

Impairment of larval development and a sex-
ratio distortion in *Litomosoides sigmodontis*
caused by *Wolbachia*- depleted microfilariae
and
localization of a filarial nematode phosphate
permease up-regulated during *Wolbachia*
depletion

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IV. Abbreviations

µg – Microgram

APS – Ammonium persulphate

bp – Base pair

BSA – Bovine serum albumin

Con – Controls

DEPC – Diethylpyrocarbonate

DMSO – Dimethyl sulphoxide

DNA – Deoxyribonucleic acid

e.g. – Example

dNTP – Deoxynucleosidetriphosphate

EDTA – Ethylene diamine tetra acetic acid

FCS – Foetal calf serum

hr(s) – Hour(s)

KO – Knockout

L – Larvae

LF – Lymphatic filariasis

Ls-actin-1 – *Litomosoides sigmodontis* actin gene

Ls-ppe-1 – *Litomosoides sigmodontis* phosphate permease gene

Ls-PPE – *Litomosoides sigmodontis* phosphate permease protein

mA – Milliampere

MF – Microfilariae

min – Minutes

ml – Millilitre

mm – Millimeter

MW – Molecular weight

nm – Nanometer

OD – Optical density

Ov-GST-1 – *Onchoverca volvulus* Glutathione S-Transferase-1

Ov-PPE – *Onchocerca volvulus* phosphate permease protein

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PBS-T – Phosphate buffered saline Tween

PCR – Polymerase chain reaction

ppe-1 – Phosphate permease gene

PPE – Phosphate permease protein

RT – Room temperature

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec – Seconds

TBS – Tris buffered saline

Tet – Tetracycline treated

qPCR – Quantitative polymerase chain reaction

WHO – World health organization

WSP – *Wolbachia* surface protein

WT – Wild type

1° Con – Primary infected control gerbils

1° Tet – Primary infected tetracycline treated gerbils

2° Con – Secondary infected control gerbils

2° Tet – Secondary infected tetracycline gerbils

IV. Definitions

Macrofilaricidal – Ability to kill adult filarial worms

Microfilaricidal – Ability to kill microfilariae

Patent infection – MF can be detected in blood. For Mongolian gerbils, patent infection is attained 3 months post-infection. For mice it is ca. 58 days post-infection.

1. Summary

Lymphatic filariasis and onchocerciasis are debilitating human diseases in developing tropical countries. Currently implemented treatment strategies are mainly effective against the microfilarial stage (first stage larvae) of the nematode parasite. Concerns regarding the increasing evidence of the development of drug resistance have stimulated the search for new drug targets and drugs. *Wolbachia* bacteria of filarial nematodes play an important role in the reproduction, development and pathogenesis of human filarial nematodes such as *Wuchereria bancrofti*, *Brugia malayi* and *Onchocerca volvulus* and are targets for the control of filarial infections.

The thesis investigates the endosymbiosis between *Wolbachia* and their host filarial nematodes. The rodent filaria *Litomosoides sigmodontis* harbour *Wolbachia* endosymbionts and the pattern of infection and migration of these developing parasites mimics that of human filarial parasites such as *W. bancrofti* and *O. volvulus*. The first aim of the thesis was to study the effect of tetracycline, which depletes *Wolbachia*, on the development of microfilariae (L1 larvae, MF) to L3 larvae in the intermediate mite host *Ornithonyssus bacoti*, and to observe the development of *Wolbachia* depleted L3 larvae in Mongolian gerbils (*Meriones unguiculatus*). Microfilaremic gerbils were treated with tetracycline for 6 weeks in drinking water (1° Tet) or left untreated (1° Con). Naive mites were then fed on the 1° Tet and 1° Con gerbils and were used to infect uninfected gerbils (2° Tet, 2° Con). Fewer female worms developed in 2° Tet gerbils but there was no significant difference in the number of male worms that developed in 2° Tet and 2° Con gerbils, resulting in a male biased sex-ratio. Although 2° Tet male worms had fewer *Wolbachia* than 2° Con males, this did not impair their development. Female worms that developed from *Wolbachia*-depleted MF had numbers of *Wolbachia* equivalent to worms from 2° Con animals. Therefore, tetracycline pre-treatment selected for female worms with higher numbers of *Wolbachia*. Male worms did not show the same dependence on *Wolbachia* for their development, in that all male worms that developed in 2° Tet gerbils had median *Wolbachia* levels significantly lower than 2° Con males. Therefore, female worms require a higher threshold of *Wolbachia* to develop from L3 into adults. The number of MF that developed into L3 larvae in naive mites after feeding on the *Wolbachia*-depleted MF in 1° Tet gerbils was also significantly lower than controls. Since the worms analyzed were only exposed to tetracycline as MF, the

experimental set-up rules out direct effects of tetracycline during larval development in the mites or 2° infected gerbils, suggesting that the initial loss of *Wolbachia* in MF was the cause of impaired larval development, not an antifilarial effect of tetracycline.

The second aim of the thesis was to study the molecular basis governing endosymbiosis between *Wolbachia* and their filarial host - *L. sigmodontis*. Predictions made from the *Wolbachia* genome of *Brugia malayi* (wBm) suggests that several metabolic pathways of *Wolbachia* and the nematode host could play an important role in endosymbiosis between them. Previously in our research group, Heider et al., 2006 studied the gene expression of *L. sigmodontis* during *Wolbachia* depletion using differential display which revealed an up-regulation of a phosphate permease gene (*Ls-ppe-1*). We hypothesized that *Ls-ppe-1* had an important role in nucleotide metabolism as depletion of *Wolbachia* induced expression of *Ls-ppe-1*, perhaps to compensate for lack of nucleotides in the absence of their endobacteria. To test this hypothesis, firstly, the regulation of phosphate permease during *Wolbachia* depletion was studied at the protein level in *L. sigmodontis* and *O. volvulus*, and secondly, the localization of phosphate permease and *Wolbachia* in *L. sigmodontis* and *O. volvulus* were investigated in untreated and antibiotic treated filarial worms. Results of the studies show the up-regulation of *L. sigmodontis* phosphate permease (Ls-PPE) both at the mRNA and protein levels and immunohistology results demonstrate that Ls-PPE is localized to areas of the worms that contain *Wolbachia*. Results also demonstrated the up-regulation of *O. volvulus* phosphate permease (Ov-PPE) at the protein level during *Wolbachia* depletion by doxycycline treatment of onchocerciasis. Ls-PPE and Ov-PPE co-localized to compartments of the worms where *Wolbachia* are in abundance. Hence, the results demonstrate that Ls-PPE and Ov-PPE are up-regulated where *Wolbachia* are located and suggests that these proteins could have a direct functional role in the symbiosis between filarial nematodes and their *Wolbachia*. Up-regulation of phosphate permease in response to *Wolbachia* depletion in filarial nematodes suggest that the functions of phosphate permease could involve provision or transportation of phosphate to the *Wolbachia* symbionts, which encode all the genes for the *de novo* synthesis of nucleotides. Further ultrastructural analysis using electron microscopy promises to bring insight into the molecular interaction between phosphate permeases and *Wolbachia*.

2. Introduction

Filarial infections such as lymphatic filariasis and onchocerciasis, caused by tissue-invading parasitic worms of the phylum Nematoda, cause a wide range of clinical signs and symptoms, including lymphoedema, hydrocele, elephantiasis or dermatitis and blindness. These filarial infections lead to severe morbidity and considerable socio-economic problems throughout the tropics. Lymphatic filariasis (LF) affects more than 120 million people (WHO, 2006) and onchocerciasis affects as many as 37-40 million people (WHO, 2007). Filariasis is still considered to be the third most important tropical disease worldwide based on Disability Adjusted Life Years (DALY) as a measure of disease burden. LF is mainly distributed in countries in Latin America, Africa and South East Asia (Fig. 1).

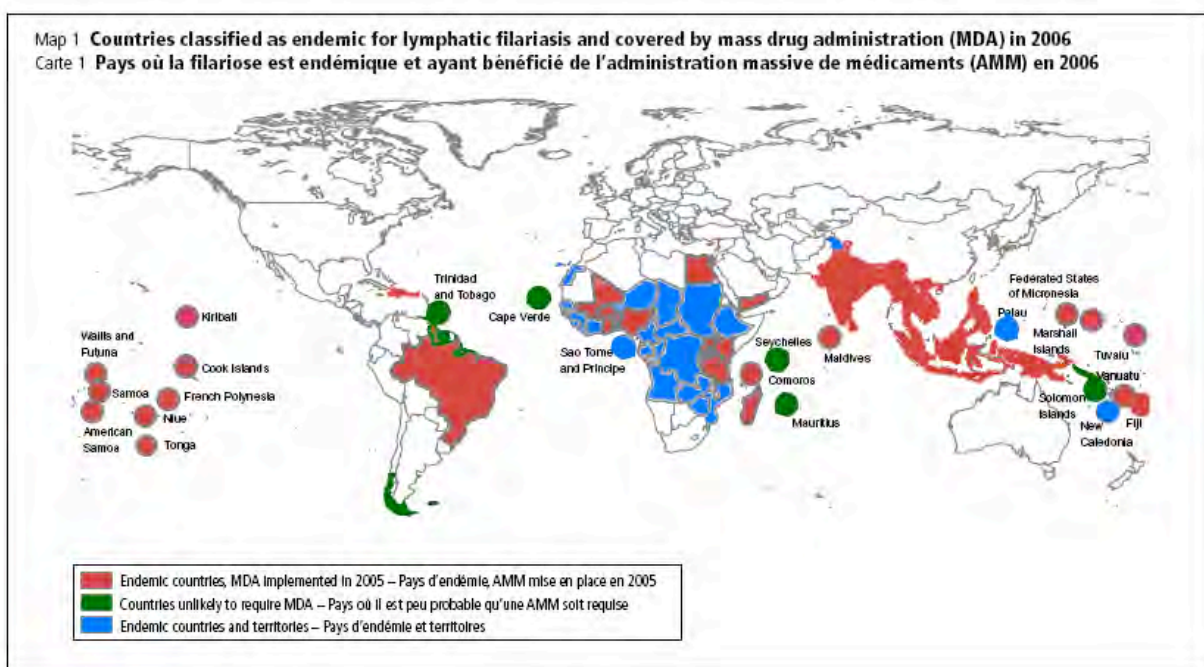


Fig. 1. Global distribution of lymphatic filariasis showing endemic areas and areas where MDA is implemented.
 Source: WHO/TDR, www.who.int/tdr/.

2.1. Lymphatic filariasis

In 2006, the World Health Organisation estimated that over 1.3 billion people are at risk of lymphatic filariasis (LF), a devastating parasitic infection spread by mosquitos (WHO, 2006). LF is caused by thread-like parasitic worms that damage the human lymphatic system. The main causative agents of LF are *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* and nearly 80% of the infection in the endemic areas is due to *W. bancrofti* (Michael and Bundy, 1997). LF is considered as one of the world's most disabling and disfiguring diseases. The disease is estimated to infect over 120 million people, with more than 40 million incapacitated or disfigured with swelling of the limbs and breasts (lymphoedema) and genitals (hydrocele), or swollen limbs with dramatically thickened, hard, rough and fissured skin (elephantiasis). LF prevents afflicted individuals from experiencing a normal working and social life, furthering the cycle of poverty (WHO, 2005; WHO, 2006). Treatment costs and loss of work time caused LF are considerable and leads to major annual economic loss in developing countries such as India (Ramaiah et al., 2000).

2.1.1. Life cycle of *Wuchereria bancrofti*

LF is largely caused by *W. bancrofti* and is spread from an infected human, i.e. someone with worms in their bloodstream, to an uninfected human by mosquitos. Adult worms live in infected human's lymphatic vessels. The adult worms are 0.2 mm wide and can be up to 10 cm long. They survive for up to 10 years. The female worms release large numbers of very small larvae called microfilariae (L1/MF) [250 to 300 μm long and 8 μm wide], which circulate in an infected person's bloodstream. When an infected human is bitten by a mosquito, the mosquito ingests the L1 larvae. The most important species of vector is *Culex quinquefasciatus*. The larvae develop in the mosquito into an infective stage (L3 larvae) and are then spread to other people via mosquito bites (Fig. 2). After a bite, the larvae pass through the skin, travel to the lymphatic vessels and develop into adult worms. Approximately 6 to 12 months after infection, MF appear in the circulation. Every day, the female worm produces numerous MF (Dreyer et al., 2000).

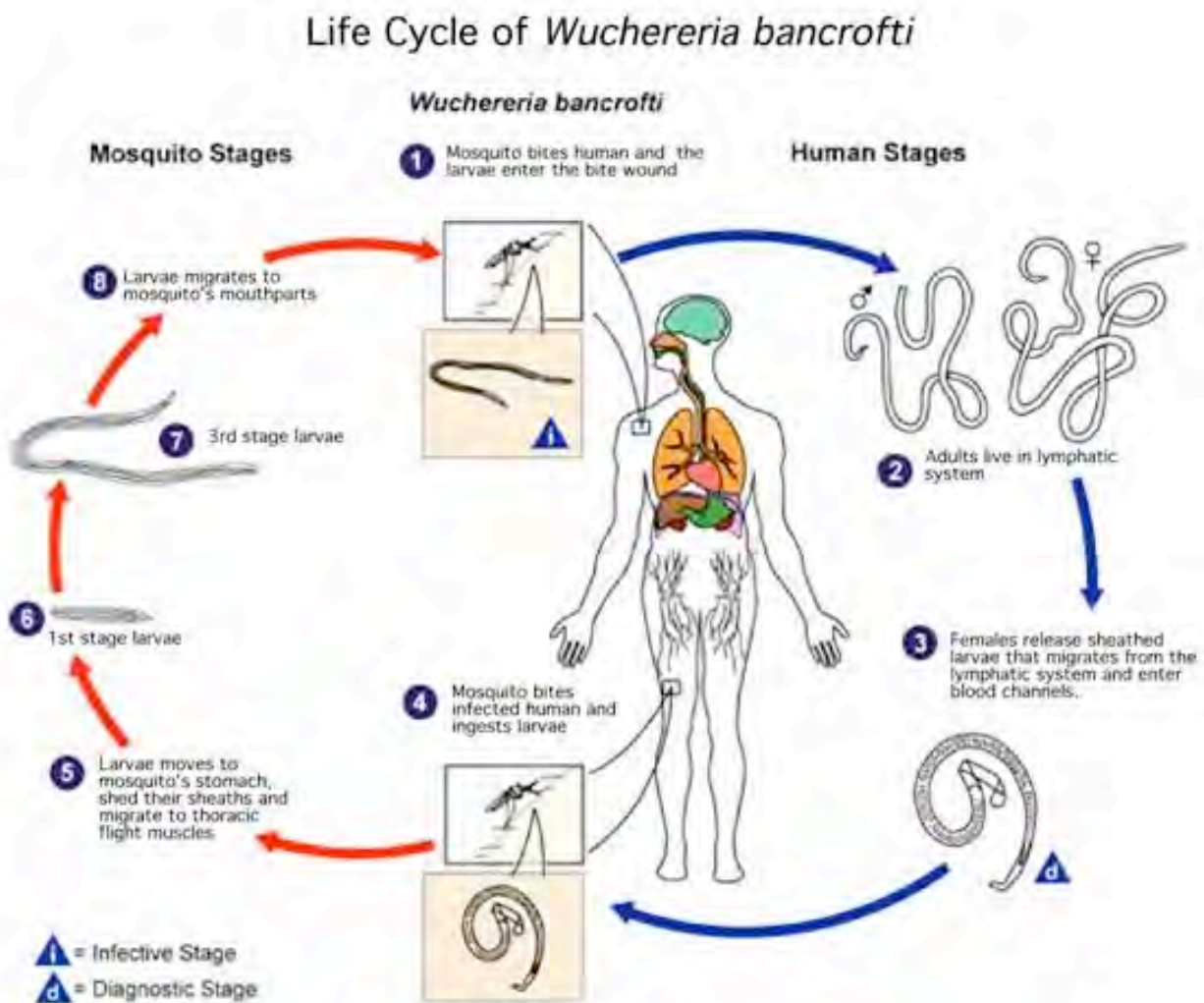


Fig. 2. Life cycle of *Wuchereria bancrofti*. Source: WHO/www.filaria.org

2.1.2. Pathogenesis of LF

The clinical manifestations associated with lymphatic filariasis can be asymptomatic or chronic. Asymptomatic individuals have detectable microfilaremia but without any outwardly discernible manifestations of lymphatic insufficiency on clinical examination, whereas chronic individuals have clinical evidence of lymphatic insufficiency or obstruction and are generally amicrofilaremic (Freedman, 1998). The adult worm induces severe immunological reaction. The basic lesion is a sterile inflammation around the worm, in and around the lymph nodes and

lymph vessels. In the case of lymphangitis, there is often retrograde inflammation (centrifugal spread). This inflammation leads to obstruction of lymph vessels, resulting in temporary lymphostasis and lymphoedema. Research findings also indicate that adult worms can themselves directly attack the lymphatics (irrespective of the immunological response). In humans with severe symptoms, low or no microfilaraemia is found in most cases, whereas humans with high microfilaraemia often have no symptoms (Freedman, 1998). The reason for this apparent paradox is that the pathology is caused by the patient's immunological response to the adult worms (Ottesen, 1984; Freedman, 1998; Dreyer et al., 2000). This is presently one of the main subjects of interest in LF research. Chronic pathological conditions include: 1. Hydrocele, where accumulation of lymph fluid occurs in the tunica vaginalis and 2. Lymphoedema, where accumulation of lymph fluid occurs in the legs, scrotum, breasts and arms which can progress to elephantiasis due to infection by opportunistic bacteria and fungi.

2.1.3. Treatment of LF

The Global Lymphatic Filariasis Elimination Program (GLFEP) aims to reduce microfilaremia levels with filaricidal drugs to a level that is too low to sustain transmission of filarial parasites to humans; and to reduce the morbidity associated with chronic filarial disease (Cox et al., 2000). Antifilarial drugs such as diethylcarbamazine (DEC) and ivermectin are predominantly active against MF, with DEC showing partial activity against adult worms (Noroës et al., 1997; Ramzy et al., 2002). Parallel hygiene management programs are implemented in endemic areas of LF where foot care hygiene training is given to lymphoedema patients, which significantly reduce the morbidity, associated with lymphoedema (Kerketta et al., 2005). Local limb care is an important intervention and together with doxycycline improves the skin integrity with loss of deep folds and knobs of lymphoedema patients (Debrah et al., 2006).

2.2. Onchocerciasis

Onchocerciasis, commonly known as river blindness, is caused by the filarial nematode, *Onchocerca volvulus* (WHO, 1987). Major pathological conditions of onchocerciasis are blindness and severe dermatitis affecting as many as 37-40 million people (WHO, 2007) and is the fourth most common cause of blindness in the world (Soboslay *et al.*, 1997) and the second

most frequent cause of preventable blindness in Africa (Hoerauf and Brattig, 2002). Severe hyperendemic onchocerciasis can cause blindness in 15% of the population and up to 40% of the adults can be visually impaired in African communities. Visual impairment is a major occupational obstacle and reduces the life span of affected persons by an average of 10 years (Kirkwood *et al.*, 1983; WHO, 1994; WHO, 1998). Hence, like lymphatic filariasis, onchocerciasis is also a major socio-economic problem. *O. volvulus* causes itching, disfiguring skin disease, serious eye lesions and blindness in parts of Africa, Latin America and the Arabian Peninsula (WHO, 1995) (Fig. 3).

