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Tissue and Stage-Specific Distribution of *Wolbachia* in *Brugia malayi*

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Abstract

**Background:** Most filarial parasite species contain *Wolbachia*, obligatory bacterial endosymbionts that are crucial for filarial development and reproduction. They are targets for alternative chemotherapy, but their role in the biology of filarial nematodes is not well understood. Light microscopy provides important information on morphology, localization and potential function of these bacteria. Surprisingly, immunohistochemistry and in situ hybridization techniques have not been widely used to monitor *Wolbachia* distribution during the filarial life cycle.

**Methods/Principal Findings:** A monoclonal antibody directed against *Wolbachia* surface protein and in situ hybridization targeting *Wolbachia* 16S rRNA were used to monitor *Wolbachia* during the life cycle of *B. malayi*. In microfilariae and vector stage larvae only a few cells contain *Wolbachia*. In contrast, large numbers of *Wolbachia* were detected in the lateral chords of L4 larvae, but no endobacteria were detected in the genital primordium. In young adult worms (5 weeks p.i.), a massive expansion of *Wolbachia* was observed in the lateral chords adjacent to ovaries or testis, but no endobacteria were detected in the growth zone of the ovaries, uterus, the growth zone of the testis or the vas deferens. Confocal laser scanning and transmission electron microscopy showed that numerous *Wolbachia* are aligned towards the developing ovaries and single endobacteria were detected in the germline. In inseminated females (8 weeks p.i.) *Wolbachia* were observed in the ovaries, embryos and in decreasing numbers in the lateral chords. In young males *Wolbachia* were found in distinct zones of the testis and in large numbers in the lateral chords in the vicinity of testicular tissue but never in mature spermatids or spermatozoa.

**Conclusions:** Immunohistology and in situ hybridization show distinct tissue and stage specific distribution patterns for *Wolbachia* in *B. malayi*. Extensive multiplication of *Wolbachia* occurs in the lateral chords of L4 and young adults adjacent to germline cells.

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Introduction

Filarial parasites infect more than 150 million people in tropical and subtropical countries and are responsible for important tropical diseases such as lymphatic filariasis (elephantiasis) and onchocerciasis (river blindness). Other filarial species are important veterinary pathogens (e.g. *Dirofilaria immitis*, the dog heartworm). Treatment of filarial infections in humans and animals is suboptimal, because available drugs do not efficiently kill adult worms. Most filarial species live in obligatory symbiosis with intracellular *Wolbachia* 2-proteobacteria. *Wolbachia* are also present in many insect species, and they are among the most widely distributed bacteria that infect invertebrates. *Wolbachia* endosymbionts are necessary for development and reproduction of filarial nematodes, and they have been validated as a target for chemotherapy [1]. Tetracycline class antibiotics are active against *Wolbachia,* and depletion of endobacteria blocks reproduction and eventually kills adult worms in some filarial species [2,3].

While *Wolbachia* DNA can be detected and quantified by PCR, microscopy provides important information on morphology and localization of bacteria in parasite tissues. Immunohistochemistry has been used for years to visualize *Wolbachia* in filarial worms, particularly in *Onchocerca volvulus* [2]. *Brugia malayi* is the only human filarial parasite that can be maintained in laboratory animals and for which all life cycle stages are relatively easily accessible. The population dynamics of *Wolbachia* during the development of *B. malayi* has been studied by quantitative PCR; for example, the number of *Wolbachia* exponentially increases soon after infection of the vertebrate host [4]. Recent studies have shown that *Wolbachia* are unevenly distributed in intrauterine embryos and that the bacteria are not always detected in germline precursor cells [5]. However, data on the histological distribution of *Wolbachia* during later development of *B. malayi* are scarce. While it is known that *Wolbachia* are present in developing embryos, the mechanism of this vertical transmission is poorly understood.

*In situ* hybridization has been used to study gene expression in filarial parasites such as *B. malayi* [6] and to detect *Wolbachia* in insects [7,8], but it has not been used before to detect *Wolbachia* in...
Author Summary

Most filarial nematodes contain Wolbachia endobacteria that are essential for development and reproduction. An antibody against a Wolbachia surface protein was used to monitor the distribution of endobacteria during the B. malayi life cycle. In situ hybridization with probes binding to Wolbachia 16S rRNA were used to confirm results. Only a few cells contain Wolbachia in microfilariae and vector stage larvae; this suggests that the bacteria need to be maintained, but may have limited importance for these stages. Large numbers of Wolbachia were detected in the lateral chords of L4 larvae and of young adult worms, but not in the developing reproductive tissue. Confocal laser scanning and transmission electron microscopy showed that Wolbachia are aligned towards the developing germline. It can be hypothesized that Wolbachia invade developing ovaries from the lateral chords. In inseminated females, Wolbachia were detected in the ovaries and embryos. In young males, Wolbachia were found in parts of the testis and in the lateral chords in the vicinity of testicular tissue but never in mature spermatisds or spermatozoa. The process of overcoming tissue boundaries to ensure transovarial transmission of Wolbachia could be an Achilles heel in the life cycle of B. malayi.

