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## Obligatory symbiotic *Wolbachia* endobacteria are absent from *Loa loa*

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### Abstract

**Background:** Many filarial nematodes harbour *Wolbachia* endobacteria. These endobacteria are transmitted vertically from one generation to the next. In several filarial species that have been studied to date they are obligatory symbionts of their hosts. Elimination of the endobacteria by antibiotics interrupts the embryogenesis and hence the production of microfilariae. The medical implication of this being that the use of doxycycline for the treatment of human onchocerciasis and bancroftian filariasis leads to elimination of the *Wolbachia* and hence sterilisation of the female worms. *Wolbachia* play a role in the immunopathology of patients and may contribute to side effects seen after antifilarial chemotherapy. In several studies *Wolbachia* were not observed in *Loa loa*. Since these results have been doubted, and because of the medical significance, several independent methods were applied to search for *Wolbachia* in *L. loa*.

**Methods:** *Loa loa* and *Onchocerca volvulus* were studied by electron microscopy, histology with silver staining, and immunohistology using antibodies against WSP, *Wolbachia* aspartate aminotransferase, and heat shock protein 60. The results achieved with *L. loa* and *O. volvulus* were compared. Searching for *Wolbachia*, genes were amplified by PCR coding for the bacterial 16S rDNA, the FTSZ cell division protein, and WSP.

**Results:** No *Wolbachia* endobacteria were discovered by immunohistology in 13 male and 14 female *L. loa* worms and in numerous *L. loa* microfilariae. In contrast, endobacteria were found in large numbers in *O. volvulus* and 14 other filaria species. No intracellular bacteria were seen in electron micrographs of oocytes and young morulae of *L. loa* in contrast to *O. volvulus*. In agreement with these results, *Wolbachia* DNA was not detected by PCR in three male and six female *L. loa* worms and in two microfilariae samples of *L. loa*.

**Conclusions:** *Loa loa* do not harbour obligatory symbiotic *Wolbachia* endobacteria in essential numbers to enable their efficient vertical transmission or to play a role in production of microfilariae. Exclusively, the filariae cause the immunopathology of loiasis in patients and the adverse side effects after antifilarial chemotherapy. Doxycycline cannot be used to cure loiasis but

it probably does not represent a risk for *L. loa* patients when administered to patients with co-infections of onchocerciasis.

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## Background

Rickettsia-like intracytoplasmatic bacteria were first observed in filarial nematodes using electron microscopy in the 1970's [1,2]. Later it was shown that these endobacteria are closely related to intracellular bacteria found in many arthropods and they were all grouped together in the genus *Wolbachia* [3,4]. Over the last few years the filarial *Wolbachia* have received increased attention since it was shown that in those filarial species that harbour them they are obligatory symbionts needed for all stages of embryogenesis (from oocytes in the ovary to microfilariae, infective larvae, male and female worms) [5]. The term 'obligatory symbiosis' was introduced for the association of *Wolbachia* with the wasp *Asobara tabida*, because following treatment with antibiotics, (which cleared the endobacteria in the insect), it became sterile due to a blockade of oocyte production [6]. Similarly, the removal of *Wolbachia* by administration of doxycycline and other antibiotics leads to an interruption of embryogenesis and probably permanent sterilisation of the female filariae [7]. Langworthy et al., reported a macrofilaricidal activity of prolonged oxytetracycline treatment against *Onchocerca ochengi* in cattle [8]. It has been shown for onchocerciasis and bancroftian filariasis that doxycycline therapy may be a new treatment strategy at least for individual patients or for small groups [9,10].

*Wolbachia* have been observed in most filarial species that have been examined. The absence of endobacteria has been reported for four species based on the results of several independent methods of investigation: *Acanthocheilonema viteae* [1,11,12], *Onchocerca flexuosa* [13,14], *Loa loa*, and very recently *Setaria equina* [15]. For the three animal parasites these findings have been accepted. The absence of endobacteria from the human parasite *L. loa* has been reported by several authors based on electron microscopy [1,16–18], on immunohistology [19] and PCR [20]. However, because of its medical importance and the small number of samples used in most studies, the statements regarding *L. loa* haven been repeatedly doubted.

For the interruption of transmission of *Onchocerca volvulus* in areas endemic for onchocerciasis and loiasis an alternative treatment with doxycycline may be useful when ivermectin and diethylcarbamazine cannot be used for onchocerciasis patients with high microfilariae loads of *L. loa* [21–23]. For such a strategy it is crucial to know whether *L. loa* have endobacteria that may cause serious adverse side effects when co-infections are to be treated.

By using electron microscopy, histology, immunohistology, and PCR, we present evidence that there are not sufficient numbers of *Wolbachia* endobacteria in *L. loa*, if any, to live in an obligatory symbiotic association.

## Methods

### *Filariae and insects*

Nematode samples from infected humans and animals were obtained with the approval of the ethics committees and regulatory authorities of all institutions and countries involved in this study.

Specimens with blood containing *L. loa* microfilariae, adult *L. loa* worms and skin biopsies extirpated by ophthalmologists and other physicians from human patients had been sent to the Bernhard Nocht Institute for diagnosis. Several specimens of a spleen extirpated from a 24-year-old German, who had been travelling in Nigeria and Cameroon, were kindly supplied by Prof. GD Burchard [24]. Further adult worms and a sample of spleen were collected in Cameroon from an experimentally infected two-year-old drill (*Mandrillus leucophaeus*) born in captivity.

