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Newly acquired *Onchocerca volvulus* filariae after doxycycline treatment

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Abstract Despite successful mass drug administration and vector control programs, the nematode *Onchocerca volvulus* is far from being eradicated. Therefore, new long-term sterilizing or macrofilaricidal drugs are needed. The depletion of *Wolbachia* endobacteria using doxycycline leads to long-term sterilizing effects and macrofilaricidal activity against female filariae of more than 60%. The worms die or degenerate 18–27 months after doxycycline. However, during this time patients may be exposed to new infections. We evaluated these newly acquired worms in onchocercomas of doxycycline-treated patients in relation to transmission using morphology, histochemistry, and immunohistology. On an average, 10% of the female filariae had been newly acquired per year in the treated groups. Our observations showed: (a) Sixty-three of 68 newly acquired worms harbored many intact *Wolbachia*, whereas none of the other surviving worms contained many bacteria. (b) Higher percentages of dead filariae than originally reported were calculated, when the new worms were excluded, indicating a stronger macrofilaricidal activity than previously reported. The difference was significant for female filariae after doxycycline treatment for 6 weeks. (c) Only newly acquired worms presented normal embryogenesis and microfilariae production after sufficient treatment. We conclude that newly acquired filariae have to be considered when evaluating drug efficacy in onchocerciasis.

Abbreviations
APR   *O. volvulus* aspartic protease
IVM   ivermectin
L3   infective larvae
mf   microfilaria (e)
WSP   *Wolbachia* surface protein

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Introduction

Onchocerciasis can lead to devastating skin disease and blindness. Ivermectin (IVM, Mectizan®) distribution within the African Programme for Onchocerciasis Control (APOC) had a marked impact on the disease, reducing prevalence of blindness and transmission of onchocerciasis. Recent surveys done by APOC indicate that approximately 37 million people may still be infected (Basañez et al. 2006), and modeling studies suggest that IVM treatments alone may not lead to onchocerciasis elimination (Alley et al. 2001). This and the risk of emergence of IVM-resistant parasites necessitate the search for alternative drugs or drug combinations, preferably with macrofilaricidal or long-

lasting sterilizing effects or a complementary vaccine (Duke 2002).

Clinical trials require optimal methods for the evaluation of drug activity, since for ethical and economical reasons reliable information should be gained from as few patients with as little risk as possible. Usually, onchocerciasis trials are carried out in endemic areas with still ongoing transmission. This implies that patients are exposed and new infections will be acquired. To evaluate the efficacy of a drug or vaccine, it is necessary to differentiate between worms present at the time of drug or vaccine administration and worms acquired during long observation periods. Such long periods are needed for the detection of long-term sterilizing or slow macrofilaricidal effects.

An example for such long observation periods is the treatment with antibiotics. *Onchocerca volvulus* and other filariae contain *Wolbachia* bacteria, which are essential endosymbionts for parasite fertility and survival. Several clinical trials with doxycycline have already been completed. The first study demonstrated that a six-week treatment with 100 mg doxycycline per day leads to the elimination of intact *Wolbachia* after 6–12 months and to long-term sterilization (Hoerauf et al. 2003). After a longer observation of 20–27 months, up to 60% of the female filariae were found dead in two recent studies (Hoerauf et al. 2008, 2009). Since these studies were performed in an area with ongoing transmission, a proportion of the worms that were still living after these longer observation periods must have been newly acquired.

Consequently, their subtraction from the number of all observed worms will change the figures previously reported for the efficacy of doxycycline. We have described the morphological and immunohistological criteria for the diagnosis of newly acquired worms in a previous report (Specht et al. 2009). Here, we assess the number of newly acquired young worms in doxycycline trials using these criteria. Our hypotheses for this study were: (a) Twelve or more months after doxycycline all *O. volvulus* filariae with many not degenerated *Wolbachia* were worms newly acquired after treatment. (b) The exclusion of the worms newly acquired after doxycycline leads to lower percentages of living worms and hence to higher percentages of dead filariae indicating a stronger macrofilaricidal efficacy. (c) Eighteen or more months after doxycycline all worms with normal embryogenesis and microfilariae (mf) production have been acquired after the treatment.