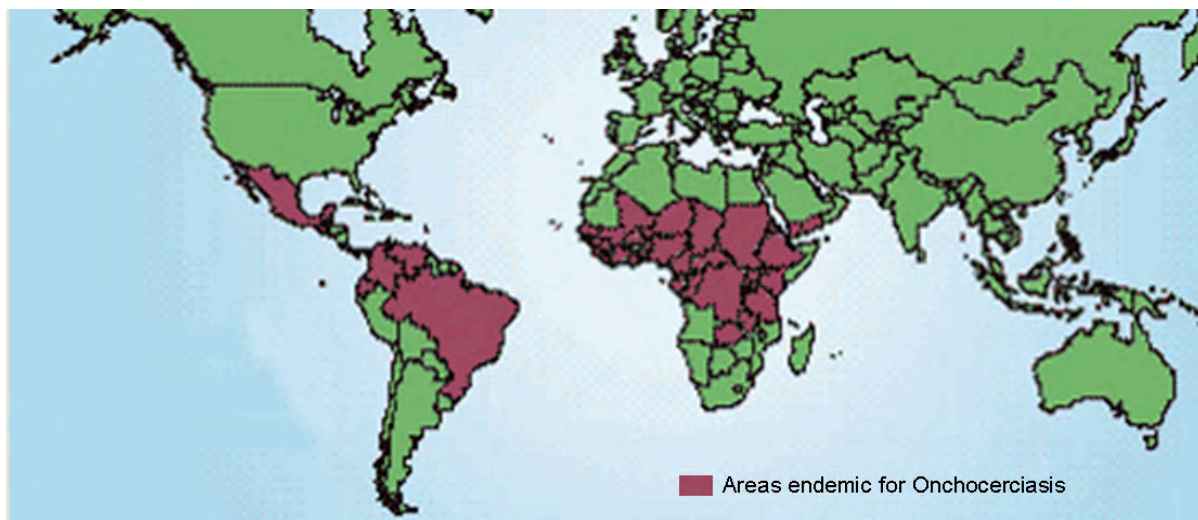


Fig. 3. Global distribution of onchocerciasis. Source: WHO/TDR, www.who.int/tdr/.

2.2.1. Life cycle of *Onchocerca volvulus*

O. volvulus is a thin nematode parasite found in humans which is transmitted by blackflies of the genus *Simulium*. The adult worms live in fibrous nodules, some of which are subcutaneous while others live deep in the connective and muscular tissues. They have a life span of around 9 – 14 years (Plaisier *et al.*, 1991b; Duke, 1993; WHO, 1995). The female adult worms produce numerous MF (250 – 300 μm in length), which migrate from the nodule to invade the skin, eyes and other organs. MF have a life span of about 6– 24 months (Duke, 1993; WHO, 1995). The MF are ingested from the skin by blood feeding *Simulium* vectors develop over 6 – 12 days. The MF develops into infective larvae (L3) in these intermediate hosts and are then inoculated into a

new host when the blackfly subsequently feeds (Fig.4). In the human host, the L3 moult twice into the adult stage. The first microfilariae (MF) produced by adult females may appear in the skin 10 – 15 months after infection (WHO, 1995). About 700 - 1600 MF per female are released into the host on average per day of a production cycle (Schulz-Key 1988; Duke, 1993; WHO, 1995).

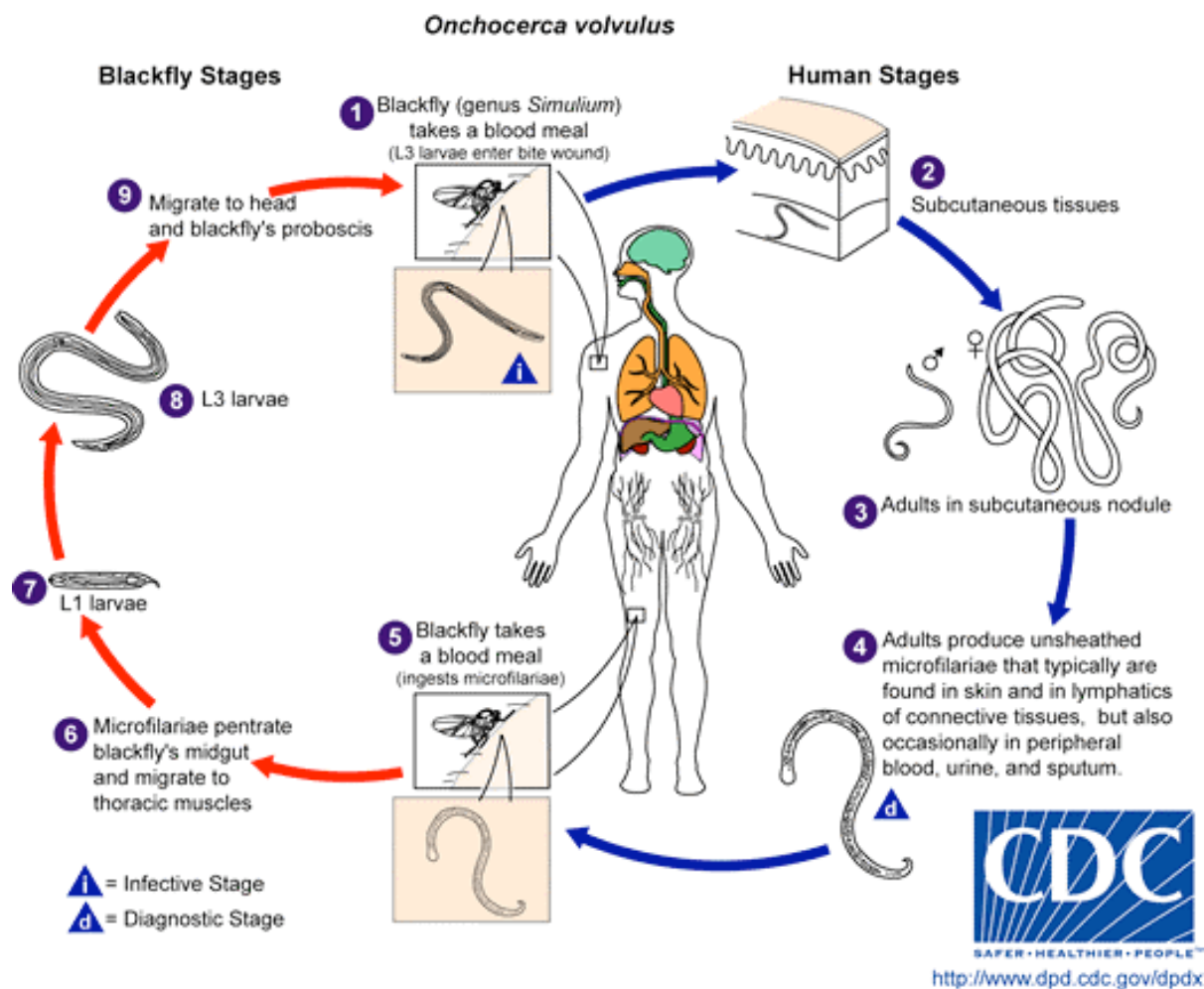


Fig. 4. Life-cycle of *Onchocerca volvulus*. Source: CDC, USA.

2.2.2. Pathogenesis of *Onchocerca volvulus*

Based on the wide spectrum of clinical manifestations associated with onchocerciasis, signs and symptoms of onchocerciasis can be categorized as dermal, lymphatic, systemic and ocular (WHO, 1995). In dermal onchocerciasis, skin lesions result from inflammatory reactions around damaged or disintegrating MF and hence the clinical manifestations vary according to the MF density in the skin, the immune responsiveness of the host, and the duration of the infection

(WHO, 1987). Onchocercomata (nodules) are major clinical symptoms associated with onchocerciasis where fibrous nodules are formed around the female worm producing MF and nodules are normally found subcutaneously or occasionally attached to the skin (WHO, 1987). In lymphatic onchocerciasis, people infected in endemic areas have enlargement of superficial lymph nodes and lymph nodes draining areas of filarial dermatitis contain small numbers of MF and long-standing infections causes severe fibrosis (WHO, 1995; Ottesen, 1995). In systemic onchocerciasis, in addition to presence of MF in skin, eyes, and lymph nodes, MF could also be seen in peripheral blood, urine, sputum, tears, vaginal smear and cerebrospinal fluid after treatment (WHO, 1987). Ocular onchocerciasis and blindness are the major pathological conditions caused by *O. volvulus* where a variety of lesions affect different parts of the eye which appears directly or indirectly related to the local death or degeneration of MF that enter the eye from the skin through conjunctiva or bloodstream (WHO, 1987; Ottesen, 1995).

2.2.3. Treatment of onchocerciasis

To eliminate the disease by vector control, the Onchocerciasis Control Programme (OCP) was established in 11 West Africa countries (WHO, 1987; 1994). The main goal of this programme was to eliminate onchocerciasis as a disease of public health. The control strategy was to interrupt the transmission of the *O. volvulus* parasites by seasonal weekly aerial application of larvicides to all the breeding sites of the vector. Though the OCP in West Africa was successful in the control of onchocerciasis, vector control could not be solely relied on due to the problems associated with environmental effects and insecticide resistance (WHO, 1995). In 1995, OCP was replaced by the African Programme for Onchocerciasis Control (APOC) where ivermectin is used for the treatment of onchocerciasis. The main goal of APOC was to establish within a period of twelve (12) years, an effective and self-sustainable community based ivermectin treatment throughout endemic areas in Africa. Several studies have demonstrated the effectiveness of ivermectin treatment in preventing the development and progression of onchocercal lesions (Remme, 1995).

2.3. *Wolbachia*

Wolbachia are gram-negative α -proteobacteria present in many invertebrates and non-insect invertebrates including nematodes, mites and spiders (Werren et al., 1995; Werren & Windsor, 2000). These intracellular bacteria were first reported within the reproductive tissues of the mosquito *Culex pipiens* by Hertig & Wolbach in 1924, and these bacteria were later named as *Wolbachia pipientis* (Hertig, 1936). For many years, *Wolbachia* were neglected until the 1970s, when Yen & Barr established that cytoplasmic incompatibility (CI) in *Culex pipiens* was associated with the presence of a rickettsial agent which was eliminated through antibiotic curing (Yen and Barr, 1971). The advent of molecular techniques, in particular polymerase chain reaction (PCR) rapidly accelerated intensive research on *Wolbachia* in the 1990s. *Wolbachia* are extremely common with 20-75% of all insect species and other arthropods being infected. 90 % of filarial nematodes harbour *Wolbachia*, including most of the agents of filariasis: *Brugia malayi*, *Brugia pahangi*, *Wuchereria bancrofti*, *Onchocerca volvulus*, *Mansonella sp.*, *Dirofilaria immitis* and *Litomosoides sigmodontis* (Bandi et al., 1998; Bandi et al., 2001; Jeyaprakash & Hoy, 2000; Plantard et al., 1999; Taylor & Hoerauf, 1999; Wenseleers et al., 1998; Werren et al., 1995; Werren & Windsor, 2000). *Wolbachia*, formerly known as rickettsiae-like organisms, are found in reproductive tissues of a wide range of arthropods and nematodes (O' Neil et al., 1992; Rousset et al., 1992; Stouthamer et al., 1999; Werren et al., 1995; Bandi et al., 1998; Taylor et al., 1999; Hoti et al., 2003; Sridhar & Pradeep, 2003). *Wolbachia* have also been found in isopods (Rousset et al., 1992) and mites (Johanowicz and Hoy, 1995).

2.3.1. Morphology of *Wolbachia*

Wolbachia have general morphological characteristics of rickettsiae (Hertig, 1936). They are dimorphic, with irregularly formed rod like (0.5–1.3 μm in length) and coccoid forms (0.25–0.5 μm in diameter). The smaller forms of bacteria also occur in aggregates as larger forms (1-1.8 μm in diameter). Usually *Wolbachia* are present in a vacuole enveloped by three layers of membranes. The outer layer is of host origin followed by the outer membrane of the bacteria and the innermost layer consists of the plasma membrane of the bacteria (Wright, 1979).

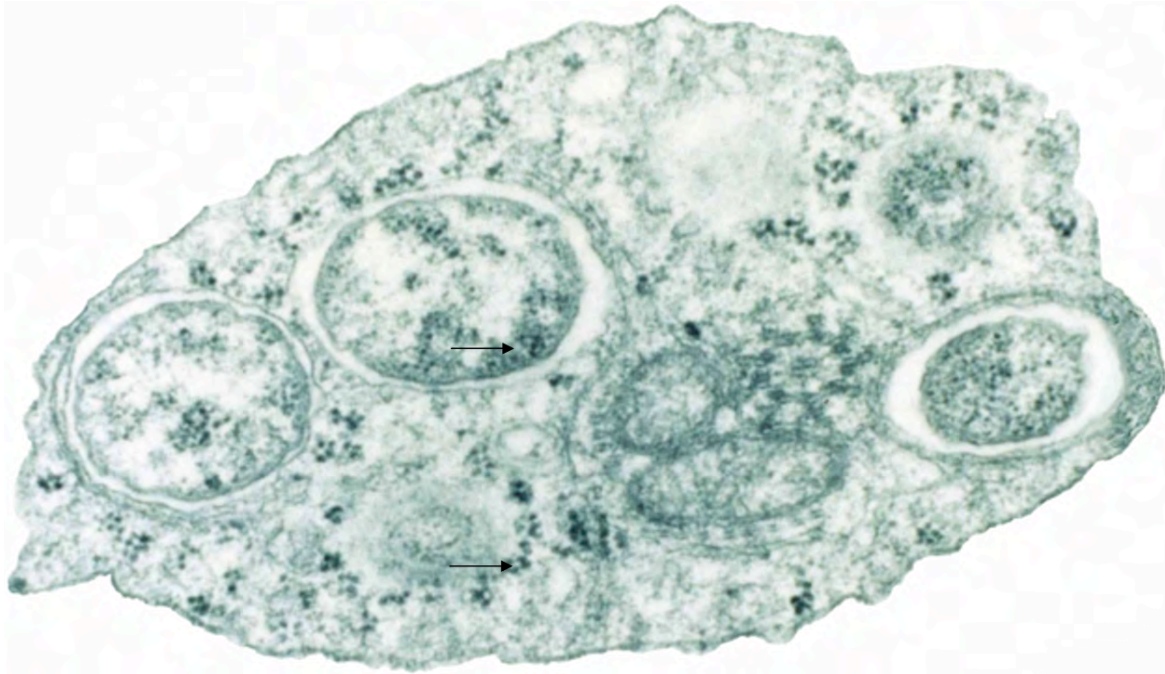


Fig. 5. Transmission electron micrograph of *Wolbachia* within an insect cell (Wu et al., 2004).

2.3.2. Phenotypes associated with *Wolbachia* infections in arthropods

Wolbachia infecting insect invertebrates are not only widespread, but they also cause a variety of physiological alterations in their host such as:

Cytoplasmic incompatibility (CI)

Cytoplasmic incompatibility (CI) occurs when uninfected females mate with *Wolbachia* infected males. This mating is incompatible and cannot produce offspring as a result of failure in karyogamy, arresting the development of early embryos in diploid insects (Tram and Sullivan, 2002). CI can either be unidirectional or bidirectional.

Unidirectional CI

Unidirectional CI occurs in mating between *Wolbachia*-infected males and uninfected females, whereas *Wolbachia*-infected females are compatible with both *Wolbachia*-infected and uninfected males. Thus, in host populations that include both infected and uninfected individuals,

CI provides a reproductive advantage to infected females since they can mate successfully with all male types. In contrast, uninfected females are incompatible with infected males, reducing their reproductive success. The advantage afforded to females by CI comes at the expense of infected males, which are incompatible with uninfected females (Hoffmann and Turelli, 1997).

♀ X ♂ = Normal Hatch Rate

♀* X ♂* = Normal Hatch Rate*

♀* X ♂ = Normal Hatch Rate*

♀ X ♂* = Reduced Hatch Rate (0-70%)
(* = *Wolbachia* infected)

Bidirectional CI

Bidirectional CI occurs when insect populations are infected with multiple strains of *Wolbachia*. For example:

A strain infected ♀ X B strain infected ♂

B strain infected ♀ X A strain infected ♂

are each incompatible.

The molecular mechanism involved in CI has not yet been completely elucidated and the mechanism of CI appears to vary between host species, e.g. in *Culex pipiens*, incompatibility appears to result from failure of sperm bearing a CI *Wolbachia* to fuse properly with female gametes that lack the same *Wolbachia*. In *Drosophila*, there is embryo development suppression at an early stage and here it is proposed that *Wolbachia* in eggs of infected females produce a substance that renders them immune to the chemical produced by sperm from infected males (Snook et al., 2000). In wasp such as *Nosonia vitripennis*, *Wolbachia* interferes with condensation of the paternal chromosomes set during the first mitotic cell division of the embryo

but the way by which CI *Wolbachia* interacts with the chromosomes of *Nosonia*, causing condensation failure is not clear (Tram et al., 2006). In addition, several other CI models have been proposed: Lock-and-key hypothesis, Titration–restitution hypothesis and Slow-motion hypothesis (Poinsot et al., 2003).

Other phenotypes associated with *Wolbachia* infections in arthropods include feminization, male killing and parthenogenesis (PI). Feminization occurs in isopod crustaceans and is a phenotype induced by *Wolbachia* where genetic males develop into fully functional females via inhibition of the production of androgen from androgenic gland. (Rousset et al., 1992). In male killing, selective killing of males is caused by *Wolbachia*. This causes a sex-ratio distortion and reduction in number of progeny. This phenotype has been reported in *Tribolium*, ladybird beetles, *Drosophila* and *Acraea* butterflies (Hurst et al., 1999). In parthenogenesis, *Wolbachia* induce asexual reproduction, resulting in all female broods. PI *Wolbachia* strains have now been found in a wide range of parasitic wasp genera, including *Trichogramma*, *Aphytis*, *Encarsia*, *Leptopilina*, and *Muscidifurax* (Stouthamer et al., 1993; Werren, 1997).

Wolbachia being a reproductive parasite in arthropods, it is generally assumed that these vertically inherited symbionts cospeciate with their host but phylogenetic evidence has shown that horizontal transfer of these bacteria must have occurred during the course of evolution because closely related bacterial strains could be found in unrelated hosts (O’Neill et al., 1992; Rousset et al., 1992; Stouthamer et al., 1993; Werren et al., 1995). In addition, *Wolbachia* can be microinjected both intra- and interspecifically in to naïve hosts (Braig et al., 1994; Grenier et al., 1998) and transfection of *Wolbachia* strains can cause different phenotypes in different hosts (Fujii et al., 2001). Also natural intra- and interspecies horizontal transfer of pathenogenetic *Wolbachia* has been observed in parasitoid wasp (Huigens et al., 2004). Recently, nearly the entire *Wolbachia* genome (>1 megabase) as short (<500 base pairs) insertions was observed in the genomes of 4 insect and 4 nematode species suggesting that the heritable lateral gene transfer occurs into eukaryotic hosts from their prokaryote symbionts (Hotopp et al., 2007). Horizontal transfer of *Wolbachia* genes across the species has attracted the attention on *Wolbachia* as new biocontrol agents where genetically modified *Wolbachia* could be introduced into pest and vector species of economic and medical relevance to suppress or modify natural populations (Zabalou et al., 2004).

2.3.3. Phylogeny of *Wolbachia* in arthropods and nematodes

The genus *Wolbachia* is phylogenetically related to the genera *Anaplasma*, *Cowdria* and *Ehrlichia* and has been assigned to the alpha 2 subclass of the proteobacteria (O'Neill et al., 1992). Unlike most other obligate intracellular bacteria, the genus *Wolbachia* forms a monophyletic clade comprising both mutualistic and parasitic lineages that showcase the diversity of symbiotic associations. In arthropods, *Wolbachia* are commonly considered as reproductive parasites as they exploit the host reproductive system to enhance their maternal transmission into next generation (Werren et al., 1997). Unlike arthropod *Wolbachia*, the *Wolbachia* bacteria of filarial nematodes are beneficial and required for host reproduction (Bandi et al., 1999; Hoerauf, et al., 1999; Hoerauf, et al., 2000; Bandi et al., 2001; Casiraghi, et al., 2001).