Materials and Methods

Parasite material

B. malayi worms were recovered from intraperitoneal (i.p.) infected jirds, 2, 5, 8 and 12 wks post infection (p.i.) as previously described [9]. Aedes aegypti mosquitoes containing different larval stages of B. malayi were available from a previous study. Parasite material was fixed either in 80% ethanol for immunohistology or in 4% buffered formalin for immunohistochemistry or in situ hybridization. At least five blocks with four or more B. malayi worms each were examined for each time point. An extensive overview about the studied material for comparative studies of different staining procedures. For some (Table S1). Up to twenty serial sections of the same block were used for comparative studies of different staining procedures. For some blocks (especially those containing young adult worms) more than 60 sections (5 μm) were cut, but only a selection of sections was examined. For the ultrastructural analysis, 18 worms (39 and 56 days p.i., Table S1) were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA, USA) in 100 mM phosphate buffer, pH 7.2 for 1 hr at room temperature.

Antibodies

A monoclonal antibody directed against the B. malayi Wolbachia surface protein (mab Bm WSP) was purified from culture supernatants kindly provided by Dr. Patrick J. Lammie, Atlanta [10]. Briefly, hybridoma supernatant was incubated overnight at 4°C with ammonium sulfate, pelleted, resuspended in water and dialyzed extensively against phosphate buffered saline. The antibody solution was concentrated to 5% of the original volume using Centricon Plus-20 columns (Millipore, Billerica, MA, USA) and the protein content was determined. A stock mab solution of 10 mg protein per ml was used to test dilution series of 1:10 up to 1:500. The best signal to background relationship was observed at a dilution of 1:100, and this dilution was used for all further experiments.

Immunohistology

The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was applied for immunostaining according to the recommendations of the manufacturer (Dako, Carpinteria, CA, USA) and as described earlier [11]. TBS with 1% albumin was used as negative control. Rabbit-anti mouse IgG (1:25; Dako) was applied as secondary antibody and was bound to the APAAP complex. As substrate for alkaline phosphatase the chromogen Fast red TR salt (Sigma) was used and hematoxylin (Merek, Darmstadt, Germany) served as the counter-stain. Sections were examined using an Olympus BX40 microscope (Olympus, Tokyo, Japan) and photographed with an Olympus DP70 microscope digital camera. For some fluorescent analysis analysis wheat germ agglutinin (WGA 633, Invitrogen, Carlsbad, CA, USA) was used as membrane stain at 200 μg/ml for 10 minutes prior to mounting.

FITC conjugated anti-mouse IgG (1:300; Sigma) was used as a secondary antibody for confocal laser scanning microscopy (LSM). Sections were examined with a Zeiss LSM 510 META (Zeiss, Jena, Germany) confocal laser scanning microscope equipped with a plan-apochromat 63× oil objective with an argon or helium/neon laser for excitation at 488 nm or 633 nm, respectively. Confocal Z slices of 0.8 μm were obtained using Zeiss LSM software. The Velocity program version 5.4.2 (Improvision, Lexington, MA, USA) was used for high resolution interactive 3D rendering. Sections were also examined using a wide field fluorescence microscope (WFTM, Zeiss Axioskop 2 MOT Plus) with plan-apochromat 100× oil, 63× or 40× objectives. Wide field fluorescence microscopy and LSM were performed at the Washington University Molecular Microbiology Imaging Facility (http://micro.imaging.wus.edu/).

rRNA probe in situ hybridization

A 424 bp fragment of the 16S rRNA gene of Wolbachia of B. malayi was amplified (forward primer 5′CAGCTGTTGTGCTGTGGATGATCCCACTT) and cloned into a dual promoter PCRII plasmid (Invitrogen). After linearization of the plasmid, probes (anti-sense) and negative controls (sense) were prepared with Megascript T7 and Sp6 high yield transcription kits according to the manufacturer’s suggested protocol (Ambion, Invitrogen). For labeling of the probe a biotin-16 dUTP mix (Roche, Indianapolis, IN, USA) was used during in vitro transcription. The plasmid template was then removed by DNase digestion (Roche). The probes were concentrated by ethanol precipitation, re-suspended in DEPC-treated water, and stored at −20°C until use.