Third stage larvae were collected from *Chrysops silacea* fed on microfilaremic human volunteers from Cameroon, who had expressed informed consent. Infective larvae from these *C. silacea* were used to infect the drill and seven months later adult *L. loa* worms and the spleen were recovered. Samples of the spleen were embedded in Paris and sent to Hamburg. Fragments of additional adult *L. loa* worms from previous experimental infections of monkeys were available in Paris. Six of these worms belonged to the human strain and three to a monkey strain.

Onchocercomas with microfilariae and adult *O. volvulus* and specimens of other filarial worms embedded in paraffin were available from several previous studies [13,25]. To test the reactivity of the antibodies against *Wolbachia* proteins from various hosts, we also examined females of the sand flea *Tunga penetrans* removed from patients in Ghana [26] and the mosquito *Culex pipiens* from a laboratory colony maintained at the Istituto di Parassitologia, Università die Roma La Sapienza.

### *Electron microscopy*

Electron micrographs that had been taken during previous studies on *L. loa* [17,18,27] and on *O. volvulus* were re-examined ([28] and unpublished studies). The *L. loa* worms had been collected in Lambarene (Gabon) during

**Table 1: *L. loa* used for histology and immunohistology**

Stage of worms	Number of worms	Quality of worms	Host	Country of origin
<b>Microfilariae</b>	dozens	in blood	human	Cameroon
	hundreds	in spleen	human	Cameroon or Nigeria
	dozens	in spleen	drill	Cameroon
<b>Female worms with embryos</b>	2	complete worms	human	unknown
	5	complete worms	drill	Cameroon
	3	fragments	human	Cameroon and Gabon
	6	fragments	monkey	Cameroon and unknown
<b>Male worms</b>	2	complete worms	human	unknown
	4	complete worms	drill	Cameroon
	2	fragments	human	Nigeria and unknown
	3	fragments	monkey	Cameroon and unknown

herniotomies or they had been sent to the Bernhard Nocht Institute for diagnosis by physicians in northern Germany. The *O. volvulus* worms had been removed from patients in Liberia. The filariae had been fixed in 2% buffered glutaraldehyde and 1% osmium tetroxide, embedded in araldite, epon or Spurr's ERL medium and processed for electron microscopy as usual. Two years ago, *L. loa* microfilariae from the blood of a Cameroonian patient were processed for immunogold electron microscopy [19,26]. For light microscopic controls, semi-thin sections had been stained with azure II and methylene-blue. Larger pieces of onchocercomas were also embedded in methacrylate and stained by methylene-blue.

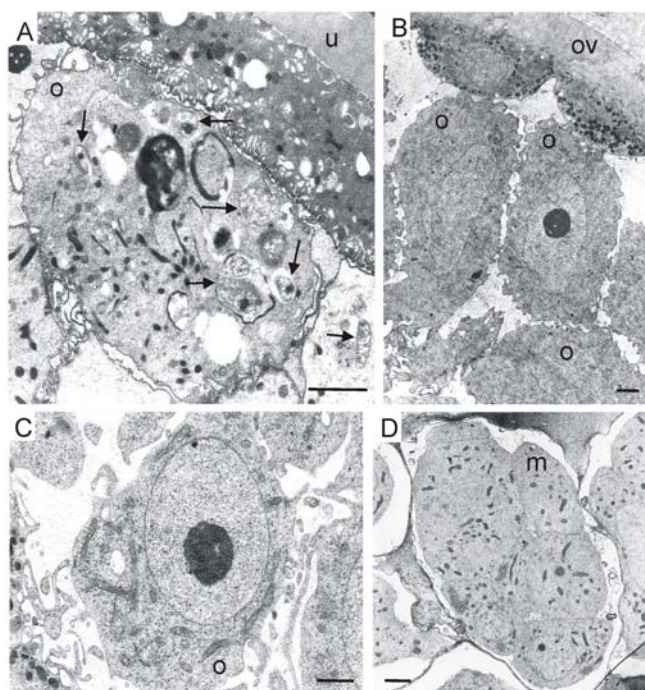
#### Light microscopy

The worms and the biopsies from organs had been fixed in 80% ethanol or 4% buffered formaldehyde. They were embedded in paraffin and stained conventionally with haematoxylin & eosin. Biopsies from organs also were stained with Giemsa or Pappenheim stain. Sections of all adult *L. loa* worms selected for this study (Table 1) and selected onchocercoma sections were stained with silver using the Warthin-Starry method conducted under strictly controlled staining conditions [29]. For immunohistology, the alkaline phosphatase anti-alkaline phosphatase (APAAP) method was applied according to the manufacturer's recommendations (Dako Diagnostika, Hamburg, Germany). Antisera against filarial, wobbachial or *Yersinia enterocolitica* proteins or against human leukocytes were used as primary antibodies. We applied as secondary antibodies anti-rabbit mouse immunoglobulins (clone MR12/53, Dako Diagnostika, Hamburg, Germany) for rabbit sera against filarial and bacterial proteins and anti-mouse antibodies for the monoclonal immunoglobulins against the proteins of human immune cells. Fast Red TR salt (Sigma, Deisenhofen, Germany) was used as chromogen and haematoxylin (Merck, Darmstadt, Germany) functioned as the counterstain.