Currently, the phylogenies of the genus *Wolbachia* are currently sorted into eight major clades (A–H), which, in the absence of a formal species description, have been named ‘supergroups’ (Lo et al., 2002). Supergroups A and B include most of the parasitic *Wolbachia spp.* so far found in arthropods (Werren et al., 1995). Supergroups C and D include the majority of the *Wolbachia spp.* found in filarial nematodes (Bandi et al., 1998) but some nematodes have been found to lack *Wolbachia spp.* (Bordenstein et al., 2003). The E supergroup consists of *Wolbachia spp.* from primitive wingless insects, the springtails (Collembola) (Vandekerckhove et al., 1999; Czarnetzki & Tebbe, 2004). The supergroup F contains *Wolbachia* bacteria of arthropods (termites) and the filarial parasite *Mansonella ozzardi* (Casiraghi et al., 2001; Lo et al., 2002). In addition, supergroup G has been proposed for *Wolbachia spp.* of certain Australian spiders (Rowley et al., 2004) and supergroup H encompasses *Wolbachia*, different from those in supergroup F, in termites. The *Wolbachia* from the filarial nematode *Dipetalonema gracile* and from the arthropod *Ctenocephalides felis* have not been designated to any of the existing supergroups (Fig. 6).

Phylogenetic analysis of a small number of genes from *Wolbachia* bacteria of *O. volvulus* (wOvo) with *Wolbachia* bacteria of *Drosophila melanogaster* (wMel), *Wolbachia* bacteria of *B. malayi* (wBm) and related anaplasmatocae has revealed that the ancestor of all the extant

Wolbachia would probably be an intracellular parasite as the root mainly falls between the arthropod clade A and the nematode clade C and D (Panaram et al., 2007). This suggests that nematode *Wolbachia* share a common ancestor before they share a common ancestor with main arthropod supergroup A and B. Further availability of genome sequence data from other *Wolbachia* clades, such as, E, F, G and H would help in tracking the evolutionary history of *Wolbachia* and especially genome information from clade F could be particularly decisive as they include *Wolbachia* bacteria from both arthropods and nematodes. The presence of closely related *Wolbachia* from arthropods and nematodes in clade F does not rule out the possibility of horizontal transfer of *Wolbachia* between nematodes and arthropods (Fenn et al., 2006).

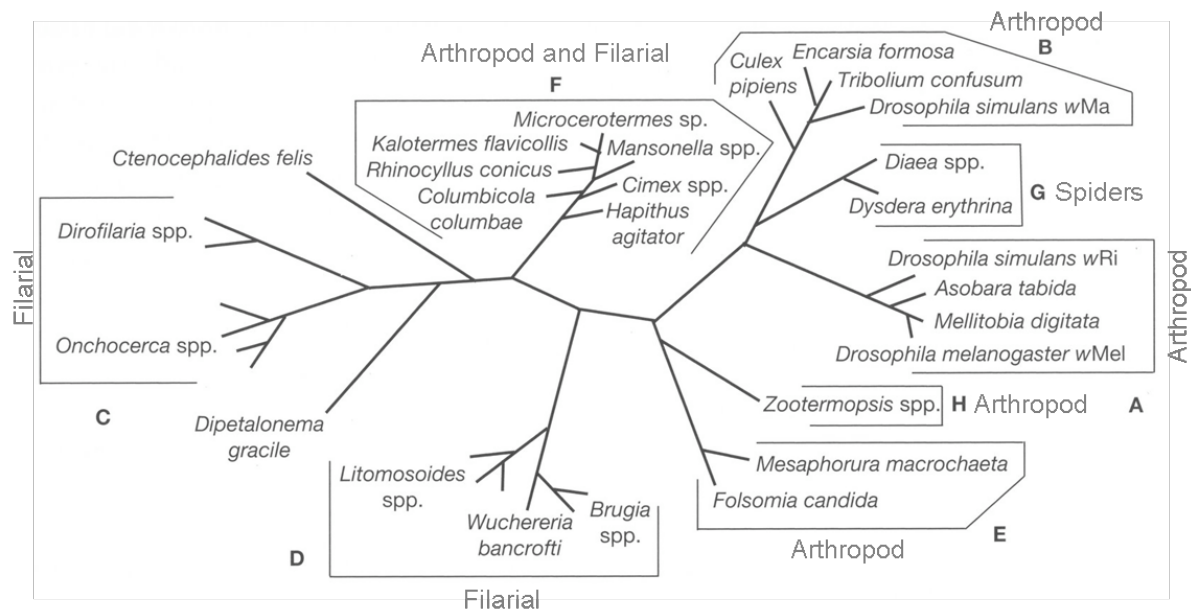


Fig. 6. Representation of *Wolbachia* spp. unrooted phylogeny based on different phylogenetic studies (Lo et al., 2007).

2.3.4. *Wolbachia* bacteria of filarial nematodes

Intracellular bacteria were first observed in filarial nematodes 30 years ago (McLaren et al., 1975; Kozek, 1977; Kozek and Marroquin, 1977). These bacteria were rediscovered in 1994 during the Filarial Genome Project funded by the World Health Organization (WHO) where the analysis of cDNA libraries generated from different life cycle stages of *B. malayi* revealed the presence of alpha-proteobacterial sequences implicating the occurrence of endobacterial DNA

(Williams et al., 2000). Phylogenetic analysis allowed these endobacteria to be identified as *Wolbachia* (Sironi et al., 1995). So far *Wolbachia* have been detected in a variety of filarial nematodes with a few notable exceptions such as *Acanthocheilonema vitea*, *Onchocerca flexuosa*, *Setaria equina* and *Loa loa* (McLaren et al., 1975; Bandi et al., 1998; Bandi et al., 2001; Taylor and Hoerauf, 1999; Hoerauf et al., 1999; Casiraghi et al., 2001; Casiraghi et al., 2004; Chirgwin et al., 2002; Egyed et al., 2002; Buttner et al., 2003; McGarry et al., 2003).

2.3.4.1. Evidence supporting the importance of *Wolbachia* in filarial nematode viability, development and fertility

In filarial nematodes, *Wolbachia* are located in the lateral cords of male and female worms and also in oocytes. But they are absent from the male reproductive system. Normally in human filarial nematodes harbouring *Wolbachia*, 100% of individual parasites contain *Wolbachia* suggesting the importance of *Wolbachia* in worm fertility and survival (Bandi et al., 1998; Taylor et al., 2000a; Taylor et al., 2000b). Hence, *Wolbachia* are potential chemotherapeutic targets for the control of filariasis. In rodent and human filarial nematodes, antibiotics such as tetracycline, doxycycline, rifampicin and azithromycin cause detrimental effect on parasite growth, development and fertility (Bosshardt et al., 1993; Genchi et al., 1998; Bandi et al., 1999; Hoerauf et al., 1999; Hoerauf et al., 2000; Langworthy et al., 2000; Smith&Rajan, 2000; Rao&Weil, 2002; Rao et al., 2002; Casiraghi et al., 2002; Chirgwin et al., 2003; Rajan, 2004). Antibiotic treatment leads to *Wolbachia* depletion in the female reproductive tract of *Dirofilaria immitis* and *Brugia pahangi* which eventually leads to degeneration of embryos, though reduced levels of *Wolbachia* often still are detected in the lateral cords (Kramer et al., 2003). Reports also suggest that antibiotic treatment leads to a 1000 fold reduction in *Wolbachia* which could still be detected by PCR in female hypodermis but not in female reproductive tract (Genchi et al., 2001). The embryonic blockade is *Wolbachia*-dependent as no similar antibiotic effects are seen in filarial nematodes devoid of *Wolbachia* (Hoerauf et al., 1999; Bandi et al., 2001; Volkmann et al., 2003). Also these effects are not seen when filarial nematodes harbouring *Wolbachia* are treated with antibiotics which are not efficient in killing *Wolbachia* (e.g. penicillin, gentamicin or ciprofloxacin) suggesting that these effects are associated with the presence of *Wolbachia* (Hoerauf et al., 2000; Taylor & Hoerauf, 2001). Recently, administration of doxycycline to *O. volvulus* and *W. bancrofti* infected patients has shown promising results of higher

macrofilaricidal and embryo toxic activity to these filarial worms and ameliorates some of the pathology in lymphatic filariasis (Hoerauf et al., 2000; Hoerauf et al., 2001; Hoerauf et al., 2003; Taylor et al., 2005; Debrah et al., 2006; Debrah et al., 2007; Hoerauf et al., 2008; Mand et al., 2008; Supali et al., 2008).

2.3.4.2. Role of *Wolbachia* in the pathogenesis of filarial infections

Wolbachia-associated molecules play a role in the host immunological response to filarial parasite invasion. Filarial infections are characterized by a humoral immune response, which results in B-cell proliferation leading to the generation of antibodies directed towards parasite and *Wolbachia*-specific antigens. So far *Wolbachia*-derived antigens such as *Wolbachia* surface protein, heat shock protein, aspartate aminotransferase, and Htr serine protease have been reported (Bazzocchi et al., 2000; Bandi et al., 2001; Punkosdy et al., 2001; Chirgwin et al., 2003; Fischer et al., 2003; Jolodar et al., 2004; Lamb et al., 2004). *Wolbachia* release during worm death can also initiate antigen specific mediation of immune regulatory factors from neutrophils and monocytes (Cross et al., 2001; Brattig, 2004; Hise et al., 2003). Drugs such as diethylcarbamazine or ivermectin kill microfilaria, which results in release of *Wolbachia* leading to adverse acute post-treatment reactions characterized by increases in plasma TNF- α , IL-6, and LPS-binding protein (Njoo et al., 1994; Turner et al., 1994), suggesting that *Wolbachia* released into the bloodstream by degenerating or dead microfilaria contribute to the acute adenolymphangitis and fever that occur after administering antifilarial drugs. Previously it was conceived that the immune response to *Wolbachia*-associated molecules mimics lipopolysaccharide (LPS)-like response through activation of the toll-like receptor 2 (TLR-2) and toll-like receptor 4 (TLR4) pathways (Brattig et al., 2004). *Wolbachia* endogen mimicking LPS appear to be involved in the eye inflammation observed in African river blindness. Infiltration of leukocytes such as neutrophils and eosinophils occurs in the cornea as a result of microfilarial invasion and death within the eye, leading to a loss of corneal transparency (Hall & Pearlmann, 1999). A recent report now suggests that the innate immune response to *Wolbachia* in *B. malayi* and *O. volvulus* are dependent on TLR-2, TLR-6, MyD88, and Mal but not on TLR-4, TRIF, or TRAM (Hise et al., 2007).

2.3.5. Genome of *Wolbachia* bacteria of *Brugia malayi* (wBm)

The Filarial Genome Project funded by the World Health Organization (WHO/TDR) was established in 1994 and in collaboration with *Wolbachia* consortium aided the annotation of complete genome of *Wolbachia* bacteria of *Brugia malayi* (wBm). wBm has a genome size of 1.1 Mb length determined by pulsed-field gel electrophoresis and restriction mapping. The genome consists of 1,080,084 nucleotides and has a GC content of 34% (Sun et al., 2001; Foster et al., 2004). Compared to most other bacteria, wBm has very low density of predicted genes and *Wolbachia* have lost a number of genes from many metabolic pathways in comparison to other α -proteobacteria. The wBm genome contains one copy of each of the ribosomal RNA genes (16S, 23S, and 5S), which do not form an operon. In comparison to other obligatory α -proteobacteria, *Wolbachia spp.* have retained an intact set of genes for translational processes and DNA replication and repair. Various functional genes encoded in *Wolbachia* genome are shown in Fig. 7.

wBm encodes complete pathways for the *de novo* biosynthesis of purines and pyrimidines. The *Wolbachia* genome also has all genes essential for the biosynthesis of fatty acids. wBm contains all the enzymes necessary for the biosynthesis of riboflavin and flavin adenine dinucleotide and it is postulated that wBm could be a main source of these coenzymes for the host nematodes as *B. malayi* genome lack genes essential for the biosynthesis of riboflavin (Ghedini et al., 2004; Ghedini et al., 2007). wBm has genes for the biosynthesis of heme and has all genes for maturation of c-type cytochromes. Heme constitutes a prosthetic group of cytochromes, catalase and peroxidase. The annotated sequence of the *B. malayi* genome reveals that they lack genes for heme biosynthesis enzymes. Heme could play a crucial role in the filarial parasite reproduction and development because of the requirement of heme in cytochromes involved in the production of steroid moulting hormones. Hence depletion of *Wolbachia* from filarial nematode could result in loss of availability of heme provided by *Wolbachia* to host nematodes which eventually result in detrimental effect on nematode viability, larval development, and reproduction (Foster et al., 2005). Genes for biosynthesis of glutathione, which has functions such as detoxification of methylglyoxal (Booth et al., 2003) and protection against oxidative stress through activation of the glutathione peroxidase–glutathione reductase system, are present in the wBm genome (Li et al., 2003; Brenot et al., 2004). wBm also has functional Type IV secretion genes essential for

successful persistence of endobacteria within their hosts (Sexton & Vogel, 2002). A similar set of genes have also been seen in the genome of *Wolbachia pipientis* wMel, an endosymbiont of *Drosophila melanogaster* (Wu et al., 2004).

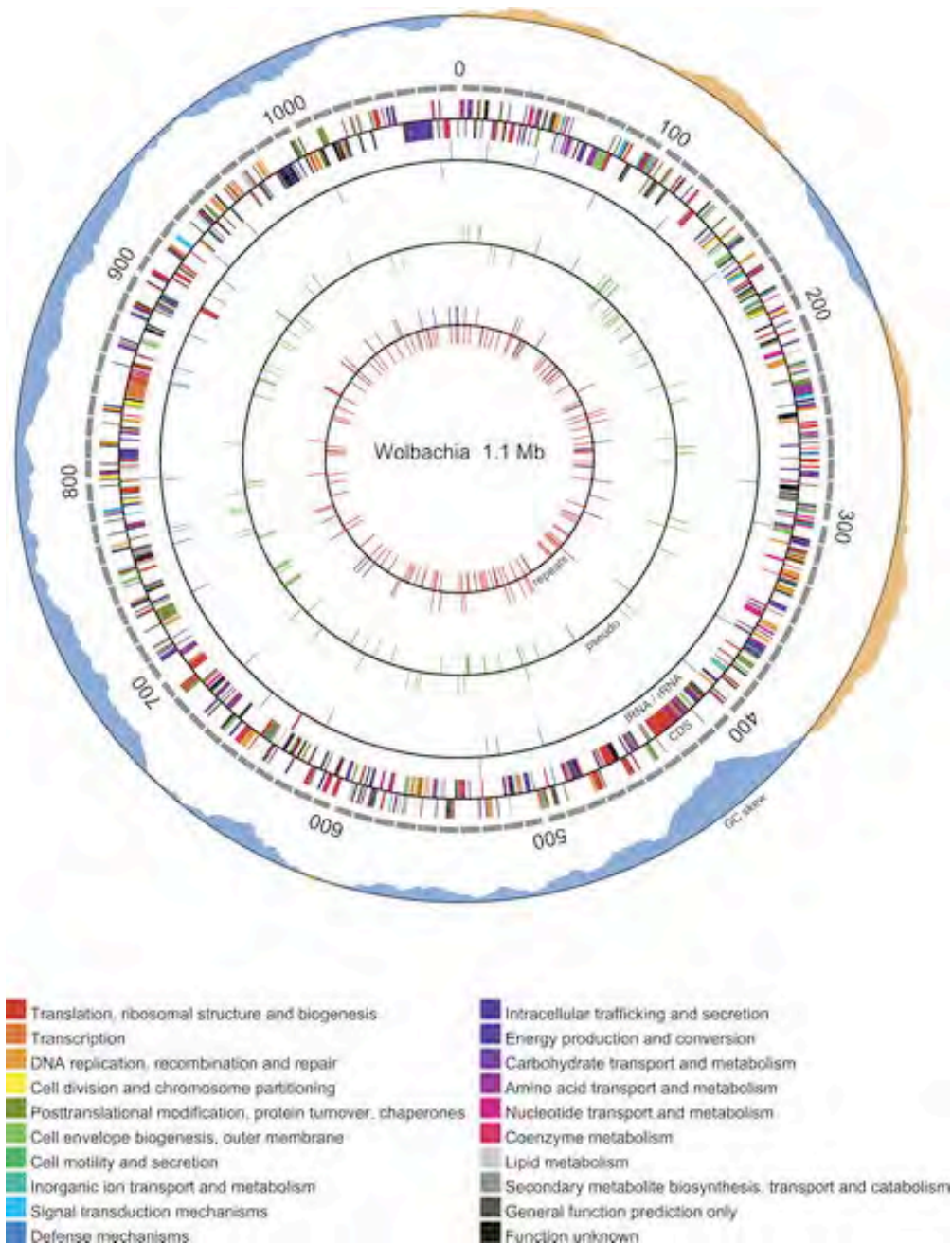


Fig. 7. Genogram of *Wolbachia* bacteria of *Brugia malayi* (wBm) (Foster et al., 2005).

Like many other endosymbionts, *wBm* lacks complete pathways for *de novo* biosynthesis of vitamins and cofactors such as Coenzyme A, NAD, biotin, lipoic acid, ubiquinone, folate, and pyridoxal phosphate, retaining only a few genes for the final steps in some of these pathways. These incomplete pathways may make *wBm* dependent upon the supply of those precursors from the host.

The completed *Wolbachia* genomes (*wBm* and *wMel*) encode limited metabolic capacity and hence the endobacteria might have a reliance on the basic metabolic pathways of their hosts. An unusual characteristic of *Wolbachia* genomes is that they contain a large proportion of repeated sequence (Wu et al., 2004; Foster et al., 2005) and in *wMel* genome, these repeats are associated with a large number of insertion elements (Wu et al., 2004). Both the *Wolbachia* genomes lack 20 genes for enzymes of cell-envelope LPS biosynthesis though a soluble endotoxin-like product of *Wolbachia* endosymbionts of filarial nematodes, including *B. malayi*, *B. pahangi*, *L. sigmodontis*, *O. volvulus*, and *D. immitis*, contribute to the immunology and pathogenesis of filarial diseases (Brattig et al., 2002; Taylor et al., 2000; Cross et al., 2001; Brattig, 2004; Hise et al., 2003; Freedman, 1998)

2.3.5.1. Genomic basis of *Wolbachia*-host parasitism and mutualism

Wolbachia endosymbionts in arthropod hosts behave as parasites while in nematode hosts, they behave as mutualists. Comparing the genomes of *Wolbachia* (*wBm* and *wMel*), it is postulated that 3 major groups of genes may be directly responsible for parasitism or mutualism: 1) Genes involved in Type IV secretion machinery and the proteins that it might secrete; 2) Genes encoding an unexpected diversity of proteins containing ankyrin-repeat motifs; 3) Genes from mobile bacteriophages (Fenn & Blaxter, 2006). The bacterial Type IV secretion systems play an important role in the pathogenesis of many bacterial species by exporting effector proteins, or 'virulence factors'. Type IV secretion systems are present in both the annotated *Wolbachia* genomes as two operons (Wu et al., 2004; Foster et al., 2005) and interestingly, one of the operons is closely related with a homologue of the *Wolbachia* surface protein (WSP) and this protein could be exported to the vacuole where *Wolbachia* are confined (Foster et al., 2005).

