.8). The low but stable transmission level of *O. volvulus* could mean that the threshold for maintaining endemicity is perhaps lower than current mathematical models predict (ATP $\geq$ 100 in West Africa [39,40], but also ATP $\geq$ 54 in Central America [41]).

Molecular vs. morphologic L3 species identification

Even though molecular techniques of identification give higher accuracy, they are less suitable for high throughput analysis due to limitations of time, cost-effectiveness and local infrastructure. They are nonetheless very useful for the detection of unknown strains and species of filarial nematodes in vector and host, such as *Onchocerca ochengi* 'Siisa' [22,23,34]. Experimental infection studies from Togo [42] and Mali [43], where *O. ochengi* microfilariae were inoculated by the vector from infected cattle, revealed shorter L3 body

lengths (Togo: 680 µm, 540 - 680; Mali: 647 µm, 540 - 810) than our observations (740 µm, 600 - 940) and previous studies from Cameroon [44]. These values, however, lie in proximity to the measurements for *O. ochengi* 'Siisa' (660 µm, 600 - 900) and may thus represent or morphologically resemble this strain. Ultimately the evolutionary relationships of *Onchocerca* parasites in humans, cattle and game animals can be compared and tested with genetic markers by generating phylogenetic trees [22,34].

Influence of closely-related animal filariae for the transmission of river blindness

Besides the climatic disparities of the two foci, which is mainly a result of different altitudes, they share similar conditions for their respective black fly populations. One major difference, however, is the disproportionately higher cattle stock density on the Adamawa plateau compared to the situation in the Sudan savannah (Fig. 1). The cattle to human ratio around the Galim focus is approx. 10:1, whereas in the Soramboum focus it ranges between 1:1 and 2:1, and was even lower in previous years, because nomadic cattle were not allowed to enter the Vina du Nord basin until 1975, and the local villagers had not kept any livestock animals, either. Nowadays, an increasing number of vagrant Bororo herdsmen arrive with their herds of zebu cattle and become settled. The inherent difference in livestock density is both culturally inherited (migrating pastoralists of the North vs. settled cattle farmers of the South) and due to biologic conditions (water and food scarcity during the dry season in the Sudan savannah; absence of tsetse flies on the Adamawa plateau, which transmit bovine trypanosomiasis). Invading *O. ochengi* L3 elicit a humoral immune reaction in humans, which cross-reacts with *O. volvulus* L3 antigens, thereby reducing transmission success [19]. The protective effect of populations under repeated antigen exposure is called premunition and well known for malaria and other infectious diseases [45,46]. On the Adamawa plateau this effect seems to be strong enough to prevent or at least complicate the regional endemicity of *O. volvulus*. The advent of nomadic herdsmen and their cattle herds in the Vina du Nord

valley is congruent with an increased transmission of animal-borne filariae, in particular *O. ochengi* (Fig. 4A). This sudden jump of animal-filariae in the vector population implies the diversion of large quantities of local *S. damnosum s.l.* to take their blood meal from cattle, thereby reducing the vector pool for humans [16]. This phenomenon has been termed zooprophylaxis and acts also as a protective trait against onchocerciasis transmission [15,20].

The important question is how the low but stable rate of onchocerciasis transmission in the Sudan savannah can be further curbed or completely stopped. Altogether, five molecularly identified *O. volvulus* L3 from two infective flies (3.85 % of total, 95% CI: 0.47 - 13.21 %) were found in the dry season of 2010 (February) and the rainy season of 2011 (June). Since yearly CDTI application rounds are given at the end of July, the late time point of occurrence after ivermectin treatment may hint to an incipient reconstitution of skin microfilariae in humans infected with *O. volvulus* 12 months prior. This would be a strong argument for the continuation or even temporary intensification of the ivermectin control program [10,36,47]. However, current political instabilities in adjacent countries and the exclusion of certain patient groups in treatment intervention programs, like nomadic people, illegal immigrants and refugees, could impede the long-term success of such measures. Ongoing monitoring of vector transmission is therefore crucial for health policy in onchocerciasis-endemic countries.