As primary antibodies for all selected *L. loa* worms (Table 1), *O. volvulus* and other filarial worms we used rabbit antisera against the following recombinant proteins: *Wolbachia* surface protein of *Dirofilaria immitis* *Wolbachia* (Wol-Di-WSP, dilution 1:1 000 – 1:4 000, [30,31]), aspartate aminotransferase of *Wolbachia* from *O. volvulus* (Wol-Ov-AAT, dilution 1:60 – 1:200, [32]), and *Y. enterocolitica* heat shock protein-60 (Y-HSP60, dilution 1:1 000, [33]), which had been supplied by Prof. IB Autenrieth, Tübingen. The specificity of the antisera against Wol-Di-WSP and Y-HSP60 to label *Wolbachia* had been shown by immunoelectron microscopy [26]. Wol-Di-WSP labelled only WSP, Y-HSP60 also labelled filarial HSP60 in mitochondria and other tissues, and Wol-Ov-AAT labelled both wobbachial and the filarial AAT especially in sperms. In addition, for selected sections we applied antibodies against human HSP60 (clone LK2, Sigma, dilution 1:5), HSP60 from *O. volvulus* *Wolbachia* (Wol-Ov-HSP, dilution 1:500, [19]) supplied by PD Dr. KD Erttmann, a catalase from *O. volvulus* *Wolbachia* (Wol-Ov-CAT, dilution 1:30 – 1:50, [14]) supplied by PD Dr. K. Henkle-Dührsen, Düsseldorf, and monoclonal antibodies against human neutrophil granulocyte elastase (Dako Diagnostika, dilution 1:150, [34]), and neutrophil defensins (Dianova, Hamburg, Germany, dilution 1:1 000 – 1:4 000, [34]). Furthermore, antibodies against proteins of eosinophil granulocytes, macrophages [34], mast cells and other filarial and wobbachial proteins were applied. As positive control a *Wolbachia*-positive onchocercoma section was included for each stained set of *L. loa* sections.

#### PCR

DNA was prepared from ethanol preserved or paraffin embedded adult *L. loa* worms using the Dneasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Microfilariae were isolated from two patients from Cameroon with confirmed loiasis by blood filtration using a 5 µm polycarbonate filter. Filters with



**Figure 1**

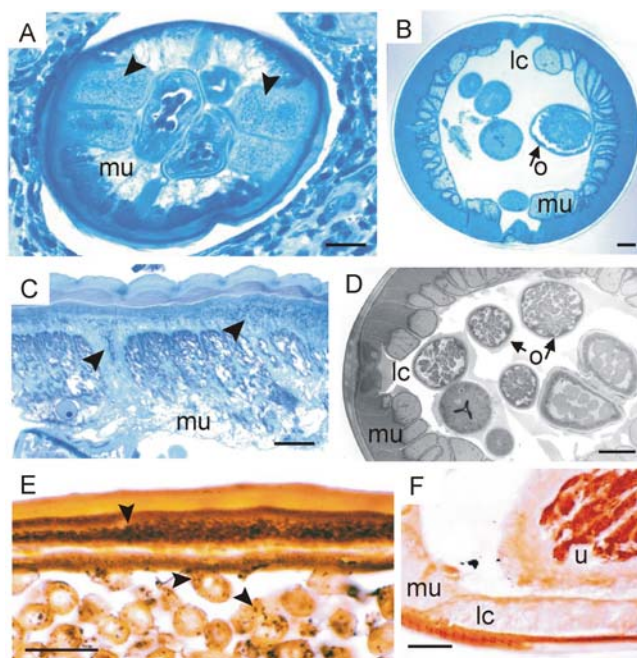
Electron micrographs of oocytes and a morula of *O. volvulus* and *L. loa*. (A) Oocyte with several intracellular *Wolbachia* bacteria (arrows) and uterus wall of *O. volvulus*. (B, C) Oocytes without any bacteria in the ovary and uterus of *L. loa*. (D) Young morula without endobacteria in the uterus of *L. loa*. m, morula, o, oocytes; ov, ovary; u, uterus; scale bar = 2  $\mu$ m.

approximately 500 microfilariae each were washed using 500  $\mu$ l TE buffer (0.01 M Tris, 0.1 mM EDTA), lysed in 250  $\mu$ l DSP buffer (0.02 M Tris, 0.05 M KCL, 2.5 mM MgCl<sub>2</sub>, 0.5% tween 20, 150  $\mu$ g/ml proteinase k) and 1  $\mu$ l template was used in a 50  $\mu$ l PCR assay. Three different sets of primers were used to amplify *Wolbachia* DNA: one *Wolbachia*-specific primer pair targeting the 16S rDNA, one primer pair targeting the gene encoding the FTSZ cell cycle protein and one primer pair targeting the gene encoding the WSP as described in detail previously [26]. These primers were known to amplify not only DNA of *Wolbachia* from filariae but also from *T. penetrans*. As positive control, DNA of untreated *O. volvulus* worms was used. In addition, to test the quality of the DNA template, the 5S rDNA of the filarial host was amplified by PCR as previously described [35].