Patients, material, and methods

Study area

The results presented here are mainly based on two doxycycline trials performed between 2003 and 2006

(Hoerauf et al. 2008, 2009). In addition, data were also considered from the first trial (Hoerauf et al. 2003) and from further dose-finding trials in 2000–2002 (unpublished data). Patients for these studies had been selected in several villages in the Central Region of Ghana. In 1999, this area was found to be hyperendemic for onchocerciasis during rapid assessment of 30 men older than 19 years per village in Upper Denkyira and Assin districts, with mf carrier rates between 47% and 90% of the examined men (R. Horstmann and D.W. Büttner). In most of the villages, IVM treatment had been administered beginning in 1999, but a reduction of the infectivity rates and the loads of infective larvae (L3) in *Simulium sanctipauli* was not observed in the trial villages between 2002 and 2006 (Kutin et al. 2004; R. Garms, Bernhard Nocht Institute, unpublished report, 2006). Transmission was ongoing during the trials.

Treatment of patients

Patients were treated with 100 or 200 mg doxycycline per day for 4, 5, or 6 weeks. Most patients took a single dose of IVM a few months after doxycycline. Control patients received matching placebos or remained untreated, except for IVM. Onchocercomas were extirpated aseptically under local anesthesia in the regional hospital in Dunkwa. Patients were nodulectomized at various follow-up time points after the start of treatment, ranging from 2–39 months (Hoerauf et al. 2003, 2008, 2009).

Immunohistology

Onchocercomas were fixed in 70–80% ethanol or 4% phosphate-buffered formaldehyde solution and embedded in paraffin using standard methods. Hematoxylin eosin (Merck, Darmstadt, Germany) was used for all nodules. For the detection of iron in the filarial tissues, Gomori's iron method was applied for all nodules selected for this study, using acid potassium ferrocyanide solution and nuclear fast red as counter stain (Luna 1968). For immunohistology, the alkaline phosphatase antialkaline phosphatase technique was applied according to the recommendations given by the manufacturer (DakoCytomation, Hamburg, Germany). An antiserum raised against lysosomal aspartic protease (APR) in a rabbit (Jolodar et al. 2004, supplied by PD Dr Norbert Brattig, Bernhard Nocht Institute) was used as primary antibody to assess vitality and to estimate worm age, diluted 1:1,000–1:4,000. To demonstrate the presence of *Wolbachia,* a rabbit antiserum against *Dirofilaria immitis Wolbachia* surface protein (WSP, Bazzocchi et al. 2000, supplied by Prof. Claudio Bandi, Milano, Italy) was used, diluted 1:200–1:4,000. As secondary antibody, mouse antirabbit immunoglobulin (clone MR12/53, Dako-

Cytomation) was applied. Fast Red TR salt (Sigma, Deisenhofen, Germany) was used as chromogen and hematoxylin (Merck) served as the counter stain. For the electron microscopic (Fig. 1 (6)) showing degenerated *Wolbachia,* standard methods were used as described previously (Pfarr et al. 2008).

Onchocercomas were analyzed for morphological and immunohistological characteristics of newly acquired young worms. All filariae were examined for vitality, productivity, presence of *Wolbachia*, and the human nodule tissue was screened for mf. Morphological criteria for the estimation of the worm age and vitality have been described (Büttner et al. 1988; Franz 1988; Schulz-Key 1988; Duke 1991 and in detail by Specht et al. 2009). The classification of female *O. volvulus* follows Duke et al. (2002) with minor modifications. Two authors (DWB and SS) examined the sections independently. When the examiners did not agree, slides were reexamined, which mostly led to a consensus. In doubtful cases, the worms were not counted as newly acquired.

Statistics

The paired *t* test was applied to test differences between previously observed and newly calculated rates of dead filariae, using GraphPad® Prism Software.