For staining, 5 μm thin paraffin sections were deparaffinized and partially digested with pepsin HCl for approximately 7 minutes. Sections were hybridized at 60°C overnight in a humid chamber with 1 μg of rRNA probe in hybridization buffer (50% formamide, 5XSSC, 0.3 mg/ml yeast tRNA, 100 μg/ml heparin, 1× Denhart’s Solution, 0.1% CHAPS and 5 mM EDTA). A stringency wash was performed at 60°C for 30 min, and detection was performed using the ‘In situ Hybridization Detection System’ (K0601, Dako) which uses alkaline phosphatase conjugated streptavidin to localize biotinylated rRNA probes. Sections were incubated for 20 min with streptavidin-AP conjugate at room temperature. BCIP/NBT substrate solution was added for 10 to 30 min to localize binding of the probes.
DNA oligonucleotide probe fluorescence-based in situ hybridization (FISH)

Sections were deparaffinized and partially digested as described above and hybridized at 37°C overnight in a dark humid chamber using 200 ng of a custom made, labeled 30-mer antisense probe targeting the 16S rRNA of Wolbachia (wBm16S as, 5’-Alexa 488-CAGTTTATCCTAGTCGT TTCCCTAAAGTC, Invitrogen). The complementary sense sequence was used as a negative control probe. One stringency wash was performed at 37°C for 30 minutes. Hybridization and stringency buffers were the same as described above. Finally sections were rinsed briefly in PBS and covered with a cover slip with ProLong Gold antifade reagent that contains DAPI (Invitrogen). This embedding reagent enables simultaneous fluorescence-based detection of condensed DNA in eukaryotic and prokaryotic organisms. Sections were examined using an Olympus-BX40 microscope equipped with the Olympus fluorescence filter 41001 (excitation 460–500 nm, emission 510–550 nm) for Alexa fluor or UN31000V2 (excitation 325–375 nm, emission 435–485 nm) for DAPI.

Transmission electron microscopy

For ultrastructural analysis fixed samples were washed in phosphate buffer, embedded in agarose, and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 hr as described previously [12]. Samples were then rinsed extensively in dH2O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA, USA) for 1 hr. Following several rinses in dH2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL, USA), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA, USA).

Results

Localization of Wolbachia in larval B. malayi

Different developmental stages were stained with mab Bm WSP (Fig. 1A, C–K). Results were confirmed by in situ hybridization or

Figure 1. Detection of Wolbachia in larval B. malayi by immunohistology or by in situ hybridization. A Clusters of Wolbachia (arrow) in single cells of a stretched intrauterine microfilaria labeled by immunohistology using mab Bm WSP. B Consecutive section to A, but Wolbachia (arrow) were detected by in situ hybridization. C Wolbachia (arrow) in single cells of a microfilaria within the midgut of A. aegypti 2 h after the blood meal. D Clustered Wolbachia in a cell of a fragment of an L2 larva in the thorax of A. aegypti 3 d.p.i. E Cross-section of L2 larvae in the thorax of A. aegypti showing a cluster of Wolbachia (arrow) in the hypodermis (7 d.p.i.) F Cross section of isolated infective L3 larvae showing only a single Wolbachia cluster (arrow) in the cells of lateral chord (14 d.p.i.) G Several clusters of Wolbachia (arrows) in the lateral chord in a longitudinal section of an infective L3 larva migrating through the abdomen of A. aegypti 14 d.p.i. H Cross-section of the anterior end of a 4th stage larva (14 d.p.i. of a jird). No Wolbachia were detected. I Cross-section of the midbody region of a 4th stage larva showing many Wolbachia (arrow) in one lateral chord. J Cross-section of the midbody region of another 4th stage larva (wide field fluorescence microscopy) showing highly condensed DNA (blue DAPI stain) in the genital primordium and numerous Wolbachia (arrow) in the lateral chord similar to I. K Longitudinal section of the midbody region of a 4th stage larva with many Wolbachia in the lateral chords (arrows). Cells in the pseudocoelomic cavity were free of Wolbachia. Ph, pharynx; gp, genital primordium; i, intestine; m, muscle. Scale bar 10 μm.

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Within spermatids or spermatozoa. The body length is approximately 1.8 cm. Spiculae are not shown.

In 12 week old females numerous Wolbachia were observed in the lateral chords and the posterior parts of the ovaries, but bacteria densities in these areas were lower than in the lateral chords adjacent to the anterior ovary and oviduct (Fig. 5B,C,E, F). Numerous Wolbachia were detected in morula stage embryos in the uterus, but only a few were detected in the lateral chords of females at that level (Fig. 5D, F, G). Intrauterine spermatozoa surrounding degenerated oocytes in the seminal receptacle were free of Wolbachia, but serial sections showed some Wolbachia in the oocytes in this area (Fig. 5E, G). Stretched microfilariae in the vagina uterina contained Wolbachia in some cells, but the numbers were low compared to those in morula stage embryos. This suggests that Wolbachia may be necessary for rapid cell division.