## Results

### Characterisation of obligatory symbiotic *Wolbachia*

*Onchocerca volvulus* represents a typical filaria that contains obligatory symbiotic *Wolbachia*. We have therefore

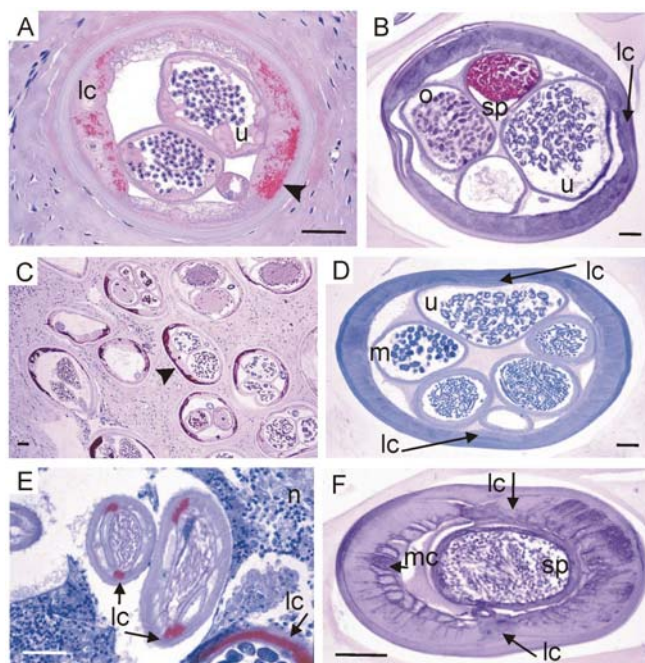


**Figure 2**

Silver and methylene-blue staining of *Wolbachia* endobacteria. (A, C) Cross sections of female *O. volvulus* show *Wolbachia* (arrow-heads) in the lateral cords of the hypodermis. (E) Longitudinal section of *O. jakutensis* with black endobacteria in hypodermis and oocytes (arrowheads). (B, D, F) No endobacteria are found in the lateral cords or in oocytes of female *L. loa* worms. lc, lateral cord; o, oocytes in the ovary; u, uterus. A – D, semi-thin sections stained with azure II and methylene-blue; E, F, sections stained with silver, Warthin-Starry method; scale bar = 25  $\mu$ m.

examined the characteristics of the obligatory symbiotic *Wolbachia* in more than 800 adult *O. volvulus* worms and in several other filaria species. It was found that each intact living worm harboured *Wolbachia* in large numbers (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5). This concerned worms from patients from Uganda, Cameroon, Benin, Togo, Ghana, Burkina Faso, Mali, Liberia, Mexico, Guatemala, Brazil, and Yemen. We observed numerous endobacteria in the hypodermis of female worms in all portions from the nerve ring to the posterior end.

In many oocytes, embryos and microfilariae the endobacteria were not detected by immunohistology. However, when the endobacteria had expressed the respective proteins, for which the antisera applied were specific, all live (intact) oocytes or morulae presented endobacteria (Figure 5A, 5C, 5E). Using electron microscopy we counted up to 14 bacteria in one oocyte section. We assume that all live embryos developing later to microfilariae harbour at

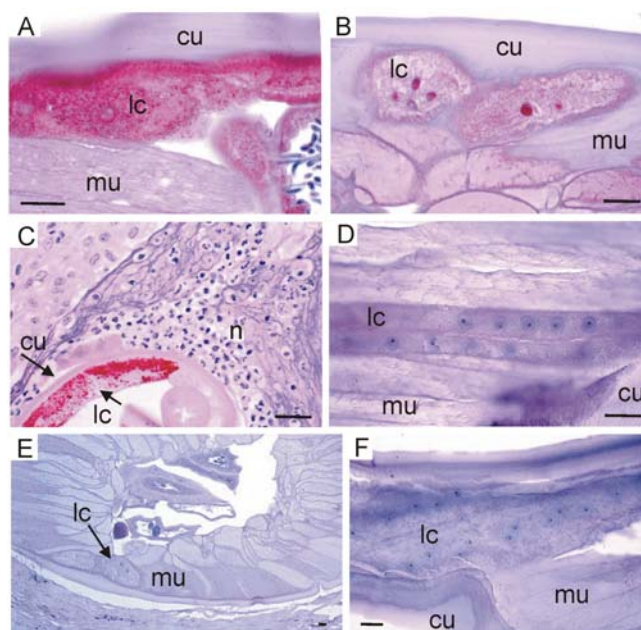


**Figure 3**

Cross sections of *Onchocerca* and *L. loa* stained with three antisera against *Wolbachia*. (A, B) Antiserum against Wol-Ov-AAT labels *Wolbachia* endobacteria (arrow-head in A) in a female *O. volvulus* but no bacteria are seen in a female *L. loa*, although sperms in the uterus are well labelled. (C, D) Antiserum against Y-HSP60 stains *Wolbachia* in the hypodermis of all sections of a female *O. volvulus* in an onchocercoma (arrow-head in C) but no bacteria can be detected in hypodermis or embryos of a female *L. loa* (D). (E, F) Antiserum against Wol-Di-WSP stains the bacteria in the lateral cords (arrows) of a male *O. dukei* (E). No bacteria are seen in the lateral or median cords of a male *L. loa* (F). lc, lateral cord; m, morulae; mc, median cord; n, neutrophil granulocytes; o, oocytes in the uterus; sp, sperms; u, uterus; scale bar = 50 µm.

least ten bacteria (and probably more since an ultra-thin section of 0.1 µm covers only a thin layer of the oocyte). As far as it can be concluded from the limited numbers of worms examined, we assume that this occurrence of numerous endobacteria found in *O. volvulus* applies also to *O. ochengi* (Figure 4C; Figure 5C), *O. dukei* (Figure 3E), *O. gibsoni* (Figure 5E), *O. fasciata* (Figure 4A), *O. jakutensis* (Figure 2E[36]), *Litomosoides sigmodontis* [12], *Wuchereria bancrofti*, *Dirofilaria immitis*, and *Dirofilaria repens*.