Results

Number of newly acquired filariae

Here, we report the results of our reexamination of the onchocercomas from two recent doxycycline trials (Hoerauf et al. 2008, 2009) using the criteria for the differentiation between young and old *O. volvulus* published previously (Specht et al. 2009). Tables 1 and 2 present the numbers and percentages of living young worms identified as newly acquired in different patient groups: a total of 68 female and 32 male filariae. The number of newly acquired males (Table 2) was probably slightly underestimated, because in nodule sections with only few male worm sections not all distinct criteria for the age estimation could be applied and some male worms near to the edge of the nodule may have been completely missed. In the treatment groups, on average 10% (median 9.0%) of the female and 9% (median 6.4%) of the male filariae were identified per year as newly acquired after doxycycline treatment. In the untreated groups on average, only 7% females and 5% male worms were identified as newly acquired per year, because the diagnosis was more difficult for untreated filariae.

Elimination of *Wolbachia* by doxycycline treatment

The elimination of *Wolbachia* by doxycycline is the precondition for sterilization and death of the filariae. Using immunohistology and PCR, we have shown that most filariae lose their endobacteria during the months after sufficient doxycycline treatment (Hoerauf et al. 2003, 2008, 2009). However, always small numbers of worms with many morphologically intact endobacteria were observed after 20–27 months and this raised the question whether they had been doxycycline-treated or were acquired only afterwards.

The number of bacteria in immature worms is limited, as it is in L3, and therefore we only saw endobacteria in three of seven immature worms (Fig. 1), which had been newly acquired during the last year before nodulectomy (according to the definition of these worms being immature). More than 19 months after doxycycline 23 nulliparous worms were identified as newly acquired. They were all *Wolbachia*-positive (Table 1, Figs. 1 (2) and (3)). During the months after, doxycycline bacteria started to degenerate in the hypodermis and then in the oocytes. Therefore, a few worms harbored small numbers of endobacteria in degenerated oocytes 11 months after treatment, but these bacteria were obviously degenerated (compare Figs. 1 (4) and (5)). The degenerated bacteria showed an irregular cytoplasm with electron dense inclusions (Fig. 1 (6)) compared to living *Wolbachia* (e.g., Fig. 1 in Pfarr et al. 2008). Eleven and more months after sufficient doxycycline treatment, the *Wolbachia*-positive male worms showed the criteria of young worms (Figs. 1 (7) and (8)) when compared to adjacent old female worms. Especially the low iron content of these young worms was striking (Fig. 1 (8)). All 17 productive female worms identified as newly acquired after doxycycline contained many morphologically intact endobacteria (Table 1, Figs. 2 (9), (10), (11) and (14)), except one worm with few bacteria. In contrast, several young fecund females were observed during the first 6 months of observation (Table 1 in Hoerauf et al. 2003) and these young as well as old females contained no *Wolbachia* since they all had been acquired before treatment