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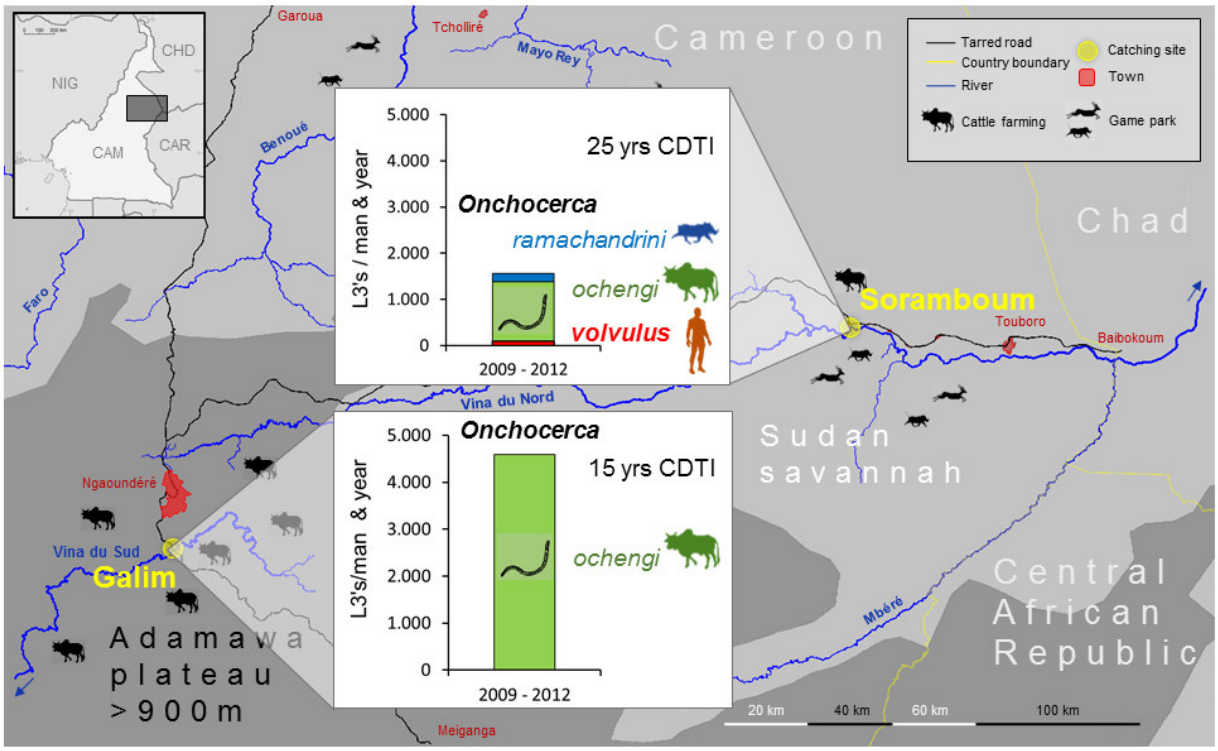