Based on these findings, we define a filaria species harbouring obligatory symbiotic *Wolbachia* as one with numerous endobacteria in each adult worm and several bacteria in each oocyte and embryo that will develop to a



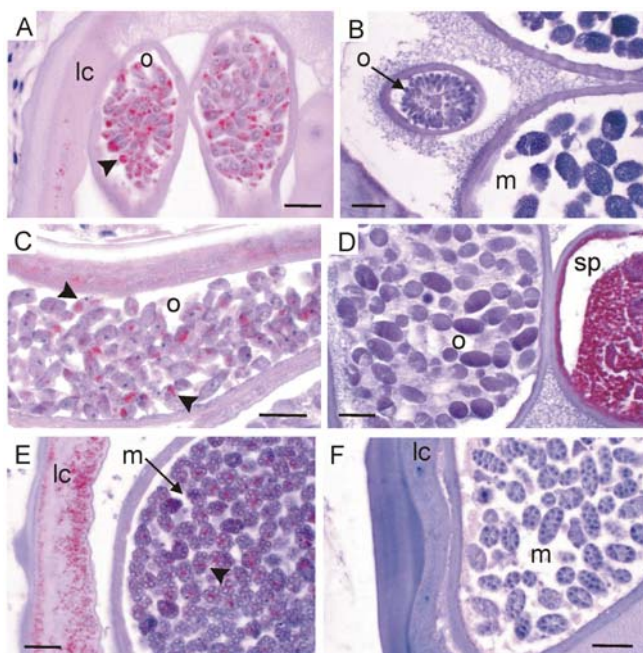
**Figure 4**

Lateral cords of *Onchocerca* and *L. loa* stained with three antisera against *Wolbachia*. (A, B) Antiserum against Wol-Ov-AAT stains numerous bacteria in a female *O. fasciata* (A). Whereas no bacteria are seen in the otherwise well stained lateral cords of a female *L. loa* from Gabon (B). (C, D) Antiserum against Y-HSP60 stains the bacteria in the hypodermis of a female *O. ochengi* to which many neutrophil granulocytes are attached (C). No bacteria are found in the lateral cords of a male *L. loa*. (D, F) No *Wolbachia* are seen in the lateral cords of a female *L. loa* from Gabon (E, cross section in skin) and in a male *L. loa* (longitudinal section) stained with Wol-Di-WSP. No neutrophil granulocytes are found attached to the cuticle of *L. loa* (E). cu, cuticle; lc, lateral cord; mu, muscles; n, neutrophil granulocytes; scale bar = 25 µm.

mature microfilaria. The studies described in the following paragraphs aimed to search for *Wolbachia* in the *L. loa* worms in numbers, as they were observed in the above-mentioned filaria species containing obligatory symbiotic *Wolbachia*.

#### Electron microscopy

Screening several dozen electron micrographs from the previous studies on *L. loa* and *O. volvulus* [17,18,27,28] we often found endobacteria in the oocytes of *O. volvulus* (Figure 1A). In contrast, no endobacteria were observed in the oocytes of the ovary (Figure 1B), the uterus (Figure 1C) or in the early morulae (Figure 1D) of *L. loa*. Immunogold electron microscopy using the anti-Y-HSP60 serum showed well-labelled mitochondria but no endobacteria in the cells of *L. loa* microfilariae.