Macrofilaricidal activity of doxycycline

Secondly, we hypothesized that taking new worms into account, this would affect the macrofilaricidal activity of doxycycline. We subtracted the numbers of newly acquired worms from those of all observed worms and recalculated new percentages of dead worms using the new figures of all worms. In all groups of untreated and doxycycline-treated patients, the corrected percentages of dead female filariae were higher than the original figures, since these included the newly acquired filariae (Table 2). The difference was
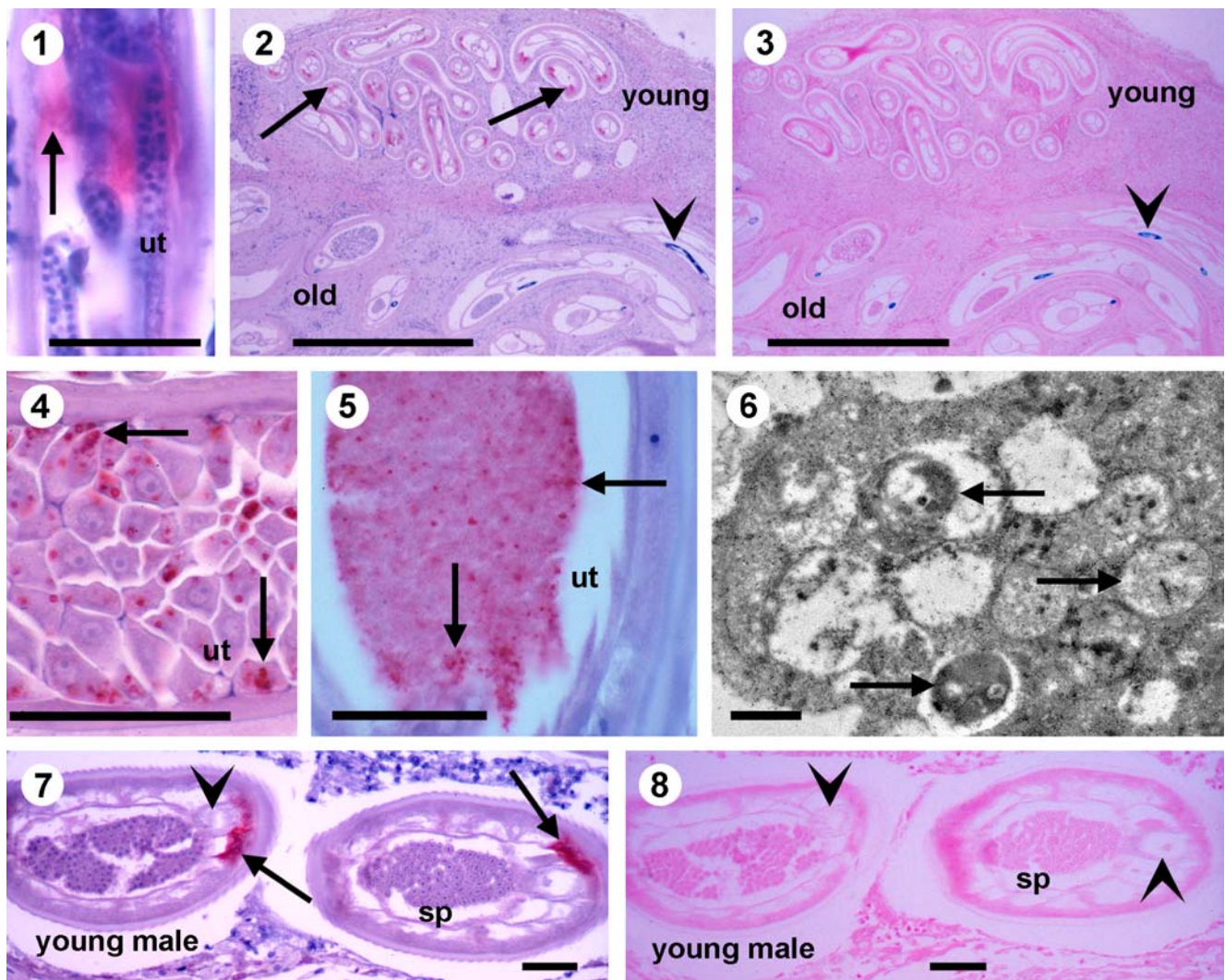
Fig. 1 *1* A *Wolbachia*-positive (*arrow*) immature female *O. volvulus*, excised 20 months after 100 mg doxycycline daily for 5 weeks, was certainly newly acquired. The uterus (*ut*) is small and empty. WSP, *scale bar*=50 μm. *2, 3* A nulliparous young *Wolbachia*-positive (*arrows*) female filaria with no iron in the gut was certainly newly acquired in contrast to the bacteria-negative, doxycycline-treated old female with much iron in the gut (*arrowheads*). Excised 11 months after 100 mg doxycycline for 6 weeks. *2* WSP. *3* Iron stain, *scale bar*= 50 μm. *4* Living endobacteria (*arrows*) in intact oocytes in the uterus (*ut*) of an untreated worm. WSP, *scale bar*=50 μm. *5* Degenerated endobacteria (*arrows*) in degenerated oocytes in the uterus (*ut*) of a doxycycline-treated female worm. Excised 11 months after 100 mg doxycycline for 6 weeks in contrast to the intact endobacteria in *4*. WSP, *scale bar*=50 μm. *6* Electron micrograph of an oocyte with degenerated *Wolbachia* (*arrows*), which show irregular cytoplasm and electron dense inclusions. Excised 11 months after 100 mg doxycycline for 6 weeks. *Scale bar*=1 μm. *7* A *Wolbachia*-positive (*arrows*) newly acquired young male worm with intact spermatogenesis (*sp*), contains no iron in the gut (*arrowheads*). Excised 27 months after 200 mg doxycycline for 6 weeks, WSP, iron stain, *scale bar*=50 mm. *8* *Wolbachia*-positive (*arrows*) newly acquired young male worm with intact spermatogenesis (*sp*), contains no iron in the gut (*arrowheads*). Excised 27 months after 200 mg doxycycline for 6 weeks, WSP, iron stain, *scale bar*=50 mm