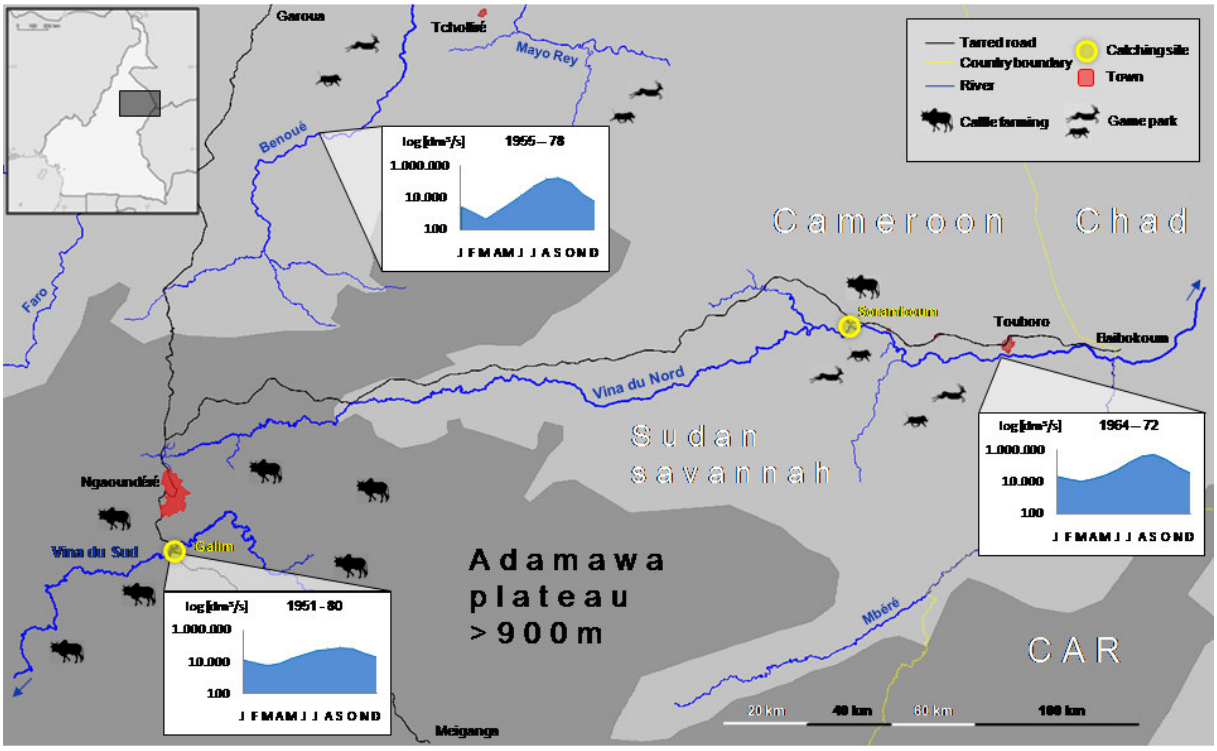
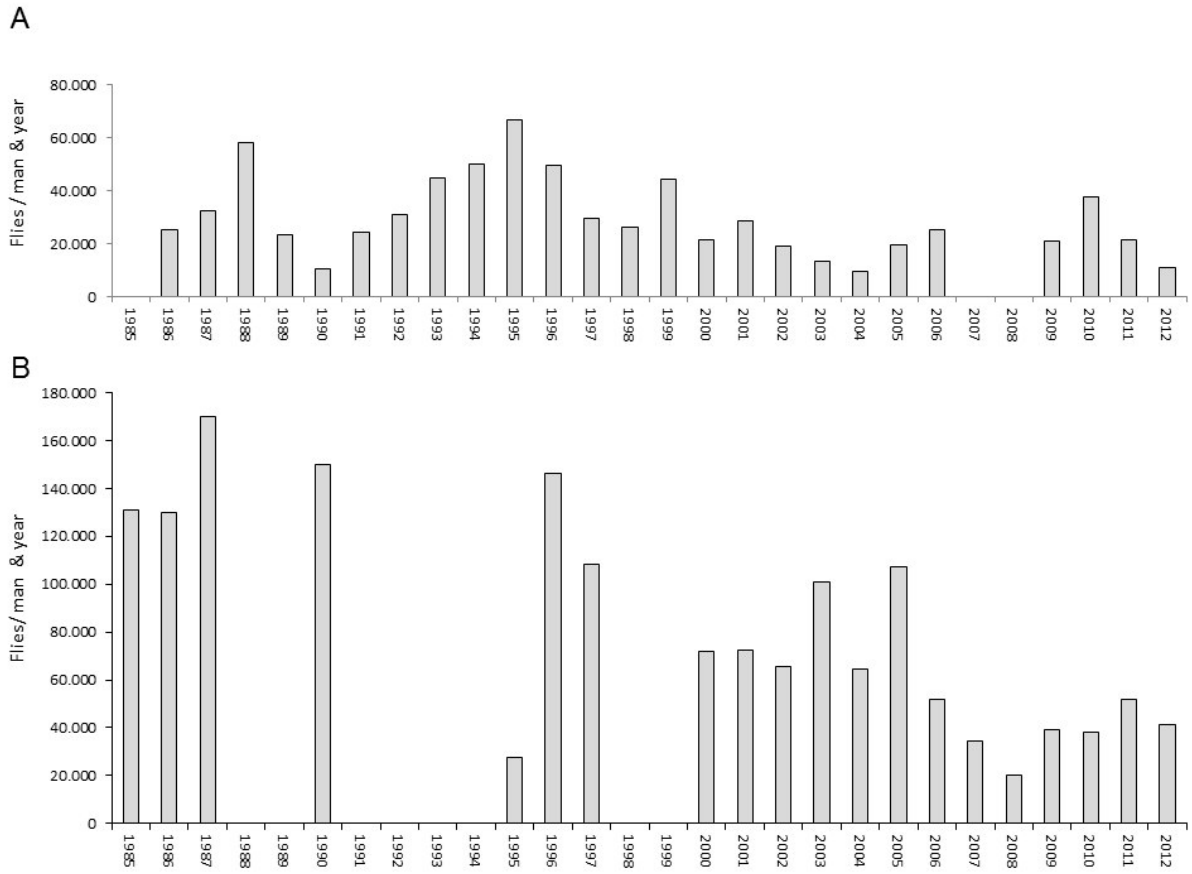


