

Both asymmetric mitotic segregation and cell-to-cell invasion are required for stable germline transmission of *Wolbachia* in filarial nematodes

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Biology Open 1, 536–547
doi: 10.1242/bio.2012737

Summary

Parasitic filarial nematodes that belong to the Onchocercidae family live in mutualism with *Wolbachia* endosymbionts. We developed whole-mount techniques to follow the segregation patterns of *Wolbachia* through the somatic and germline lineages of four filarial species. These studies reveal multiple evolutionarily conserved mechanisms that are required for *Wolbachia* localization to the germline. During the initial embryonic divisions, *Wolbachia* segregate asymmetrically such that they concentrate in the posteriorly localized P₂ blastomere, a precursor to the adult germline and hypodermal lineages. Surprisingly, in the next division they are excluded from the germline precursor lineage. Rather, they preferentially segregate to the C blastomere, a source of posterior hypodermal cells. Localization to the germline is accomplished by a distinct mechanism in which *Wolbachia* invade first the somatic gonadal cells close to the ovarian distal tip cell, the nematode stem cell niche, from the hypodermis.

This tropism is associated with a cortical F-actin disruption, suggesting an active engulfment. Significantly, germline invasion occurs only in females, explaining the lack of *Wolbachia* in the male germline. Once in the syncytial environment of the ovaries, *Wolbachia* rely on the rachis to multiply and disperse into the germ cells. The utilization of cell-to-cell invasion for germline colonization may indicate an ancestral mode of horizontal transfer that preceded the acquisition of the mutualism.

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Key words: *Wolbachia*, Bacteria, Filarial nematodes, Mutualism, Parasite, Symbiosis

Introduction

Filarial parasites cause debilitating diseases such as lymphatic filariasis (caused by *Brugia malayi*, *Brugia timori*, and *Wuchereria bancrofti*) and onchocerciasis (River Blindness, caused by *Onchocerca volvulus*), with a billion people at risk, and over 120 million infected individuals in tropical areas (Taylor et al., 2005). With the exception of *Loa loa*, these human parasites harbor the *Wolbachia* endosymbionts. These alpha-proteobacteria are widely distributed among arthropods, but restricted to the family of Onchocercidae in the nematodes (Werren et al., 1995; Ferri et al., 2011). In arthropods, the *Wolbachia* are maternally inherited and have developed an array of strategies based on the manipulation of their host reproduction to spread through populations (Serbus et al., 2008). *Wolbachia* have been classified in different supergroups, defined by multilocus sequence typing (MLST) based on five conserved and rapidly evolving genes (Baldo and Werren, 2007). Some supergroups are specific to arthropods (A,B,E,H,I,K), others to nematodes (C,D,J), and one supergroup encompasses both phyla (F) (Lo et al., 2002; Casiraghi et al., 2005).

In filarial nematodes, *Wolbachia* are mutualistic and required for fertility and survival (Hoerauf et al., 2000; Fenn and Blaxter,

2004). Their removal by antibiotic treatments from *Brugia malayi* or from *Onchocerca volvulus* leads to extensive apoptosis (Landmann et al., 2011). In addition, the *Wolbachia* released from the nematodes into the human body trigger an inflammatory reaction that underlies the lymphedema and corneal occlusion associated with these neglected diseases (Taylor et al., 2000; Turner et al., 2006; Debrah et al., 2009; Turner et al., 2009). For these reasons, *Wolbachia* have become major drug targets, since the current treatments do not kill adult worms (Slatko et al., 2010).

Experimental studies and genome analysis suggest *Wolbachia* contribute to the biosynthesis of diverse metabolic pathways, among them nucleotides, riboflavin, FAD and heme biosynthesis (Foster et al., 2005; Wu et al., 2009; Strübing et al., 2010). *Wolbachia* also help the worms to escape the host's immune system, and therefore confer longevity (Hansen et al., 2011). In most adult filarial species harboring *Wolbachia*, the endosymbionts are present in two sites, in the female gonad, from which they are vertically transmitted to the progeny, and in the hypodermis forming the lateral chords. Hypodermal chords are a site of intense metabolism, providing nutrients to

developing germ cells and growing embryos in the female gonads. Chords harbor the highest *Wolbachia* titer in the worm, and the strong evolutionary conserved tropism of *Wolbachia* for this tissue suggests participation to the metabolism. *Wolbachia* removal leads indeed to cytoskeleton defects in the chords, and apoptosis occurs in the gonads in a non-cell autonomous manner (Landmann et al., 2011). It has been recently suggested that the *Wolbachia* present in the lateral chords initially show a tropism for both the female and the male gonads, but remain only in the adult female reproductive apparatus (Fischer et al., 2011). A thorough characterization of the cellular and molecular basis of the *Wolbachia* transmission to the chords and subsequently to the germline will lead to the identification of potential drug targets.

Here we take advantage of newly developed whole-mount immunofluorescent techniques for localizing *Wolbachia* in filarial nematodes, and a recent cocladogenesis study of *Wolbachia* in a wide range of filarial species to identify conserved mechanisms of *Wolbachia* soma and germline transmission during nematode development (Ferri et al., 2011). We examine *Wolbachia* transmission in four filarial species, all of which belong to the secernentean class of nematodes. An important feature of this class of nematode is a fixed and almost identical embryonic lineages (Skiba and Schierenberg, 1992; Malakhov, 1994; Dolinski et al., 2001; Lahl et al., 2003). Significantly *Caenorhabditis elegans* with its defined lineage is a member of this class and provides a framework for the studies described here.

We previously reported the asymmetric transmission of *Wolbachia* in the *B. malayi* embryo, whose embryonic development resembles to that of *C. elegans* (Landmann et al., 2010). In secernentean nematodes, embryogenesis consists of mitotic divisions following a fixed lineage, encompassing gastrulation. Divisions then stop (i.e. at about 550 cells in *C. elegans*) and morphogenesis is achieved by a dramatic remodeling of the hypodermal epithelium, transforming a ball of cells into a worm-shaped embryo (Sulston et al., 1983). Specifically, we demonstrate that *Wolbachia* supergroups C and D follow the same evolutionary-conserved pattern of asymmetric segregation during embryogenesis to reach a subset of hypodermal cells prior to morphogenesis. They rely on cell fusion to colonize the lateral chords, before invading the posterior pseudocoelom in both male and female, but enter only the female distal ovary. *Wolbachia* may rely on engulfment to enter into the somatic gonad, as suggested by the associated cortical F-actin disruption. We then highlight the importance of the ovarian rachis for *Wolbachia* multiplication and spread into the germline, identifying therefore new cell mechanisms crucial for sustaining the mutualistic association between *Wolbachia* and its nematode host.

Materials and Methods

Specimens

Brugia malayi specimens were supplied alive and *Dirofilaria immitis* adults were supplied frozen, by TRS Laboratories (Athens, Georgia), and by the Filariasis Research Reagent Resource Center at the University of Georgia and funded by the National Institutes of Health. *Litomosoides sigmondontis* specimens were raised in jirds, *Meriones unguiculatus*, at the Muséum National d'Histoire Naturelle, Paris, France (C.M.). *Onchocerca dewittei japonica* adults were recovered from a Japanese wild boar in Feb. 2011 in Oita prefecture, Japan (S.U.). *Dirofilaria immitis* adult living worms were kindly provided by Prof. Wieslaw Kozek, University of Puerto Rico. Worms obtained alive were fixed approximately 1 to 3 days after their removal from the host, while worms fixed prior to shipping were typically used 1 to 2 weeks after reception.

Stainings and immunostainings

Procedures for propidium iodide (PI) and phalloidin stainings, as well as immunostainings in embryos, larval and adult stages were performed as described in detail in (Landmann et al., 2010). Briefly, worms were either cut (for immunostainings) or not (for PI and phalloidin stainings), placed in PBST with 3.2% paraformaldehyde for fixation, with an equal volume of heptane, under rotation for 10 minutes. After a brief centrifugation, they were rinsed in PBST for 5 minutes, and treated in RNase (1 mg/mL) in PBST overnight, before proceeding to immunostainings. Small larval stages were freeze cracked after fixation and before RNase treatment.

