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Tissue distribution and transmission routes for the Tsetse fly endosymbionts

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Abstract

The tsetse fly *Glossina* is the vector of the protozoan *Trypanosoma brucei* spp., which causes Human and Animal African Trypanosomiasis in sub-Saharan African countries. To supplement their unbalanced vertebrate bloodmeal diet, flies permanently harbor the obligate bacterium *Wigglesworthia glossinidia*, which resides in bacteriocytes in the midgut bacteriome organ as well as in milk gland organ. Tsetse flies also harbor the secondary facultative endosymbionts (S-symbiont) *Sodalis glossinidius* that infects various tissues and *Wolbachia* that infects germ cells. Tsetse flies display viviparous reproductive biology where a single embryo hatches and completes its entire larval development in utero and receives nourishments in the form of milk secreted by mother's accessory glands (milk glands). To analyze the precise tissue distribution of the three endosymbiotic bacteria and to infer the way by which each symbiotic partner is transmitted from parent to progeny, we conducted a Fluorescence *In situ* Hybridization (FISH) study to survey bacterial spatial distribution across the fly tissues. We show that bacteriocytes are mono-infected with *Wigglesworthia*, while both *Wigglesworthia* and *Sodalis* are present in the milk gland lumen. *Sodalis* was further seen in the uterus, spermathecae, fat body, milk and intracellular in the milk gland cells. Contrary to *Wigglesworthia* and *Sodalis*, *Wolbachia* were the only bacteria infecting oocytes, trophocytes, and embryos at early embryonic stages. Furthermore, *Wolbachia* were not seen in the milk gland and in the fat body. This work further highlights the diversity of symbiont interactions in multipartner associations and supports two maternal routes of symbiont inheritance in the tsetse fly: *Wolbachia* through oocytes, and, *Wigglesworthia* and *Sodalis* by means of milk gland bacterial infection at early post-embryonic stages.

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Disclosures

None.

Keywords

Tsetse fly; *Glossina*; Endosymbiont; FISH; Transmission

1. Introduction

The tsetse fly (Diptera: Glossinidae) is the vector of the protozoan *Trypanosoma brucei* spp., that causes the Human African Trypanosomiasis (HAT), commonly called sleeping sickness that is endemic in 36 sub-Saharan African countries. This disease first affects the haemolymphatic system, then targets the central nervous system and leads to death. Although the number of new cases of HAT reported in 2009 has encouragingly dropped below 10,000, for the first time in 50 years, the disease is still considered by the World Health Organization (WHO) as a public health problem (Simarro et al., 2008). WHO is encouraging efforts and innovative approaches with the goal of, not only controlling HAT, but also working to its final eradication. One approach is to explore the tsetse fly interaction with their symbiotic bacteria as an emerging strategy because endosymbiotic bacteria have been shown to potentially affect the insect's capacity to vector trypanosomes (Aksoy et al., 2008; Weiss et al., 2011). Furthermore, paratransgenic technology, where the modified symbionts expressing trypanocidal compounds, could also be used to manipulate host functions and lead to the control of trypanosomiasis (Durvasula et al., 1997; Aksoy et al., 2008).