Figure 1: Study area



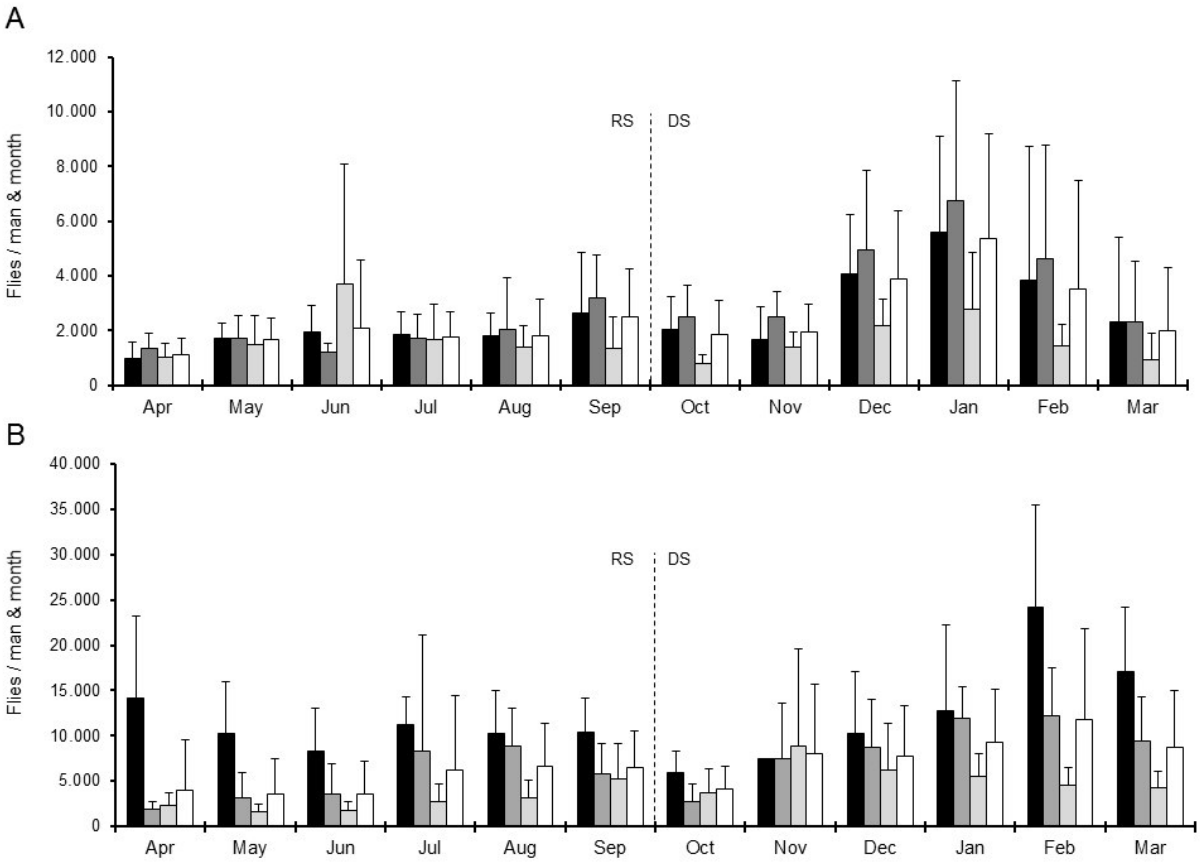
Overview map of the study sites (yellow circles) from two river basins in Northern Cameroon. The village Galim at the Vina du Sud river near Ngaoundéré is on the Adamawa highland plateau, an area above 900 m altitude (dark grey area) with intense cattle husbandry. The village Soramboum at the Vina du Nord river towards Touboro is located in the Sudan savannah (light grey area), a region not often frequented by cattle herds and with high wildlife density. Diagrams of hydrological data showing the mean monthly water discharge from three river basins is taken from Olivry [26]. CAR: Central African Republic.