significant for the female worms treated 6 weeks ($p=0.050$). The corrected percentages were also higher for males except for the three groups with 0%. Combining all doxycycline-treated groups examined after 20–39 months into one group, the difference between the previous and the corrected percentages was significant for the female filariae ($p=0.0018$, paired *t* test). For the male worms, the difference showed only a trend for higher rates of dead worms, but it was not significant ($p=0.073$ for all and $p=$

0.094 for 6 weeks). Hence, the macrofilaricidal activity of doxycycline was higher in the previous studies than originally reported (Hoerauf et al. 2008, 2009).

Cessation of embryogenesis and mf production

In our previous reports, we had observed that a small number of filariae presented still normal embryogenesis and mf were found in the human nodule issue and the skin up to

Table 1 Embryogenesis and presence of *Wolbachia* in newly acquired *O. volvulus*

Treatment group	Months after study onset	Number of patients/nodules	Number of living female worms				Number of newly acquired	Number containing many *Wolbachia*
			Newly acquired					
			All	Immature	Oocytes only	Embryos/ mf		
Placebo[a]	6	13/14	29	0	1	0	1	1
	20	16/51	70	1	4	6	11	10
	27	7/38	61	0	3	2	5	5
Doxycycline[a, b] 4 weeks	20	7/36	27	0	1	1	2	2
	27	7/23	20	0	4	2	6	6
	39	2/13	12	0	1	1	2	2
Doxycycline[a, b] 6 weeks	6	11/19	32	0	0	1	1	0
	20	13/62	42	3	5	3	11	9
	27	5/27	15	1	3	1	5	5
	39	2/7	9	0	2	2	4	4
Not treated [c]	27	10/41	51	1	5	0	6	5
Doxycycline[c] 5 weeks	20	12/49	34	1	2	2	5	4
	27	13/47	49	0	5	4	9	9

[a] Hoerauf et al. (2008)

[b] 39 months after treatment: unpublished data

[c] Hoerauf et al. (2009)

39 months after doxycycline (Hoerauf et al. 2008, 2009). Our hypothesis was that these productive worms had not been treated since they had been newly acquired after doxycycline. Because of the long prepatency, newly acquired worms need a period of one to several years to produce mf. We observed 24 productive new females among the newly acquired worms (Table 1; Fig. 2 (14)). One of them was doubtful since it was seen already after 6 months. But we found no old productive worms after sufficient doxycycline treatment for 5 or 6 weeks (see Table 4 in Hoerauf et al. 2008 and Table 3, 2009). Twenty to 27 months after sufficient doxycycline treatment, only in those few nodules containing newly acquired *Wolbachia*-positive productive worms numerous bacteria-positive mf were found adjacent to these female worms (Figs. 2 (14) and (15)). Since we did not detect *Wolbachia*-positive mf later than 6 months after doxycycline in nodules without new females, we assume that mf was only produced by new females, and the sterilization of the female worms present at the time of doxycycline treatment was complete.