Propidium iodide stains both host and bacterial DNA. We favored propidium iodide since it preferentially stains the bacterial DNA, which appears as bright foci compared to the much larger host nuclei. In addition PI does not have the issue of penetration as do antibodies, allowing whole-mount stainings. The specificity has been established (Landmann et al., 2010), and we show again colocalization of PI foci with an anti-*Wolbachia* *ftsZ* antibody and anti-WSP (supplementary material Fig. S4).

Antibodies

A chicken polyclonal anti-WSP antibody was kindly provided by J. Foster, New England Biolabs, and used at a 1:100 dilution to confirm that propidium iodide-stained DNA foci were corresponding to *Wolbachia* (supplementary material Figs S3, S4).

A rabbit polyclonal anti-*Wolbachia* *ftsZ* antibody used at a 1:500 dilution was raised against the following conserved peptide sequence (accession number of *Wolbachia* *ftsZ*: CAT19364) and affinity-purified: eelqkyvdtlivpqnlfirvanekttf-sdafkladnvlhigirgvtldmvmplglnldfadietvmsemkamigtgeaedraisaeeainplld.

Microtubule stainings were performed using the monoclonal DM1 α antibody (Cell Signaling Technology) at a dilution of 1:100.

Microscopy and Imaging:

Confocal microscope images were captured on an inverted photostage (DMIRB; Leitz) equipped with a laser confocal imaging system (TCS SP2; Leica) using an HCX PL APO 1.4 NA 63 oil objective (Leica) at room temperature. 3-D tissue reconstructions were performed using the Velocity5 software.

Results

Investigating the transmission of different *Wolbachia* supergroups at the tissue and cell levels required the use of a reliable technique we could apply in several filarial nematode species. We opted mainly for a combination of fluorescent phalloidin and propidium iodide (PI) throughout this study, revealing the cortical actin, therefore the cell membranes, as well as the total DNA respectively. The identity of the *Wolbachia*, appearing as DNA foci, was confirmed by specific immunostainings (cf. Material and Methods).

Wolbachia mitotic asymmetric segregation pattern during embryogenesis is conserved across species of Onchocercidae harboring the supergroups C and D

We have previously described the segregation of *Wolbachia* supergroup D during the embryogenesis of *Brugia malayi* (Landmann et al., 2010). In the *B. malayi* zygote, the *Wolbachia* concentrate to the posterior pole and become asymmetrically inherited by the germline precursor lineage, successively in P₁ and P₂ (Fig. 1A). In the 12-cell stage *B. malayi* embryo however, the highest *Wolbachia* titer is always observed in the C blastomere, with a minor concentration in its sister, the germline precursor P₃ (Fig. 1A, arrow and arrowhead respectively; supplementary material Movie 1). This raises the question of the evolutionary conservation of this transmission mechanism based on asymmetric segregation. To address these issues, we decided to look at the behavior of two *Wolbachia* supergroups during early embryogenesis in four species of Onchocercidae. *Brugia malayi* and *Litomosoides sigmondontis*, two species raised in laboratory rodent hosts carrying the *Wolbachia* supergroup D, while *Dirofilaria immitis* and *Onchocerca dewittei japonica*, harboring the supergroup C,

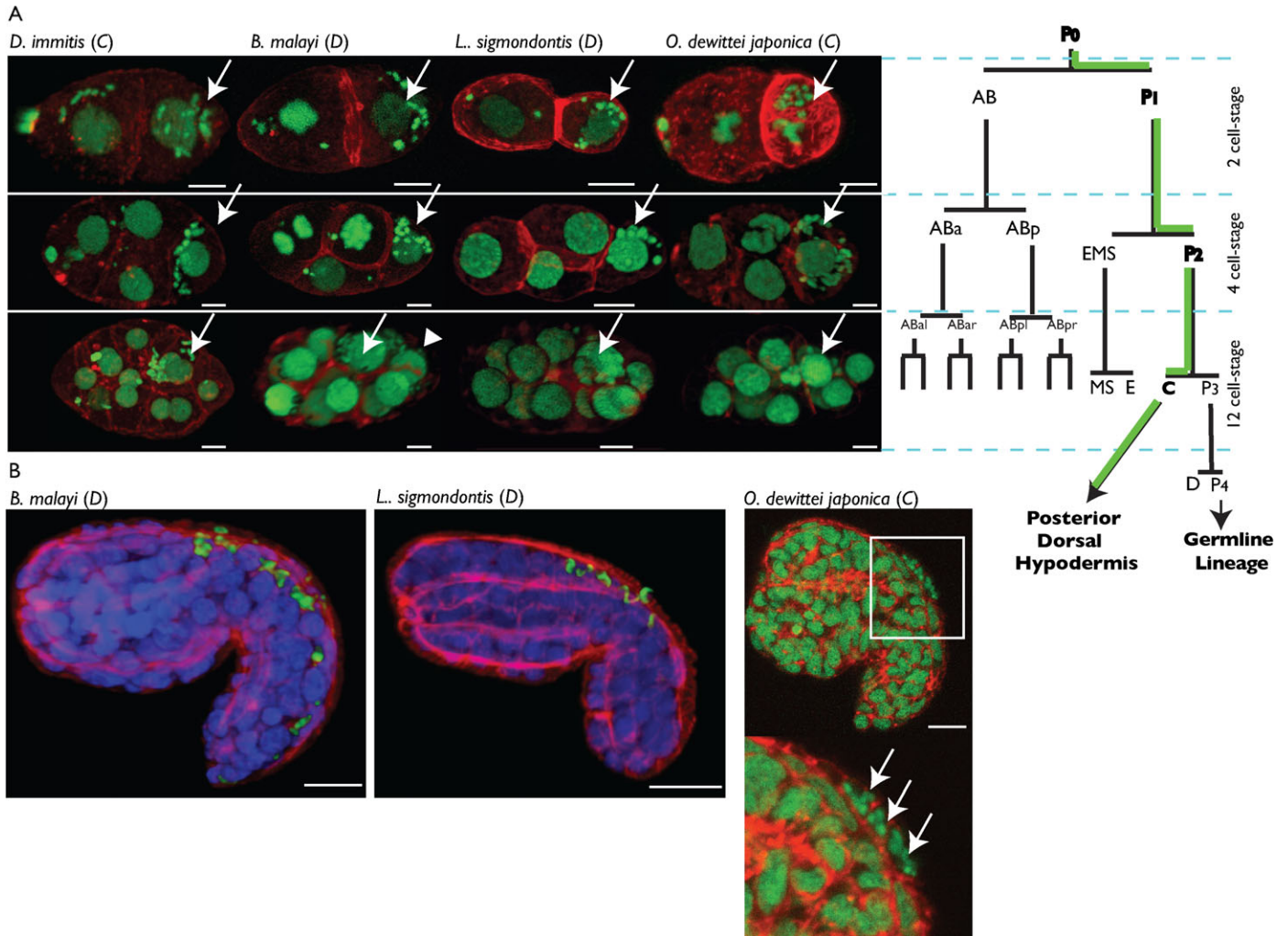


Fig. 1. *Wolbachia* supergroups C and D segregate into the C blastomere and its hypodermal progeny during filarial embryogenesis. (A) Propidium iodide -green- and phalloidin -red- stainings of 2, 4 and 12-cell stage embryos. For sake of simplicity, the *C. elegans* lineage nomenclature was used to name the early blastomeres, based on the division pattern. The green path on the lineage tree shows the *Wolbachia* asymmetric segregation. On confocal images, arrows point to the blastomeres indicated in bold fonts on the lineage tree, and the arrowhead points to the P₃ blastomere in the *B. malayi* 12-cell stage. In all species the AB lineage divides ahead of the posterior lineage. Anterior to the left. (B) Immunostainings of *B. malayi* and *L. sigmondontis* embryos in early elongation with an anti-WSP (green), PI -blue- and phalloidin -red-, cf. supplementary material Movies 3, 4. *O. dewittei japonica* is stained as in (A), arrows point to *Wolbachia*. Anterior to the right, dorsal part to the top. Scale bar = 5 μm.

were collected from their natural hosts, *Canis lupus* and *Sus scrofa leucomystax* (Japanese wild boar) respectively. We observed that in all four species, *Wolbachia* follow the same segregation pattern, leading to a highest titer in the C blastomere at the 12-cell stage (Fig. 1A). Unlike in *B. malayi*, the presence of *Wolbachia* was not always detected in the germline precursor P₃ in *D. immitis* and *L. sigmondontis*, but sometimes in the C blastomere only. More strikingly, in the *O. dewittei japonica* 12-cell stage embryo, the *Wolbachia* segregated exclusively into the C blastomere (Fig. 1A, n > 10 embryos; supplementary material Movie 2). In this species the *Wolbachia* asymmetric segregation during early embryogenesis is perfect, unlike in *B. malayi*, where we showed the variable presence of *Wolbachia* in other early blastomeres (Landmann et al., 2010) (supplementary material Movie 1).