Figure 2: Annual Biting Rates



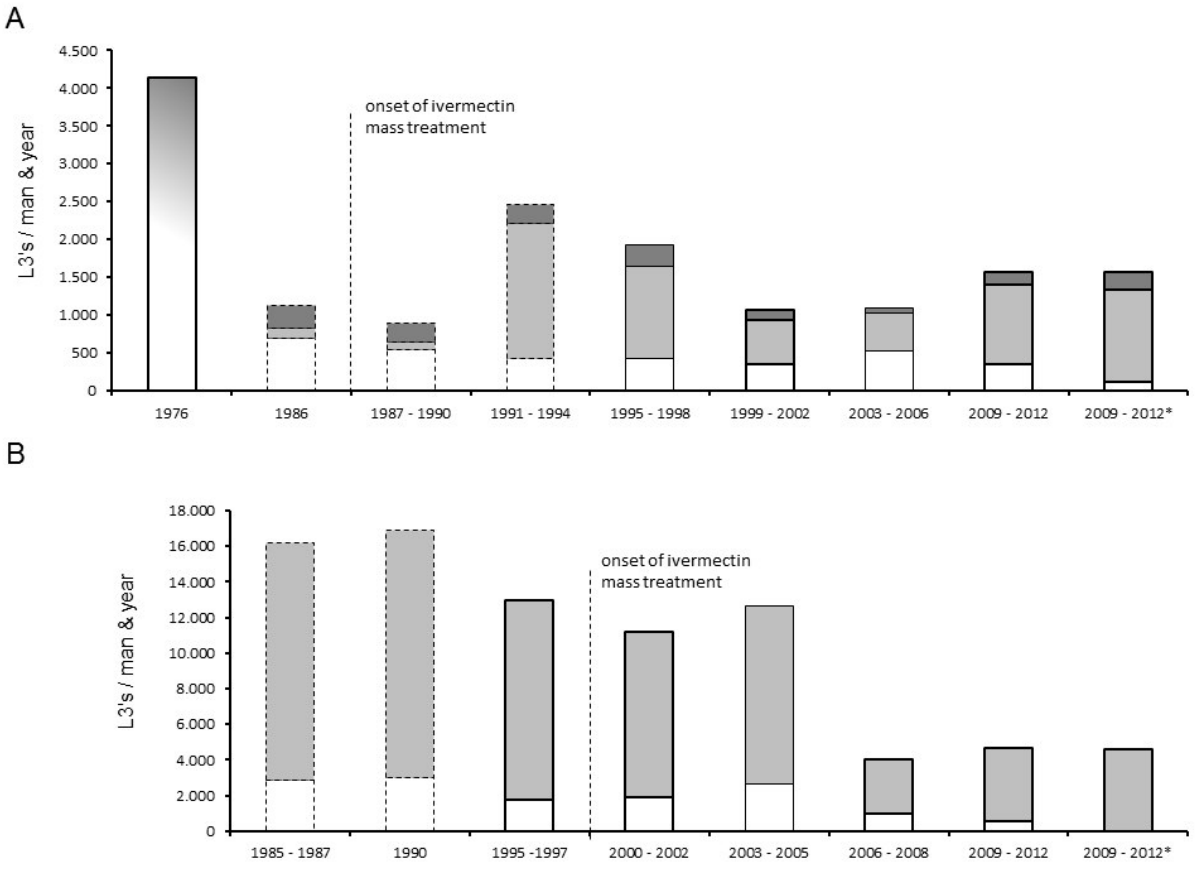
Annual biting rates of *Onchocerca* spp. from two onchocerciasis foci in North Cameroon. Each data point starts at the beginning of the rainy season (April) till the end of the dry season (March of the following year). Years with no data are left blank. **A** Soramboum, Vina du Nord. Epidemiological data prior to 1998 was published before and modified to fit this graph. Data from 1976 was taken from Touboro, 30 km further downstream. **B** Galim, Vina du Sud. Epidemiological data prior to 1997 was published before and modified to fit this graph.

Figure 3: Monthly Biting Rates



Mean monthly biting rates of *Onchocerca* spp. from two onchocerciasis foci in North Cameroon. Whiskers show the standard deviation. Black bar: 1984 - 1993; dark grey bar: 1994 - 2003; light grey: 2004 - 2012; white bar: 1984 - 2012. RS: rainy season; DS: dry season. **A** Soramboum, Vina du Nord. **B** Galim, Vina du Sud.