Detection of Wolbachia during the development of adult female *B. malayi*

In order to understand the distribution of Wolbachia in adult worms it is crucial to recall the anatomy and development of reproductive organs of filarial worms [13,14]. The genital opening (vulva) lies close to the anterior end of the female worm, approximately at the level of the esophagus (Fig. 2A, B). The vagina leads into the bifurcated uterus which ends in the seminal receptacles. Theses organs are linked by oviducts with two ovaries that have an anterior growth zone, a maturation zone in the receptacles. Theses organs are linked by oviducts with two ovaries.

Figure 2. Schematic drawing the anatomy of adult stage *B. malayi* and distribution of Wolbachia. Hypodermis, muscles, median chords as well as nerve and secretory-excretory system are not shown. Proportions are estimates. Tissues and organ systems are simplified and the midbody region is interrupted for clarity. The lateral chords are shown dorsally and ventrally instead of laterally. **A** Adult microfilaria producing female (12 weeks p.i.). The body length is about 4 cm. Wolbachia (red dots) are localized mainly in the lateral chords, ovary and developing embryos. The lateral chords are the head (up to the vulva) of the worm rarely contain Wolbachia. **B** Adult stage, immature female (5 weeks p.i.) a few days after the 4th molt. The body length is approximately 1.8 cm. Wolbachia are mainly localized in the lateral chords. Occasionally Wolbachia are attached to the ovaries or single endobacteria can be found within the ovary. **C** Adult spermatozoa producing male worm (12 weeks p.i.) with a total body length of about 2.5 cm. Spiculae are not shown. Wolbachia are localized in the lateral chords; Wolbachia remnants can be detected in parts of the vas deferens but not within spermatids or spermatozoa.

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which occurs in developing embryos but not in stretched microfilariae. The distribution of *Wolbachia* in the lateral chords was often asymmetrical, and this depended on the proximity to the reproductive system, body region and on the age of the worm.

Detection of *Wolbachia* during the development of adult male *B. malayi*

The genital opening of the male worm lies at the posterior end and forms with the anus a cloaca (Fig. 2C). This is in stark contrast to the anatomy of females. A single *vas deferens* leads into a seminal vesicle that is connected to the testis; this can be subdivided into a growth zone, a maturation zone, and a germinative zone. In parallel to the distribution of *Wolbachia* in females, large numbers of endobacteria were observed in the lateral chords of 5 week old males, while the growing sections of the testes in the midbody region were free of *Wolbachia* (Fig. 6A). However, *Wolbachia* were present in 5 week males near the testes (Fig. 6B–E) and in the middle part of the testis itself (Fig. 6 F–J). No *Wolbachia* were detected within the *vas deferens* by immunohistology (Fig. 7A, D). In contrast, *Wolbachia* 16S rRNA was detected by *in situ* hybridization in the testis tissue surrounding the spermatocytes and in the periphery of the *vas deferens* that contained spermatids (Fig. 7B, C, E). *Wolbachia* were never observed in the spermatids or the spermatozoa.

Comparison of morphological detection methods

Comparison of four different methods on consecutive sections (Figs. 3 D–G; 6 C–E; 6 F, J; 7 A, B, 7 D–G) revealed almost identical staining patterns for *Wolbachia* by immunohistology with mab Bm WSP, by *in situ* hybridization (using FISH and RNA *in situ* to detect Bm *Wolbachia* 16S rRNA), and by DAPI staining. Differences between immunohistology and 16S rRNA *in situ* detection were occasionally observed (Figs. 5B, C; 7 A, B; 7 D, E). In these cases *in situ* hybridization detected a strong *Wolbachia* 16S rRNA signal, while no or very little *Wolbachia* surface protein was detectable by immunohistology. This may indicate a small difference of gene expression pattern or of gene product stability of both markers, but was not noticed as confounding factor. In addition, the intestine of...
**Figure 4. Detection of *Wolbachia* in female *B. malayi* at 5 weeks p.i. by advanced microscopy techniques.** Confocal laser scanning microscopy (LSM) and wide field fluorescence microscopy (WFFM) were used together with mab Bm WSP (green) to label *Wolbachia* and a WGA 633 stain (red) to visualize membranes. WFFM contained also a filter for DAPI to detected concentrated DNA. A Cross-section showing large amounts of *Wolbachia* in the lateral chords close to the ovaries (arrow). Some *Wolbachia* were also detected in the hypodermis. For full scans of LSM images and 360° rotation see suppl. material (LSM). B Another cross-section showing a more advanced stage of infection with endobacteria attached to the ovary membrane (arrow head) and already in the ovaries (arrow) (LSM). C Longitudinal section showing *Wolbachia* lining up in the lateral chords in the vicinity of the ovary (arrow) or within one ovary branch (arrow head)(LSM). D WFFM showing *Wolbachia* approaching ovaries with highly condensed chromatin (blue DAPI stain). E Another cross-section showing *Wolbachia* (arrows) attached to the ovaries (blue DAPI stain). One ovary branch is already *Wolbachia* (arrow head) infected while the other is still free (WFFM). F Close-up of a longitudinal section showing *Wolbachia* (arrow) directly at the ovary membrane (red) (WFFM). G Another close-up showing *Wolbachia* in the lateral chords close to the ovary (arrow) or already in the somatic cells of the ovary (arrow head) (WFFM). Staining for WSP provides a characteristic donut shaped pattern of a *Wolbachia* surface protein. Ov, ovary; i, intestine. Scale bar 30 μm.