To confirm the identity of the single infected blastomere in the *O. dewittei japonica* 12-cell stage embryo, we compared the

Wolbachia localization after morphogenesis had begun in *B. malayi*, *L. sigmondontis*, and *O. dewittei japonica* (Fig. 1B). When elongation begins, the C hypodermal progeny is located at the posterior dorsal surface of the embryo (Sulston et al., 1983) (Fig. 2A,B). During early embryogenesis in *B. malayi*, the *Wolbachia* in blastomeres other than C almost always disappear, in contrast to the hypodermal progeny of C. As a consequence, almost only the posterior dorsal surface contains *Wolbachia* (supplementary material Movies 3, 4). In *O. dewittei japonica*, strictly only these cells harbor the endosymbionts. (Fig. 1B).

The comparison of *Wolbachia* distribution during early embryogenesis in several filarial species shows that: i. *Wolbachia* supergroups C and D rely on similar evolutionary-conserved mechanisms to segregate into embryonic hypodermal cells derived from the C blastomere, and ii. *Wolbachia* presence in *B. malayi* P₃ and P₄ germline precursors is due to imperfect asymmetric segregation. Taken together, these data show that in

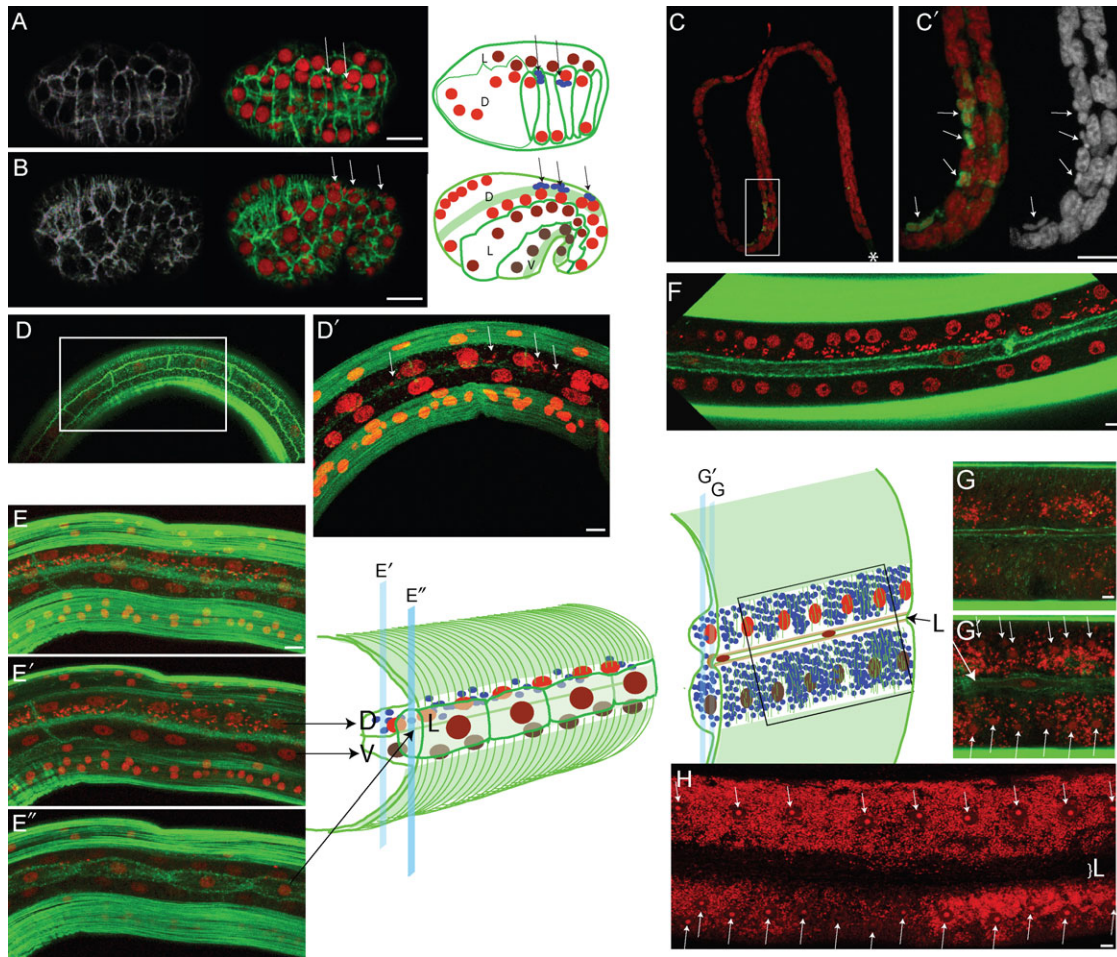


Fig. 2. Hypodermal cell fusion correlates with *Wolbachia* dispersion in *B. malayi* lateral chords. All stages were stained with propidium iodide -red- and phalloidin -green-, except for (C,C') where the anti-ftsZ is in green, and phalloidin was not used in (C,C'). (A) top view of the embryonic dorsal intercalation. (B) Lateral view of early elongation. In (A,B) arrows point to *Wolbachia*. In addition to cell membranes, the schematic drawing in (B) shows the underlying muscle precursors -green stripes-, and the *Wolbachia* -arrows pointing to blue foci-. D = dorsal hypodermis -red nuclei-; L = lateral hypodermis -burgundy nuclei-; V = ventral hypodermis -brown nuclei-. (C) Microfilaria stained with ftsZ, the star indicating the anterior. (C') is a magnification of (C). (D,D') Posterior lateral chord of a L3 at 3 dpi showing the hypodermal circumferential actin bundles reaching the unfused lateral hypodermal cells in (D), and (D') the dorsal and ventral hypodermal syncytia underneath the lateral cells, with few *Wolbachia* in the dorsal syncytium (arrows). (E) Confocal merge of a posterior lateral chord in a L3 at 7 dpi, separating muscle quadrants. (E') deep view showing the dorsal and ventral syncytia, with *Wolbachia* populating the dorsal hypodermis. (E'') surface view showing the yet unfused lateral hypodermis. (F) Merge of surface sections of a 30 dpi immature worm, showing the fused lateral syncytium, while the *Wolbachia* are still confined in the dorsal syncytium. (G,G') section of an adult female lateral chord, showing the *Wolbachia* in the basal compartment (G), and a deeper view showing the ventral/dorsal syncytium chord with nuclei (arrows) surrounded by numerous *Wolbachia* (G'). The thin lateral hypodermal syncytium ("L") does not fuse with the ventral/dorsal syncytium. Positions and depth of the G and G' confocal images are indicated on the schematic drawing. (H) Adult female lateral chord, arrows point to the chord nuclei, "L" indicates the position of the lateral hypodermal syncytium. Note that in adult, the *Wolbachia* can occupy most of the lateral chord cytoplasm. Scale bar = 5 μ m.

both supergroups, *Wolbachia* are not maintained in the embryonic germline precursors, but are inherited by blastomeres destined for a hypodermal fate.

The hypodermal cells derived from C are the source of all *Wolbachia* in larval and adult syncytial chords

We focused on the laboratory strain *B. malayi* to follow the *Wolbachia* invasion of hypodermal tissues in embryonic, larval and adult stages. In secernentean nematodes, such as *B. malayi* and *C. elegans*, embryonic morphogenesis is driven through contractions of hypodermal circumferential actin bundles leading to epithelial cell shape changes (Zhang et al., 2010). Prior to embryonic morphogenesis, the major hypodermis is composed of two rows of dorsal cells, separated from ventral hypodermal cells

on either side of the embryo by lateral hypodermal cells. The posterior left and right dorsal hypodermal cells derived from C, that contain *Wolbachia*, intercalate in a process similar to that in *C. elegans* (Fig. 2A) (Chisholm and Hardin, 2005). When morphogenesis occurs soon after, the *Wolbachia* remain exclusively located in the apical compartment of the posterior dorsal hypodermis, above the nuclei and under the actin bundles (Fig. 2B, arrows, $n > 100$).