Discussion

To fully evaluate macrofilaricidal drug efficacy in human onchocerciasis, worms present at the time of treatment have to be differentiated from those acquired afterwards, because longer trial periods of a few years need to be adhered to, during which the treated individuals are still being exposed to L3. In the present study, we used morphological, histochemical, and immunohistological criteria for the diagnosis of potentially newly acquired worms from older ones in doxycycline treated patients. Doxycycline leads to long-term sterilization by elimination of *Wolbachia* endosymbionts (Hoerauf et al. 2003, 2008).

At 18–39 months, no endobacteria were found in worms that met the criteria of older worms and must therefore have already been present during treatment. We have no evidence that there is any recovery of endobacterial populations in worms arising from a few bacteria that we may not have detected. But this can only be definitely proven with a longer observation until all sufficiently treated filariae have died. In contrast, all worms that were described at that time point as young, including fecund ones, contained many morphologically intact *Wolbachia*. We have to admit that scanning for worms with many bacteria during histological examination has facilitated the detection of newly acquired worms in the doxycycline treatment groups as the higher percentages show. However, all of them were confirmed as young worms based on other criteria than the endobacteria.

A slightly but significantly higher macrofilaricidal activity than originally reported was observed after doxycycline treatment, when newly acquired worms were considered.

Table 2 Corrected percentages of dead worms based on number and percentages of newly acquired worms

Treatment group	Months after study onset	Number of patients	Number of female worms							Number of male worms						
			All no.	Living			Dead% [e]		Corrected[d,e]	All no.	Living			Dead		Corrected[d,e]
				All no.	New no.	New %	no.	%			All no.	New no.	New %	no.	%	
Placebo [a]	6	13	35	29	1	3	6	17	18%	8	8	0	0	0	0	0%
	20	16	93	70	11	16	23	25	28%	27	25	2	8	2	7	8%
	27	7	78	61	5	13	17	22	23%	26	25	2	8	1	4	4%
4 weeks doxycycline[a, b]	20	7	55	27	2	7	28	51	53%	28	21	2	10	7	25	27%
	27	7	39	20	6	30	19	49	58%	16	9	6	67	7	44	70%
	39	2	27	12	2	17	15	56	60%	9	9	3	33	0	0	0%
6 weeks doxycycline[a, b]	6	11	41	32	1	3	9	22	23%	18	18	0	0	0	0	0%
	20	13	111	42	11	26	69	62	69%	31	20	4	20	11	35	41%
	27	5	38	15	5	33	23	61	70%	17	14	2	14	3	18	20%
	39	2	17	9	4	44	8	47	62%	8	6	2	33	2	25	33%
Not treated [c]	20–27	10	61	51	6	12	10	16	18%	21	20	4	20	1	5	6%
5 weeks doxycycline[c]	20	12	79	34	5	15	45	57	61%	20	13	1	8	7	35	37%
	27	13	83	49	9	18	34	40	46%	28	24	4	17	4	14	17%

[a] Hoerauf et al. (2008)

[b] 39 months after treatment: unpublished data

[c] Hoerauf et al. (2009)

[d] Percentage of dead worms calculated from the number of all worms minus the number of newly acquired worms