Ankyrin-repeat domains play an important role in protein-protein interaction (Bork, 1993) and there are around 110 different ankyrin-repeat-containing (ANK) in *D. melanogaster Wolbachia* (wMel). ANK present in bacteria are implicated to play a crucial role in host-pathogen interactions (Caturegli et al., 2000). wMel has 23 ANK genes (Wu et al., 2004), of which some of them are secreted, in contrast wBm has only 9 ANK genes and several of these genes appear to be pseudogenes (Foster et al., 2005), and of those seeming to be functional, only two are orthologues of wMel ANK proteins. *Wolbachia* ANK genes might also be involved in regulating the host cell cycle and presence of more ANK genes in arthropod *Wolbachia* and less ANK genes in nematode *Wolbachia* could attribute to the phenotypes they cause in their hosts (Fenn & Blaxter, 2006). Analysis of wMel reveal the presence of *Wolbachia*-specific bacteriophages (WO) in contrast, wBm does not contain WO prophages (Foster et al, 2005) and low number of ANK genes are related to lack of WO prophages in wBm. WO bacteriophages might also influence the parasitic nature of arthropod *Wolbachia*. Further analysis of the wBm genome could assist in identification of metabolic pathways that account for *Wolbachia*'s mutualism with *B. malayi*. wBm has a complete set of genes for the biosynthesis of riboflavin and heme (Foster et al, 2005) whereas *B. malayi* lack these genes and cannot synthesize riboflavins or heme endogenously. *Wolbachia* could be a source of heme for enzymes participating in biosynthetic pathways of hormones or other metabolites needed during moulting (Foster et al., 2005, Pfarr and Hoerauf, 2006). However, some nematodes including *Caenorhabditis elegans* lack the ability to synthesize heme but still are not dependent on an endosymbiont as a source of this metabolite. Occurrence of *Wolbachia* negative filarial nematode (Fenn & Blaxter, 2004; Werren et al., 1997; Bordenstein et al., 2003; Casiraghi et al, 2001; Grobusch et al., 2003) also suggests that there might not be a special role for *Wolbachia* in providing essential nutrients, although these bacteria might supplement other nutrients in restrictive environment.

2.3.6. Targeting *Wolbachia* as control of filariasis

Conventional strategies to combat filariasis have included vector control in the presence or absence of antiparasitic drugs (Hougard et al., 2001; Richards et al, 2001; Mackenzie et al., 2002; Ottesen, 2003). Anti-parasitic drugs such as diethylcarbamazine, albendazole, and ivermectin have been recently used for prevention of filarial infections. These drugs are primarily efficient against microfilariae (MF) with little effect on adult worms. Hence, repeated

administration of these drugs is required in endemic areas to prevent new infections (Campbell, 1991; Richards et al., 2001; Hoerauf et al., 2003). Repetitive drug administration can also lead to a possibility of development of drug resistance (Prichard, 1994; Prichard, 2001). In the last two decades, no new drugs have been developed which are both microfilaricidal and macrofilaricidal efficient in permanently sterilizing or killing the adult worms. This has required a search for new drugs for the control of filariasis especially targeting *Wolbachia* as these bacteria play a vital role in nematode fertility and pathogenesis. *Wolbachia* endosymbionts play a crucial role in the development and reproduction of their nematode hosts and also contribute significantly to the pathogenesis of filarial infection. *Wolbachia* are also known to contribute to the major adverse reactions observed after anti-parasitic treatment. Many reports suggest that antihelminthic drug causes severe systemic inflammatory responses such as fever, headache, dizziness and enlargement of lymph nodes which could be associated to the release of *Wolbachia* from dead MF (Boreham & Atwell, 1983; Francis et al., 1985; Cross et al., 2001; Supali et al., 2008). Hence, use of anti-*Wolbachia* chemotherapy against filarial parasites has paved the way to a novel approach for filarial disease control and eradication.

2.4. Antibiotic treatment in murine filariasis

Antibiotics active against Rickettsiaceae, particularly tetracyclines, rifampicin and chloramphenicol, were effective in reducing the filarial larval molt (from L3 to L4) and larval development *in vitro* (Smith and Rajan, 2000; Rao et al., 2002). The symbiosis between filarial nematodes and *Wolbachia* bacteria in rodent mouse model was exploited to study the efficacy of antibiotic therapy of filariasis. Previous studies have shown effects of antibiotics on filarial nematodes in experimental animal models (Bosshardt et al., 1993; Roa et al., 2002; Bandi et al., 1999; Hoerauf et al., 1999; Hoerauf et al., 2000; Langworthy et al., 2000; Townson et al., 2000). Depletion of *Wolbachia* after tetracycline treatment resulted in filarial sterility because of interruption of embryogenesis and also inhibited larval development and adult worm viability (Volkman et al., 2003). The success obtained with anti-*Wolbachia* therapy in experimental animal models lead to human clinical field trials.

2.4.1. Tetracyclines

Tetracyclines are primarily bacteriostatic affecting multiplying micro-organisms and are believed to inhibit the bacterial protein synthesis. The site of action of these antibiotics is the bacterial ribosome (Sande and Mandell, 1992). The tetracyclines gain access to the ribosome by directly passing through the lipid bilayer by an active transport system that pumps tetracyclines through the cytoplasmic membrane. In the bacterial cell, it binds to the ribosome 30S subunit and prevents access of aminoacyl t-RNA to the acceptor site on the m-RNA-ribosome complex. This inhibits the addition of amino acids to the growing peptide chain thereby blocking prokaryotic protein synthesis (Sande and Mandell, 1992). Doxycycline, a semisynthetic derivative of the antibiotic tetracycline, is efficient against *Wolbachia*.

2.4.2. Anti-*Wolbachia* therapy in human filariasis

Doxycycline was used to test the hypothesis that elimination of *Wolbachia* could be beneficial in reducing human filarial infections. Patients with onchocerciasis infections received a 6 week course of daily doxycycline treatment (100 mg/day). Treatment resulted in depletion of *Wolbachia* in worms, and caused extensive degeneration of embryos by 4 months posttreatment (Hoerauf et al., 2000). Loss of *Wolbachia* resulted in sterilization of female worms leading to significant low or no microfilaridemia (Hoerauf et al., 2000). Use of doxycycline in combination with ivermectin also lead to significant reduction in microfilaridemia following lower levels of *Wolbachia* in adult worms (Hoerauf et al., 2001; Hoerauf et al., 2003). A double-blind, randomised, placebo-controlled field trial of doxycycline (200 mg per day) for 8 weeks in 72 individuals infected with *W. bancrofti* resulted in significant activity against adult worms (macrofilaricidal activity) and microfilaraemia (microfilaricidal activity) (Taylor et al., 2005). Another double-blind, placebo-controlled trial of a 6-week regimen of 200 mg/day doxycycline showed that anti-*Wolbachia* treatment lead to amelioration of supratesticular dilated lymphatic vessels and with an improvement of pathology in lymphatic filariasis patients (Debrah et al., 2006). Recently, a shorter 4-week regimen of doxycycline seems to be sufficient in killing adult *W. bancrofti* (Debrah et al., 2007). In brugian filariasis, a 6-week regimen of doxycycline (100 mg/day) followed by diethylcarbamazine (6 mg/kg) plus albendazole (400 mg) lead to a decrease in microfilaremia and reduced adverse reactions to antifilarial treatment in *B. malayi*-infected

patients (Supali et al., 2008). Targeting *Wolbachia* of filarial nematodes with doxycycline have macrofilaricidal activity and reduces adverse reactions and early stages of lymphatic pathology and hence these endosymbionts represents an ideal target for the development of new antifilarial chemotherapies.

2.5. *Wolbachia* and filarial nematode endosymbiosis and *Litomosoides sigmodontis* phosphate permease (*Ls-ppe-1*)

As discussed above, several anti-rickettsial antibiotics deplete *Wolbachia*, leading to worm sterility and inhibition of larval and adult worm development. However, the molecular mechanism governing the endosymbiosis between *Wolbachia* and their filarial nematodes is still unclear. The annotated *wBm* genome suggests that *wBm* could provide essential metabolites to the nematode and vice versa. Microarray is a technique which could be used to discover genes which might play an important role in the endosymbiosis but alternatively RNA differential display can also be used which has an advantage of over microarrays that no prior sequence information is required to discover genes that are differentially expressed in response to antibiotic treatment.

RNA differential display technique was used to discover nematode gene that are up-regulated in response to *Wolbachia* depletion. Several genes were found to be differentially up-regulated in response to *Wolbachia* depletion and one of the up-regulated genes (*Ls-ppe-1*) had homology to the phosphate permease family of proteins which has orthologues in *Caenorhabditis elegans*, *Acanthocheilonema viteae*, *O. volvulus* and *B. malayi*. In comparison to control (untreated) worms, there was threefold up-regulation of *Ls-ppe-1* at the mRNA level in tetracycline treated worms. In female worms, the *Ls-ppe-1* up-regulation showed a bimodal pattern whereas in male worms there was only an increase in expression at days 3 and 6 of tetracycline treatment (Heider et al., 2006).

The up-regulation of *Ls-ppe-1* was shown to be *Wolbachia*-dependent as *A. viteae* devoid of *Wolbachia* showed no up-regulation of *ppe-1* when treated with tetracycline and also there was no up-regulation of *Ls-ppe-1* in response to heat shock or oxidative stress, suggesting that the death of *Wolbachia* does not cause the up-regulation (Heider et al., 2006). The full-length cDNA

of *Ls-ppe-1* is 1729 bp large and has an open reading frame of 523 amino acids. Three variant transcripts of *Ls-ppe-1* have been identified. They have the same coding potential but differ in the 3' untranslated region after the poly (A) signal. The deduced protein sequence of *Ls-ppe-1* (Ls-PPE) has an N-terminal signal sequence with a predicted cleavage site between amino acid positions 49 and 50. Ls-PPE has 12 predicted transmembrane helices suggesting that Ls-PPE is a membrane bound protein (Heider et al., 2006). *Ls-ppe-1* up-regulated in response to *Wolbachia* depletion could play an important role in the nematode biology and in the endosymbiosis between *Wolbachia* and their filarial nematodes hosts (Pfarr and Hoerauf, 2006).

2.6. Rodent filarial nematode-animal model

The life cycle of the rodent filarial nematode *Litomosoides sigmodontis*, a close relative of human filarial parasites, can be feasibly maintained in rodents. *L. sigmodontis* is the only filarial parasite that produces patent infection in laboratory mice and the pattern of infection and migration of developing parasites mimics that of human filarial parasites such as *W. bancrofti* and *O. volvulus* (Hoerauf et al., 1999). Like most human filarial nematodes, *L. sigmodontis* worms also harbour *Wolbachia* endosymbionts (Fig. 8). During a blood meal, mites (*Ornithonyssus bacoti*) carrying infective L3 larvae feed on BALB/c mice, injecting L3 larvae into the mice which then develop into male and female adult worms [L3-L4-L5]. Later, the fertilized female worms start producing numerous microfilariae which are taken up by mites during subsequent blood meal where they undergo moulting to develop into L3 larvae [L1-L2-L3] (Hoerauf et al., 1999). This nematode-animal model provides an excellent platform to study the endosymbiosis involved between *Wolbachia* and their filarial hosts.

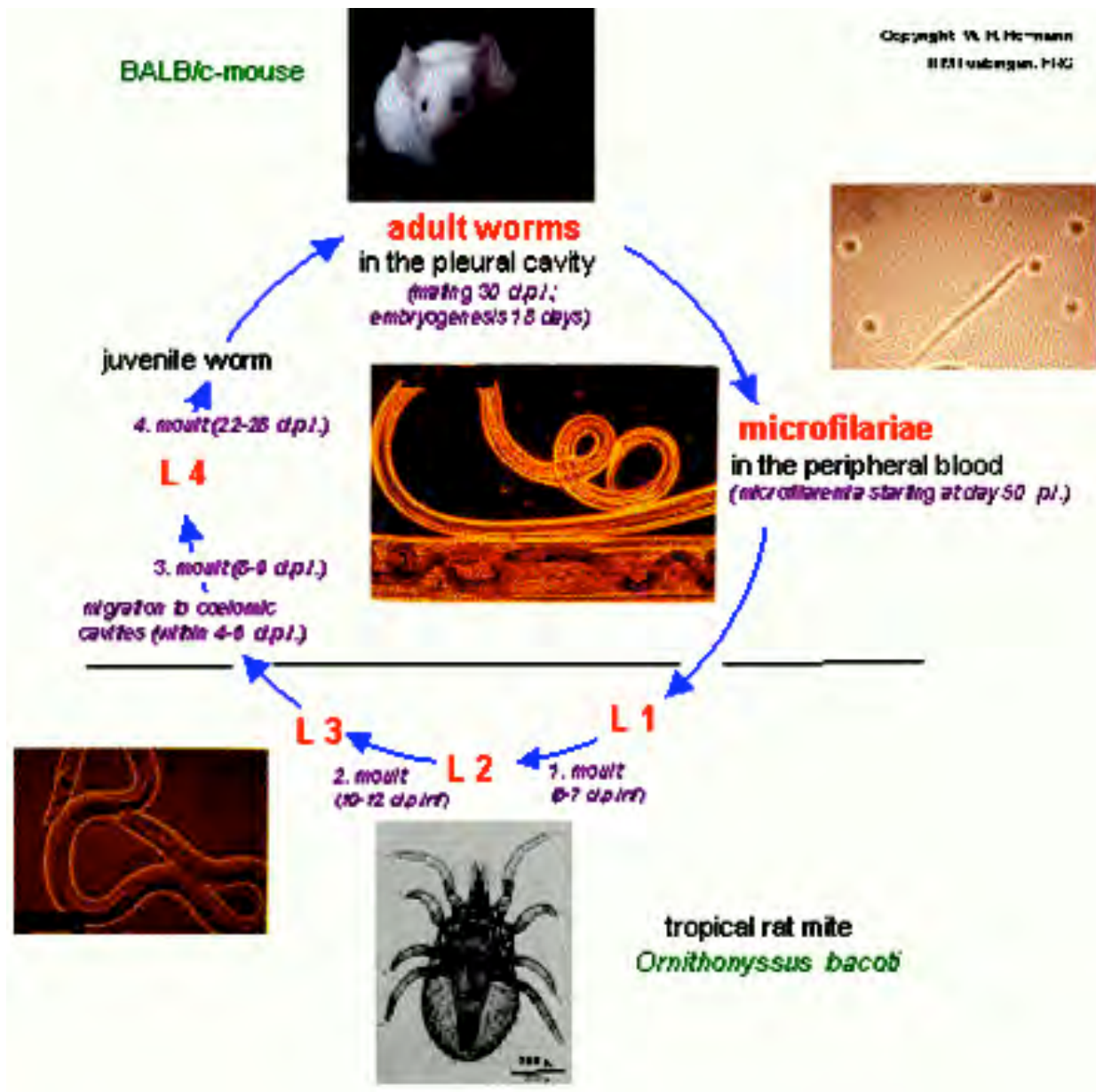


Fig. 8. Life-cycle of *Litomosoides sigmodontis* (Courtesy: Institute for tropical medicine, Tuebingen, Germany).

3. Aims of the thesis

Filarial infections are major causes of morbidity in tropical developing countries and development of resistance to current drugs has required the search for new drugs. *Wolbachia* are considered excellent targets for the discovery of new anti-filarial drugs because depletion of *Wolbachia* in rodent filarial nematodes results in inhibition of larval moulting (from L3 to L4) *in vitro* (Smith and Rajan, 2000; Rao et al., 2002) and this effect is also seen *in vivo* where filarial sterility occurs due to interruption of embryogenesis resulting in inhibition of larval and adult worm development (Hoerauf et al., 1999; Volkmann et al., 2003). Similarly, anti-*Wolbachia* therapy in human filariasis has met with great success where depletion of *Wolbachia* has macrofilaricidal activity (Hoerauf et al., 2001; Hoerauf et al., 2003; Taylor et al., 2005; Debrah et al., 2006; Supali et al., 2008). Hence, anti-*Wolbachia* therapy has proven to be an effective treatment strategy in combating filariasis. But still the exact molecular mechanism governing the endosymbiosis between *Wolbachia* bacteria and their filarial nematodes is unclear. Also it is unknown what the precise role *Wolbachia* have in the development of male and female adult worms. The annotation analysis made from the *Wolbachia* genome sequence suggests that several biochemical pathways of *Wolbachia* can deliver essential metabolites to the nematode hosts, and vice versa. Study of mRNA gene expression in *Litomosoides sigmodontis* during *Wolbachia* depletion revealed up-regulation of several nematode genes and one gene showed homology to a phosphate permease gene (*Ls-ppe-1*) (Heider et al., 2006). Studying regulation of *Ls-ppe-1* at protein level and structural localization of this protein and *Wolbachia* can bring an insight into role of *Ls-ppe-1* in *Wolbachia*-nematode endosymbiosis.

Therefore, the goals of the present thesis were:

1. To ascertain the role of *Wolbachia* in the development of male and female adult worms.
2. To demonstrate the up-regulation of *L. sigmodontis* phosphate permease (Ls-PPE) and *Onchocerca volvulus* phosphate permease (Ov-PPE) at the protein level during *Wolbachia* depletion and to compare its localization with respect to that of *Wolbachia* in filarial worms.

4. Materials and Methods

4.1. Chemicals

Advanced protein reagent solution (ADV01)	Cytoskeleton, Denver, USA
Agarose	Fermentas. St. Leon-Rot, Germany
Ampicillin	Sigma-Aldrich, Munich, Germany
1-bromo-3-chloro-propane	Sigma-Aldrich, Munich, Germany
Bromophenol blue	Sigma-Aldrich, Munich, Germany
BSA	Roth, Karlsruhe, Germany
Circle Grow	Q-Biogene, Cambridge, United Kingdom
DEPC-water	Ambion, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Biomol, Hamburg, Germany
Ethylene diamine tetra acetic acid	Sigma-Aldrich, Munich, Germany
FCS	PAA, Cölbe, Germany
Guanidine hydrochloride	Sigma-Aldrich, Munich, Germany
IPTG	Sigma-Aldrich, Munich, Germany
Isoforene	Abbot, Wiesbaden, Germany
Isopropanol	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
LB Agar	Sigma-Aldrich, Munich, Germany
Methanol	Merck, Darmstadt, Germany
PMSF	Sigma-Aldrich, Munich, Germany
Sodium dihydrogen phosphate	Merck, Darmstadt, Germany
Sodium Chloride	Roth, Karlsruhe, Germany
Sodium Hydroxide	Merck, Darmstadt, Germany
RPMI-1640 media with L-glutamine	Sigma-Aldrich, Munich, Germany
Buffer tablets 1/1	Merck, Darmstadt, Germany
Hydrochloric acid	Sigma-Aldrich, Munich, Germany
SOC medium	Invitrogen, Karlsruhe, Germany
Tetramisole Hcl	Sigma-Aldrich, Munich, Germany
Tris-hydrochloride	Roth, Karlsruhe, Germany
Trizol®	Invitrogen, Karlsruhe, Germany

TWEEN [®] 20	Sigma-Aldrich, Munich, Germany
X-GAL	Roth, Karlsruhe, Germany

4.2. Equipment used

Centrifuges	Hettich Mikro 20'', Tuttlingen, Germany Hettich, Universal 32R, Tuttlingen, Germany
Cuvettes	Eppendorf, Hamburg, Germany
Disposable pipettes	Copan Innovation, Brescia, Italy
Digital balance	Sartorius AG, Goettingen, Germany
Digital pH meter	Mettler Toledo, Columbus, USA
Electrophoresis set up for agarose gels	Bio-Rad, Munich, Germany
ELISA plates	Greiner Bio-one, Frickenhausen, Germany
Eppendorf 5810 R, 5417 R and 5415 D	Eppendorf, Hamburg, Germany
Eppendorf BioPhotometer	Eppendorf, Hamburg, Germany
Gel blot system	Bio-Rad, Munich, Germany
Glass-glass homogenizer	Sartorius BBI Systems, Melsungen, Germany
Heating block model III Thermostat 5320	Eppendorf, Hamburg, Germany
Ice machine AF-80	Scotsman, Vernon Hills, USA
Incubator	Heraeus, Düsseldorf, Germany
Microscope	Carl Zeiss, Cologne, Germany
Needles	B. Braun, Melsungen, Germany
Neubauer cell counter	Brandt, Wertheim, Germany
Object slides	Engelbrescht, Germany
Laminar flow system	Kendro, Langenselbold, Germany
Magnetic stirrer	IKA-Labortechnik, Staufen, Germany
Polyacrylamide electrophoresis set-up	Bio-Rad, Munich, Germany
Thermocycler	MWG Primus, Ebersberg, Germany
Reichert-Jung 1140 Autocut microtome	Leica Microsystems, Wetzlar, Germany
Rotor gene 6000	Corbett Research, Sydney, Australia
Spectra Max340 Microwell Reader	Molecular Devices, Sunnyvale, USA

Syrings (1ml)	B. Braun, Melsungen, Germany
Trans-blot semi dry transfer cell	Bio-Rad, Munich, Germany
Vortexer	IKA-Labortechnik, Staufen, Germany
Water purifier Milli-Q plus	Millipore, Schwalbach , Germany

4.3. Bacterial strain

1. DH5 Fé: (F⁻/endA1 hsdR17 (rk-mk+) supE44 thi-1 recA1 gyrA (NaIr) relA1 (lacZYAargF) u169 (m80lacZ M15) (Invitrogen, Karlsruhe, Germany).