Once morphogenesis is completed, a motile prelarval stage called microfilaria (mf) is released from the worm's uterus into the vertebrate host. In mf, nuclei density is very high (Fig. 2C), and because nuclei are tightly surrounded by actin-rich muscle quadrants, phalloidin staining does not allow a good visualization of hypodermal cortical actin (data not shown). We were therefore

unable to follow the *Wolbachia* in the microfilaria hypodermal cells. We therefore chose to stain the *Wolbachia* in mf with an anti-ftsZ (Fig. 2C,C'). FtsZ concentrates at the septum when the bacterium divides, but is otherwise localized around the bacterial DNA. In mf, the bacteria are always located from the anterior 1/2 to the 3/4 of the worm's length ($n > 200$), where hypodermal cells derived from the C embryonic blastomere are expected to be found, based on *C. elegans* development (Sulston et al., 1983). We detected at most a few dozen of *Wolbachia*, and in addition *Wolbachia* localize identically in mf of the two other species we observed, *L. sigmondontis* and *D. immitis* (data not shown).

Multiplication of *Wolbachia* follows hypodermal cell fusion

To establish the onset of *Wolbachia* proliferation and population of the hypodermal tissues, we selected different larval developmental time points. After blood-meal infection of the mosquitos, the mf develop to second-stage (L2) and then third-stage larvae (L3). We did not observe a significant increase in *Wolbachia* number or distribution during this phase (data not shown). Upon mosquito biting, the L3 larvae penetrate in the vertebrate host. We next scored the *Wolbachia* density at different days post-vertebrate host infection (dpi), along the larvae antero-posterior axis (Table 1). A massive *Wolbachia* multiplication takes place between 3 and 7 dpi, and correlates with a spread along the A-P axis, in the hypodermis ($n > 10$ for each dpi).

To better understand the relationship between the *Wolbachia* multiplication and the lateral chords morphogenesis, we followed the major changes during the worm hypodermis development. When embryonic morphogenesis begins, three rows of hypodermal cells are present on either side of the embryo, such as in *C. elegans* (Fig. 2B) (Zhang et al., 2010). One peculiarity of filarial nematodes is that the hypodermal dorsal and ventral nuclei migrate towards and below the lateral hypodermis (Fig. 2D,D' at 3dpi, Fig. 2E–E'' at 7 dpi), to create in larval stages thick lateral chords isolating two wide submedian muscle quadrants on each side. As in *C. elegans*, cell fusion regulates the formation of mature hypodermis (Alper and Podbilewicz, 2008). Dorsal cells on one part and ventral cells on the other part fuse to create two syncytia, located under the lateral hypodermis in *B. malayi*. We observed that during larval development, the lateral hypodermal cells divide and provide additional nuclei to the dorsal syncytium by fusion as in *C. elegans* (data not shown).

Table 1. Qualitative scoring of *Wolbachia* density in L3 and L4 *B. malayi* larvae. L3 (at 3, 7 and 11 days post-infection -dpi-) and L4 (at 14 dpi) were stained with propidium iodide and phalloidin, the *Wolbachia* localization along the A-P axis was established by confocal microscopy, and their density, established as propidium iodide foci, was scored into arbitrary categories ranging from 0 to 4 as follows: 0 = no *Wolbachia*;

1 = few, scattered *Wolbachia*; 2 = numerous *Wolbachia*; 3 = very numerous (i.e. ~100(s) around each nucleus), 4 = the cytoplasm is filled with *Wolbachia*.

	A	1/4 length	2/4	3/4	4/4	P
					7/8	8/8
3 dpi		0	0	1	0	0
7 dpi		0	0	2 to 4	0 to 3	0
11 dpi		0	0	3 to 4	1 to 3	0 to 1
14 dpi		0	1 to 3	3 to 4	1 to 4	0 to 2

These two syncytia are clearly visible in L3 at 3 dpi before the *Wolbachia* density increases (Fig. 2D', compare with the dorsal syncytium at 7 dpi in Fig. 2E'). *Wolbachia* colonize first the dorsal syncytium of the lateral chords (Fig. 2E'). In 30 dpi young adults, the very elongated and thin lateral cells have fused to create their own syncytium above the secretory-excretory canal, along the equator (Fig. 2F). In mature adults, the apical surface of the dorsal and ventral rows increase, creating larger lateral chords. The lateral syncytium is embedded in the fused "dorsoventral" syncytium, again in a manner similar to *C. elegans* (compare Fig. 2E to Fig. 2E'', with Fig. 2G,G', and with (Alper and Podbilewicz, 2008)). We previously described that in some adults, half of a chord is *Wolbachia*-infected, very likely to be the dorsal part (Landmann et al., 2010). The thinness of the hypodermis under the lateral syncytium (similar to what is observed above the muscle quadrants) may act as a barrier to *Wolbachia* dispersion, which accumulate basally around the dorsal and ventral nuclei and are excluded from the equatorial line (Fig. 2H). Collectively these findings indicate that all the *Wolbachia* originate from the embryonic C blastomere's hypodermal progeny, and that hypodermal cell fusion precedes *Wolbachia* multiplication and dispersion in the lateral chords, first in the dorsal rows in the posterior of the larva, and second along the entire chords.

There is no *Wolbachia* in the *B. malayi* gonadal primordium in L3 larvae

Wolbachia are mutualistic in filarial nematodes, and their presence in the female germline is mandatory to ensure their transmission and the survival of the progeny (Landmann et al., 2011). In contrast, the adult male germline is devoid of *Wolbachia* (Kozek, 1977; Landmann et al., 2010). To understand how and when the endosymbionts colonize the female, but not the male, germline, we followed the development of the gonad in *B. malayi* larval stages and young adults, and confirmed the critical observations in *L. sigmondontis*.

While it is very difficult to locate the gonadal primordium (GP) in microfilariae without a specific germline marker due to the nuclei density, it is possible to identify the GP in L3 larval stages taken from mosquitoes about 14 days after their mf-infected blood meal. Although the precise nature of these cells is not yet established, the GP must contain primordial germ cells, surrounding somatic gonad precursors, and a leading tip cell similar to the *C. elegans* distal tip cell, known to be critical for proliferation and migration of the GP (Kimble and Crittenden, 2005; Byrd and Kimble, 2009). The developing germ cells are flanked by somatic gonad precursors and their basal membrane, and in both sexes the GP appears as a bag of 10 cells in L3 larvae from mosquitoes. In these larvae the GP has already migrated toward the anterior in the female, where it reaches the junction between the developing oesophagus and intestine (Fig. 3A,C–C''), while it is located in the male mid-body (Fig. 3B,D–D'') (Bain, 1972). The GP contacts the body wall only in female, where the gonad anchor cell will induce the vulva differentiation from a group of competent hypodermal cells during the remaining larval development (supplementary material Fig. S1). Based on DNA and actin staining to localize the GPs, none of the observed L3 GPs ever contained any *Wolbachia* ($n > 100$, Fig. 3C–C'',D–D''; supplementary material Movie 5). In L3 larvae, the apical part of the lateral chords embedded between wide muscle