[e] Difference between observed and corrected percentages of all dead worms was significant (females: $p=0.0002$, males: $p=0.07$, paired t test) and all doxycycline-treated groups (females: $p=0.002$).
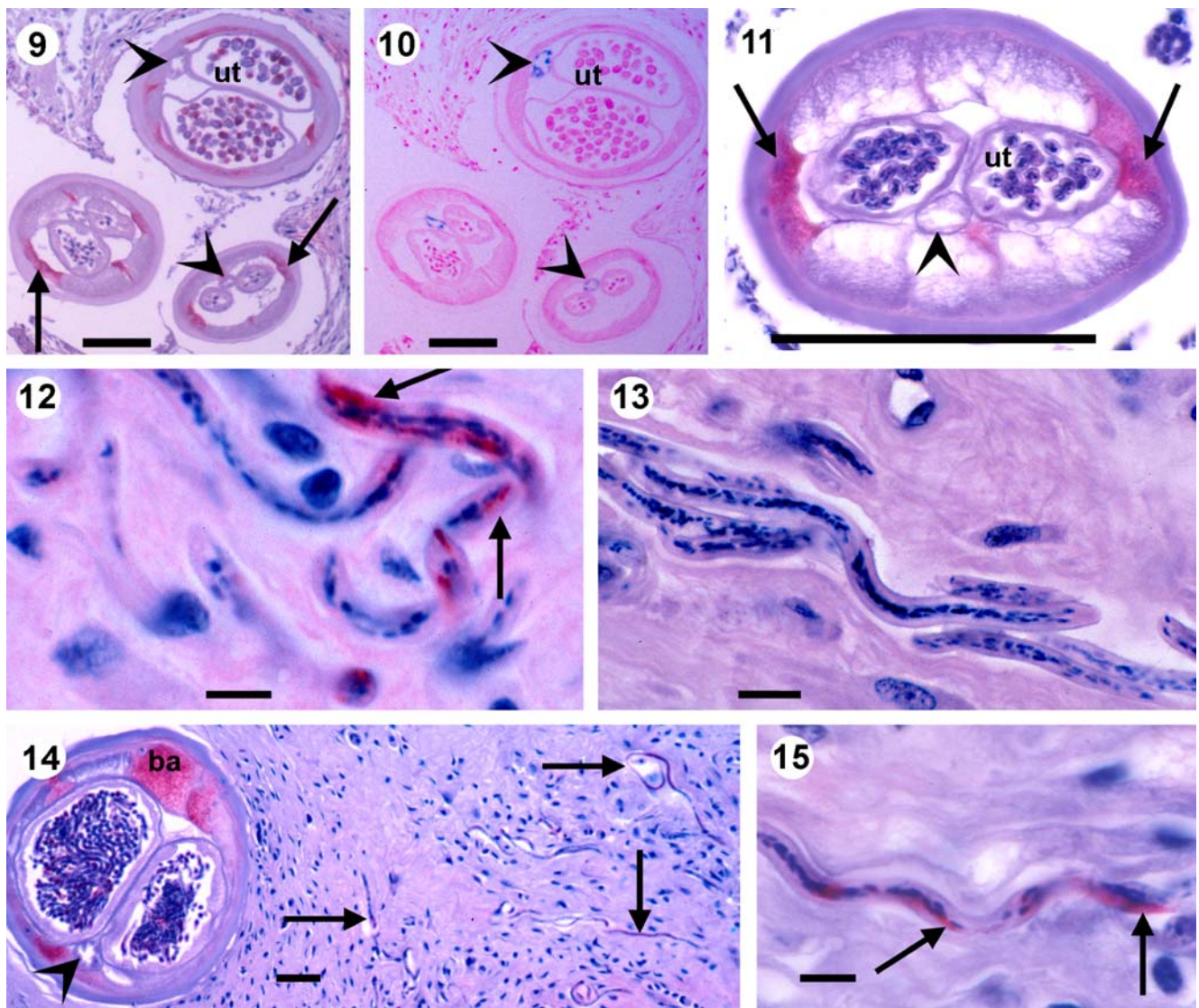
Fig. 2 A newly acquired young female worm full of endobacteria-positive morulae in the uterus (*ut*) and with sparsely iron in the gut (*arrowheads*). Excised 27 months after 200 mg doxycycline daily for 6 weeks. *9* WSP, *10* iron stain, *scale bar*=100 μm. *11* A newly acquired young productive female worm with mature mf in the uterus (*ut*) and a light gut (*arrowhead*) contains *Wolbachia* (*arrows*). Excised 27 months after 100 mg doxycycline for 5 weeks. WSP, *scale bar*=100 μm. *12* A *Wolbachia*-positive mf in a nodule from an untreated patient. WSP, *scale bar*=10 μm. *13* Two months after 100 mg doxycycline daily for 6 weeks are still mf in the nodule but they no longer harbor endobacteria. WSP, *scale bar*=10 μm. *14* Adjacent to a newly acquired, young, endobacteria-positive (*ba*) female worm with a light gut (*arrowhead*) are many *Wolbachia*-positive mf (*arrows*). Excised 27 months after 100 mg doxycycline for 5 weeks. WSP, *scale bar*=50 μm. *15* Detail of a *Wolbachia*-positive mf from *14*. WSP, *scale bar*=10 μm