4.4. Animals

Mongolian gerbils- *Meriones unguiculatus* (WT) and IL-5-deficient BALB/c mice of different age were used for the experiments which were raised in the animal house of Institute for Medical Microbiology, Immunology and Parasitology, University of Bonn. All animal experiments were performed according to the laws prescribed by the German Federal Government and the ethical committee.

4.5. Standard buffers

Table 1

Name of the buffer	Ingredients
Phosphate buffered saline (PBS)	50 mM Na ₂ HPO ₄ /NaH ₂ PO ₄ (pH 7.4) 150 mM NaCl
Tris-(hydroxymethyl)-aminoethane buffered saline (TBS)	20mM Tris-HCl (pH 7.4) 150 mM NaCl
50X TBE	2M Tris-base 89 mM Boric Acid 100mM EDTA (pH 8.0)

Table 2
Destaining solutions

Destaining solution 1	Destaining solution 2
50%(v/v) Methanol	10%(v/v) Methanol
10%(v/v) Acetic acid	10%(v/v) Acetic acid
40%(v/v) Water	80%(v/v) Water

4.6. Polymerase chain reaction (PCR) and other molecular biology reagents

DNase	Ambion, Darmstadt, Germany
DNase Inhibitor	Ambion, Darmstadt, Germany
dNTPs	Qiagen, Hilden, Germany
HotStarTaq® DNA polymerase	Qiagen, Hilden, Germany
Magnesium chloride (MgCl ₂)	Qiagen, Hilden, Germany
Oligo-dTs	Qiagen, Hilden, Germany
Reverse Transcriptase (RT)	Qiagen, Hilden, Germany
RNase Inhibitor	PeqLab, Erlangen, Germany
Sybr Green®	Roche, Mannheim, Germany
10x Buffer	Qiagen, Hilden, Germany
Lithium chloride	Ambion, Darmstadt, Germany
Linear acrylamide	Ambion, Darmstadt, Germany

Primers

<i>Ls-ppe-1</i> Forward	5'-AGGCCAAGTTTACTGGCTGTT _{3'}
<i>Ls-ppe-1</i> Reverse	5'-CTGGATGTTTCGACAACGAAGT _{3'}
<i>Ls-actin-1-1</i> Forward	5'-GTGCTACGTTGCTTTGGACT _{3'}
<i>Ls-actin-1-1</i> Reverse	5'-GTAATCACTTGGCCATCAGG _{3'}

Ls-*FtsZ* Forward 5'-CGATGAGATTATGGAACATATAA₃'

Ls-*FtsZ* Reverse 5'-TTGCAATTACTGGTGCTGC₃'

Primers were synthesized in Qiagen, Hilden, Germany.

Taqman Hybridization 6-FAM-CAGGGATGGGTGGTGGTACTGGAA-TAMRA

Probe was synthesized in Operon Biotechnologies, Cologne, Germany.

4.7. Animal models

Two rodent nematode models, a) infection of Mongolian gerbils (*Meriones unguiculatus*) with *L. sigmodontis* and b) infection of mice with *L. sigmodontis* were used in the study. The Mongolian gerbil *L. sigmodontis* model was used to study the L1 to L3 development and subsequent sex-ratio distortion in adult worms following infection of the intermediate mite hosts with *Wolbachia*-depleted *L. sigmodontis*. The mouse *L. sigmodontis* model was used to study the depletion of *Wolbachia* and regulation of phosphate permease (Ls-PPE) at the protein level. Worms from this model were also used in the localization of *Wolbachia* and Ls-PPE using immunohistology.

4.7.1. Infection cycle of *L. sigmodontis*

The infection cycle of *L. sigmodontis* was maintained by passage through cotton rats (*Sigmodon hispidus*) and mites (*Ornithonyssus bacoti*). Mice and gerbils were infected naturally through the mite vector. The infected mites were maintained under 80-90% humidity and at 26-28°C. Naïve mites were kept in a glass flask filled with 10 cm high layer of bedding material and were covered with a removable sieve and 10 days prior to infection of mice or gerbils, they were allowed to have a blood meal over night on infected cotton rats with microfilareamia of more than 1000 MF/μl blood. Afterwards the mites which fed on cotton rats were collected in bedding material. Mites were kept for 10 days during which the MF (L1) ingested by the mites developed into infective stage 3 larvae (L3) which are then transmitted to mice or gerbils during a blood meal as described above for cotton rats (Fig. 8).

4.7.1.1. *L. sigmodontis* Mongolian gerbil model and sex-ratio distortion study

Mongolian gerbils (*Meriones unguiculatus*) are fully permissive to *L. sigmodontis* infection allowing normal development and reproduction of these filarial nematodes. In comparison to

BALB/c mice, *L. sigmodontis* infection in Mongolian gerbils is characterized by higher microfilaraemia and worm load and hence makes this system ideal for studying sex-ratio distortion.

Primary infection and tetracycline treatment

Mongolian gerbils were maintained at the Institute for Medical Microbiology, Immunology and Parasitology, University Clinic Bonn, Germany. Gerbils (8-12 weeks old) were infected with *Litomosoides sigmodontis* by mites (*Ornithonyssus bacoti*) carrying infective L3 larvae as described above (Hoerauf et al., 1999). Tetracycline treatment was started 3 months post-infection when MF were detectable in the blood. Before tetracycline treatment, the MF count was determined using Neubauer cell counter (Hoerauf et al., 1999). The microfilaraemic blood was then used to extract MF DNA for *Wolbachia* quantitative PCR (qPCR) to assess the initial *Wolbachia* load per MF. All the treated animal groups, designated as “1° Tet”, received tetracycline dihydrochloride (tetracycline-HCl, Sigma-Aldrich, Taufkirchen, Germany) orally at 0.5% (w/v) in drinking water for 6 weeks. The medicated drinking water was prepared fresh daily. Controls, designated as “1° Con”, were infected but left untreated. These treatment groups were considered primary infections (Fig. 9).

In each primary infection experiment, both 1° Tet and 1° Con groups consisted of 5 gerbils, 2 males and 3 females. At the end of treatment, blood was collected from gerbils for monitoring microfilaraemia as described above. DNA was then extracted from blood-borne MF for qPCR. One and 3 months post tetracycline treatment, blood was collected from 1° Con and 1° Tet gerbils to monitor microfilaraemia and *Wolbachia* levels to evaluate tetracycline treatment.

Infection of intermediate host-mites with *Wolbachia*-depleted larvae

After confirming the depletion of endobacteria from the MF by tetracycline treatment, the 1° Tet and 1° Con gerbils were used to infect naïve mites (*O. bacoti*) as described previously (Hoerauf et al., 1999). Populations of mites that fed on 1° Tet gerbils and 1° Con gerbils were designated as “Tet” or “Con” mites, respectively.

Monitoring development of larvae (L1-L3) in intermediate mite hosts

After 14 days, 100 mites from Tet and Con mites were dissected for the presence of L3 larvae to monitor the success in molting from L1 to L3. Additionally, 50 L3 larvae isolated from Tet and Con mites were pooled in batches of 10. DNA was extracted from the pooled L3 larvae and used for quantification of *Wolbachia* using qPCR.

Secondary infection of gerbils with *Wolbachia*-depleted larvae

The remaining Tet and Con mites were used to infect naïve gerbils designated as secondary infections (2° Tet, 7 gerbils; 2° Con, 7 gerbils) (Fig. 9). These 2° Tet and 2° Con gerbils were reared for 3 months to study the outcome of adult worm development. Before necropsy, blood was collected from Tet and Con gerbils to monitor microfilaraemia and *Wolbachia* levels in MF.

Worm recovery at necropsy

Three months after the secondary infection, the gerbils were euthanized and the worms taken out of the pleural cavity. Worms were washed twice in sterile PBS to remove blood cells and other debris. Worms were separated by sex, counted and their length was measured. Worms were then frozen at -20 °C for later DNA extraction.

L. sigmodontis Mongolian gerbils Model and Tetracycline Treatment

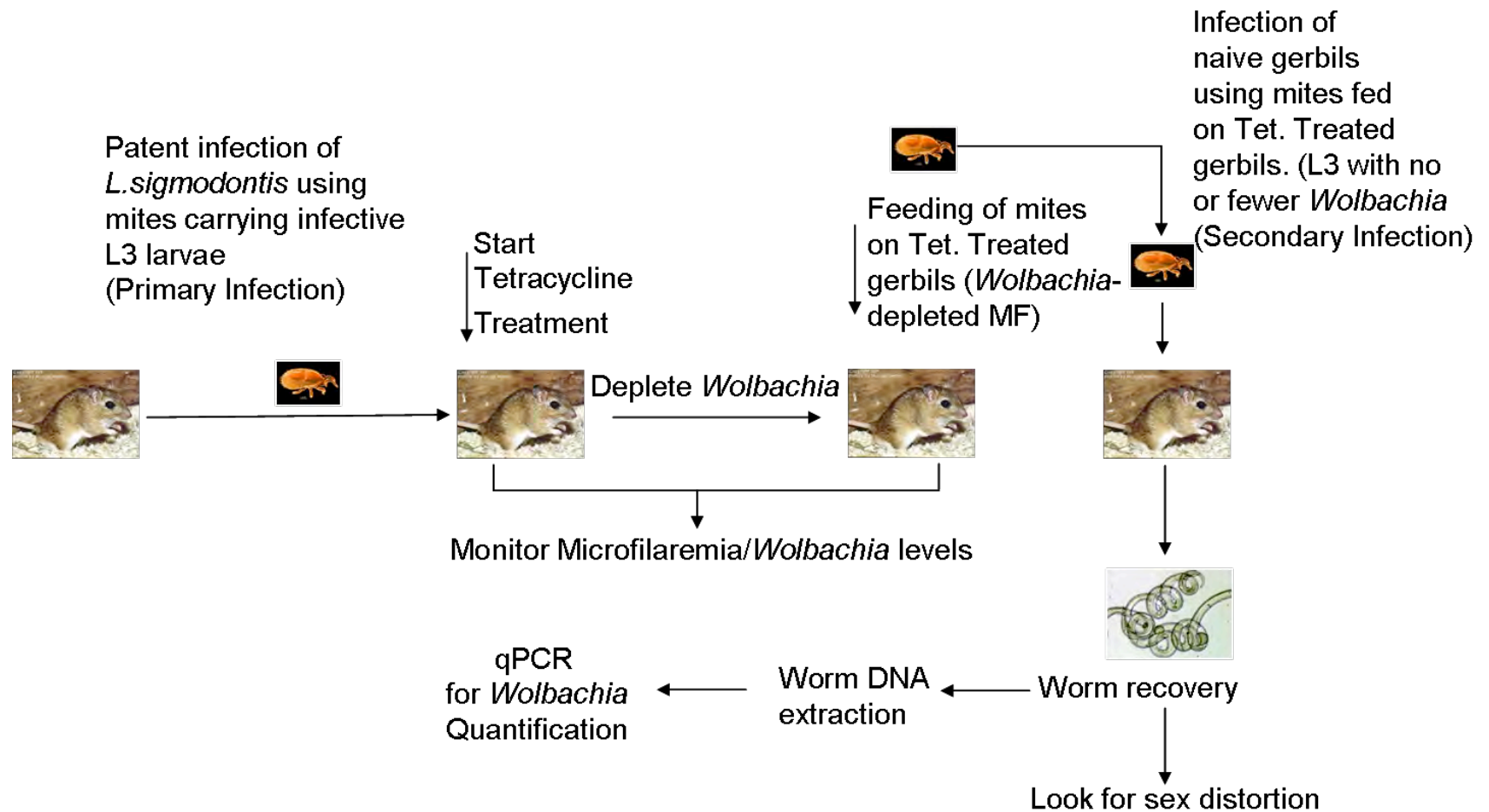


Fig.9. *L. sigmodontis* Mongolian gerbils model and tetracycline treatment.

4.7.1.2. *Wolbachia* depletion and *Ls-ppe-1* regulation study

IL-5-deficient BALB/c mice are fully permissive for *L. sigmodontis* infection allowing development and reproduction of filarial worms and hence were used in the study. IL-5-deficient mice have up to 200-fold higher parasite load and prolonged patency in comparison to wild-type mice (Volkmann et al., 2003). This extended filarial infection in IL-5-deficient BALB/c mice allows prolonged therapeutic regimes of more than 2 weeks which is not possible in wild-type BALB/c mice. Another advantage of using IL-5-deficient BALB/c mice instead of wild-type BALB/c mice is the reduction of inflammatory nodule formation around the adult worms which facilitates easier recovery for further parasitological and molecular analyses. IL-5-deficient BALB/c mice (6–8 weeks old) were infected with *L. sigmodontis* by mites (*Ornithonyssus bacoti*) carrying infective L3 larvae as described above (Hoerauf et al., 1999).

Tetracycline treatment

After infection of mice with infective L3 larvae, it takes 2 months for the mice to attain patency when released L1 larvae are detected in the blood. Tetracycline treatment was started at the onset of a patent infection, i.e. at day 58 post-infection, mice were intraperitoneally injected with 50 mg tetracycline per kg body weight per day (Hoerauf et al., 1999). Nematodes were collected from the pleural cavities of mice on days 6, 15 and 36 of the treatment (Fig. 10). Adult worms were washed thrice in 1x PBS to get rid of blood and other debris. Worms were then separated based on sex and some worms were snap frozen in liquid nitrogen and stored at -80°C for further analysis such as RNA extraction. The rest of the worms were stored in 4% paraformaldehyde in PBS for immunohistological studies.

L. sigmodontis Mouse model and Tetracycline Treatment

Patent infection of
L. sigmodontis
using mites carrying
infective L3 larvae

Intraperitoneal injection of 50 mg tetracycline/kg body per day for 36 days

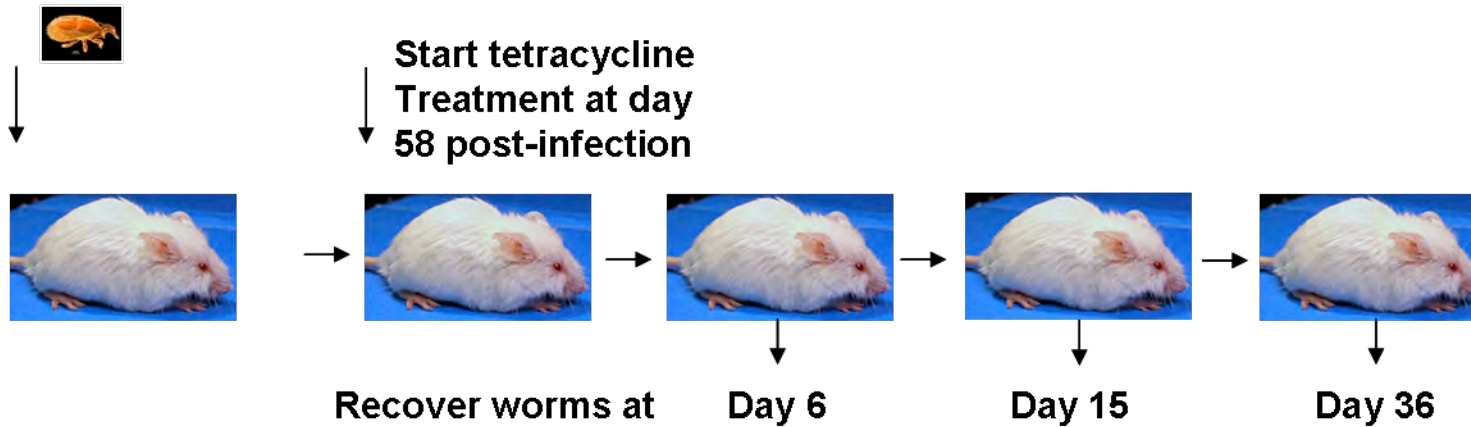


Fig.10. *L. sigmodontis* mouse model and tetracycline treatment

4.8. Production of anti-peptide antiserum against *L. sigmodontis* phosphate permease (Ls-PPE) and *Onchocerca volvulus* phosphate permease (Ov-PPE)

4.8.1. Anti-peptide antiserum production

Anti-peptide antiserum production methodology employs the use of synthetic peptide antigens to target specific antigenic epitopes. Raising anti-serum to immunogenic peptides is the simplest method of obtaining antibodies against non-isolated proteins, for example, against putative protein sequences derived from DNA sequence information. This approach is also useful when the isolation of the antigen is difficult or time consuming, or when the antigen is a member of a large protein family. Anti-peptide sera production was done under contract by Eurogentec in Belgium.

4.8.1.1. Peptide design

A combination of online prediction software, BepiPred 1.0 Server, NetCTL 1.2 Server and NetMHC 3.0 Server, were used to select immunogenic peptide sequences. The overall aim was to identify regions of the protein that were most likely to be accessible on the surface of the full length protein. Based on the deduced amino acid sequence of *L. sigmodontis* phosphate permease (Ls-PPE) and *O. volvulus* phosphate permease (Ov-PPE), two potential peptide candidates each for Ls-PPE and Ov-PPE were chosen (Table 3).

Table 3: Potential peptide used for peptide immunization

Protein	Peptides used for immunization
Ls-PPE	CQASKTENVESSTVIK ILPTDNRATDNRTMKC
Ov-PPE	CMRKGVIDLAVYNGSE DTANSSFGTSVGSKVLK

4.8.1.2. Coupling of peptides to carrier protein

In order to elicit a strong immune response against the peptides, the peptides were coupled to carrier protein. Normally carrier proteins such as KLH, OVA, BSA or THY are used to increase the molecular weight of peptide antigen making them capable of eliciting a strong immune response. In the immunization protocol, 5 mg of each peptide were coupled to KLH carrier protein.

4.8.1.3. Testing pre-immune rabbit serum for cross reacting nematode antibodies

Before starting immunization schedule, 10 pre-immune rabbit sera were tested for cross reacting nematode antibodies by Western blot (5 rabbit sera for Ls-PPE and 5 rabbit sera for Ov-PPE). The serum showing least cross reactivity to nematode antigen in Western blot was chosen for immunization. Out of 5 rabbits tested, two rabbits each for Ls-PPE and Ov-PPE were picked for immunization with carrier protein coupled peptides.

4.8.1.4. Immunization schedule

The immunization schedule used an 87-day protocol with 4 immunizations at day 0, day 14, day 28 and day 56. Bleeds were collected at 4 time points on day 0, day 38, day 66 and final bleed on day 87 (Fig. 11).

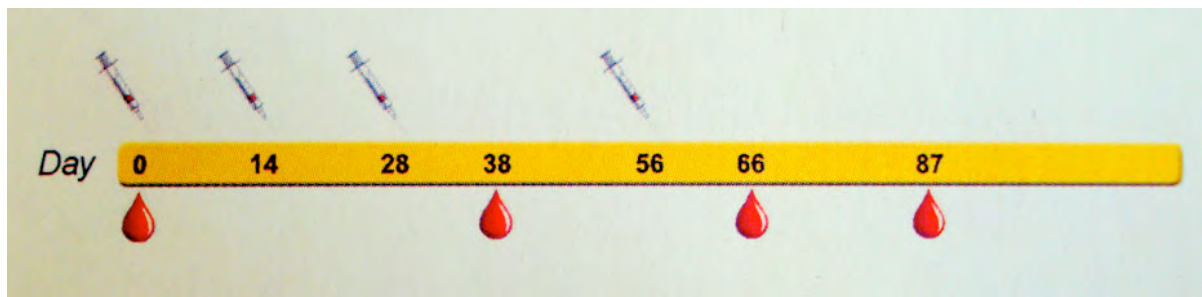


Fig. 11. Immunization schedule

Bleeds were analysed using Western blot to check for presence of anti-peptide antibodies against Ls-PPE and Ov-PPE.