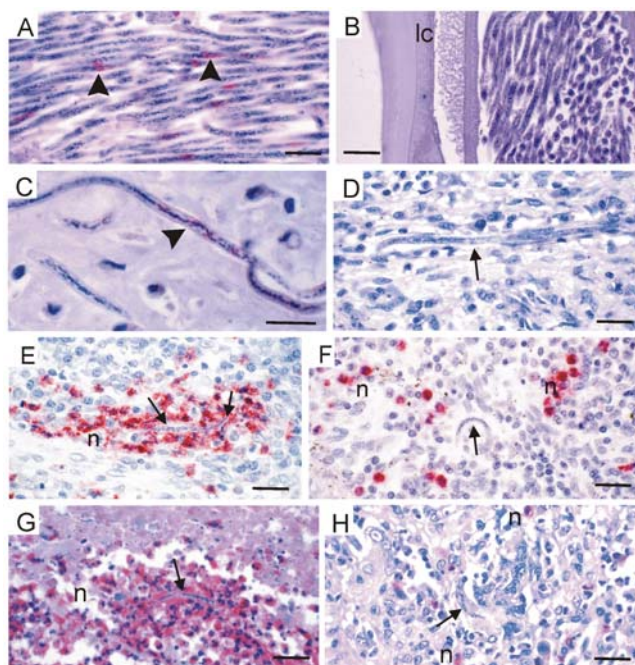


**Figure 5**

Oocytes and young morulae of *Onchocerca* and *L. loa* stained with three antisera against *Wolbachia*. (A, B) Antiserum against Wol-Di-WSP stains the *Wolbachia* (arrowhead in A) in all primary oocytes in the ovary of *O. volvulus* whereas no bacteria are seen in the oocytes in the ovary or in the morulae of a *L. loa* female (B). (C, D) Antiserum against Wol-Ov-AAT stains the *Wolbachia* (arrow-heads in C) in all mature oocytes in the uterus of *O. ochengi* but no bacteria are seen in the uterus of *L. loa*. The *L. loa* worm indicates its reactivity to the antiserum by well-labelled sperms. (E, F) Antiserum against Y-HSP60 stains the *Wolbachia* (arrow-head in E) in the lateral cord and in all young morulae of an *O. gibsoni* female but no bacteria can be detected in the morulae or in the lateral cord of a *L. loa* female (F). lc, lateral cord; m, morulae; o, oocytes; sp, sperms; scale bar = 25µm.

### Histology

In semi-thin sections stained with azure II and methylene-blue we detected granular structures in the hypodermis (Figure 2A,2C), oocytes and embryos of dozens of *O. volvulus* worms but never any in *L. loa* worms (Figure 2B,2D). Using silver staining of paraffin sections, we found endobacteria-like granules in consecutive sections of *O. volvulus* and *O. jakutensis* (Figure 2E) precisely where *Wolbachia* were seen after labelling with specific antisera against *Wolbachia* antigens. In contrast, none of the *L. loa* worms selected for this study (Table 1) displayed such silver-stained granules (Figure 2F). Furthermore, no endobacteria-like granules stained by haematoxylin or Giemsa stain were seen in the hypodermis of *L. loa*.



**Figure 6**

*Wolbachia* in microfilariae of *Onchocerca* but not of *L. loa* and the reaction of neutrophils. (A – D) Antiserum against Wol-Di-WSP stains the *Wolbachia* (arrowheads) of microfilariae in the uterus of *O. volvulus* (A) and in an onchocercoma (C) whereas no bacteria are seen in intact microfilariae of *L. loa* in the uterus (B) or in human spleen tissue (D). (E – H) Neutrophil granulocytes are attracted to degenerating microfilariae (arrows) of *O. volvulus* in lymph node tissue 24 hours after a dose of ivermectin (E, anti-neutrophils elastase) or in an onchocercoma of an untreated patient (G, anti-neutrophils defensins). Neutrophils are not attracted by degenerated *L. loa* microfilariae (arrows) in spleen tissue from a human patient not treated with microfilaricidal drugs (F, anti-neutrophils elastase) or from an experimentally infected monkey (H, anti-neutrophils defensins). n, neutrophil granulocytes; scale bar = 25 µm.

### Immunohistology

The reactivity of *Wolbachia* with our antisera was examined in various hosts. The antiserum against Wol-Di-WSP reacted strongly with *Wolbachia* belonging to wolbachial clade C: *D. immitis*, *D. repens*, *O. volvulus*, *O. gutturosa*, *O. dukei*, *O. gibsoni*, *O. fasciata*, *O. armillata*, *O. ochengi*, *O. jakutensis*, *O. tarsicola*; to clade D: *Brugia malayi*, *Brugia pahangi*, *L. sigmodontis*, *W. bancrofti*, and three other filarial species and with the *Wolbachia* from the insects *Cx. pipiens* (clade B) and *T. penetrans*. The antiserum against Y-HSP60 reacted well with all the *Wolbachia* of the above-mentioned filariae and *T. penetrans* (except that it was not tested with those of *B. pahangi* and *Cx. pip-*

iens). We conclude that these two antisera react with all *Wolbachia*. The other antisera were examined with some or most but not all of the above mentioned *Wolbachia*.

Having shown the suitability of these anti-wolbachial sera, *L. loa* worms were compared with *O. volvulus* and other *Wolbachia*-positive filariae. Using these antisera against *Wolbachia* we easily detected endobacteria in many filariae in the hypodermis of male (Figure 3E) and female worms (Figure 3A,3C; Figure 4A,4C; Figure 5E). We found the *Wolbachia* well labelled by the antisera in the oocytes of the ovary (Figure 5A) and in portions of the uterus (Figure 5C), in zygotes, in morulae (Figure 5E) or in other embryos and microfilariae (Figure 6A), when the respective wolbachial proteins had been expressed. However, to interpret these photos and other previously published photos [12,14,32,36–39] it has to be taken into account that it would have been easy to present as many photos from other sections of the same worms, in which no endobacteria occurred or where the endobacteria had not expressed the respective proteins. Such non-reactive endobacteria were often well stained by haematoxylin, Giemsa or toluidine-blue.