Tsetse flies can host up to three endosymbiotic bacteria, which play various functions associated with reproduction, immunity and insect fitness (Pais et al., 2008; Attardo et al. 2008, Alam et al. 2011). *Wigglesworthia glossinidia*, a member of the *Enterobacteriaceae* family, is an obligate mutualistic bacterium, mainly located within a specialized organ called the bacteriome that surrounds the anterior midgut of the tsetse (Aksoy, 1995). Based on the coding capacity of *Wigglesworthia* genome (Akman et al., 2002; Rio et al., 2012), and the dietary supplementation experiments (Nogge, 1981), it is thought that an important role for *Wigglesworthia* is to provide nutrients to their tsetse host diet such as vitamins that are lacking or at low amounts in the vertebrate blood. It was also shown that the presence of *Wigglesworthia* during the development of immature progeny is essential for the immune system to function properly in adults (Weiss et al., 2011). Furthermore, this obligate endosymbiont impacts host vector competence. The *Trypanosoma* infection prevalence in flies is significantly higher in adults that have completed their juvenile development in the absence of *Wigglesworthia* (Pais et al, 2008). *Sodalis glossinidius*, the secondary endosymbiont (S-symbiont), also a member of the *Enterobacteriaceae*, is rather wide spread in numerous tissues in the fly (Attardo et al., 2008). *Sodalis* seems to affect the host longevity and may also influence host's ability to establish trypanosome infections (Dale and Moran, 2006). Some tsetse populations harbor a third endosymbiotic bacterium belonging to the genus *Wolbachia*, a *Rickettsia*-like parasitic bacterium known to infect many arthropods (Cheng et al., 2000). This bacterium was shown recently to induce strong cytoplasmic incompatibility in the tsetse flies, resulting in the arrest of the developing embryo when infected males were crossed with uninfected females (Alam et al., 2011).

While the tsetse symbiont functions are being deciphered, less is known about the precise tissue localization and the way by which each symbiotic partner is transmitted from parent to offspring. The tsetse reproduction is unique among insects as it is viviparous and females carry and nourish their progeny during the entire larval development. A single oocyte develops at a time. After ovulation and fertilization, the embryo hatches within the uterus, while a second oocyte begins its development in the second ovary. During the intrauterine development, the mother nourishes the offspring by milk produced by differentiated

accessory gland (named the milk gland), which empties into the uterus (Attardo et al., 2008). Therefore, the progeny can acquire the symbiont infections either through germ line cells, or through milk secretions of the mother at early larval stages during their maturation in the uterus.

The aim of this work was to understand how tsetse endosymbiotic bacteria are transmitted from one generation to the other, by analyzing the precise localization of the endosymbionts in host tissues associated with reproduction. To this end, we have used Fluorescent *In Situ* Hybridization (FISH) by using specific fluorescent oligoprobes designed to match specifically with 16S rRNA of the different bacteria.

2. Materials and methods

2.1. Fly rearing

The *Glossina morsitans morsitans* fly colony is maintained in the insectary at Yale University. Newly emerged flies are separated by sex and mated at 3-4 days post-eclosion. Flies are maintained at $24 \pm 1^\circ\text{C}$ with 50-55% relative humidity, and receive defibrinated bovine blood every 48h using an artificial membrane system

2.2. Tissue dissection

A total of 25 tsetse flies from the laboratory colony were dissected and ten were randomly picked up for histological experiments (see Table 1). Different tissues of the reproductive tract and tissues in contact with embryo and larval progeny were dissected and fixed for FISH studies. The tissues included the reproductive tract containing the uterus, the ovaries and the oocytes, progeny from flies (embryos and larvae from the first to third instars), tissues surrounding the reproductive tract that are the milk gland and the fat body, and the midgut bacteriome organ. All tissues were dissected in phosphate buffer saline (PBS) and placed directly into 4% paraformaldehyde for fixation.

2.3. Sample preparation

Fixative solution was replaced several times with PBS and tissues were embedded in agar prior to the dehydration step. Samples were dehydrated through a graded ethanol series, from ethanol 70° to absolute ethanol, then moved into butanol and stored at 4°C until the following step. Afterwards, tissues were embedded in Paraplast and cut on a microtome at $5\mu\text{m}$ thick sections. Sections were placed on poly-lysine coated slides, dried overnight at 37°C and kept at 4°C until used for FISH.