4.9. Nematode preparation

4.9.1. *Litomosoides sigmodontis*

L. sigmodontis adult worms were isolated from the pleural cavities of infected rodent hosts (*Meriones unguiculatus* (WT) and IL-5 deficient BALB/c mice). Worms were washed thrice in PBS to get rid of blood and other debris. For RNA and DNA extraction, single adult female worms or 10 female worms pooled, were taken in individual eppendorf tubes and were frozen immediately in liquid nitrogen and stored at -80°C. For immunohistology, adult female worms were stored in 4% formaldehyde at RT.

4.9.2. *Onchocerca volvulus*

O. volvulus worm nodule samples were kindly provided by Dr. Sabine Specht. Nodules were extirpated from doxycycline-treated and untreated control patients at 4 months post 6 weeks doxycycline treatment was used for the study (Hoerauf et al., 2003).

4.10. Nematode protein extraction

O. volvulus nodule proteins were extracted from the phenol-ethanol supernatant obtained after the extraction of RNA and DNA using Trizol reagent. Proteins were precipitated from the phenol-ethanol supernatant with isopropanol (0.8 ml of isopropanol was used per 1 ml of Trizol reagent used in initial RNA extraction). The samples were stored for 10 min at 15 to 30°C and the protein precipitates were sedimented at 12,000 x g for 10 min at 8 °C. The protein pellet was washed thrice with 2 ml of 0.3 M guanidine hydrochloride in 95% ethanol. During each wash, the protein pellet was stored for 20 min at 30 °C and centrifuged at 7,500 x g for 5 min at 8 °C. Later, the protein pellet was air dried for 10 min and dissolved in 1% SDS by pipetting at 50 °C. Any insoluble material was sedimented by centrifugation at 10,000 x g for 10 min at room temperature and the supernatant transferred to fresh tubes. Proteins were then subjected to protein estimation and SDS-PAGE gel electrophoresis.

L. sigmodontis adult female worms were solubilised in 1 ml of extraction buffer [1% SDS, 500 mM NaCl, 5mM EDTA and 1mM PMSF]. Eppendorf tubes were vortexed briefly to bring whole worms into extraction buffer. Worms were cut into minute pieces using small sterile scissors and then homogenized with a glass–glass homogenizer at 1200 rpm. Tubes containing worm extract were heated at 95°C for 5 min with intermediate vortexing every minute. Tubes were then centrifuged for 15 min at 12, 000 x g at RT. After centrifugation, supernatant containing the soluble protein was collected into fresh eppendorf tubes and the pellet containing the insoluble proteins was resuspended with 1 ml of extraction buffer. Proteins were then subjected to protein estimation and SDS-PAGE gel electrophoresis. If not immediately used, proteins were frozen in small aliquots at -20°C until further use.

4.10.1. Protein estimation

Protein estimation was performed using is a colorimetric assay determining the protein concentration following detergent solubilisation. The principle of the assay is based on the gold standard Bradford assay (Bradford, 1976) where an absorbance shift in the dye coomassie when the previously red form coomassie reagent changed and stabilized into coomassie blue by the binding of protein. Different concentrations of universal protein standard, bovine serum albumin (BSA) such as 0.125; 0.25; 0.5; 1.0 and 2.0 mg/ml and test protein samples were solubilised with Advanced protein reagent solution (ADV01). 10µl of protein standard, protein samples and negative control were added in triplicates in a 96 well ELISA plate. To this 300 µl of 1X ADV01 assay reagent was added and incubated in dark at 37°C for 30 min. The plate was read at 590 nm using Spectra Max340 microwell reader. The protein concentrations were calculated in µg/ml using the following formula

$$\text{Protein concentration } (\mu\text{g/ml}) = (\text{measured OD}) \times (37.5) \times 30^*$$

* 30 is the dilution of the protein in ADV01 solution (10 µl in 300 µl).

4.11. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins are charged molecules and hence general electrophoresis techniques such as PAGE cannot be used to measure their molecular weight because their mobility in the gel is influenced by both charge and size. In order to overcome this, protein samples are treated with sodium dodecyl sulfate (SDS) so that they have a uniform charge and then their electrophoretic mobility depends primarily on size. The percentages of separating gels and stacking gels were prepared as shown in the following tables.

4.11.1. Reagents for 10% Separating Gel

Total volume: 15ml

Table 4

Ingredients	Separating gel
H ₂ O	6.25 ml
30% Acrylamide	5.0 ml
4x Lower Tris	3.75 ml
TEMED	30 μ l
10% APS	30 μ l

4.11.2. Reagents for 5 % Stacking Gel

Total volume: 10 ml

Table 5

Ingredients	Stacking gel
H ₂ O	6.5 ml
30% Acrylamide	1.3 ml
4x Upper Tris	2.5 ml
TEMED	30 μ l
10% APS	30 μ l

2-5 μ g of total nematode protein was analysed using SDS-PAGE. The protein samples were denatured in one fourth the volume of 4x Laemmli sample buffer (4% SDS, 0.5% bromophenol blue, 1% β -mercaptoethanol, 0.5 % glycerol, 0.5 M Tris-HCl pH 6.8) at 95°C for 5 min. The samples were centrifuged for 10 min at 13000 rpm and then were loaded to 10 % SDS-PAGE gel which was submerged in an apparatus containing running buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, and pH 8.3). A constant voltage of 100 V was applied to the gel until the tracking dye entered the separating gel. Then the voltage was increased to 150 V until the dye reached the bottom of the gel and proteins were blotted onto PVDF membranes using Trans-blot semi dry transfer cell following manufacturer's protocol.

4.11.3. Coomassie staining

The gel was stained with commercially available stain; Rotiphorese Blau R (Carl Roth GmbH, Karlsruhe, Germany) for 20 min. The ready to use Rotiphorese Blau R stain contains Coomassie Brilliant Blau B, methanol and acetic acid. The gel was destained with destaining solution 1 until bands became visible, then destained with destaining solution 2 until background disappeared, later dried and photographed when necessary.

4.12. Western Blot

Western blot is a robust technique in biochemistry used to detect a protein by using antibody specific to that protein. Western blot also gives information about the molecular weight of the protein. The complex protein mixture (such as a cell lysate, extract or a purified protein preparation) is fractionated on a SDS-PAGE gel. After electrophoresis, proteins are transferred either to nitrocellulose membrane, polyvinylidene fluoride (PVDF) membrane or nylon membrane. Specific antibodies (primary antibodies) are used to detect specific protein antigens on the membrane. Further these primary antibodies are probed with chemical or fluorescent labelled secondary antibodies.

After SDS-PAGE, proteins were transferred onto a PVDF membrane with a pore size of 0.45 μm (Schleicher & Schuell) using a Trans-blot semi dry transfer cell. The gel and membrane were pre soaked in transfer buffer containing Tris/glycine/methanol (10 mM Tris, 100 mM glycine, and pH 8.5 and 20 % methanol). Protein transfer was carried out at 0.8 mA/cm² for 1 hr. After transfer, the membrane was washed twice with TBS (20 mM Tris, 137 mM NaCl, pH 7.6). To ensure proper loading and transfer of proteins to the membrane, the membrane was stained for 20 min with Ponceau S, a reversible protein stain (Carl Roth GmbH, Karlsruhe, Germany). The membrane was washed for 10 min with PBS containing 0.5 % Tween 20 (PBS-T) to remove the Ponceau S stain. Then the membrane was blocked with 5 % Non-fat milk in PBS-T at RT for 1 hr or over night at 4°C. After blocking, the membrane was incubated with 1:250 or 1:2000 dilution of primary antibody overnight at 4°C depending on the protein to be detected. Dilution of 1: 250 and 1:2000 was used for the detection of WSP and Ls-PPE/Ov-PPE, respectively. Then the membrane was washed thrice for 10 min with PBS-T to remove unbound antibodies. After

washing, the membrane was incubated for 1 hr at RT with 1:3000 dilution of goat anti-rabbit IgG secondary antibody (Bio-Rad, Munich, Germany) prepared in PBS-T containing 0.5% Non-fat milk. The membrane was washed thrice for 10 min with 1X PBS-T to remove unbound antibodies. Then the membrane was placed either in Alkaline Phosphatase Blue Membrane Solution (Sigma-Aldrich, Munich, Germany) for 10 min to visualise bound antibodies or in Immun-star AP substrate (Bio-Rad, Munich, Germany) for 5 min for chemiluminescent detection where the blots were wrapped in a heat-sealable bag and exposed to X-ray film at RT for 2 min. X-ray film was developed after exposure.

4.13. Molecular biology methods

4.13.1. Extraction and quantification of nucleic acids

DNA extraction

DNA was extracted from MF, L3 larvae and adult worms using the QIAamp DNA Mini Kit following manufacturer's protocol for tissue, except that the worms were incubated overnight at 56 °C. DNA from 1000 MF and single adult worms was eluted in 200 µl of AE buffer (10 mM Tris, 0.5 mM EDTA, pH 9) while DNA from 10 L3 larvae was eluted in 50 µl of AE buffer.

RNA extraction

L. sigmodontis adult female worms from the pleural cavity of mice were collected and 10 female worms were pooled together in eppendorf tubes. Tubes were frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from 10 female nematodes using Trizol reagent following manufacturer's protocol. Ten female worms were suspended in 800 µl of Trizol reagent and were finely cut with scissors and later homogenized with a glass-glass homogenizer at 1200 rpm. 80 ml of 1-bromo-3-chloro-propane was added to separate the homogenate into RNA-containing aqueous and DNA and protein-containing organic phases. DNase treatment was performed for 30 min at 37°C to avoid genomic DNA contamination. Nucleases were removed with RNeasy clean up kit (Qiagen, Hilden, Germany).

The nucleic acid sample was diluted in nuclease free water or TE buffer and the absorbance was then measured at 260 and 280 nm in an Eppendorf BioPhotometer. Diluents were used as a blank to calibrate the spectrophotometer.

4.13.2. Reverse transcription

The extracted RNA was reverse transcribed into complementary DNA (cDNA) following OmniScript Reverse Transcriptase Kit manufacturer's protocol in a 20 μ l reaction.

Table 6: Reagents for reverse transcription

Reagents	Vol. in μ l
10x Buffer	2
dNTP	2
Oligo Nucleotide	1
Reverse Transcriptase (RT)	1
RNase-Inhibitor	0.33

The above reaction mix was set up with 2 μ g of RNA was incubated for 1 hr at 37 °C and then cooled to 4°C. Reverse transcribed cDNA was diluted to 1:3 with DEPC water and further used for quantitative PCR (qPCR).

4.13.3. Polymerase chain reaction amplification of *Ls-ppe-1*, *Ls-actin-1* and *Ls-FtsZ* genes

In order to generate plasmid standards for quantitative PCR (qPCR), normal PCR was performed using cDNA as template to amplify the following genes, *Ls-actin-1* (*L. sigmodontis* actin gene), *Ls-ppe-1* (*L. sigmodontis* phosphate permease gene) and *Ls-FtsZ* (*L. sigmodontis* Wolbachia cell-division protein gene).

Table 7: PCR recipe (50µl reaction)

Reagents	Vol. in µl
H ₂ O	35.5
10x Buffer	5
MgCl ₂ (25mM)	3
dNTP (40mM)	1
Forward primer (300nM)	1.5
Reverse primer (300nM)	1.5
HotStarTaq® (250 U)	0.5
cDNA	2

The above reaction mix was set and placed in PCR thermal cycler. *Ls-ppe-1* and *Ls-FtsZ* gene amplifications were performed under the following conditions: initial denaturation at 95°C 15 min, 35 cycles of 94°C 10s; 58°C 15s followed by a final extension of 72°C 15 s. For *Ls-actin-1* the conditions were identical except for the use of an annealing temperature of 52°C.

10 µl of PCR product was run on 2 % agarose gel and stained with ethidium bromide. Bands were visualized under UV light. PCR bands were excised from gel and DNA was extracted using DNA Clean & Concentrator-5 Kit, Zymo Research, Orange, USA. Purified PCR products were used for bacterial cloning.

4.13.4. Cloning of PCR product

PCR products of *Ls-actin-1*, *Ls-ppe-1* and *Ls-FtsZ* were cloned using pCR[®]4-TOPO-TA[®] cloning kit following manufacturer's protocol. TOPO-TA[®] cloning reaction mix is described in following table.

Table 8: TOPO-TA[®] cloning reaction mix

Reagents	Vol. in μl
Salt solution	1
Sterile Water	2
pCR [®] 4TOPO [®] -Vector	1
PCR-Product	2

The reaction mix was gently mixed and incubated for 5 min at RT. 2 μl of the TOPO[®] cloning reaction mix was added to a vial of One Shot[®] Chemically Competent *E.coli* and mixed gently and incubated on ice for 5 min. Heat-shock of cells at 42°C for 30 sec was given and cells were immediately transferred to ice. 250 μl of S.O.C medium was added to cells and incubated at 37°C for 1 hr in a shaker cum incubator. 10-50 μl of cells were plated on LB agar-plates containing 100 mg/ml ampicillin, 100 $\mu\text{g/ml}$ IPTG and 100 $\mu\text{g/ml}$ X-Gal which were incubated at 37°C overnight.

4.13.4.1. Selection of transformants

Transformants were selected based on their ability to grow on ampicillin agar plates as transformed vector contains a gene for the resistance against ampicillin and also insertion of foreign gene in TOPO[®] vector results in insertional inactivation of the *LacZ* gene which results in formation of white colonies in IPTG/X-Gal plates. Hence white colonies were picked and colony PCR was performed with same set of primers used previously to screen for positive clones.

4.13.4.2. Plasmid DNA preparation and purification

Positive bacterial clones for *Ls-actin-1*, *Ls-ppe-1* and *Ls-FtsZ* screened using colony PCR were cultured in 4 ml of Circle Grow[®] liquid medium containing 10 $\mu\text{g/L}$ ampicillin. Cells were incubated at 37°C overnight with shaking at 300 rpm/min. On the next day, cells were briefly centrifuged to pellet the cells and plasmid DNA was extracted using Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Plasmids were sequenced at MWG Biotech AG (Germany) and sequence for *Ls-actin-1*, *Ls-ppe-1* and *Ls-FtsZ* was confirmed by BLAST search.

4.13.4.3. Preparation of plasmid standards for quantitative PCR (qPCR)

Copy number of plasmid containing gene of interest was calculated using following formula

$$\frac{6 \times 10^{23} \text{ (Copies/mol)} \times \text{Plasmid concentration}}{\text{Molecular weight}_{\text{MW}} \text{ (g/mol)}} = \text{Copies}/\mu\text{l}$$

Plasmids were serial diluted so as to get a standard for quantitative PCR (qPCR) and during each qPCR run, a serial dilution of linearized plasmid containing *Ls-actin-1*/*Ls-ppe-1*/*Ls-FtsZ* gene was used to make a standard curve. The gene/ μl in nematode DNA was calculated from the plasmid standards.

4.13.5. Quantitative PCR (qPCR) for *Ls-actin-1*, *Ls-ppe-1* and *Ls-FtsZ* gene

Quantification of *Ls-actin-1* and *Ls-ppe-1* was performed using Sybr Green assay whereas quantification of *Ls-FtsZ* gene was performed using Taqman assay. The following are the qPCR recipe for *Ls-actin-1*, *Ls-ppe-1* and *Ls-FtsZ* gene.

Table 9: qPCR recipe for *Ls-actin-1* gene (10 μl reaction)

Reagents	Vol. in μl
H ₂ O	4.4
10x Buffer	1
MgCl ₂ (25mM)	0.6
dNTP(40mM)	0.05
<i>Ls-actin-1</i> forward primer (900nM)	0.9
<i>Ls-actin-1</i> reverse primer (900nM)	0.9
SYBR® Green I (1:1000)	0.1
HotStarTaq®	0.05
Nucleic acid	2

Table 10: qPCR recipe for *Ls-ppe-1* gene (10 µl reaction)

Reagents	Vol. in µl
H ₂ O	5.6
10x Buffer	1
MgCl ₂ (25mM)	0.6
dNTP(40mM)	0.05
<i>Ls-ppe-1</i> forward primer (300nM)	0.3
<i>Ls-ppe-1</i> reverse primer (300nM)	0.3
SYBR® Green I (1:1000)	0.1
HotStarTaq®	0.05
Nucleic acid	2

Table 11: qPCR recipe for *Ls-FtsZ* gene (10 µl reaction)

Reagents	Vol. in µl
H ₂ O	5.0
10x Buffer	1
MgCl ₂ (25mM)	1.2
dNTP(40mM)	0.05
<i>Ls-FtsZ</i> forward primer (300nM)	0.3
<i>Ls-FtsZ</i> reverse primer (300nM)	0.3
Taqman HybeProbe (5 µM)	0.1
HotStarTaq®	0.05
Nucleic acid	2

The above reaction mix was set and performed on RotorGene 3000 (Corbett Research, Australia). PCR reactions were performed in triplicate. *Ls-ppe-1* and *Ls-FtsZ* gene amplifications were performed under the following conditions: initial denaturation at 95°C 15 min with 35 cycles of 94°C 10s; 58°C 15s followed by a final extension of 72°C 15 s. For *Ls-actin-1* the conditions were similar except for the use of an annealing temperature of 52 °C.

4.14. Immunohistology

4.14.1. Embedding tissues prior to paraffin processing

L. sigmodontis adult worms were embedded in agar-gelatin. 4 % agar in distilled water at 40°C was mixed with a 5 % (w/v) solution of gelatin in distilled water at a 1:1 ratio to generate a slightly viscous 2 % agar: 2.5 % gelatin solution. The temperature of this medium was used at approximately 40°C to avoid exposing tissues to excessive heat. Worms were arranged or oriented as desired on a clean surface and molten agar-gelatin mixture was poured over the top of the worm tissues and was allowed to solidify. The agar-gelatin solidifies around the fixed worm tissue (pre-embedding) and the fixed worms were taken into plastic cassettes. *O. volvulus* worm nodules were directly taken into plastic cassettes without pre-embedding procedure. The plastic cassettes containing worm tissue or nodule were subjected to routine dehydration and paraffin infiltration procedures, followed by finally embedding the agar-gelatin-encased tissue in paraffin wax.

4.14.2. Sectioning, deparaffining and rehydration.

Paraffin embedded worms were cut into fine sections of 0.5 µM using a Reichert-Jung 1140 Autocut microtome (Leica Microsystems, Wetzlar, Germany). The sections were placed on glass slides and incubated over night at 40°C for proper drying and fixing of sections to the glass slides. Then the sections were placed in Xylene for 5 min to deparaffinize and then were placed serially in 100%, 96%, 80%, 70% and 50% of ethanol for 5 min each and then followed by rehydration in distilled water for 5 min.

4.14.3. Immunostaining

The deparaffinised and rehydrated worm sections were rinsed with distilled water and thereafter with TBS. The sections were briefly placed in 0.1% BSA for 5 min and then incubated with 10% BSA for 5 min. After incubation, BSA was tapped off and the sections were placed in 0.1% BSA for 5 min. Then the sections were either incubated for 1 hr with rabbit anti-Ls-PPE or anti-Ov-PPE serum diluted optimally in 0.1% BSA at a dilution of 1:2000 or anti-WSP serum diluted in

0.1% BSA at a dilution of 1:250. After incubation, the antibody was tapped off and the slides were washed in TBS for 5 min to remove unbound antibodies. Then the sections were incubated with monoclonal mouse anti-rabbit immunoglobulins (DAKO Code No. M 0737, Dako Cytomation, Glostrup, Denmark) diluted at 1:25 in 0.1% BSA for 30 min. After incubation, the antibody was tapped off and slides were washed in TBS for 5 min. Then the slides were incubated with polyclonal rabbit anti-mouse immunoglobulins (DAKO Code No. Z 0259, Dako Cytomation, Glostrup, Denmark) diluted 1:25 in 0.1% BSA for 30 min and followed by washing in TBS for 5 min, slides were incubated with APAAP, mouse monoclonal (alkaline phosphatase anti-alkaline phosphatase) (DAKO Code No D 0651, Dako Cytomation, Glostrup, Denmark) diluted 1:50 in 0.1 % BSA for 30 min. After incubation, the complex was tapped off and the slides were washed in TBS for 5 min. Later the slides were incubated with Sigma Fast Red substrate (Sigma-Aldrich, Munich, Germany) for 15-20 min. After incubation, the substrate was tapped off and the slides were rinsed in distilled water and counter stained with Mayer's hemalum solution for 10 min. Then the slides were washed in running tap water and coverslips mounted with Aquatex for observation with a microscope.