Before we selected the *L. loa* worms for our study, we examined them with several antisera against filarial proteins. Antisera against the ankyrin-related protein [40] and glutathione S-transferase of *O. volvulus* did not react with any of the *L. loa* worms. Y-HSP60 cross-reacting with filarial proteins [36,39], reacted weakly with *L. loa* proteins. However, using immuno electron microscopy, mitochondria of microfilariae were distinctly labelled by this antibody. Good cross-reactivity was achieved by the antiserum against Wol-Ov-AAT, which labelled the *Wolbachia*-free sperms of *L. loa* and with two other antisera. All *L. loa* worms that did not react well with Wol-Ov-AAT were excluded as unsuitable for immunohistology. Those that were well labelled (Figure 3B; Figure 4B; Figure 5D) were selected for the study. As shown in Table 1, seven complete female *L. loa* worms producing embryos and microfilariae, six complete male worms, and some fragments of 14 other adult worms were examined. Each complete worm had been cut into small pieces before embedding in one block that was cut until nothing was left. All sections were analysed by either anti-Wol-Di-WSP or anti-Y-HSP60, except for a few sections stained with anti-Wol-Ov-AAT or by the silver method. This procedure yielded about 20 slides from each *L. loa* worm, each slide containing many worm sections. No *Wolbachia* was detected in any of these sections (Figure 3B, Figure 3D,3F; Figure 4B,4D,4E,4F; Figure 5B,5D,5F; Figure 6B,6D). Furthermore, no *Wolbachia* were observed in *L. loa* when antisera against human HSP60, Wol-Ov-HSP60, Wol-Ov-CAT and another antiserum were applied.

*Wolbachia* could easily be observed with all antisera in microfilariae in onchocercomas (Figure 6C), skin, and lymph nodes. In contrast, no *Wolbachia* were found in several hundred *L. loa* microfilariae from the spleen of a patient and of an experimentally infected monkey. Sections of *L. loa* microfilariae from a sample of human blood were also non-reactive with the antisera.

#### **Neutrophil granulocytes as an indicator of *Wolbachia***

Previously it has been shown that the accumulation of neutrophil granulocytes around adult *O. volvulus* worms is dependent on *Wolbachia* [36]. Such accumulation could also be seen around the other endobacteria-positive filariae: *O. jakutensis* [36], *O. dukei* (Figure 3E), *O. ochengi* (Figure 4C), *O. gibsoni*, and *D. repens* in subcutaneous nodules of human patients. In contrast, no neutrophil granulocytes were found around the two female *L. loa* worms in skin biopsies from patients (Figure 4E). Since live adult *L. loa* are mobile these observations were not sufficient for a conclusion. Therefore, degenerated microfilariae in the spleen were examined. We had previously shown the activity of neutrophils against *O. volvulus* microfilariae in onchocercomas [41] and in the skin after treatment with diethylcarbamazine [34]. Such activity of neutrophils against microfilariae could clearly be shown after staining with antisera specific for proteins of neutrophils (Figure 6E,6G). However, neutrophils present in human and monkey spleen tissue, where degenerating *L. loa* microfilariae could be observed, were not attached to the parasites (Figure 6F,6H). The degenerated or disintegrating microfilariae were attacked only by eosinophil granulocytes, macrophages and small giant cells, as shown previously by Duke [42]. We conclude from these findings that the microfilariae of *L. loa* do not contain sufficient numbers of *Wolbachia*, if any, to attract neutrophil granulocytes.

#### **PCR**

The 5S rDNA spacer of *L. loa* was amplified from all samples included in the study, indicating the absence of significant PCR inhibitor in the samples. Using the *Wolbachia* 16S rDNA primers, the *ftsZ* primers and the *wsp* primers, PCR products of about 530 bp, 510 bp and 490 bp, respectively, were obtained in all *O. volvulus* samples, which functioned as controls. In contrast, no bands were visible on the ethidium bromide stained agarose gel when the DNA from the three males and six female *L. loa* worms and two batches of *L. loa* microfilariae samples were tested.

#### **Discussion**

Positive findings, when *Wolbachia* endobacteria are detected by any of the methods used in this study, need only a few worms to demonstrate the reproducibility of the results. Negative findings, such as the absence of *Wolbachia*,

can only be presented with a certain probability and even this evidence requires larger numbers of worms and repeated experiments. Negative electron microscopic findings of *Wolbachia* in *Loa* microfilariae have been based on observations of "several hundred" [1] or "many thousand" sections [16]. These numbers are needed since large portions of bacteria-positive microfilariae are free of *Wolbachia*. The screening of adult *L. loa* worms has to focus on microfilariae producing female worms since they would harbour the largest numbers of endobacteria in oocytes and embryos. Using electron microscopy we observed up to 14 bacteria in oocytes of *O. volvulus*, eight or more bacteria were found in an oocyte of *W. bancrofti* [43] and electron micrographs of *D. immitis* showed 15 – 25 bacteria in embryos [1]. In *Mansonella ozzardi* microfilariae, ten or more bacteria were reported [44]. Since all these figures are based on single ultra-thin sections, and regarding our light microscopic observations of several bacteria clusters in oocytes and microfilariae, we estimate that oocytes, embryos and microfilariae of filaria species with essential numbers of *Wolbachia* for vertical transmission harbour 30–50 or more endobacteria. Assuming only one or ten *Wolbachia* per embryo and a minimal number of 50,000 oocytes and embryos in an 8 cm long *L. loa* female worm (we calculated more than 200,000 based on the number of more than 100 embryos per cross section) a microfilariae producing *L. loa* female would harbour 50,000 or 500,000 endobacteria plus those in the hypodermis, if essential numbers would be present. These figures indicate that electron microscopic searches for endobacteria should focus on the oocytes, zygotes and young morulae as far as possible and not on the hypodermis.