2.4. Fluorescent In Situ Hybridization (FISH)

Sections were dewaxed in methylcyclohexane 2 times for 10 min each followed by 2 times for 10 min in absolute ethanol. To permeabilize the membranes, slides were coated on a hotplate set at 60°C with a few drops of 70% acetic acid, rinsed with PBS after 1min incubation, dehydrated through a graded ethanol series and left on the bench until complete dryness. For deproteinization, slides were incubated in hydrochloric acid 0.01N with pepsin 0.1mg/ml for 10 min at 37°C in a waterbath, rinsed with PBS, dehydrated in ethanol and air-dried. Then sections were prehybridized for 30min at 45°C in the prehybridization buffer, made with 79% of hybridization buffer (NaCl 0.9M; Tris 20mM; EDTA 5mM, in water, pH 7.2), 20% of Denhardt solution (Ficoll 5g; PVP 5g; Bovine serum Albumin 5g in 500ml water), and 1% SDS. Hybridization step was performed for 3h at 45°C by coating slides with $100\mu\text{l}$ of prehybridization buffer with $1\mu\text{g}$ specific labelled probes targeting *Sodalis*, *Wolbachia* or *Wigglesworthia*. From this step on, all slides were kept in dark. After hybridization, sections were rinsed in hybridization buffer with SDS 0.1% 2 times at 45°C , followed by a quick rinse in PBS and in water. The slides were air-dried and mounted with

an aqueous mounting medium made for fluorescence (Gel Mount) with DAPI (3µg/ml). Slides are kept in dark at 4°C until observation on an epifluorescence microscope (Olympus IX81). Images were captured using an F-ViewII camera and the CellF software (Soft Imaging System).

2.5. Probe designing

Oligoprobes were labelled at 5' with TAMRA. They were designed to match the 16S rRNA sequence of *Sodalis*, *Wolbachia* and *Wigglesworthia*, with a minimum of 6 mismatches between 2 different probes. Probe sequences are as follows: *Wigglesworthia*: 5'-ACG ATA CTC TAG TTT ATT AG-3'; *Sodalis*: 5'-ACG AGA CTC TAG CCT GCC AG-3'; *Wolbachia*: 5'-AAT CCG GCC GAR CCG ACC C-3' and 5'-CTT CTG TGA GTA CCG TCA TTA TC-3'. *Wolbachia* cells were labelled with two probes to increase the signal of these minute bacteria. All probes were purchased at Eurogentec France SA.

3. Results

To better understand how tsetse endosymbionts are transmitted from the mother to her progeny, we have examined the symbiont distribution across mother's tissues involved in reproduction (*i.e.* the reproductive tract and tissues nourishing developing progeny), and within the progeny at early developmental stages. To this end, we have used FISH experiments with probes designed to match specifically with the 16S rRNA of each endosymbiont to avoid any cross-hybridization. All probes were labeled with a fluorescent tail so that bacteria can be analyzed by fluorescence microscopy. Probe specificity was tested by hybridizing each probe with tissues housing separately *Wigglesworthia*, *Sodalis* and *Wolbachia*. Figure 1 shows, for example, the specificity of *Sodalis*, and *Wolbachia* probes with regards to *Wigglesworthia*. Table I summarizes the samples analyzed in this study and the presence/absence of each symbiont across insect tissues and organs.

3.1. The bacteriome tissue houses *Wigglesworthia* only

As an obligate endosymbiont, *Wigglesworthia* is contained in bacteria-bearing host cells called the bacteriocytes, which are grouped to form the bacteriome organ. This tissue is located in the anterior midgut forming two lobes surrounding the gut. FISH experiments with the *Wigglesworthia* probe showed strong signals from bacteriome sections from the female gut. Each bacteriome lobe is formed by different bacteriocyte layers (Fig. 1). Remarkably, applying probes of S-symbionts failed to show any signal on the same sections, suggesting a monoinfection status of tsetse bacteriocytes with the primary endosymbiont (P-symbiont) *Wigglesworthia*.