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*B. malayi* was sometimes nonspecifically labeled by immunohistology because of endogenous alkaline phosphatase (e.g. Fig. 5A, F–H). This did not occur with *in situ* staining.

The mab Bm WSP immunohistology assay detects a protein on the surface of *Wolbachia*, and it is possible that this protein is not present on all *Wolbachia* cells. In contrast, the *in situ* hybridization assay detects expression of 16S rRNA in the cytoplasm of *Wolbachia*. Small subunit rRNA is known to be highly expressed during the exponential growth phase of bacteria, and that has been used as marker for viability [15]. Therefore the *in situ* assay is an excellent marker for *Wolbachia* growth, and it may be suitable for assessing both the presence and viability of *Wolbachia*. DAPI staining, which detects A-T rich regions in DNA, is an easy and quick method to detect *Wolbachia* in the lateral chords, since this syncytial tissue usually does not contain condensed filarial chromosomes (Figs. 3G; 7G). However, it is difficult to identify *Wolbachia* by DAPI staining in areas with condensed filarial chromosomes such as ovaries or in spermatozoids within the *vas deferens* (Figs. 3G; 7G). This problem can be solved by combining the DAPI stain for condensed DNA with immunohistology (Figs. 2J, 4D–G; 6H). This permits visualization of *Wolbachia* in the vicinity of filarial nuclei.

**Confocal laser scanning microscopy**

Confocal laser scanning microscopy was studied to use the three dimensional distribution of *Wolbachia* in larvae and in developing reproductive tissue of young adult worms. Although *Wolbachia* numbers were increasing in the lateral chords in 4th stage larvae, no *Wolbachia* were observed in developing reproductive organs in L4. The higher resolution of LSM confirmed heavy *Wolbachia* loads in the lateral chords of young female worms (5 weeks) and relatively few endobacteria in the hypodermis (Fig. 4A). Entire oocytes could be examined for *Wolbachia* from primary oocytes by DAPI staining. The confocal examination of the distal end of the ovaries in 5 week old females confirmed the absence of *Wolbachia* from primary oocytes. A full LSM scan and rotation of the section show that *Wolbachia* were present also in the hypodermal pouches that form longitudinal lines in 5 week old female worms (video S1). A membrane stain helped to demonstrate that some *Wolbachia* were attached to the external membrane around the proximal oovaries while other bacteria were actually in the oovaries (Fig. 4B, C). A full LSM scan and rotation of the section show that *Wolbachia* were always in the vicinity of large clusters of *Wolbachia* in the lateral chords adjacent to the ovaries in developing adult female worms (Fig. 4B, C). Wide field fluorescence microscopy using FITC labeled mab Bm WSP with a membrane stain and an overlay of the DAPI nuclear stain showed that *Wolbachia* were attached to the ovary membranes (Fig. 4D, E, F, G). It is possible that these endobacteria invade the ovaries of young females from the lateral chords. *Wolbachia* distribution in the developing ovaries was not uniform; in some cases, one branch was infected while the other branch was *Wolbachia* free (Fig. 4E).

