Medical and Veterinary Entomology (1991) **5**, 293–297

Development of *Onchocerca volvulus* microfilariae injected into *Simulium* species from Cameroon

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Abstract. Microfilariae (mff) of the savanna and forest strains of *Onchocerca volvulus* (Leuckart) were injected intrathoracically into adult females of *Simulium damnosum* Theobald *sensu stricto*, *S.sirbanum* Vajime & Dunbar, *S.squamosum* Enderlein and *S.mengense* Vajime & Dunbar. Nine days post infection (pi) 27–29% of the savanna mff and 31–38% of the forest strain had developed to third-stage larvae (L3), irrespective of the fly species, size or injection dose (5, 10 or 15 mff). Savanna flies supported the development of forest *O.volvulus* better than forest flies, in contrast to the results after *per os* infections. Therefore, in these four species of the *S.damnosum* complex from Cameroon, the peritrophic membrane is considered to be the main factor limiting the success rate of microfilarial development following the ingestion of blood infections, while the fly's haemolymph and intracellular environment play minor roles.

Key words. Onchocerciasis vectors, *Simulium damnosum* complex, *Onchocerca volvulus* strains, intrathoracic injection, vector competence, Cameroon.

Introduction

Microfilariae (mff) of the nematode parasite *Onchocerca volvulus* (Leuckart), which causes human onchocerciasis, seem capable of developing in virtually all blackfly species (Diptera: Simuliidae) which come to feed on man. In Africa more than twenty taxa of the *Simulium damnosum* Theobald complex are reported to be vectors of onchocerciasis (Crosskey, 1987), although there is significant variation in the proportion of ingested microfilariae that develop to the infective third-stage larvae (L3).

In Cameroon, mff of the forest strain developed much better in blackflies from the forest than in those from the savanna and vice versa (Duke *et al.*, 1966). Within half a minute after the ingestion of a fly's bloodmeal, the epithelial cells of the posterior midgut secrete a peritrophic membrane which envelops the ingested blood (Lewis, 1953). Some of the mff manage to penetrate the membrane before it is completely hardened, but the majority are imprisoned and die (Laurence, 1966). By injection of mff into the fly's thorax the limiting effect of the peritrophic membrane can be overcome. Nevertheless, the proportion of intrathoracically injected mff that develop to L3s in nearctic or palaearctic species of *Simulium* ranges from only 2% to 28% (Reid, 1979; Ham & Bianco, 1983). By injection it is possible to examine to what extent either the peritrophic membrane or the haemolymph of the fly contributes to the observed differences in the vector competence.

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Materials and Methods

Skin biopsies were taken at the iliac crest and calves of highly infected volunteers from Barombi Mbo, near Kumba, in the rain forest (RF) and Sora Mboum, near Touboro, in the Sudan savanna (SS). For isolation of mff, skin snips were each incubated for 1–3 h in 100 µl of medium, consisting of 1.04 g RPMI (Serva) diluted in 80 ml of sterile filtrated, demineralized water complemented with 20% of heat-inactivated fetal calf serum, 0.2 g $CaCO_3$, 200 µl Penstrep® and 100 µl amphotericine B®, adjusted to pH 7.3–7.5 by NaOH and HCl. Prior to the incubation of mff the medium was centrifuged for 5 min at 1500 U/min and stored for up to 5 days at 4°C.

Simulium pupae were collected from rivers near Kumba (RF), Touboro (SS) and Ngaoundere in the Guinea savanna (GS) and larvae from the same breeding sites were fixed in Carnoy's for cytotaxonomical identifications (Vajime & Dunbar, 1975). Adult flies, hatched in a breeding cage in the laboratory (Wenk & Raybould, 1972), were classified according to their morphology (as described below) and the morphometric results were compared to the cytotaxonomical findings. The newly emerged flies were fed on a 10% (w/v) sugar solution. A total of 104 female *Simulium* from the Sudan savanna and Guinea savanna habitats were taken to Kumba for injection.

Glass capillaries with a tip diameter of 30 µm were used for injections. 5, 10 or 15 mff were drawn up into the needle in a minimum of medium (0.5–1 µl) and were injected through the pleural membrane into the thorax of a CO_2-narcotized *Simulium* fly.