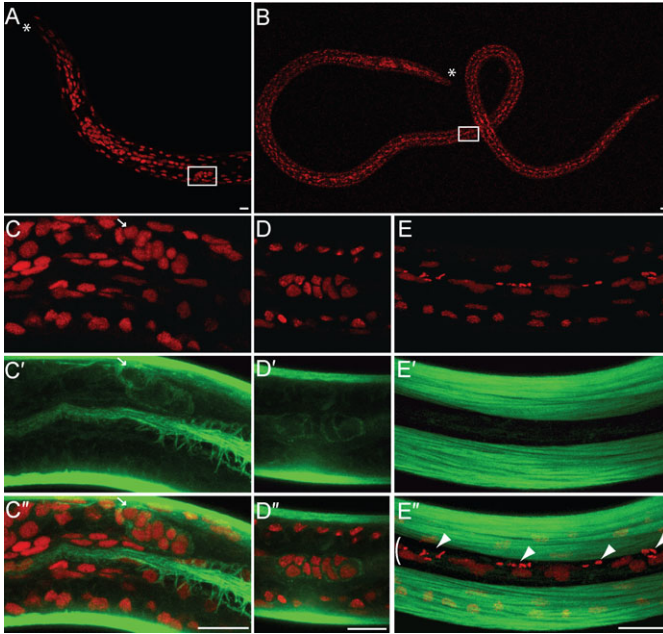


Fig. 3. The *Wolbachia* are absent from the gonadal primordium in *B. malayi* male and female L3 larvae. *B. malayi* L3 larvae stained with PI-red- and phalloidin-green-. (A) anterior part of a female L3 larva and (B) male L3 larva, all obtained from mosquitoes 14 days after their mf-infected blood meal. The gonadal primordium (GP, white box) is anterior in the female and in the mid-body in the male. The anterior is marked by an asterisk. (C–C'') female GP (arrow pointing toward the anchor cell) located at the junction between the developing oesophagus and intestine, and apposed to the hypodermis. (D–D'') male GP. (E–E'') surface detail in the posterior half of a L3 larva, where the *Wolbachia* (arrowheads) localize in a lateral hypodermal chord (bracket). All the images are merges of confocal sections, the anterior of the worms being oriented to the left. Scale bar = 10 μm .

quadrants is still narrow, while these chords run deep toward the central axis of the worm. Most if not all of the *Wolbachia* are located in the posterior half of the larvae, strictly in the dorsal row of lateral hypodermal chords (Fig. 3E–E'').

Wolbachia invade only the female distal gonad from lateral chords, in L4 larval stages

The female GP is anchored to the developing vulva in the anterior (Fig. 4A). The distal part has split into two ovaries migrating towards the posterior, led by a distal tip cell capping each ovary (Fig. 4B). The few germ cells located in the distal ovaries are all connected by a central actin-rich structure called the rachis, forming a syncytium (Fig. 4B). At this 8 to 10 dpi stage begins the germ cell mitotic proliferation. In contrast to the female where the distal gonad migrates toward the posterior, the distal testis is already anterior (Fig. 4C), and in the male the proximal somatic gonad elongates toward the posterior, led by a “linker cell” (Fig. 4D). We never observed any *Wolbachia* in the *B. malayi* male gonad, at any level, from the distal to the proximal end (Fig. 4C,D, $n > 50$ males). In the *B. malayi* female, very few *Wolbachia* are present in the migrating distal ovaries. More specifically, they are observed in the distal tip cell or in the closely associated somatic cells forming the gonad (Fig. 4B, $n > 50$ females). This suggests that the L4 larval stage (at 8 to 10 dpi) is the earliest stage during which the *Wolbachia* appear in the female gonad. We observed an identical behavior in *L. sigmondontis* L4 larvae (data not shown).

In 8 to 10 dpi L4 larvae, the *Wolbachia* are found in the posterior $\frac{1}{2}$ of the worm's length in the lateral chords, and are almost or completely absent from the anterior of the worm (Fig. 4E). Using whole mount larvae, we were able to observe the pseudocoelomic fluid in which the organs develop, and found free *Wolbachia* almost exclusively in the posterior (Fig. 4D). There, the *Wolbachia* concentrate in the basal compartment of the lateral chords, and cross the chords through the basal lamina to access the pseudocoelom in both male and female (Fig. 4F).

Taken together, these data show that the endosymbionts cross the lateral chords basal membrane to enter the pseudocoelom in the posterior of male and female L4 larvae, but the gonad tropism is female-specific.

Only the distal somatic ovary is favorable to *Wolbachia* multiplication in young adult gonads

We then followed how *Wolbachia* colonize the germline in young adult (Fig. 5). At 30 dpi, the ovaries are not yet coiled, but the distal tips are in the posterior of the female. The *Wolbachia* are present in large amount in the somatic gonad, the epithelium surrounding the germ cells (Fig. 5A,B; supplementary material Movie 6). This indicates that they first replicate in these cells before infecting the germ line. We observed only their presence in the vicinity of the distal tip, at no more than 300 μm from it ($n > 30$, Fig. 5C). In addition, the entry into somatic cells, for example in the somatic gonad epithelium (Fig. 5D) or into the distal tip cell from the somatic gonad epithelium (Fig. 5B) is associated with a local cortical actin weakening (Fig. 5B,D, double arrows; supplementary material Fig. S2). At this stage, the vast majority of germ cells and the connecting rachis are still devoid of bacteria. In 30 dpi males, the distal testis located in the anterior has often reached its adult morphology. The male distal tip cell makes a U-shaped turn (Fig. 5E). *Wolbachia* are often present in the adjacent anterior lateral chords, but never in the distal testis ($n > 30$, Fig. 5F). We could not detect any bacteria in the male gonad, even in the proximal gonad, in spite of numerous *Wolbachia* in the surrounding lateral chords (Fig. 5G; supplementary material Movie 7).

Wolbachia presence has been previously reported to be transient in the *B. malayi* male gonad, by *in situ* hybridization techniques, at 5 weeks post infection (Fischer et al., 2011). We therefore extended our observation to this stage in both female and male ($n > 50$, Fig. 6). In 5wpi female, the *Wolbachia* are no longer restricted to the somatic cells of the distal gonad, but are found in all the distal germ cells (Fig. 6A–C). In contrast, we never found any *Wolbachia* within the male gonad by using a direct propidium iodide detection at any level observed, neither in the germ cells, before or during meiosis starting at this stage (Fig. 6D–F) nor in the proximal somatic gonad, the *vas deferens* (Fig. 6G; supplementary material Movies 8–10).

These observations support a *Wolbachia* tropism for the gonad that is specific to the female. In the 5wpi female, the bacteria are almost never detected beyond $\sim 700 \mu\text{m}$ from the distal tip of the ovary (Fig. 6A, “ovary NO. 1”; supplementary material Movie 11). Unlike in the distal tip where they first multiply in the surrounding somatic gonad, the few *Wolbachia* leading the invasion of the germ cells toward the proximal end of the ovary are detected in the center of the gonad, often in actin-rich areas devoid of nuclei, the rachis (supplementary material Movie 11). This raised the question of the *Wolbachia* colonization of the germ cells in the mature ovary.