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Ultrastructural studies of Wolbachia in developing reproductive tissue

Studies of the midbody region of 5 week old worms by transmission electron microscopy confirmed the presence of Wolbachia in the vicinity of developing reproductive tissues. Numerous rod-shaped and spherical Wolbachia were detected in the lateral chords in females, especially in adult worm tissues that are adjacent to developing ovaries. In some areas the hypodermal chord tissue was loose and vacuolized (Fig. 8A). The epithelial cells surrounding the basal lamina of the ovaries were occasionally also strongly vacuolized indicating tissue degeneration, and small, electron dense Wolbachia were detected in these vacuoles (Fig. 8B). Occasionally extracellular Wolbachia were seen in the pseudocoelemic cavity docking to the edge of the ovaries (Fig. 8C, D) or attached to the outer ovarian tissue (Fig. 8E, F). While most of the Wolbachia in the lateral chords were rod-shaped or spherical and up to 1 μm in length and 0.5 μm in diameter, the endobacteria in the pseudocoelemic cavity were condensed, bacillary in shape and only 0.15 to 0.5 μm in length (Fig. 8G–I). Within the ovaries, these small Wolbachia forms were observed in large vacuoles or in loose ovarian tissue (Fig. 8G, I) either as single bacteria or in groups (Fig. 8H).

In 5 week old male worms large clusters of large, rod-shaped or spherical Wolbachia were observed in the lateral chords in the vicinity of the testis (Fig. 9A). Small, bacillary Wolbachia forms were sometimes observed in the testis tissue. At the caudal end of the testis, close to the transition to the vas deferens, Wolbachia were observed in the inner tissue, sometimes in the vicinity of peripheral spermatids (Fig. 9B, C, D). These spermatids can be easily identified and differentiated from mature spermatozoa by their compact membranous organelles and the absence of major sperm protein complexes. Large amounts of membranous material were observed in the lumen between the spermatids and the inner testis epithelium. This material resembles degenerating Wolbachia (Fig. 9B, E–G) as they have been described previously [16]. Wolbachia were unambiguously identified in the reproductive tissue of young male worms, but not in the spermatids or spermatozoa.

Discussion

Immunohistology has been extensively used to study Wolbachia and their clearance following chemotherapy in O. volvulus. Compared to O. volvulus, mature B. malayi have a
Wolbachia in Brugia malayi

Figure 6. Detection of Wolbachia in immature male B. malayi at 5 weeks p.i. In A and C Wolbachia were labeled by mab Bm WSP. A This cross-section of the midbody region shows primary spermatogonia in the testis and numerous Wolbachia (arrow) in the lateral chords. B LSM of a consecutive section of A. Individual clusters of Wolbachia (arrow) can be identified. C Two cross-sections more distal compared to A with Wolbachia in the lateral chords (arrow) and in tissue attached to the testis (arrow head). D Consecutive section to C examined by LSM. E Consecutive section to C stained by in situ rRNA hybridization confirming the staining pattern. F–J Serial cross-sections showing many Wolbachia in the lateral chords (arrowheads) and in the spermatogonia (arrows). F mab Bm WSP. G Also mab Bm WSP but LSM. H Same staining as G but WFFM with DAPI stain of testis. I 16S in situ rRNA hybridization. J 16S oligonucleotide FISH. te, testis. Scale bar 25 μm.
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thinner hypodermis and less pronounced lateral chords, and this can make the detection of Wolbachia more difficult. Our results demonstrate that the distribution and density of Wolbachia vary in different tissues and developmental stages. Our results are consistent with those from a PCR study that reported low amounts of Wolbachia DNA in vector stages and larger amounts in mammalian stages [4]. McGarry and co-workers reported an exponential increase in Wolbachia DNA in transmitted B. malayi L3 larvae as early as 7 days p.i. We detected large amounts of endobacteria by histology in the lateral chords of the midbody region in L4 larvae (14 d.p.i.). More Wolbachia were present in young adult worms at 35 d.p.i. in most parts of the lateral chords and also in an uneven distribution in the hypodermis.

Observations on Wolbachia density and tissue localization may lead to hypotheses regarding their potential function in filarial worms. Antibiotic treatment experiments have suggested that Wolbachia may play a crucial role in the molting process of filarial parasites [17,18,19,20]. It appears clear that if Wolbachia have a direct function during molting, this function does not require localization in the vicinity of the filarial cuticle, since our localization results show that Wolbachia are not located near the cuticle during or immediately after molting. The distinct age and tissue specific distribution patterns of Wolbachia suggest also that the bacteria are not likely to be needed for housekeeping functions in all cell types of filarial nematodes. The absence of Wolbachia in the filarial nervous system, muscles, or the digestive systems suggests that Wolbachia are not needed for these functions. In adult worms the majority of mitochondria can be found in the periphery of the lateral chords, while the majority of Wolbachia are localized in or near the reproductive system. The differential distribution of Wolbachia and mitochondria within the lateral chord of filarial parasites has been reported previously [21]. Especially to the female worms the localization of Wolbachia in the lateral chords in vicinity of the reproductive system implies an important role of endobacteria for embryogenesis and intrauterine development. In agreement with this hypothesis tetracycline treatment to deplete Wolbachia in developing filarial worms has been shown to affect mainly females and causes a male-biased sex-ratio [20,22].