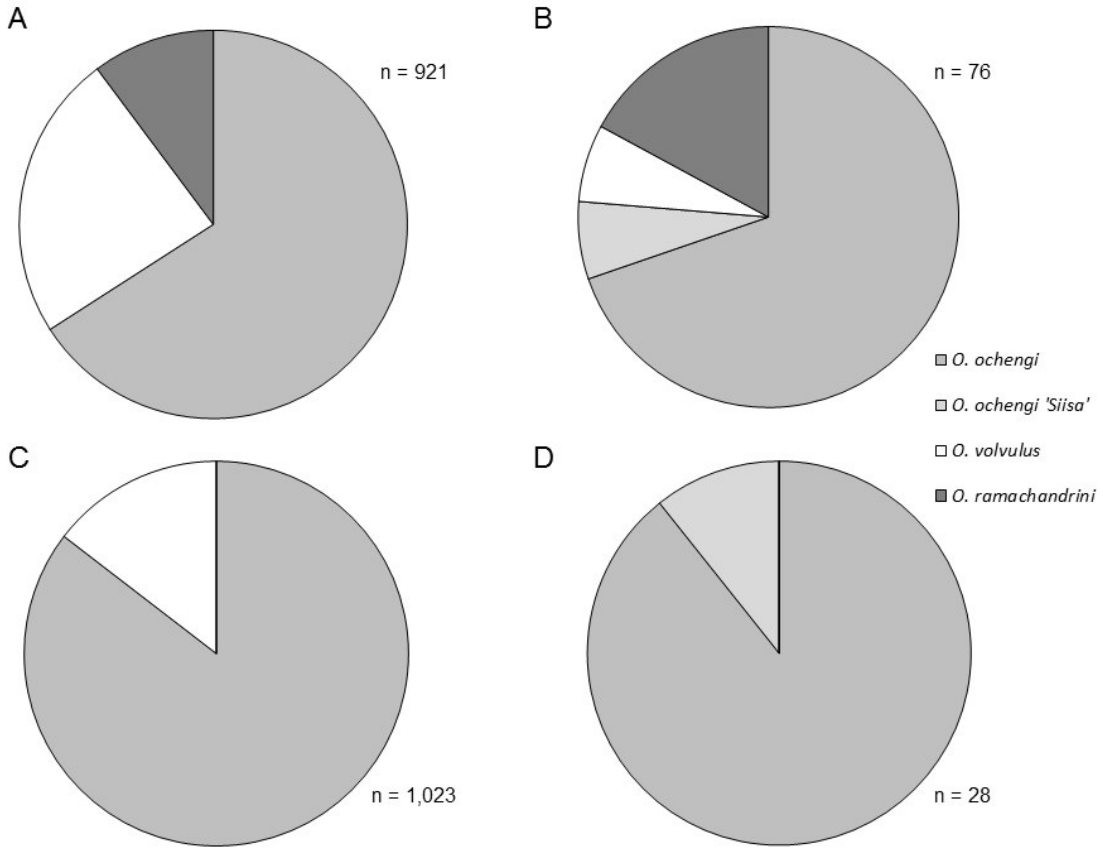
Figure 4: Annual Transmission Potentials



Annual transmission potentials of *Onchocerca* spp. from two onchocerciasis foci in North Cameroon. Data is given at the beginning of the rainy season (April) till the end of the dry season (March of the following year). Dark grey: *O. ramachandrini*; light grey: *O. ochengi*; white: *O. volvulus*. Insufficient fly dissection data from 1986, 1989 - 1992 in Soramboum, and from 1985 - 1987, 1990 and 1997 in Galim have been extrapolated. In 1976 (color transition of white, light grey to dark grey) no species discrimination was done. ATP with available MTP data ≥ 80 % have borders in bold; those with data 80 % > x ≥ 30 % have normal borders, and those with data < 30 % have borders with a dotted line. The dotted line marks the beginning of ivermectin mass treatments. The asterisk indicates the introduction of PCR-based methods for species discrimination in the vector. Prior to 1990, the L3 species was determined according to the following body length criteria: *O. volvulus* ≤ 750 µm; 750 µm < *O. ochengi* < 900 µm; *O. ramachandrini* ≥ 900 µm. **A** Soramboum, Vina du Nord.

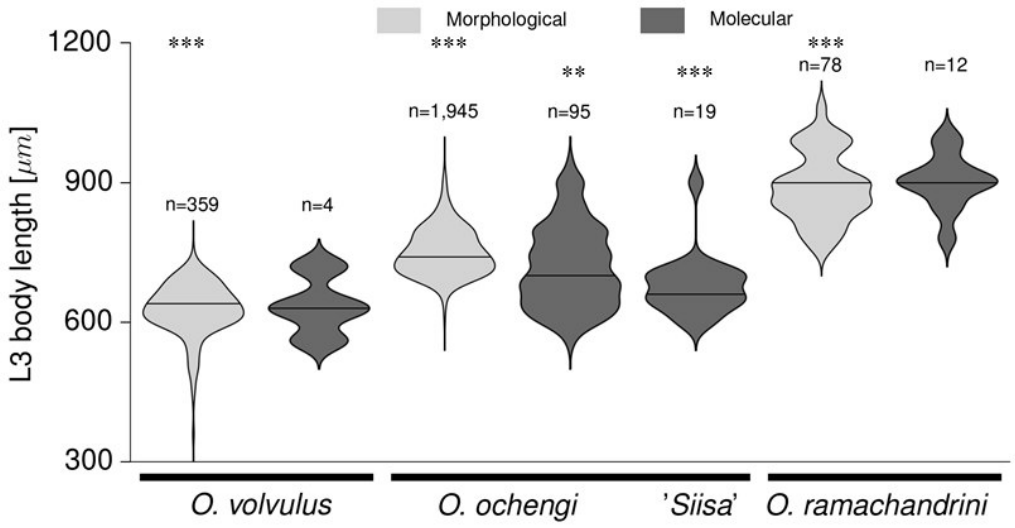
Epidemiological data prior to 1998 was published before and modified to fit this graph. Data from 1976 was taken from Touboro, 30 km further downstream. **B** Galim, Vina du Sud. Epidemiological data prior to 1997 was published before and modified to fit this graph.