3.2. *Wigglesworthia* does not infect reproductive tissues

FISH analysis of the female reproductive tract with *Wigglesworthia* probe failed to reveal any signal from ovaries and uterus (Fig. 2A). Surrounding adipocytes were also free of *Wigglesworthia*. Accordingly, the examination of oocytes, trophocytes and embryos did not show any *Wigglesworthia* inside these germ cells (Fig. 2B). However, *Wigglesworthia* was abundant in the lumen of the mother's milk gland tubules (Fig. 2C), while some bacterial cells were also observed in the canal leading to the secretory cell vacuoles (Fig. 2C), where the milk products are produced and secreted to feed the developing progeny in the uterus. *Wigglesworthia* could also be found extracellularly in cavities within the fat body in larva (Fig. 2D). As for the progeny, infection with *Wigglesworthia* seems to occur late during development. *Wigglesworthia* was detected in the first instar larvae, at low amounts surrounding the intestine (Fig. 2E). The bacterial density increased in the bacteriome of the third instar larvae (Fig. 2F). Interestingly, *Wigglesworthia* does not infect the larval intestine but remains confined to the second layer of cells that surround the gut making up the

bacteriome, similar to that described in *Blochmannia* association with the carpenter ant *Camponotus floridanus* (Stoll et al., 2010). Taken together, these data indicate that *Wigglesworthia* constitutes two separate sub-populations in adult females: the first strictly intracellular within the bacteriocytes in the adult gut and the second free-living in the lumen of milk glands and inside fat body cavities in larva.

3.3. *Sodalis endosymbionts* are in the reproductive tract and infect milk gland cells and lumen

As was the case for *Wigglesworthia*, no *Sodalis* cells were detected in oocytes and embryos (Fig. 3A). However, unlike *Wigglesworthia*, *Sodalis* cells are spread throughout the reproductive tract. They were shown to infect the oviduct (Fig. 3B), all over the uterine muscle tissue (Fig. 3C), in the fat body (Fig. 3D), and even in spermathecae sheath cells (Fig. 3D). Moreover, *Sodalis* can be detected in the lumen of the milk gland (Fig. 3E), intracellular within the secretory cells (Fig. 3E), or in both compartments. The milk gland lumen was shown to house either *Sodalis* or *Wigglesworthia*, while both endosymbionts can cohabit in very limited cases, suggesting a possible competition between these symbionts.

In young larval progeny, the S-symbiont *Sodalis* can be detected in the first instar larvae, as the P-symbiont *Wigglesworthia* (Fig. 3F). However, while *Wigglesworthia* invades the bacteriocyte cells and differentiates into the bacteriome organ, *Sodalis* cells form aggregate islands throughout the fat body and gut (Fig. 3F).

3.4. *Wolbachia* infects early oocytes and embryos

Wolbachia endosymbiont is the only bacterium that infects embryos prior to intrauterine development. *Wolbachia* was seen in the developing oocytes, being at both poles as a thin layer, within the yolk, just above the follicular cells (Fig. 4A). In intimate connection with the oocytes, four out of five trophocytes analyzed were highly infected with *Wolbachia* (Table I, Fig. 4B). Apart from these cells, *Wolbachia* was not detected in the fat body and in the uterus (Fig. C). Trophocytes could represent a *Wolbachia* reservoir, intimately connected to oocytes. When oocytes move down through the uterus to hatch into an embryo, *Wolbachia* can still be detected at both poles of the embryo, constituting aggregates on the follicular cell layer (Fig. 4D). Later in second instar larvae, *Wolbachia* was seen sporadically in aggregates (Fig. 4E). Contrary to the nutritional endosymbionts *Wigglesworthia* and *Sodalis*, *Wolbachia* was not detected in the milk gland (Fig. 4F). It is unlikely that milk secretions provide a route of transmission for *Wolbachia*.

4. Discussion

Most insects subsisting on nutrient unbalanced diets owe their survival to long-term resident microbes that provide them with nutrients either lacking in the diet or which the host is incapable of synthesizing (Douglas, 1998; Moran, 2006). Symbionts with obligate functions in the host biology are termed primary, while S-symbionts refer to more recently established symbionts, whose functions remain less understood. They are thought to provide their hosts with ecological benefits, such as resistance to parasites (Oliver et al., 2002; Oliver et al., 2005; Moreira et al., 2009), or to broaden food plant range of their host (Tsuchida et al., 2004). *Wolbachia* S-symbionts rarely behave as a mutualist and often manipulate host reproduction, for their own transmission (Bourtzis, K. and S. O'Neill. 1998). In insects living with multipartner symbionts, such as aphids, whiteflies and tsetse flies, interactions between symbionts and their hosts are still poorly understood. How symbionts infect specific host tissues and how they insure their transmission across host generations remain challenging questions both at the ecological and the molecular levels.