The Wolbachia genome in B. malayi encodes complete pathways for the biosynthesis of nucleotides, riboflavin, flavin adenine dinucleotide and heme, which are missing or incomplete in the filarial genome [23]. A high demand for gene products (which may not be taken up from the mammalian host) from these pathways might be especially necessary during the development of the reproductive system in young adult worms. Furthermore, the phylogenetically old and tight association of filarial nematodes with Wolbachia during reproduction may have led to additional interdependencies that account for their mutualistic relationship. As hypothesized for Wolbachia in insects, it is possible that Wolbachia in filarial nematodes are especially important for premeiotic mitosis, meiosis, and meiosis associated processes [24,25,26,27].

A recent study examined the dynamics of Wolbachia during intrauterine embryogenesis of B. malayi using Caenorhabditis elegans embryogenesis as a framework for the analysis [5]. Asymmetric Wolbachia segregation was observed that could explain the concentration of Wolbachia in the hypodermal chords. The early differential distribution of Wolbachia within embryonic cells corresponds well with the strong tissue specific distribution in later development described in our study. However, the authors also hypothesized that the asymmetric segregation pattern may be
Figure 7. Detection of Wolbachia in male B. malayi at 12 weeks p.i. In A and D Wolbachia were labeled by mab Bm WSP. A Two cross-sections demonstrating Wolbachia in the lateral chords (arrows). One section is in the midbody region showing developed spermatogonia and numerous Wolbachia in the lateral chord, while the other section is in the more muscular distal end of the worm showing spherical spermatids in the vas deferens and fewer Wolbachia in the lateral chord. B Consecutive section to A, but stained with 16S rRNA in situ hybridization. In contrast to A some staining for Wolbachia rRNA is also detected in the testis (arrowheads) and at the border of the vas deferens (arrow heads). C Cross-section of the distal part of another male worm showing Wolbachia 16S rRNA labeling at the epithelium of the vas deferens (arrowhead) and in the lateral chord (arrow). Spermatozoa were never labeled. D–G Consecutive cross-section through the terminal end showing the vas deferens containing fully developed spermatids in transition to spermatozoa. Wolbachia are detected in the lateral chord (arrow), but not in the spermatids. D mab Bm WSP. E 16S rRNA in situ hybridization. Similarly to B, staining is also observed at the border of the vas deferens (arrowheads). F 16S oligonucleotide FISH. Although Wolbachia can be easily identified in the lateral chord, granular staining at the membrane of the vas deferens is difficult to recognize. G DAPI stain, again Wolbachia can be easily identified in the lateral chord, but granular staining at the membrane of the vas deferens is hard to differentiate. Sp, spermatozoa; te, testis; vd, vas deferens. Scale bar 25 μm.
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Wolbachia in Brugia malayi

responsible for the presence of Wolbachia in the female germline [5]. This is in contrast to our results which clearly demonstrate the absence of Wolbachia in male and female reproductive tissue from the third stage larvae to the young adult worms. Since it is difficult or impossible to identify the germline cells or gender of microfilariae, vector stage first stage larvae, and second stage larvae of B. malayi, we cannot be sure when during development Wolbachia are lost in these cells.

The terminal ends of Brugia ovaries form the germinative zones which contain the mitotic growing oogonia [28]. Our study showed that these areas were free of Wolbachia in growing, young adult worms. Our results suggest that Wolbachia from adjacent lateral chords may cross tissue zones to infect cells in maturation zone 1 (which mainly contains primary oocytes in the pachytene stage of meiotic prophase I) and in maturation zone 2 (which contains oocytes in the remaining phases of meiosis I). The germinative zones of the ovaries seem to be populated by Wolbachia over a period of approximately three weeks following the L4–L5 molt. Large numbers of Wolbachia were present in the maturation zones of eight week or older female worms, while the attached growth zones which contain the secondary oocytes and the oviducts contained lower numbers of Wolbachia (see Fig. 5E). Fertilization precedes meiosis II in filarial nematodes [28]. Wolbachia were detected in secondary oocytes surrounded by spermatozoa and unfertilized oocytes within the seminal receptacle in mature females (see Fig. 5F, G).