Still, not all worms present during doxycycline treatment were dead after 18–39 months. This observation period might therefore not be long enough for all filariae to die. We believe that they will die later as degenerated filariae after long years of vector control do (Albiez et al. 1987; Büttner et al. 1990). However, we cannot formally exclude that a few of them may recover in spite of their severe degeneration. On the other hand, survival of a small number of worms is accepted for other anthelmintic drugs, e.g., for schistosomes (WHO 1995).

Embryogenesis and mf production were interrupted by doxycycline treatment. It was clearly visible that 11–39 months after treatment, all *Wolbachia*-positive mf were found adjacent to an endobacteria-harboring female with the characteristics of a newly acquired worm and all these mf were bacteria-positive. We assume that all the endobacteria-positive mf seen 18 months and later had been produced by newly acquired filariae. The life expectancy of mf varies, but on average 18 months is assumed (Plaisier et al. 1991). Probably, none or only few

doxycycline-treated mf survived after 20 and more months. This is probably also true for the mf in the skin (Hoerauf et al. 2008, 2009). The results confirm a long-term sterilizing effect of doxycycline. Since the treated surviving females had either degenerated oocytes or empty uteri, the sterilization is probably permanent. Further, these severely degenerated filariae will probably die and not recover.

We assume that 9–10% of the living worms observed at the follow-up examinations had been newly acquired annually since the transmission was ongoing in the trial villages (Kutin et al. 2004; R. Garms, Bernhard Nocht Institute, unpublished report, 2006). This percentage is sufficient to replenish the number of worms dying naturally, which may be about 10% each year, if we assume an average life expectancy of 10 years. Plaisier et al. (1991) report 9–11 years as average life expectancy in different villages in the savannah areas. We estimated similar figures based on the decline of worms in the upper part of the body in children (Büttner and Weiss 1984) and after vector control in Burkina Faso and Ghana (Büttner et al. 1988, 1990). In Burkina Faso, we nodulectomized patients after interruption of transmission in 1977, 1978, 1980, 1983, and 1987 and analyzed their worm loads, which decreased continually. After these 11 years, the burden of living worms per person was only 10% of that in 1977 and some of these worms had obviously been acquired in neighboring Ivory Coast (Albiez et al. 1987). Our assumption that about 10% of worms die annually is based on these figures. The approximately 10% of the adult worm population, which die each year in chronic infections, will more or less be replenished. Schulz-Key and Soboslay (2000) found that such stable parasite loads are maintained in communities with longstanding immunity despite the different intensities of transmission. Thus differing proportions of transmitted L3 will develop into adult worms, varying between less than 0.1% and 10% or more, whereas most L3 are eliminated by the host. Stable *O. volvulus* loads in highly endemic foci had been shown to be an important mechanism for parasite density regulation by Duerr et al. (2003). These findings support our present results that within 2–3 years roughly 20–30% of the adult filariae are newly acquired worms.

Conclusions

We found our three hypotheses confirmed. (a) All the 61 adult and two of the immature of the 68 newly acquired filariae harbored many intact endobacteria, whereas after 12 and more months of doxycycline treatment, none of the other surviving worms harbored many endobacteria. (b) Higher percentages of dead filariae than originally reported were calculated, when the newly acquired worms were

excluded; therefore, the real macrofilaricidal activity of doxycycline is higher than originally reported. This difference was significant for the group of female filariae after sufficient doxycycline treatment for 6 weeks. (c) Only worms identified as newly acquired presented normal embryogenesis and mf production, and all mf observed in the nodule tissue harbored *Wolbachia* 18 and more months after sufficient doxycycline treatment. We conclude further that newly acquired filariae have to be considered for proper evaluation of drug efficacy in onchocerciasis.

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