While insect P-symbionts are often strictly associated with bacteriocyte cells, sometimes constituting the bacteriome organ, S-symbionts exhibit a large variety with regards to tissue localization and transmission across insect generations. In aphids, S-symbionts could be found in bacteriocyte-like cells, different from those housing the P-symbiont *Buchnera* (Fukatsu et al., 2000; Gomez-Valero et al., 2004), or they can be free-living in the hemolymph (Tsuchida et al., 2005). In whiteflies, the P-symbiont *Portiera* can co-inhabit with the five associated S-symbionts within the same bacteriocyte (Gottlieb et al., 2008). In the mealybugs, an intriguing symbiotic inclusion of the S-symbiont inside the P-symbiont was reported (von Dohlen et al., 2001).

The tsetse fly house three endosymbiotic bacteria that play separate functions in the host and behave differently in terms of interaction with the host tissues. Although previously studied with PCR amplification technique, symbiont localization was here examined with Fluorescent *In Situ* Hybridization that permits specific and precise localization of endosymbiotic bacteria. Two striking results are provided by this work:

Nutritional symbionts, *Wigglesworthia* and *Sodalis*, infect somatic cells while *Wolbachia* infects germ cells

There seems to be two kinds of bacterial space antagonisms, one between nutritional endosymbionts and *Wolbachia* (i.e. soma *versus* germen), the other between *Wigglesworthia* and *Sodalis* (i.e. bacteriome *versus* milk gland), although coexistence of *Wigglesworthia* with *Sodalis* is not excluded in the milk gland lumen. Although cellular mechanisms driving this tissue distribution are not known, a correlation could be made between the space localization and the functional potentialities of each endosymbiont. *Wigglesworthia* and *Sodalis* functions have been described. *Wigglesworthia* are thought to supply the host with vitamins lacking in the blood diet and it remains to be seen if they also interact with the biosynthesis of milk gland components (Akman et al., 2002; Pais et al., 2008; Attardo et al. 2008). Data presented here further support the nutritional functions of these endosymbionts on the host development. The P-symbiont *Wigglesworthia*, which infects both the milk gland lumen and the bacteriome organ, may play a pivotal role in new-hatched progenies, as well as in supplying the mother diet with essential components. Remarkably, the increase of *Wigglesworthia* density in the bacteriome during post-embryonic development (Fig. 2F) suggests that there may be metabolic exchanges between *Wigglesworthia* and the developing progeny. Less is known about the functions of *Sodalis*, which infects the milk gland tissue, both present intracellularly and in the lumen. While every tsetse individual harbors and depends on *Wigglesworthia* for nutrient supplementation and fecundity, many natural populations of tsetse lack *Sodalis* (Geiger et al., 2005; Lindh and Lehane, 2011).

The absence of nutritional symbionts from the oocytes and embryos, and their presence in the milk gland, argues in favor of their maternal inheritance by means of bacterial infection of new-hatched embryos and larvae through milk secretions. *Sodalis* is spread throughout the reproductive tract, infecting the muscular cells of the uterus and the oviduct, as well as the layer surrounding the spermathecae. Alternative routes of transmission are hence not excluded for this S-symbiont. Future electron microscopy analyses will help to get more details on these ways of symbiont transmission. It also remains to be seen if *Sodalis* infects male gonadal tissue, thus at the present time paternal transmission route for *Sodalis* cannot be excluded.