The picture was similar in male worms. Wolbachia were not observed in the germinative zone of the testis. It is possible that Wolbachia from the lateral chords infect the primary spermatocytes in maturation zone 1, which are mostly in the pachytene stage of prophase of meiosis I [29]. This report is the first detection of Wolbachia in primary spermatocytes of developing male filarial nematodes. Although mature male worms have been previously examined for Wolbachia, prior studies did not report infection of the testis [30]. This is not contradictory to our findings, since Wolbachia appear to only infect the testis of immature adult stage B. malayi males and such worms were not studied previously. The spermatocytes of the adjacent growth zone and maturation zone 2 are difficult to differentiate morphologically, but larger secondary spermatocytes that have completed meiosis and the spherical spermatids which enter the vas deferens can be distinguished. Wolbachia were never seen in the spermatids or the mature spermatozoa. However, out in situ hybridization results clearly indicated the presence of Wolbachia 16S rRNA in the periphery of the seminal vesicle. This was confirmed by electron microscopy that showed Wolbachia in the inner epithelium of the testis or vas deferens, but not in the spermatids. These data may suggest that high Wolbachia densities are correlated with condensed chromatins
and Wolbachia may be involved in chromosome segregation of filarial nematodes.

Our ultrastructural studies of young adult B. malayi confirm that Wolbachia are highly pleomorphic. This pleomorphism was recognized shortly after the discovery of endobacteria in filarial nematodes, and it has been suggested that Wolbachia may have a Chlamydia-like lifecycle with small dense bodies as potential infectious forms [30,31]. Chlamydia and filarial Wolbachia both have an obligatory intracellular life style and a small genome size due to the loss of a number of essential biosynthetic pathways. Both bacterial groups lack cell walls but retained a functional lipid II biosynthesis pathway [32]. It is also possible that Wolbachia share the requirement of Chlamydia for host cell sphingolipids supplied by the host cell Golgi apparatus and multivesicular bodies for activation [33]. Clearly, further studies are needed to assign functions to different morphological forms of Wolbachia during the filarial life cycle.

Based on our results we hypothesize that the genital primordium in larval B. malayi is devoid of Wolbachia and that reproductive tissues in young adult worms become infected with Wolbachia from adjacent lateral chords which have many Wolbachia. Prior studies have shown that newly introduced Wolbachia can cross several tissue planes and infect the germline in Drosophila [34]. This could be also the case in filarial Wolbachia, and it is possible that similar host signals trigger the germline tropism of Wolbachia in filarial worms and Drosophila. Previous studies have shown that a Wolbachia htrA serine protease can be found outside bacterial cells in filarial parasites. This protease and other secreted bacterial proteins may be involved in tissue invasion [35]. In addition to tissue lysis, motility of Wolbachia may be necessary for the bacteria to cross tissue boundaries. Actin-based motility occurs in Rickettsia and many other intracellular bacteria [36]. Orthologs of genes essential for actin-based motility have been found in the Wolbachia genome. Additional work will be needed to study the localization and timing of expression for these genes [23,37].

Our ultrastructural results confirmed the presence of large clusters of Wolbachia in the lateral chords in the vicinity of the ovaries and in the outer ovary epithelium as previously described [30,38]. The new finding reported here is the detection of extracellular Wolbachia in the pseudocoelomic cavity in young females and the presence of Wolbachia in testis of developing male worms. In summary, this study shows the value of histological techniques such as immunohistology and in situ hybridization to study the tissue distribution of Wolbachia during the life cycle of filarial nematodes. Wolbachia infection was found to be highly cell and tissue specific. No Wolbachia were found in the developing reproductive organs in fourth stage larvae and freshly molted adult worms, which had heavy Wolbachia loads in the lateral chords. Wolbachia were detected in reproductive tissues with the onset of oocyte and sperm development, and infection of oocytes results in transovarial transmission of Wolbachia to the next generation.
Supporting Information

Table S1  Summary table for the parasite material used for the present study. Each slide was thoroughly examined and numerous pictures were taken. If a slide contained more than one block section, all sections were analyzed.

Video S1  Full rotation of a cross-section of a 5 week old female B. malayi. Wolbachia were detected in the hypodermal lateral chords and the hypodermis. Confocal laser scanning microscopy stained with mab BmWSP and WGA 633 (red) to visualize membranes. Compare Fig. 6A.

Video S2  Similar to video S1 but Wolbachia are already attached to the membrane of the one ovary branch. Note these attached bacteria can be noticed only from one side of the section. A few additional endobacteria are already in the ovary. The second ovary branch is still devoid of Wolbachia. Compare Fig. 6B.

Video S3  Longitudinal section of a 5 week old female B. malayi. Wolbachia are lining up at the lateral chords and some Wolbachia in one ovary branch. Confocal laser scanning microscopy stained with mab Bm WSP and WGA 633 (red) to visualize membranes. Compare Fig. 6C.

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Author Contributions
Conceived and designed the experiments: KF WLB GJW PUF. Performed the experiments: KF WLB. Analyzed the data: KF WLB DJ GJW PUF. Contributed reagents/materials/analysis tools: WLB DJ. Wrote the paper: KF GJW PUF.

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