The main problem for immunohistology is the reactivity of the wolbachial proteins with the antisera. The proteins may not have been expressed or they may have been destroyed by processing the specimens for microscopy. To overcome these problems and to achieve a rather high degree of probability for the absence of *Wolbachia* in *L. loa*, we used 27 adult worms from different sources, and applied several anti-wolbachial antisera that had resulted in reliable findings for 21 filarial or insect hosts of *Wolbachia*. The analysis of seven microfilariae producing female worms that were completely examined should especially provide a high degree of probability that we did not miss *Wolbachia*, even if they would have been present in only small numbers.

In insects, the density of *Wolbachia* can vary between different host populations. For example, in the mosquito *Aedes albopictus* a higher density of *Wolbachia* was found in populations from Houston (USA) compared to those from Mauritius or Koh Samui (Thailand) [45]. Furthermore, a changing *Wolbachia* density is also observed after

experimental transinfection to a novel host, and it was suggested that too high densities in the ovaries may result in reduction of reproductive fitness [46]. However, in arthropods a minimal level of bacterial infection is assumed to be required to cause effects like cytoplasmatic incompatibility or for vertical transmission in an obligatory symbiosis [6,47].

The completely different molecular biological analysis of nine adult *L. loa* worms and two batches of microfilariae by PCR comprises by itself a high degree of probability that the negative result is correct. As calculated above, in an adult worm at least 50,000 – 500,000 copies of the *Wolbachia* target sequence should be present, which can be easily amplified by a single PCR using 35 cycles. It cannot be excluded however, that the primer sets used did not hybridise to the target sequences, though our previous results have shown that these primers amplified *Wolbachia* DNA of all species that have been examined so far including the sand flea *T. penetrans* [26]. Furthermore, our results are in line with those reported from PCR analysis of microfilariae from two patients infected with *L. loa* [20].

Our results agree with the previous reports on the absence of *Wolbachia* in *L. loa* worms [1,16–20]. Other filaria species for which the absence of endobacteria has been shown by at least two independent methods are *O. flexuosa* [13,14], *A. viteae* [1,11,12], and *S. equina* [15]. Less certain is the absence in *Acanthocheilonema setariosa* (reported as *Dipetolonema setariosum*, [1]). Possibly no obligatory symbiotic *Wolbachia* occur in *Mansonella perstans* since Fischer and co-workers did not detect any *Wolbachia* DNA examining three batches of microfilariae by PCR using the same primers as for *L. loa* (unpublished data). In contrast, among the filariae infecting man are *O. volvulus*, *W. bancrofti*, *B. malayi*, *Brugia timori*, *M. ozzardi*, *D. repens*, and *D. immitis* bacteria-positive species [48,49].

The medical significance of the *Wolbachia* endobacteria concerns their role in the immunopathology [50–52] including adverse side effects after antifilarial chemotherapy [53,54] and the feasibility of antifilarial treatment using already registered antibiotics [7,9]. These aspects have been discussed in detail in recent meetings and they have been summarised in several reviews [4,5,55,56].

## Conclusions

Using electron microscopy, histology, immunohistology and PCR, no direct evidence was found that *L. loa* filariae harbour *Wolbachia* endobacteria in numbers required for vertical transmission of the bacteria or embryogenesis of the filariae. These findings exclude only the occurrence of obligatory symbiotic filarial *Wolbachia*. They do not exclude that endobacteria in small numbers may occasionally be detected in *L. loa*, e.g. acquired from any infected



vectors. Even if they would be detected in a local population of *L. loa*, they would not be obligatory for microfilariae production. Hence, only the filariae and not any *Wolbachia* cause the immunopathology of loiasis patients or the adverse side effects after antifilarial chemotherapy seen in patients with high microfilarial loads [21–23]. This is confirmed by the lack of any reaction of neutrophil granulocytes to *L. loa* microfilariae.

Unlike onchocerciasis and lymphatic filariasis, loiasis cannot be treated by antibiotics. This statement is in accordance with the findings of Brouqui et al., [20].

The African Programme for the Control of Onchocerciasis (APOC) uses ivermectin to eliminate onchocerciasis as a public health problem [57]. In a few African countries, co-endemicity of onchocerciasis and loiasis exists [58,59]. Co-infected patients with high *L. loa* microfilarial loads cannot be treated with ivermectin without a risk of serious side effects. Since the number of such patients is very small, the onchocerciasis of these individuals can probably be treated with doxycycline without any additional risk due to their loiasis, because doxycycline acts mainly in an indirect manner, eliminating the *Wolbachia* from the *O. volvulus*.

### Competing interests

None declared.

### Author's contributions

DWB conceived the study, participated in drafting the manuscript, and carried out the morphological analysis of *L. loa* and *Onchocerca*.

SW performed the monkey experiments to collect *L. loa* worms and spleen.

CB produced the rabbit antiserum against Wol-Di-WSP and examined the reactivity of it for *Wolbachia* of filariae and of *Culex*.

OB collected and identified *L. loa* worms.

PF participated in drafting the manuscript, produced the rabbit antiserum against Wol-Ov-AAT, examined the reactivity of it for filariae and sand fleas, and performed the PCR analysis.

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