Figure 5: L3 species proportion



Species composition of infective third-stage larvae from *S. damnosum s.l.* at two foci in North Cameroon. White: *O. volvulus*; light grey: *O. ochengi* 'Siisa' type; medium grey: *O. ochengi*; dark grey: *O. ramachandrini*. **A-B** Soramboum, Vina du Nord. **C-D** Galim, Vina du Sud. Left side (A, C): morphological identification. Right side (B, D): PCR-based identification.

Figure 6: L3 body lengths



Violin plot of the L3 body lengths of identified *Onchocerca* spp. in *S. damnosum s.l.* from North Cameroon. Species identification is based on morphological (light grey) and molecular-genetic (dark grey) characteristics. Two genotypes of *O. ochengi*, *O. ochengi s. str.* and *O. ochengi* 'Siisa', are shown based on their mitochondrial clades. ***$P < 0.001$; **$0.01 > P > 0.001$; *$P < 0.05$.

Table 1: Entomologic parameters and proportion of filarial stages from *Simulium damnosum s.l.* at two different sites in Northern Cameroon.

Location		RS 2009		DS 2009/10		RS 2010		DS 2010/11		RS 2011		DS 2011/12		RS 2012		DS 2012/13		Total RS		Total DS	
Soramboum/ Vina du Nord	flies caught	84		5,776		11,403		3,692		1,613		1,344		561		772		13,661		11,584	
	dissected (%[a])	84	*100.0*	2,141	*37.1*	4,236	*37.1*	2,636	*71.4*	1,393	*86.4*	1,128	*83.9*	561	*100.0*	772	*100.0*	6,274	*45.9*	6,677	*57.6*
	parous (%[b])	34	*40.5*	1,067	*49.8*	2,687	*63.4*	1,844	*70.0*	1,216	*87.3*	829	*73.5*	437	*77.9*	504	*65.3*	4,374	*69.7*	4,244	*63.6*
	infected (%[c])	4	*11.8*	84	*7.9*	303	*11.3*	101	*5.5*	128	*10.5*	45	*5.4*	40	*9.2*	19	*3.8*	475	*10.9*	249	*5.9*
	infective (%[d])	3	*8.8*	44	*4.1*	234	*8.7*	59	*3.2*	77	*6.3*	24	*2.9*	25	*5.7*	11	*2.2*	339	*7.8*	138	*3.3*
	L1 (%[e])	0	*0.0*	30	*14.6*	54	*8.4*	52	*28.6*	47	*16.5*	40	*34.5*	8	*9.6*	6	*24.0*	109	*10.7*	128	*24.2*
	L2 (%[e])	1	*20.0*	73	*35.4*	156	*24.2*	24	*13.2*	80	*28.1*	19	*16.4*	20	*24.1*	2	*8.0*	257	*25.3*	118	*22.3*
	L3 (%[e])	4	*80.0*	103	*50.0*	434	*67.4*	106	*58.2*	158	*55.4*	57	*49.1*	55	*66.3*	17	*68.0*	651	*64.0*	283	*53.5*
Galim/ Vina du Sud	flies caught	812		3,069		987		1,660		1,379		4,402		813		715		3,991		9,846	
	dissected (%[a])	757	*93.2*	1,783	*58.1*	947	*95.9*	1,014	*61.1*	962	*69.8*	2,168	*49.3*	773	*95.1*	542	*75.8*	3,439	*86.2*	5,507	*55.5*
	parous (%[b])	407	*53.8*	1,049	*58.8*	515	*54.4*	645	*63.6*	601	*62.5*	1,453	*67.0*	422	*54.6*	391	*72.1*	1,945	*56.6*	3,538	*64.2*
	infected (%[c])	35	*8.6*	199	*19.0*	73	*14.2*	114	*17.7*	72	*12.0*	225	*15.5*	35	*8.3*	50	*12.8*	215	*11.1*	588	*16.6*
	infective (%[d])	12	*2.9*	86	*8.2*	44	*8.5*	53	*8.2*	19	*3.2*	84	*5.8*	8	*1.9*	27	*6.9*	83	*4.3*	250	*7.1*
	L1 (%[e])	54	*45.8*	408	*40.6*	28	*12.3*	146	*35.8*	109	*39.6*	402	*42.0*	62	*48.4*	59	*29.4*	253	*33.8*	1,015	*39.5*
	L2 (%[e])	32	*27.1*	219	*21.8*	78	*34.2*	108	*26.5*	98	*35.6*	272	*28.4*	34	*26.6*	49	*24.4*	242	*32.3*	648	*25.2*
	L3 (%[e])	32	*27.1*	378	*37.6*	122	*53.5*	154	*37.7*	68	*24.7*	283	*29.6*	32	*25.0*	93	*46.3*	254	*33.9*	908	*35.3*