Flies were kept individually in tubes (diameter 1.5 cm) at 21.5±2°C in constant darkness and high humidity. They were fed on 10% (w/v) sugar solution containing preservative 0.2% (w/v) nipagine® = methyl–4-hydroxybenzoate, on cotton balls replaced every second day.

Dead or moribund flies were examined for the colour of the setae on the postcranium, the scutellum and the wing tufts. They were classed 'pale' (only pale setae), 'dark' (only dark setae) and 'mixed' (all other flies). The length of the thorax was measured under the dissecting microscope (50×). Flies were dissected in demineralized water, using a stereomicroscope at 20× magnification, and were checked under a compound microscope (63×) for *O. volvulus* larvae.

The correlation of the infection success (L3/mff) with the thoracic length was tested with the Spearman rank correlation test.

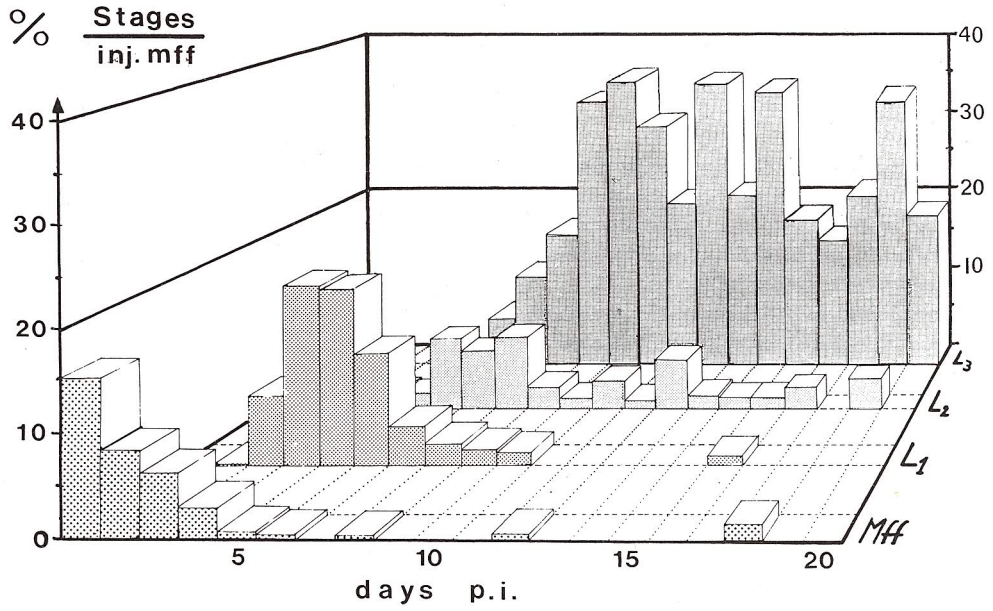

Fig. 1. Development of *O. volvulus* microfilariae in *Simulium damnosum s.l.* following intrathoracic injection.

Table 1. Success of injecting *O.volvulus* microfilariae into *Simulium damnosum sensu lato*. Species of blackfly: *da, S.damnosum*; *si, S.sirbanum*; *sq, S.squamosum*; *me, S.mengense*. Strains of *O.volvulus*: SS, Sudan savanna; RF, rainforest.

Colour class	Breeding site	Species	Flies inj.	mff/ fly	Flies surviving ≥ 9 days p.i.			
					No. flies (%)	Infective flies	L3/ inf. fly	L3/mff
Pale	SS Vina du Nord	*da/si*	17	5 SS	7 (41.2)	71.4%	2.00	28.6%
	SS Vina du Nord	*da/si*	63	10 SS	32 (50.8)	84.4%	3.15	26.6%
	SS Vina M. Oldiri	*da/si*	64	10 RF	18 (28.1)	88.9%	3.99	35.5%
	GS Vina du Sud	*sq*	33	10 RF	6 (18.2)	83.3%	4.60	38.3%
	RF Kake	*da*	111	15 RF	38 (34.2)	76.3%	6.90	35.1%
Mixed	RF Bile/Nyoke	*sq*	79	10 RF	46 (58.2)	45.6%	2.43	11.1%
	RF Menge	*sq*	4	10 RF	4 (100)	75.0%	4.33	32.5%
	RF Kake	*sq*	49	15 RF	17 (34.7)	82.4%	5.71	31.4%
Dark	RF Meme	*me*	46	10 RF	14 (30.4)	92.9%	4.08	37.9%

Table 2. Success of injecting other *Simulium* spp. with strains of *O.volvulus* (as for Table 1).