5. Results

5.1. Sex-ratio distortion study

To dissect between a direct effect of tetracycline and the absence of *Wolbachia* on larval moulting and adult worm development, I depleted *Wolbachia* from MF, knowing that tetracycline has no effect on MF viability in blood (Chirgwin, et al., 2003; Debrah, et al., 2006). Hence, to study the effect of tetracycline, which depletes *Wolbachia*, on the development of microfilariae (L1 larvae, MF) to L3 larvae in the intermediate mite host (*O. bacoti*), and to observe the development of *Wolbachia*-depleted L3 larvae in gerbils; microfilaremic gerbils were treated with tetracycline for 6 weeks in drinking water (1° Tet) or left untreated (1° Con) and designated as primary infections. Naive mites were then fed on the 1° Tet and 1° Con gerbils and were used to infect uninfected gerbils, designated as secondary infections (2° Tet, 2° Con). Hence, the gerbils were infected with mites carrying L3 larvae that developed from *Wolbachia*-depleted MF.

5.1.1. Six weeks oral tetracycline treatment leads to a persistent depletion of *Wolbachia* and lower microfilaremia

Pre-treatment blood was collected from patent gerbils and microfilaraemia was determined before the start of tetracycline treatment. Gerbils were treated with tetracycline orally and microfilaraemia was monitored at the end of treatment (6 weeks), and 1 and 3 months post-treatment to assess the efficiency of tetracycline treatment. At the end of treatment, there was no significant difference in microfilaraemia in 1° Tet and 1° Con gerbils, but 1° Tet gerbils at 1 and 3 months post-treatment had significantly fewer MF (11- and 5-fold differences, respectively; Table 12, column 3). Although at 3 months post-treatment the fold difference in the number of MF/ μ l blood decreases, this is due to a decrease of MF in the 1° Con gerbils and not an increase of MF in the 1° Tet gerbils. After 15 months, 1° Con gerbils also became amicrofilaremic (Fig. 12). DNA was extracted from 1000 MF and the *Wolbachia* load was quantified by qPCR. At the end of oral tetracycline treatment, 1° Tet gerbils had microfilariae with levels of *Wolbachia* 8.5-fold lower than controls. The 1° Tet MF collected at 1 and 3 months post-treatment had 11- and 6-fold fewer *Wolbachia* than 1° Con MF, respectively. In all cases, the differences in

endobacteria levels were significant (Table 12, column 5).

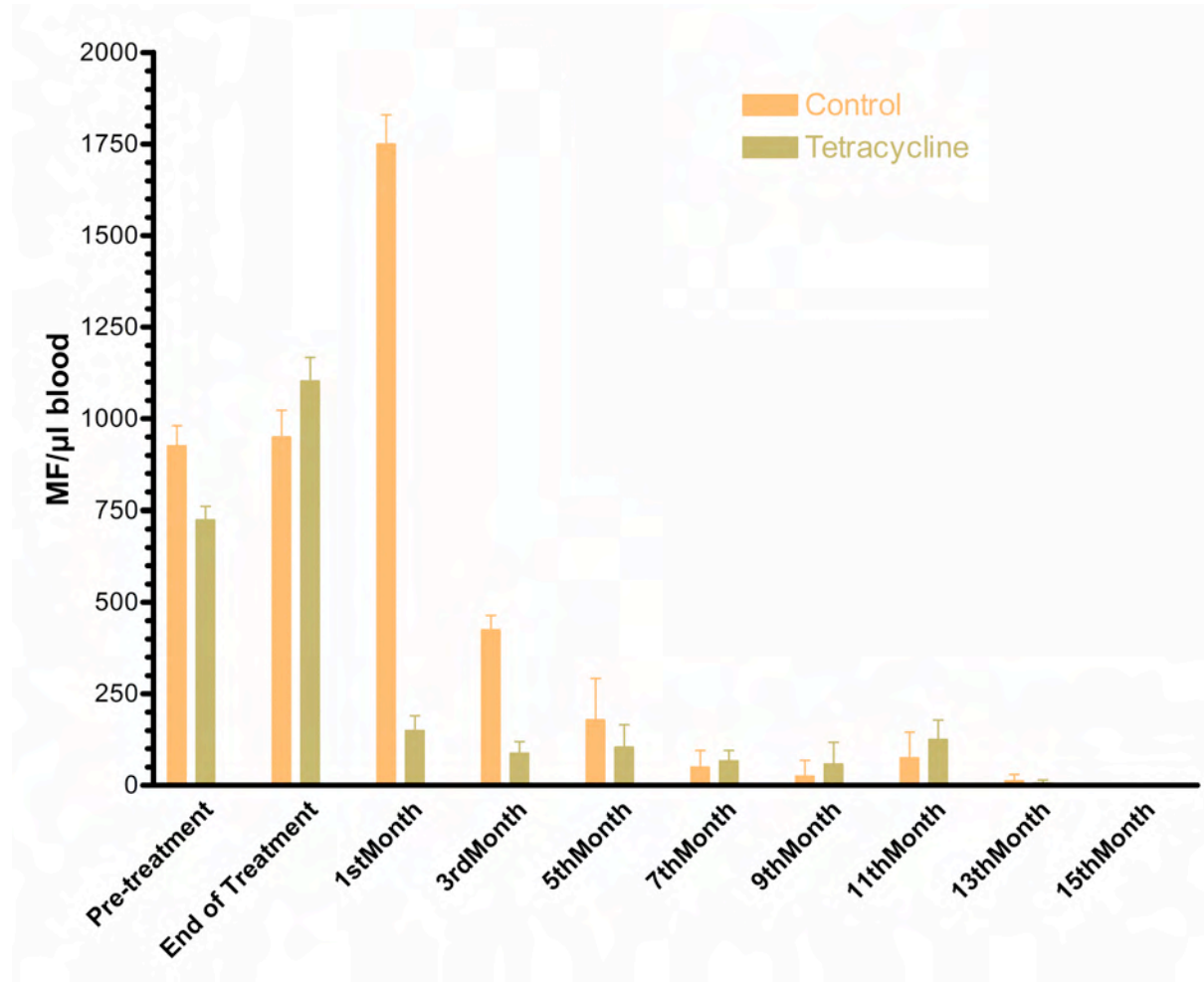


Fig. 12. Microfilaraemia in primary infected gerbils followed to 15 months post infection. At the end of 6 weeks tetracycline treatment, there is no significant difference in microfilaraemia in 1° Tet and 1° Con gerbils, but 1° Tet gerbils at 1 and 3 months post-treatment had significantly fewer MF. At 15 months post treatment, both 1° Tet and 1° Con gerbils became amicrofilaraemic.

Table 12: Microfilaremia and *Wolbachia* levels before and after tetracycline treatment of gerbils infected with *L. sigmodontis*

Treatment	Time	MF/ μ l median (25 th ; 75 th percentiles)	<i>P</i> value ¹	<i>Ls-FtsZ</i> MF median (25 th ; 75 th percentiles)	<i>P</i> value ¹
Control (N=5)	Pre-treatment	925 (706.5; 1163)		61 48.5; 108.5	
	6 weeks ²	950 (643.5; 1283)		34 (20.8; 42.7)	
	1 mo. p.t. ³	1750 (643.8; 2063)		58 (25.2; 60.6)	
	3 mo. p.t.	425 (325; 1038)		86.06 (47.5; 124.8)	
Tetracycline (N=5)	Pre-treatment	725 (531.5; 1250)	0.69	66 (47; 88)	0.69
	6 weeks	1112 (587; 1169)	0.69	4 (1.2; 6.5)	<0.01*
	1 mo. p.t.	150 (112.5; 362.5)	<0.01*	5.2 (3.1; 10.3)	0.03*
	3 mo. p.t.	88 (66.2; 112.5)	<0.01*	14.8 (7.5; 45.4)	0.02*

¹ Mann-Whitney U Test, $P \leq 0.05$, comparing microfilaremia and *Wolbachia* levels in tetracycline treated gerbils to untreated controls. The results are representative of 3 experiments.

² End of oral tetracycline treatment.

³ p.t.: Post treatment.

* Results are statistically significant.

5.1.2. Fewer L3 develop from *Wolbachia*-depleted L1 larvae

Six weeks after tetracycline treatment naive mites were fed on the 1° Tet or 1° Con gerbils. From each infection, 100 mites were collected and dissected for infective L3 larvae. Con mites had an infection rate of 54% while Tet mites had an infection rate of 9% (Table. 13, column 2). Although there was no difference in microfilaremia (Table 12, column 3) in the gerbils used to infect both groups of mites, the percentage of mites with MF that successfully developed into L3 larvae was significantly lower in the Tet mites. Quantification of *Wolbachia* levels in these larvae showed that the L3 larvae from Tet mites had significantly lower *Wolbachia* levels than L3 larvae from Con mites (Table 13, column 4). Despite the significantly reduced number of *Wolbachia* (Table 13, column 5), L3 larvae were able to develop in the intermediate host, suggesting that the larvae that developed still had *Wolbachia* levels above a minimum threshold needed for larval development.

5.1.3. Microfilaraemia and *Wolbachia* levels in MF from secondary infected gerbils

After confirming a significant reduction in *Wolbachia* levels in L3 larvae from Tet mites, Tet and Con mites were used to infect naive gerbils (2° Tet and 2° Con gerbils). After three months, blood was collected from 2° Tet and 2° Con gerbils to monitor microfilaraemia and *Wolbachia* levels in the MF. All 2° Con gerbils were microfilaraemic. Unexpectedly, five of the 2° Tet gerbils were microfilaraemic. 2° Tet gerbils infected with *Wolbachia*-depleted L3 larvae had significantly fewer circulating MF in comparison to 2° Con gerbils which were infected with L3 larvae containing more *Wolbachia* (Table. 13, column 6). Assessing the *Wolbachia* levels in MF from 2° Tet and 2° Con gerbils revealed that MF from 2° Tet gerbils had significantly fewer (12-fold) *Wolbachia* in comparison to MF from 2° Con gerbils (Table. 13, column 8). Again suggesting that the L3 larvae that successfully developed in the intermediate mite host contained a minimum number of *Wolbachia* to develop into sexually mature adult worms.

Table 13: Tetracycline treatment after primary infection leads to impaired L1-L3 development in mites, reduced *Wolbachia* levels in L3 larvae, low microfilaraemia and reduction of *Wolbachia* in MF in secondary infected Tet gerbils.

Treatment	% of infective L3 in mites (n=100)	<i>P</i> value ¹	<i>Ls-FtsZ</i> /L3 median (25 th ; 75 th percentiles) (N=5)	<i>P</i> value ²	MF/ μ l median (25 th ; 75 th percentiles) (N=5/7)	<i>P</i> value ³	<i>Ls-FtsZ</i> /MF median (25 th ; 75 th percentiles) (N=5/7)	<i>P</i> value ⁴
Control	54		2140 (602.5; 2900)		912.5 (737.5; 1413)		61.8 (41.46; 113)	
Tetracycline	9	<0.0001*	40 (35; 55)	0.04*	100 (62.5; 412.5)	<0.01*	5.2 (2.13; 32.6)	0.02*

¹Mites fed on control and tetracycline gerbils were dissected 14 days after infection and examined for L3 larvae. Significant difference was determined by the Fischer's exact test, $P \leq 0.05$.

²Fifty L3 larvae from Con and Tet mites were pooled in batches of 10 (N=5), DNA was extracted and *Ls-FtsZ* was quantified by qPCR.

³Con and Tet mites were used to infect naive gerbils with *L. sigmodontis* (N=7, secondary infection). Microfilaraemia was assessed at 3 months post-treatment.

⁴Quantification of *Ls-FtsZ* gene in MF from secondary infected Con and Tet gerbils.

* Result is statistically significant, Mann-Whitney U Test, $P \leq 0.05$. Results are representative of 3 experiments.

5.1.4. Depletion of *Wolbachia* from MF causes a sex-ratio distortion in adult worms from 2° Tet gerbils

Adult worms were recovered from the pleural cavities of 2° Tet and 2° Con gerbils and the adult worms were analyzed. Fewer female worms developed from *Wolbachia*-depleted L3 larvae in 2° Tet gerbils than in 2° Con gerbils. However, there was no significant difference in male worm number between 2° Tet and 2° Con gerbils. This led to an 8-fold lower female: male ratio in 2° Tet gerbils compared to 2° Con gerbils (Fig. 13). There was no significant difference in the male and female worm length between both groups (Fig. 14 A, Fig. 14 B).

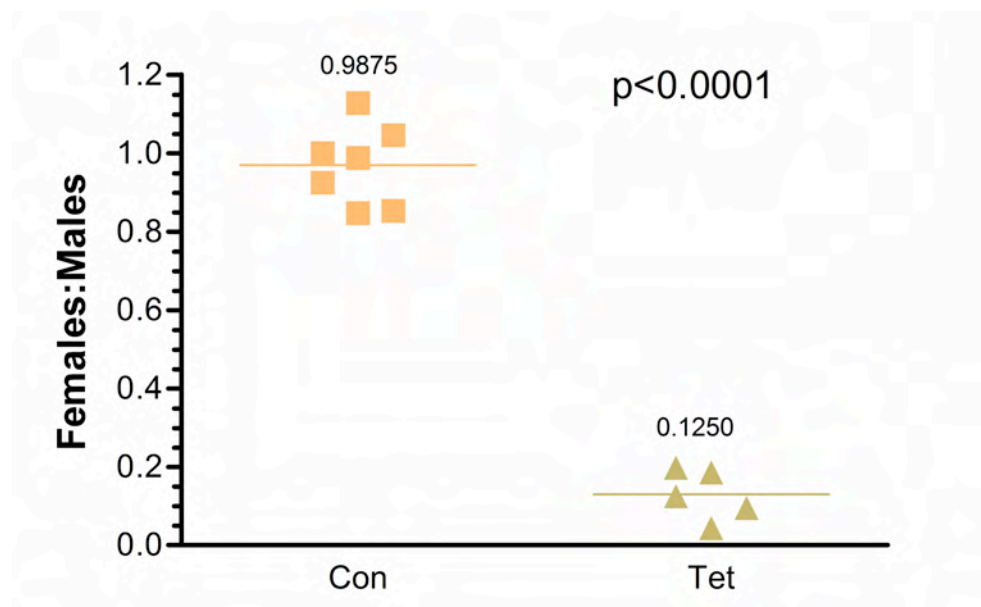


Fig.13. The female: male ratio is significantly lower in 2° Tet gerbils infected with *Wolbachia*-depleted L3. Naive mites took blood meals on gerbils infected with *L. sigmodontis* treated with tetracycline for 6 weeks (1° Tet) or untreated gerbils (1° Con) from the same infection. The mites were then used to infect naive gerbils. After 3 months, adult worms were recovered from the pleural cavity and counted. Fewer female worms developed from *Wolbachia*-depleted L3 larvae than from control L3 larvae in secondary infected gerbils ($P < 0.0001$), leading to an 8-fold lower female: male ratio. Significance was determined using the Mann-Whitney U Test, $P \leq 0.05$. The graph is representative of 3 experiments. Squares and triangles represent one infected gerbil. The line represents the median value.

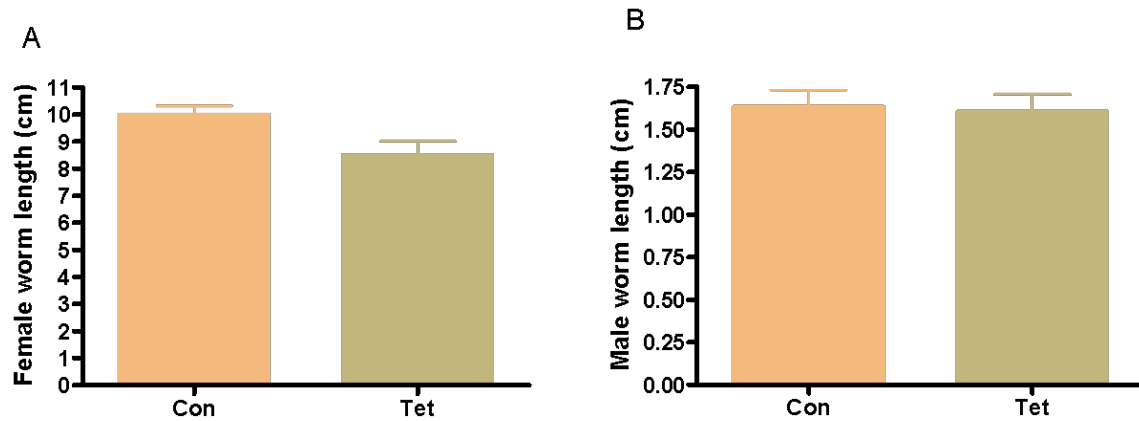


Fig. 14. Adult worm length in secondary infected control and tetracycline treated gerbils. Mites which took blood meals on gerbils infected with *L. sigmodontis* treated with tetracycline for 6 weeks (1° Tet) or untreated gerbils (1° Con), were used to infect naive gerbils. After 3 months, adult worms recovered from pleural cavity were separated based on sex and the length of male and female worms was measured using. A) Female worm length and B) Male worm length. There is no significant difference in male and female worm length between Con and Tet groups. Significance was determined using the Mann-Whitney U Test, $P \leq 0.05$. The graph is representative of 3 experiments.

5.1.5. *Wolbachia* levels in male and female worms developed in 2° Con and 2° Tet gerbils

Naive mites took blood meals on gerbils infected with *L. sigmodontis* treated with tetracycline for 6 weeks (1° Tet) or untreated gerbils (1° Con) from the same infection. The mites were then used to infect naive gerbils (2° Con, 2° Tet). After 3 months, adult worms were recovered from the pleural cavity. DNA was extracted and *Wolbachia* levels were quantified by real-time PCR. *Ls-FtsZ* levels measured in male worms showed a wide range of distribution that differed by 3 logs. There were significantly fewer *Wolbachia* in male worms from 2° Tet gerbils compared to those of male worms from 2° Con gerbils. In the female worms that developed in 2° Tet and 2° Con gerbils the range of *Wolbachia* distribution was ~1 log. There was no significant difference in the number of *Wolbachia* in female worms from 2° Tet gerbils, although there was a trend of more *Wolbachia* in the few female worms that developed (Fig. 15).