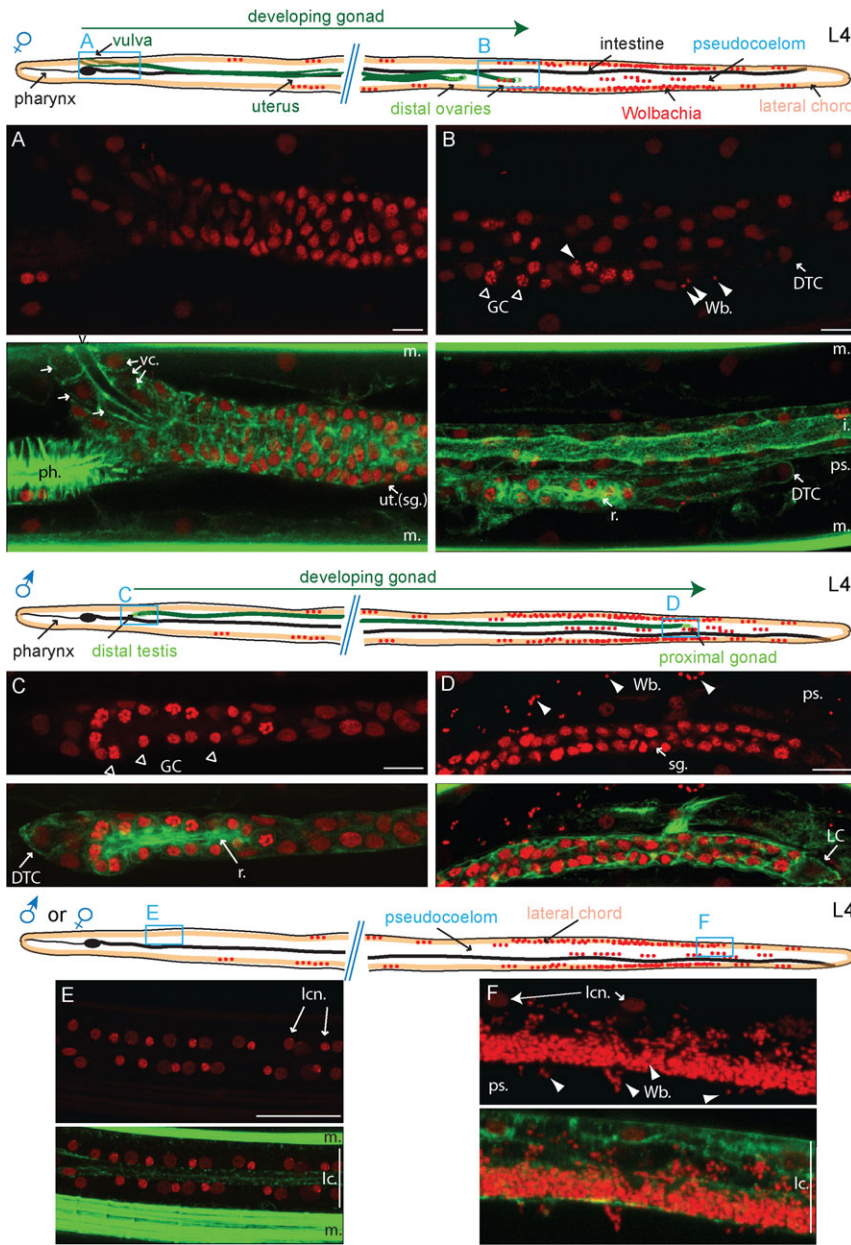


Fig. 4. The *Wolbachia* start invading the female ovary distal tips from the adjacent posterior lateral chords in L4 larvae, but are absent from the L4 male gonad. *B. malayi* L4 larvae (8 to 10 dpi) stained with propidium iodide -red- and phalloidin -green-. The localization of the images taken is indicated on the schematic drawings. (A,B) anterior -proximal- part, and posterior -distal- part of the female gonad respectively. (C,D) anterior -distal- part and posterior -proximal- part of the male gonad respectively. (E) Top down view of a typical anterior lateral chord. (F) Longitudinal view of a typical posterior lateral chord. DTC, distal tip cell; GC, germ cells; i., intestine; sg., somatic gonad; lc., lateral chord; lcn, lateral chord nuclei; LC, linker cell; m., muscle; ps., pseudocoelom; r., rachis; ut., uterus; v., vulva; vc, vulval cells; Wb, *Wolbachia*; All images are oriented with the L4 larvae anterior part to the left. Scale bar =20 μ m.

The rachis, *Wolbachia* route to the proximal germline

In secernentean nematodes, proliferating and maturing germline nuclei are organized as a syncytium. Nuclei are surrounded by a membrane forming a basal peduncle, connected to the rachis by a pore. Until cellularization takes place, all the germline nuclei share the same cytoplasm. To optimize *Wolbachia* transmission to all germ cells, it is tempting to think that they populate the oogoniae before cellularization. We observed ovaries of several species of adult filarial nematodes, and found indeed *Wolbachia* present in the syncytial gonad (Fig. 7). We found however two modes of transmission. In *B. malayi* (Fig. 7A), as well as in *D. immitis* and *O. dewittei japonica* (supplementary material Fig. S3), *Wolbachia* are present around all the germline nuclei in the syncytial ovary. We also found in these species numerous amount of *Wolbachia* in the rachis, suggesting that the rachis either favors their multiplication, and/or serve as a route to infect the

proximal syncytium in young adults and newly born germline nuclei in older adults (Fig. 7B, arrowhead). While the rachis is always enriched in filamentous actin, the peduncles are enriched in microtubules that surround the nuclei, as observed in *B. malayi* (supplementary material Fig. S4).

Instead of being sinuous (i.e. like in *B. malayi*), and extremely branched (i.e. in *D. immitis*), *L. sigmondontis* rachis is a simple tube around which peduncles emanate as a rosette (Fig. 7D–G). In contrast to the previous species, all the *Wolbachia* of the distal syncytial ovary are found in the rachis, as revealed with the propidium iodide staining (Fig. 7D,E). We confirmed that the propidium iodide staining, marking the entire distal rachis, corresponds to a massive *Wolbachia* population, using a specific anti-WSP antibody (supplementary material Fig. S5). Prior to cellularization, the cytoplasmic peduncles containing the germline nuclei elongate. Progressively, *Wolbachia* leave the rachis and

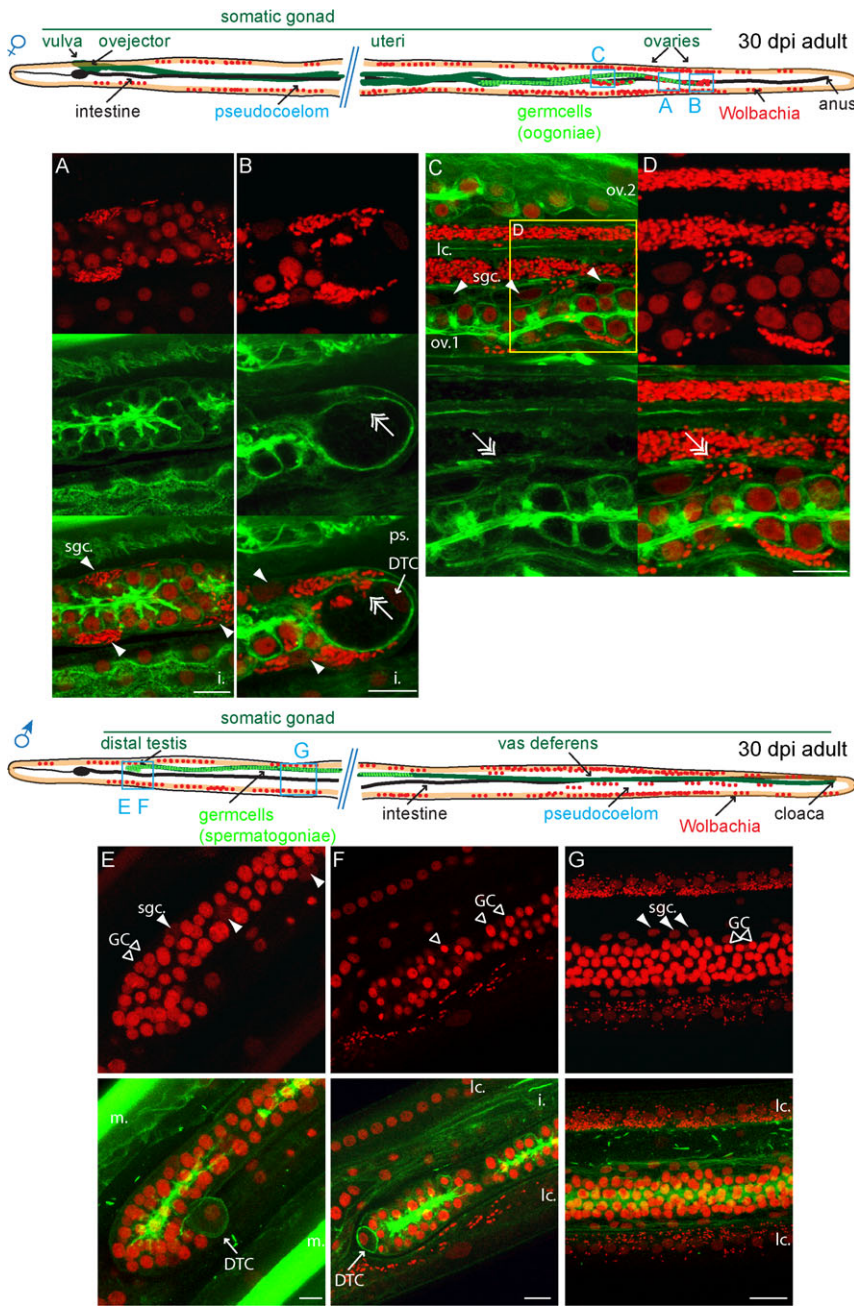


Fig. 5. Cortical actin disruption upon *Wolbachia* entry into the female somatic gonad. *B. malayi* 30 dpi young adults stained with propidium iodide -red- and phalloidin -green-. The localization of the confocal images is indicated on the schematic drawings. (A–D) female. (A,B) tip of the most distal ovary. (C) Confocal section at the level of the first ovary, at about 300 μm from the distal ovary tip, and at the surface of the second ovary. The two ovaries are separated by the basal part of a lateral chord. Arrowheads point to the sgc, the somatic gonad cells, double arrows to *Wolbachia*-associated cortical actin disruption. The split and merged D panels are an enlargement from, and indicated on (C). DTC, distal tip cell; i, intestine; lc., lateral chord; ov.1, most distal ovary; ov.2, most proximal ovary. (E–G) male. Open arrowheads point to the GC, germ cells. m, muscles. Scale bar = 10 μm.

disperse into the rosette (Fig. 7F,G). After cellularization, all the germcells contain *Wolbachia*. These observations stress the crucial role of the rachis in the *Wolbachia* population of the oocytes, therefore for the transmission to the progeny.