Fly species	Breeding sites	Flies inj.	mff/ fly	Flies surviving ≥ 9 days p.i.			
				No. flies (%)	Infective flies	L3/ inf. fly	L3/mff
S.hargreavesi	SS Vina du Nord	3	10 SS	1 (33.3)	100%	1.00	10.0%
	SS/GS	5	10 RF	4 (80.0)	100%	4.25	42.5%
S.cervicornutum	GS Vina du Sud	2	10 RF	1 (50.0)	100%	4.00	40.0%
S.kenyae	RF Bile/Bl.water	25	5 RF	8 (32.0)	0%	0.00	0.0%

Results

6585 microfilariae were injected into 594 flies in twenty-seven experiments. Totals of 170 mff, 242 sausage stages, 140 L2s and 769 L3s were recovered from dead flies, amounting to 20% of the mff injected. Mff formed sausage stages 2 days p.i., L2s were found after 4 days and the first infective L3 after 6 days p.i. After 9 days most of the larvae had developed to the infective stage (Fig. 1). In most experiments the mean number of larvae recovered per fly increased during the first 7 days p.i., and decreased in flies dying after 12 days p.i.

The proportion of injected mff which had developed to the L3 stage in flies surviving for 9 or more days p.i. was 27–29% for the savanna strain and 31–38% for the forest strain, independent of the injection dose and the fly's colour class (Table 1). The 'mixed haired' flies (*S. squamosum*) from the Nyoke and Bile breeding sites (RF) were the only exception to this rule, giving rise to only 11% L3/mff, while those from Menge and Kake were within the normal range (≈32% L3/mff). The forest strain of *O.volvulus* developed apparently better in the savanna flies than in the forest flies (Table 1). Within each colour class, the success of injection (L3/mff) was independent of the fly's thoracic length ($P>0.05$).

In fifteen male flies from Bile (*S.squamosum*), each injected with 5 mff, a total of eight L3s were produced (40% L3/mff ≥7 days p.i.). *O.volvulus* also developed well in *S.hargreavesi*

Gibbins and *S.bovis* De Meillon, but failed to develop in *S.kenyae* De Meillon (Table 2). Twenty-five *S.kenyae* injected with a total of 125 mff gave rise to only three sausage stages and four L2s.

Discussion

The time needed for larval development after intrathoracic injection was equal to a control group of flies infected *per os* and kept under the same conditions (data not shown). It did not differ between the forest and the savanna strains of *O.volvulus*. Low infection rates in flies dying early have been described previously for both *per os* infection (Duke, 1962) and intrathoracical injection (Barbiero & Trpis, 1985). Up to 30% of the mff and sausage stages may have been missed in our observations, as the flies were not stained prior to dissection, but there seems also to be a differential mortality of the flies. A decrease in the number of infective larvae in old flies has often been reported (Duke, 1962; Nelson & Pester, 1962) and is usually interpreted as the loss of infective larvae from the fly during sugar-feeding. In our experiments there was a marked increase in mortality of the flies at the age when large numbers of third stage larvae appeared, 8–9 days p.i. (not seen in flies that contained no larvae despite injection). Therefore it seems more reasonable to explain this decrease by differential mortality of highly infected flies as shown, for example, with *Wuchereria bancrofti* Cobbold in *Culex* mosquitoes (Zielke, 1977).

After *per os* infection, forest microfilariae developed well in flies from the rain forest and Guinea savanna, but showed little or no development in those from the Sudan savanna (Duke *et al.*, 1966). Forest flies usually have dark or mixed setae, while those from the savanna have pale ones but, since 1984, pale flies have also been observed in the Cameroon forest. In wild-caught flies the highest mean numbers of infective larvae were observed in *S.mengense* in the forest (5.5 per infective fly), followed by forest *S.squamosum* (4.4), Guinea savanna *S. squamosum* (3.3), Sudan savanna *S.damnosum* (3.0) and *S.sirbanum* (1.9) (Renz *et al.*, 1987). In contrast, there were no marked differences in the developing infection rates after intrathoracic injection. Savanna flies were not only able to develop the forest strain of *O.volvulus*, but also produced the highest proportion of infective larvae. Therefore, the peritrophic membrane should be regarded as the main barrier against larval development in the Cameroon vectors, while the haemolymph or the intracellular milieu plays only a minor role in the fly's defence against *O.volvulus* microfilariae.