The facultative endosymbiont *Wolbachia*, which induces a strong cytoplasmic incompatibility in the tsetse flies (Alam et al. 2011), was not detected in the milk gland secretions, or in any other somatic tissues surrounding the uterus. Its transmission to progeny is unlikely to be transuterine. *Wolbachia* was however seen in trophocyte cells, which are connected to the oocytes by intracellular bridges for the transfer of

ribonucleotides, mitochondria, and lipid spheres. This, along with the permanent infection of the oocytes by *Wolbachia* are in line with the reproductive disorder caused by this bacterium, and highly suggests *Wolbachia* transmission *via* mother germ cells.

While symbiont distribution is in agreement with the respective functions of each symbiont, it remains unclear whether endosymbionts target specifically their tissues or whether the host exerts tissue selection through manipulating cell functions such as metabolism or immune system? Alternative possibilities would involve interactions between symbiont partners, which can either compete or act in a synergistic way to promote their coexistence within the same tissue.

***Wolbachia* is strictly intracellular while *Wigglesworthia* and *Sodalis* are both intracellular and extracellular**

The observation that nutritional symbionts constitute intra- and extracellular sub-populations is intriguing in terms of bacterial subsistence and host immune signaling. The mechanisms by which the host perceives and keeps endosymbionts surviving within the bacteriocytes were investigated recently. In both the cereal weevils *Sitophilus* and the tsetse fly *Glossina*, authors showed that bacteriocytes exhibit adapted local immune responses to intracellular symbiotic bacteria (Heddi et al., 2005; Anselme et al., 2006; Anselme et al. 2008; Wang et al., 2009). While the experimental challenge of the P-symbiont of *Sitophilus* into the larval hemolymph results in drastic induction of genes encoding antimicrobial peptides (AMP), only few immune genes are expressed in the bacteriocyte cells (Anselme et al. 2008). In the tsetse fly, a similar bacteriocyte immunomodulation was shown to rely on the expression of the *pgrp-lb* gene (Wang et al., 2009). The amount of *pgrp-lb* transcripts correlated with *Wigglesworthia* density, and *pgrp-lb* gene inhibition with RNAi led to the synthesis of AMP by the bacteriocytes (Wang et al., 2009). Further molecular investigations in weevils showed that bacteriocytes selectively express one AMP, the *coleopteracin A*, which inhibits symbiont cell cytokinesis and prevents bacterial evasion from the bacteriocytes to insect tissues (Login et al., 2011). Moreover, modifications of symbiont outer membrane protein (Omp) were also demonstrated as microbial adaptation that mediates host tolerance to symbiotic bacteria (Weiss et al., 2009). In the present study, the finding that both *Sodalis* and *Wigglesworthia* were found extracellularly raises the questions of whether the host expresses locally attenuated immune response similar to that of the bacteriocyte cells, or the endosymbionts manage to avoid host systemic immune response. The fact that both *Wigglesworthia* and *Sodalis* were not seen freely in the hemolymph, but constitute aggregates in separate body cavities, goes in line with the first hypothesis. On the other hand, the second hypothesis is supported by studies which show that *Sodalis* is resistant to host derived AMP (Hu and Aksoy, 2005) and the recent work of Pontes et al. (2011), which showed that the regulator *phoP* was involved in *Sodalis* resistance to insect derived AMPs. In contrast, facultative endosymbiont *Wolbachia* was not seen extracellularly and infects only few cells, namely the trophocytes and oocytes. Similar to the nutritional endosymbionts, either host cells express attenuated immune response against *Wolbachia* or this bacterium has the ability to overcome host immune response. At the present time, these questions remain speculative since data concerning *Wolbachia* interactions with insect immune system still remain controversial (Bourtzis et al., 2000; Teixeira et al., 2008; Kambris et al., 2009; Moreira et al., 2009; Bian et al., 2010).