Discussion

To define transmission mechanisms of *Wolbachia* during filarial nematode development, we took advantage of newly developed whole-mount immunofluorescent techniques as a complementary approach to the traditional histological methods commonly used in filarial nematode research (Landmann et al., 2010). An accurate robust transmission of *Wolbachia* to the female germline is critical in filarial nematodes because they rely on the presence of the endosymbionts for their growth, development, fertility and long term survival (Slatko et al., 2010; Landmann et al., 2011;

Tamarozzi et al., 2011). We previously described the asymmetric segregation of *Wolbachia* during *Brugia malayi* early embryogenesis to the posterior blastomeres, which produce posterior hypodermal cells as well as the germline lineage (Landmann et al., 2010). However a lack of markers prevented lineage tracing of *Wolbachia* through late embryogenesis and into the microfilaria. The fixed secernentean embryonic lineage and the presence of *Wolbachia* in the germline precursors led us to postulate that localization of *Wolbachia* to the adult ovary may occur through segregation with the germline lineage during nematode development. However recent work in *B. malayi* has produced convincing data that *Wolbachia* do not fate map to the germline through their mitotic segregation pattern, and germline localization requires invasion from neighboring somatic cells (Fischer et al., 2011).

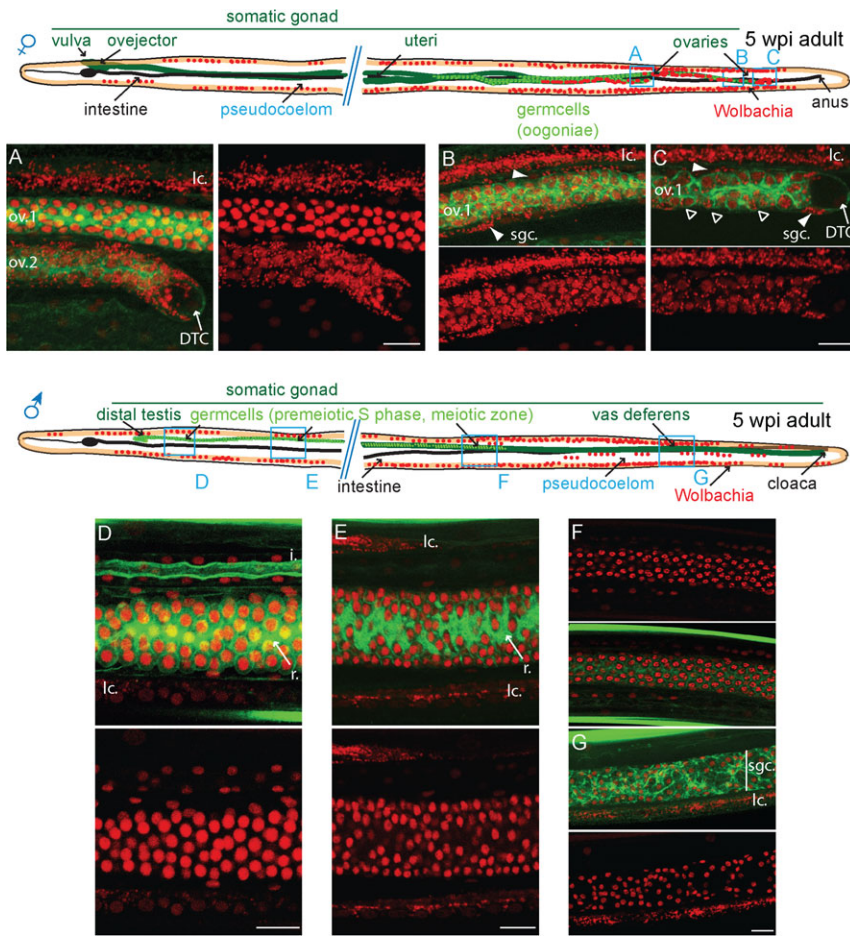


Fig. 6. Wolbachia tropism for the somatic distal gonad is female-specific. *B. malayi* 5wpi adults stained with propidium iodide -red- and phalloidin -green-. The localization of the confocal images is indicated on the schematic drawings. (A–C) female. (A) distal tip of the second ovary “ov.2”, located at 700 μm of the most posterior ovary “ov.1”. (B,C) posterior distal ovary. Arrowheads point to infected somatic gonad epithelial cells “sgc”. In (C) open arrowheads point to infected germline. (D–G) male. (D) zone of mitotic proliferation, (E) premeiotic S phase zone, (F) meiotic zone, (G) vas deferens. I, intestine; lc., lateral chords; r, rachis. Scale bar = 10 μm.

To explore this issue further we examined the transmission of different *Wolbachia* supergroups in multiple nematode species. We find that *Wolbachia*, regardless of their supergroup or the filarial host, rely on multiple evolutionarily conserved mechanisms to reach the ovary. During embryogenesis through the motile pre-larval microfilarial stage, *Wolbachia* distribution in the nematode is determined entirely through asymmetric segregation patterns during host mitosis. In all nematode species examined, this leads to an asymmetric concentration of *Wolbachia* in the C founder blastomere. As development progresses, *Wolbachia* concentrate to the C hypodermal progeny. Segregation with the C lineage results in *Wolbachia* populating the posterior lateral chords in L3 and L4 larval stages. Once the *Wolbachia* titer becomes high in the posterior lateral chords, the bacteria cross the basal membrane of the chords, diffuse in the posterior pseudocoelom in both males and females, but invade only the distal gonad of female worms, by crossing the cell membrane of somatic gonad epithelium, as previously observed using electron microscopy (Taylor and Hoerauf, 1999).

In the embryo, *Wolbachia* rely on mitotic division to asymmetrically segregate to hypodermal blastomeres. The transmission to hypodermal blastomeres by mitotic division is stereotypical in the four species we studied. Based on lessons from *C. elegans*, another secernentean nematode with an identical early lineage, it is likely that the *Wolbachia* integrate, directly or indirectly, posterior polarity cues or their downstream

effectors to segregate into the P₁ and P₂ blastomeres, and that these cues are conserved across the Onchocercidae family of filarial parasites (for a review on embryonic polarity establishment in *C. elegans*, cf. (Munro and Bowerman, 2009)). Surprisingly when P₂ divides, they no longer segregate in the posterior P₃ germline precursor, but rather concentrate into the more dorsal-anterior C founder cell, suggesting that *Wolbachia* localization is likely to not depend on germline determinants.

Hypodermal cell fusion precedes the spread of *Wolbachia* in lateral chords

We find a major increase of the bacterial titer in larval chords between 3 and 7 dpi (cf. Table 1), consistent with previous quantitative PCR data (McGarry et al., 2004). In the larval lateral chords, *Wolbachia* first spread along the anterior-posterior axis. It is only at later stages that the bacteria multiply to fill most of the hypodermal syncytium of the lateral chords. Cell fusion therefore precedes *Wolbachia* dispersion and multiplication in the hypodermis. Mechanisms leading to formation of hypodermal syncytia have been characterized in *C. elegans* (Alper and Podbilewicz, 2008). Disturbing cell fusion may prevent *Wolbachia* multiplication in human filarial parasites.