[a] Percentage of the caught fly population. [b] Percentage of dissected fly population. [c] Percentage of parous fly population. [d] Percentage of parous flies population with infective L3 larvae. [e] Percentage of development stages found in infected flies. DS: dry season (October – March); RS: rainy season (April – September); L1: first-stage larvae; L2: second-stage larvae; L3: infective third-stage larvae.

Table S1: Infection rate of female nulliparous flies and aquatic larvae of *Simulium damnosum s.l.* at two different sites in Northern Cameroon.

Location		DS 2009/10		RS 2010		DS 2010/11		RS 2011		DS 2011/12		RS 2012		Total	
Galim,	mean monthly biting rate	4.907		1.660		3.900		2.459		6.224		1.874		3.504	
Vina du Sud	flies dissected	1.783		947		1.014		962		2.168		773		7.647	
	nulliparous (%)	734	*41,2*	432	*45,6*	369	*36,4*	361	*37,5*	715	*33,0*	352	*45,5*	2.963	*38,7*
	with mermithids (%)	37	*5,0*	19	*4,4*	2	*0,5*	17	*4,7*	34	*4,8*	8	*2,3*	117	*3,9*
	with fungus round form (%)	0	*0*	1	*0,2*	0	*0*	0	*0*	1	*0,1*	0	*0*	2	*0,1*
	with malpighian nematode (%)	5	*0,7*	1	*0,2*	4	*1,1*	3	*0,8*	5	*0,7*	0	*0*	18	*0,6*
	with planidium larva (%)	0	*0*	3	*0,3*	1	*0,1*	0	*0*	0	*0*	0	*0*	4	*0,1*
	larvae dissected													250	
	with mermithids (%)													14	*5,6*
	with fungus round form (%)													1	*0,4*
	with malpighian nematode (%)													2	*0,8*
Soramboum,	mean monthly biting rate	1.844		3.662		2.579		1.738		1.803		711		2.056	
Vina du Nord	flies dissected	2.141		4.236		2.636		1.393		1.128		561		12.095	
	nulliparous (%)	1.085	*50,7*	768	*18,1*	771	*29,3*	176	*12,6*	299	*26,5*	124	*22,1*	3.223	*26,6*
	with mermithids (%)	1	*0,1*	0	*0*	1	*0,1*	0	*0*	0	*0*	0	*0*	2	*0,1*
	with fungus round form (%)	4	*0,4*	1	*0,1*	0	*0*	1	*0,6*	0	*0*	0	*0,0*	6	*0,2*
	with malpighian nematode (%)	1	*0,1*	3	*0,4*	2	*0,3*	2	*1,1*	0	*0*	2	*1,6*	10	*0,3*
	with planidium larva (%)	0	*0*	6	*0,1*	1	*0,04*	0	*0*	0	*0*	0	*0*	7	*0,1*

Location		RS 1995		DS1995/96		RS 1996		DS1996/97		Total	
Galim,	mean monthly biting rate	1.552		3.084		11.145		13.230		7.253	
Vina du Sud	flies dissected	574		800		2.564		2.050	*26,5*	5.988	
	nulliparous (%)	210	*36,6*	247	*30,9*	534	*20,8*	544	*48,2*	1.535	*25,6*
	with mermithids (%)	1	*0,5*	0	*0*	5	*0,9*	3	*0,6*	9	*0,6*
	with fungus round form (%)	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
	with malpighian nematode (%)	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
	with planidium larva (%)	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*

Lebenslauf

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