In conclusion, FISH experiments using specific probes designed against the tsetse fly endosymbionts have led to precisely determine the selective localization of endosymbionts throughout the host tissues. Whereas the *Wolbachia* facultative symbiont was restricted to the germ cells, the nutritional symbionts *Wigglesworthia* and *Sodalis* were either located in somatic cells or extracellular in body cavities. This work further highlights the diversity of

symbiont localization in multipartner associations and supports two maternal routes of symbiont inheritance in the tsetse fly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlight

- Three symbionts and a DNA virus reside in different tsetse tissues.
- Availability of Gmm^{Wig-} and Gmm^{Apo} enables analysis of intercommunity dependencies.
- The symbiont densities are tightly regulated through host development.
- There is cooperative interaction between *Wigglesworthia* and *Sodalis*
- DNA virus is commensal without displaying pathogenesis in the absence of symbionts.

Figure 1.

Hybridization of bacteriome sections obtained from the mother gut with probes from *Wigglesworthia* (left), *Sodalis* (middle) and *Wolbachia* (right). Only *Wigglesworthia* shows a strong signal from the bacteriocyte cells (see arrows).

Blue signal: nucleus stained with DAPI; red-pink signal: TAMRA-labeled probe; brownish-green signal: non-specific signal from background autofluorescence.

Figure 2.

Tissue distribution of *Wigglesworthia* endosymbiont. (A) *Wigglesworthia* is absent from the mother reproductive tract (left) and from the adipocytes (right). (B) It is also absent from the oocyte (left), from the trophocyte (left) and from the embryo (right). (C) It infects the milk gland lumen and could be free-living in larval cavity structures (D). (E) *Wigglesworthia* is also seen at the first instar larvae where they infect the bacteriome during formation (F, left) and at the third instar larvae with well differentiated bacteriome (F, right).

Blue signal: nucleus stained with DAPI; red-pink signal: TAMRA-labeled probe; brownish-green signal: non-specific signal from background autofluorescence.

Figure 3.

Tissue distribution of *Sodalis* endosymbiont. (A) Hybridization with *Sodalis* specific probe failed to show any signal from the oocytes (left), trophocytes and from the embryo (right). However, *Sodalis* was present in the oviduct (B), the uterus (C), the adipocytes (D, left), and in the spermathecae (D, right, see arrows). In the milk gland, *Sodalis* could be found in the lumen (E, left) or intracellularly within is the milk gland cells (E, right). In the progeny, *Sodalis* infects the first instar larvae as bacterial aggregates within the fat body (F). Blue signal: nucleus stained with DAPI; red-pink signal: TAMRA-labeled probe; brownish-green signal: non-specific signal from background autofluorescence.

Figure 4.

Tissue distribution of *Wolbachia* endosymbiont. (A) *Wolbachia* infecting both poles of oocytes (see arrows); (B) relative high amount of *Wolbachia* in the trophocytes; (C) Fat body (left) and uterus (right) are devoid of *Wolbachia*; (D) *Wolbachia* infecting both poles of embryos; (E) *Wolbachia* in a 2nd instar larva; (F) No *Wolbachia* was detected in the milk gland (D).

Blue signal: nucleus stained with DAPI; red-pink signal: TAMRA-labeled probe; brownish-green signal: non-specific signal from background autofluorescence.

Table 1
Infection of tsetse fly tissues with *Wigglesworthia*, *Sodalis*, and *Wolbachia* endosymbiotic bacteria.

		<i>Wigglesworthia</i>		<i>Sodalis</i>		<i>Wolbachia</i>	
Symbiotic Cells	Bacteriome	3/3	100%	0/3	0%	0/3	0%
Reproductive tract	Uterus – spermathecae -oviduct	0/9	0%	9/9	100%	0/9	0%
	Trophocytes	0/5	0%	0/5	0%	4/5	80%
Tissues in contact	Milk gland cells	0/9	0%	8/8	100%	0/8	0%
	Milk Gland lumen	8/9	89%	3/8	38%	0/8	0%
	Fat body	0/9	0%	7/8	88%	0/8	0%
Progeny	Oocyte	0/5	0%	0/5	0%	5/5	100%
	Embryo	0/3	0%	0/2	0%	3/3	100%
	larvae	7/7	100%	7/7	100%	5/7	71%

For each bacterium, the left column shows the number of infected tissues per the number of analyzed sections, right column gives the percentage of positive samples.