In the L4 larva, *Wolbachia* invade the ovary from the lateral chords, crossing cell membranes

We found that *Wolbachia* colonize the distal tip of the gonad from adjacent chords. This was recently reported (Fischer et al.,

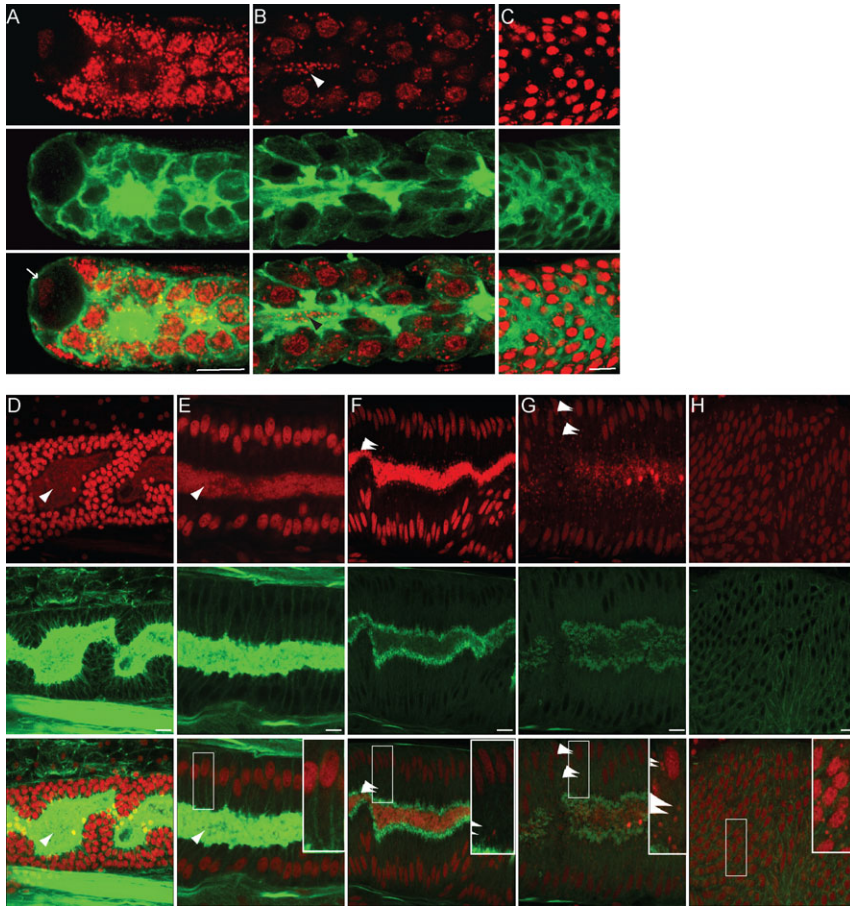


Fig. 7. Two modes of population in the syncytial ovary. Longitudinal confocal sections of *B. malayi* and *L. sigmondontis* adult ovaries stained with propidium iodide -red- and phalloidin -green-. (A–C) *B. malayi* female ovary. (A) distal tip, the arrow points to the DTC, (B) more proximal ovary, the arrowheads point to the *Wolbachia* in the rachis, (C) cellularization zone. (D–H) *L. sigmondontis* ovary, from distal to proximal ends. Arrowheads point to the *Wolbachia*. (D–G) syncytial ovary, (H) cellularization. Scale bar = 5 μm.

2011), but in contrast to the conclusions of this study based on *in situ* hybridized sections, we never detected *Wolbachia* in the male germline at any developmental stages. Our data, based on direct detection of bacterial DNA strongly argue for a strict ovarian tropism. An anatomical peculiarity of filarial nematodes starting in the fourth larval stage is the different localization of the distal gonad in male and female worms. In the male, the distal testis resides in the anterior, while in the female the two ovaries have extended in the posterior. The highest density of *Wolbachia* occurs in the posterior hypodermis adjacent to the point where *Wolbachia* populate the female gonad. This posterior high density may be a factor linked to their release into the posterior pseudocoelom only, occurring in both males and females. In the female gonad, the *Wolbachia* enter only the distal tip. We find that the gonad invasion coincides with the beginning of germline mitotic proliferation around 8 dpi, a process under the control of the distal tip cell (DTC) in nematodes. This cell acts as a stem cell niche, signaling a germline stem cell-like pool to divide via a notch pathway (Hubbard, 2007). It is possible that the surrounding somatic gonad epithelium may also be influenced by the DTC, and express in response specific cell surface receptors, absent from the male gonad and from the proximal female ovary, and used by the *Wolbachia* for docking. It was demonstrated in *Drosophila* that newly introduced *Wolbachia* show a tropism for the somatic stem cell niche of the ovary, before invading the germline (Frydman et al., 2006). Similarly, we found in *B. malayi* a bacterial proliferation in the distal

somatic gonad, suggesting again evolutionarily conserved mechanism for the gonad tropism.

Two mechanisms of cell-to-cell transmission entry can be envisaged. Local membrane lysis, by secretion of a serine protease as previously suggested, could be used by *Wolbachia* to cross membranes (Fischer et al., 2011). Alternatively, manipulation of the host cytoskeleton and trafficking could be envisaged. For instance, internalization after engulfment has been described for many intracellular pathogens. After engulfment, bacteria transiently disassemble the actin network to pass this otherwise impermeable physical barrier. This step is crucial for cell invasion (Carabeo, 2011). We indeed observe a local cortical actin disruption associated with internalized *Wolbachia*, suggesting that they manipulate the actin dynamics at the cell cortex. Future studies will be needed to clarify whether they secrete actin depolymerization factor(s), or if they increase the activity of endogenous depolymerization factors, as *Salmonella* do (Dai et al., 2004).

A mode of transmission unique to filarial nematodes

Despite the obligate mutualism between *Wolbachia* and their filarial hosts, the vertical transmission to the female to its progeny follows a circuitous route. First segregating with and then away from the germline lineage, requiring cell invasion mechanisms to become established in the germline. In insects such as *Drosophila*, *Wolbachia* germline localization requires a direct concentration at the embryonic pole cells, the germline

precursors. However recent studies demonstrate that *Wolbachia* are also capable of reaching the germline through a distinct somatic route requiring cell-cell invasion. The somatic stem-cell niche tropism in flies could reflect an ancient and conserved mechanism of germline invasion by horizontal transfer that may reinforce the direct transmission through the germline precursors (Frydman et al., 2006). Similarly, the presence of *Wolbachia* in hypodermal cells of filarial nematodes may reflect an ancestral somatic tissue-preference after horizontal transfer in the arthropod vector or the vertebrate host.

In a second time, the successful transfer to the progeny could have allowed the development of diverse mutualisms across Onchocercidae carrying the *Wolbachia* supergroups C and D. Support for a common ancestor's transmission pattern in different *Onchocercidae*, rather than a similar parallel evolution of infection mechanisms, comes from the absence of recombination in *Wolbachia* supergroups C and D, carried by different species of nematodes, unlike in arthropod specimens, hosting sometimes several supergroups (Casiraghi et al., 2001; Casiraghi et al., 2003; Bordenstein et al., 2009).

In addition to the hypodermal fate and the distal somatic gonad cells, we also demonstrate that the rachis is a favorable environment for bacterial replication. In insects, *Wolbachia* are present in Golgi-related host vesicles (Cho et al., 2011).

Wolbachia-enriched cells must support their high membrane demand. While the *C. elegans* rachis has been described to be enriched in Golgi apparatus (Abi-Rached and Brun, 1975), *D. immitis* rachis seems devoid of such organelles, rather located around the germline nuclei (Lee, 1975). Differences among nematodes species may account for the different localizations of *Wolbachia* we observed in distal ovaries. We noticed microtubules running between the rachis and the peduncles, as described in *Ascaris* (Foor, 1967), and the *Wolbachia* may use a microtubule cytoskeleton to reach the oogoniae, as they do in *Drosophila* (Ferree et al., 2005).

This study suggests a number of new cellular processes, which could deliver potential drug-targets against filarial diseases. Future efforts will focus on the mechanisms of recognition of the distal somatic gonad cell surface leading to the ovarian population, and on the identification of factors required for *Wolbachia* replication.

Acknowledgements

This work was supported by the Sinsheimer Laboratories Insurance fund (433317-09577), and by the Bill and Melinda Gates Foundation for financial support of the AWOL consortium through a grant awarded to the Liverpool School Of Tropical Medicine.

Competing Interests

The authors declare that there are no competing interests. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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