As regards the other species of *Simulium*, surprisingly *S.hargreavesi*, which is not known to feed on man, developed *O.volvulus* better than did any member of the *S.damnosum* complex. One specimen, presumably *S.cervicornutum* Pomeroy, produced four L3s in one specimen (Table 2). In the non-anthropophilic *S.kenyae*, development of *O.volvulus* mff terminated at the sausage or the second stage. Thus *S.kenyae* seemed to be entirely refractory, whereas all the other six species studied in this mode appeared to have vector potential for *O.volvulus*.

Acknowledgments

This investigation received financial support from the Commission of the European Community (TSD 007/D) and from the filariasis component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (I.D. 870089). We are grateful to the MESRES, Yaoundé, for granting research permits, and to the Chief of the Medical Research Station in Kumba, Dr Moyou, for providing laboratory facilities. Mr D. Ekale provided invaluable technical assistance during the injection and dissection procedures.

References

Barbiero, V.K. & Trpis, M. (1985) A field method for the procurement of infective larvae of *Onchocerca volvulus*. *American Journal of Tropical Medicine and Hygiene*, **34**, 731–734.

Crosskey, B.W. (1987) A taxa summary for the *Simulium damnosum* complex, with special reference to distribution outside the control areas of West Africa. *Annals of Tropical Medicine and Parasitology*, **81**, 181–192.

Duke, B.O.L. (1962) Studies on factors influencing the transmission of onchocerciasis. II. The intake of *Onchocerca volvulus* microfilariae by *Simulium damnosum* and the survival of the parasites in the fly under laboratory conditions. *Annals of Tropical Medicine and Parasitology*, **56**, 255–263.

Duke, B.O.L., Lewis, D.J. & Moore, P.J. (1966) *Onchocerca–Simulium* complexes. I. Transmission of forest and Sudan-savanna strains of *Onchocerca*

volvulus, form Cameroon, by *Simulium damnosum* from various West-African bioclimatic zones. *Annals of Tropical Medicine and Parasitology,* **60**, 318–336.

Ham, P.J. & Bianco, A.E. (1983) Development of *Onchocerca volvulus* from cryopreserved microfilariae in three temperate species of laboratory-reared blackflies. *Tropenmedizin und Parasitologie*, **34**, 137–139.

Laurence, B.R. (1966) Intake and migration of the microfilariae of *Onchocerca volvulus* (Leuckart) in *Simulium damnosum* Theobald. *Journal of Helminthology,* **40**, 337–342.

Lewis, D.J. (1953) *Simulium damnosum* and its relation to onchocerciasis in the Anglo-Egyptian Sudan. *Bulletin of Entomological Research,* **43**, 597–645.

Nelson, G.S. & Pester, F.R.N. (1962) The identification of infective filarial larvae in *Simuliidae*. *Bulletin of the World Health Organization,* **27**, 473–481.

Reid, G.D.F. (1979) The development of *Onchocerca volvulus* in two temperate blackfly species, *Simulium ornatum* Meigen and *Simulium lineatum* Meigen. *Annals of Tropical Medicine and Parasitology,* **73**, 577–581.

Renz, A., Barthelmess, C. & Eisenbeiss, W. (1987) Vectorial capacity of *Simulium damnosum s.l.* populations in Cameroon. *Tropenmedizin und Parasitologie,* **38**, 344–345.

Vajime, C.G. & Dunbar, R.W. (1975) Chromosomal identification of eight species of the subgenus *Edwardsellum* near and including *Simulium (Edwardsellum) damnosum* Theobald (Diptera: Simuliidae). *Tropenmedizin und Parasitologie,* **26**, 111–138.

Wenk, P. & Raybould, J.N. (1972) Mating, blood feeding and oviposition of *Simulium damnosum* Theobald in the laboratory. *Bulletin of the World Health Organization,* **47**, 627–634.

Zielke, E. (1977) On the escape of infective filarial larvae from the mosquitoes. *Tropenmedizin und Parasitologie*, **28**, 461–466.

Accepted 28 November 1990