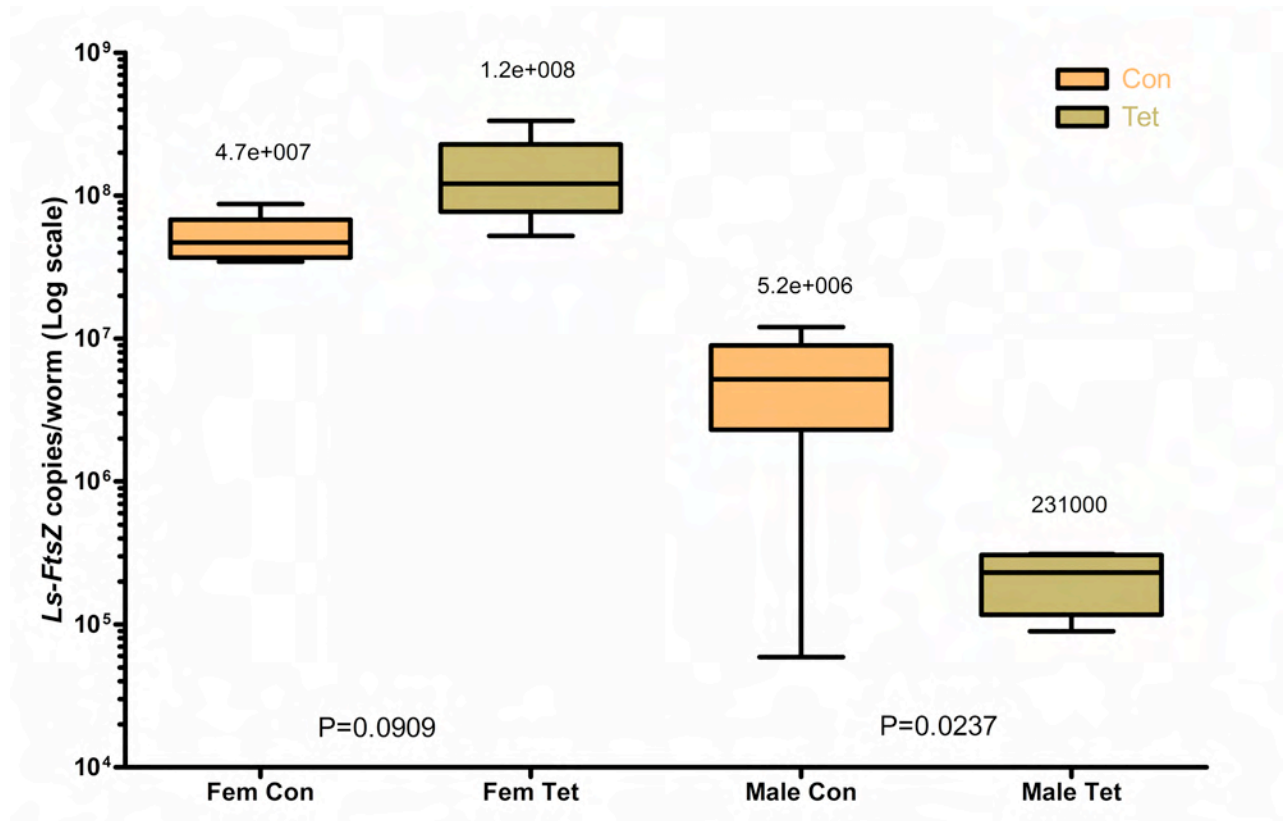


Fig. 15. *Wolbachia* levels from female and male worms that developed in 2° Con and 2° Tet gerbils. Comparison of *Wolbachia* levels in 2° Tet and 2° Con female worms shows no significant difference ($P=0.09$) whereas the *Wolbachia* levels in 2° Tet male worms were significantly lower than in 2° Con male worms ($P=0.02$). The line in the boxes represents the median (numerical value of which is given above each box). The top and bottom of the boxes represents the 75th and 25th percentiles, respectively; the lines below and above the boxes represent the minimum and maximum values. Significance was determined using the Mann-Whitney U Test, $P \leq 0.05$. The graph is representative of 3 experiments.

5.2. *Wolbachia* depletion and *L. sigmodontis* phosphate permease (*Ls-PPE*) regulation study

The information from the annotated *wBm* genome suggests that important metabolites might be provided by *wBm* to nematodes and vice versa. One way to identify and characterize these metabolic pathways would be to study the expression of genes in nematodes during *Wolbachia* depletion. RNA differential display was used to discover genes in *L. sigmodontis* that are differentially regulated in response to *Wolbachia* depletion during tetracycline treatment. Several genes were up-regulated in response to *Wolbachia* depletion and one of the up-regulated genes (*Ls-ppe-1*) had homology to the phosphate permease class of proteins (Heider et al., 2006).

The aim of this part of the work was to study the regulation of *Ls-ppe-1* gene at the protein level and to investigate a possible co-localization of this protein to *Wolbachia* in *L. sigmodontis* adult worms using immunohistology.

5.2.1. Depletion of *Wolbachia* in *L. sigmodontis* during tetracycline treatment

IL-5-deficient BALB/c mice having patent infection with *L. sigmodontis* were treated intraperitoneally with tetracycline. Worms were collected on days 6, 15 and 36 of treatment. *Wolbachia* levels were determined by *Ls-FtsZ* qPCR. To quantify the worm load, a house keeping gene of *L. sigmodontis*, *Ls-actin-1* was also measured by qPCR. There was no significant reduction of *Wolbachia* in worms collected on day 6 of tetracycline treatment and *Wolbachia* levels in worms collected on day 15 of tetracycline treatment showed reduction in *Wolbachia* in comparison to control worms, but this reduction was not statistically significant. In comparison to controls, significant depletion of *Wolbachia* of 97% was observed at day 36 of tetracycline treatment (Fig. 16A) and no significant difference in *Ls-actin-1* levels was observed in both the groups during the entire treatment period (Fig. 16B).

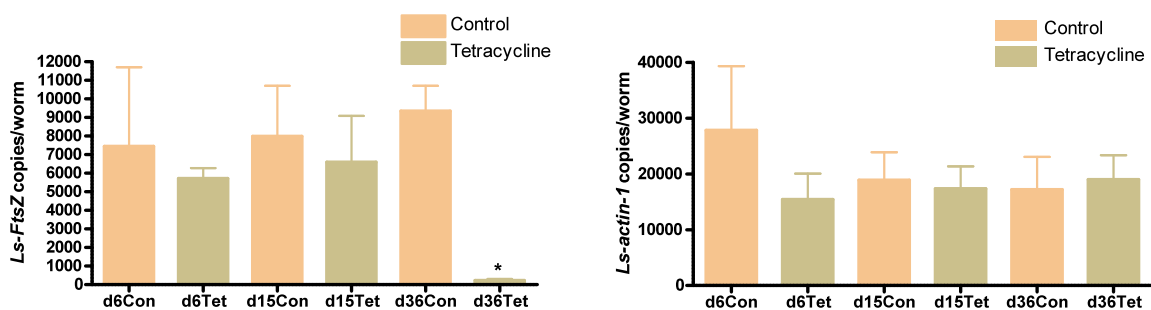


Fig. 16. Tetracycline treatment leads to significant depletion of *Wolbachia* bacteria in adult worms at day 36 of tetracycline treatment (Tday36). A) *Ls-FtsZ* and B) *Ls-actin-1* copy numbers were determined for each time point by qPCR. Significance was calculated by the Mann–Whitney U test, * denotes a significant difference between tetracycline-treated *L. sigmodontis* and untreated controls ($p < 0.05$).

5.2.2. Expression levels of *Ls-ppe-1* during *Wolbachia* depletion by tetracycline treatment

Ls-ppe-1 levels were measured in *L. sigmodontis* worms collected on day 6, 15 and 36 of tetracycline treatment using qPCR. Levels of actin gene of *L. sigmodontis*, *Ls-actin-1*, was measured to quantify the worm load. In comparison to control, 74 % up-regulation of *Ls-ppe-1* mRNA was observed in worms collected on day 36 of tetracycline treatment (Fig. 17A) and no significant difference in *Ls-actin-1* levels was observed (Fig. 17B). Notably on these days there was also reduction in levels of *Wolbachia* (Fig. 16A) and confirms previously published work (Heider et al., 2006).

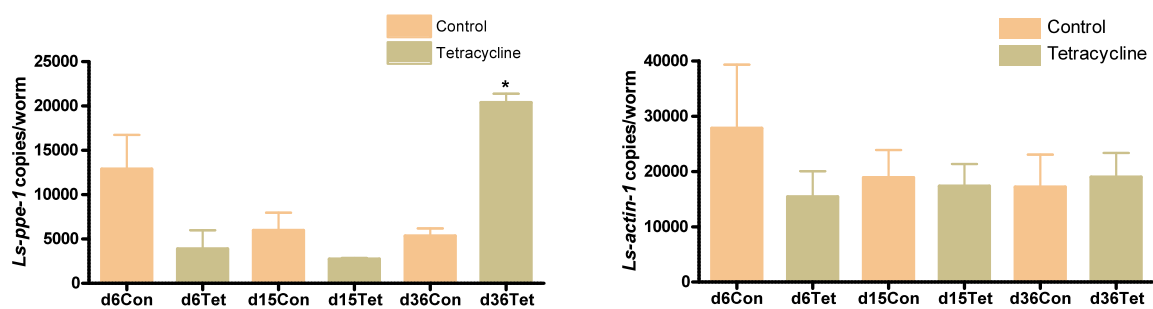


Fig. 17. Up-regulation of *Ls-ppe-1* during *Wolbachia* depletion by tetracycline treatment in *L. sigmodontis* adult female worms. A) *Ls-ppe-1* and B) *Ls-actin-1* copy numbers were determined for each time point by qPCR. Significance was calculated by the Mann–Whitney U test, * denotes a significant difference between tetracycline-treated *L. sigmodontis* and untreated controls ($p < 0.05$).

5.2.3. Testing of anti-Ls-PPE serum

In order to study the regulation of Ls-PPE at protein level, anti-Ls-PPE serum was developed. Two rabbits were immunized with Ls-PPE peptides coupled to carrier protein KLH and bleeds were collected from rabbits. 2 μ g of *L. sigmodontis* protein was separated on SDS-PAGE and transferred to PVDF membrane. Western blot was performed to test the anti-Ls-PPE serum at different dilutions (1:250, 1:500, 1:1000 and 1:2000). A protein of the predicted molecular weight of 53 KDa corresponding to *L. sigmodontis* phosphate permease (Ls-PPE) was observed (Fig.18). A serum dilution of 1:2000 showed a clear band and had the least background, whereas no bands were observed in blots incubated with pre-immune serum at the same dilution (Fig. 18).

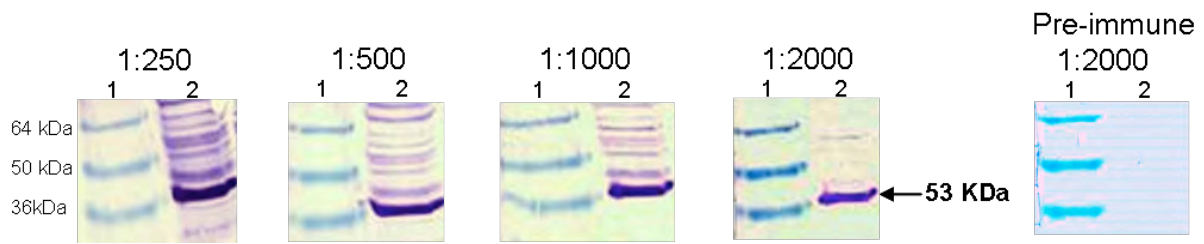


Fig. 18. Detection of Ls-PPE using anti-Ls-PPE serum.

2 μ g of *L. sigmodontis* proteins were separated on SDS-PAGE gel and transferred to PVDF membrane. Lane 1: Protein marker and Lane 2: *L. sigmodontis* protein extract. Blots were incubated with 1:250, 1:500, 1:1000 and 1:2000 dilutions of anti-Ls-PPE serum and 1:2000 dilution of pre-immune serum followed by goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase. Specific proteins were detected by incubating the blot in Alkaline Phosphatase Blue Membrane Solution (Sigma-Aldrich, Munich, Germany).

5.2.3.1. Testing the specificity of anti-Ls-PPE serum

To test the specificity of anti-Ls-PPE serum against Ls-PPE, a competitive Western blot was performed by competing the anti-serum with the soluble peptides (100 μ g/ml) used for immunization. Blots incubated with anti-Ls-PPE serum without the peptides showed a band of 53 KDa (Fig. 19, lane 1), whereas blots incubated with anti-Ls-PPE serum plus the soluble peptides showed no bands (Fig. 19, lane 2), suggesting that the serum was specific for Ls-PPE peptides.

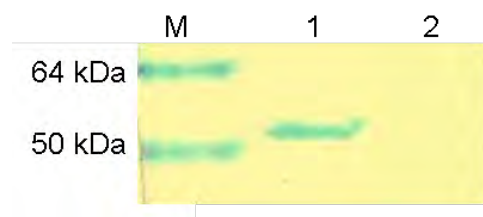


Fig. 19. Anti-Ls-PPE serum is specific for Ls-PPE peptides. 2 μ g of *L. sigmodontis* protein was separated on a 10% SDS-PAGE gel and then transferred to nitrocellulose membrane. Lane 1: Blot incubated with a 1:2000 dilution of anti-Ls-PPE serum. Lane 2: Blot with 1:2000 dilution of anti-Ls-PPE serum plus free peptides. Blots were incubated with goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase and Ls-PPE was detected by incubating the blot in Alkaline Phosphatase Blue Membrane Solution (Sigma-Aldrich, Munich, Germany).

5.2.4. Up-regulation of Ls-PPE during *Wolbachia* depletion by tetracycline treatment at the protein level

To study the expression levels of Ls-PPE during *Wolbachia* depletion, IL-5-deficient BALB/c mice with a patent infection of *L. sigmodontis* were treated with tetracycline intraperitoneally for 30 days. Worms were recovered on days 6, 15 and 36 and expression of Ls-PPE at the protein level was investigated by Western blot using anti-Ls-PPE serum. Up-regulation of Ls-PPE was observed on days 15 and day 36 of tetracycline treatment, as was seen at the mRNA level (Fig. 20).

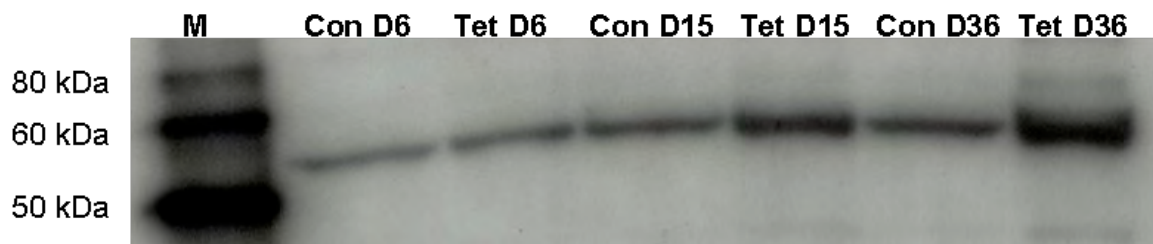


Fig. 20. Up-regulation of Ls-PPE at protein level on day 15 and day 36 of tetracycline treatment. 2 µg of soluble *L. sigmodontis* protein from worms from different time points of tetracycline treatment was separated on a 10% SDS-PAGE gel and then transferred to PVDF membrane. Anti-Ls-PPE serum was used to detect Ls-PPE followed by goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase and a chemiluminescent Western blot was performed. Ls-PPE was found to be up-regulated on day 15 and day 36 of tetracycline treatment.

5.2.5. Ls-PPE is not up-regulated during treatment of *L. sigmodontis* with gentamycin, a drug known to be inefficient in killing *Wolbachia*

Gentamycin is known to be inefficient in curing *Wolbachia* in *L. sigmodontis* (Heider et al., 2006). Hence to test the hypothesis of Ls-PPE up-regulation is dependent on *Wolbachia* depletion, IL-5-deficient BALB/c mice with patent infection of *L. sigmodontis* were treated with gentamycin intraperitoneally for 30 days and worms were collected on day 15 and day 36 post-gentamycin treatment. Nematode proteins were extracted from control and gentamycin treated worms and a Western blot was performed to analyse Ls-PPE up-regulation. There was no up-regulation of Ls-PPE observed on days 15 and day 36 of gentamycin treatment (Fig. 21).

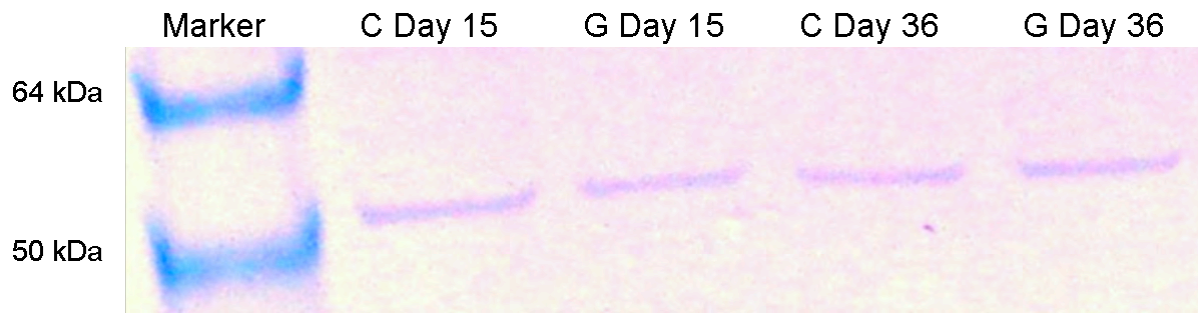


Fig. 21. Ls-PPE is not up-regulated at the protein in *L. sigmodontis* during gentamycin treatment.

2 μ g of soluble *L. sigmodontis* protein from worms from different time points of gentamycin treatment was separated on a 10% SDS-PAGE gel and then transferred to PVDF membrane. Blot was incubated with anti-Ls-PPE serum followed by goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase. Ls-PPE protein was detected by incubating the blot in Alkaline Phosphatase Blue Membrane Solution (Sigma-Aldrich, Munich, Germany). Ls-PPE was not found to be up-regulated at the protein level on day 15 and day 36 of gentamycin treatment.

5.3. *Onchocerca volvulus* phosphate permease regulation study

The phosphate permease of *L. sigmodontis* (Ls-PPE) found to be up-regulated in response to *Wolbachia* depletion, have putative orthologues in the human pathogenic filarial nematode *O. volvulus* (Heider et al., 2006). In addition to filarial growth and reproduction, endosymbiotic *Wolbachia* in *O. volvulus* also play an important role in the pathogenesis of onchocerciasis and are ideal targets for chemotherapy (Hoerauf, 2000; Taylor et al., 2005). Hence, it was worthwhile to study the regulation of phosphate permease in *O. volvulus* (Ov-PPE) during *Wolbachia* depletion in doxycycline treated onchocerciasis patients and to discover a possible role of Ov-PPE in nematode-*Wolbachia* endosymbiosis.

5.3.1. Testing of anti-Ov-PPE serum and its specificity

Potential immunogenic Ov-PPE peptides were coupled to carrier protein KLH, two rabbits were immunized with Ov-PPE peptides, and bleeds were collected. 2 μ g of soluble *O. volvulus* nodule proteins were run on SDS-PAGE and transferred to PVDF membrane. To test for anti-peptide Ov-PPE serum and its specificity, blots were incubated with 1: 2000

dilution of anti-Ov-PPE serum +/- 100 µg/ml of the soluble peptides used for the immunizations. Blots incubated with 1: 2000 dilution of anti-Ov-PPE serum without free peptides had a protein at the predicted MW of 52 KDa corresponding to Ov-PPE (Fig. 22, lane 1) and blots incubated with anti-peptide Ov-PPE serum plus the soluble peptides showed no bands (Fig. 22, lane 2) suggests that the anti-serum is specific for Ov-PPE peptides.

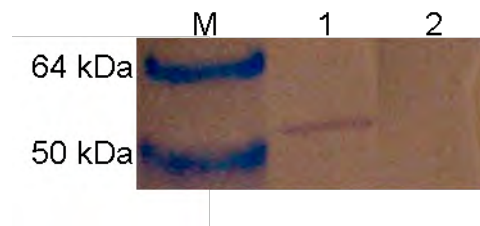


Fig. 22. Anti-Ov-PPE serum is specific for Ov-PPE peptides. 2µg of *O. volvulus* nodule proteins were separated on a 10% SDS-PAGE gel and then transferred to PVDF membrane. Lane1: Blot incubated with 1:2000 dilution of anti-Ov-PPE serum alone. Lane 2: Blot with 1:2000 dilution of anti-Ov-PPE serum plus free peptides. Blots were incubated with goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase and Ls-PPE was detected by incubating the blot in Alkaline Phosphatase Blue Membrane Solution (Sigma-Aldrich, Munich, Germany).

5.3.2. *O. volvulus* GST-1 western blot assay for the normalization of worm content in human *O. volvulus* nodules

O. volvulus Glutathione S-Transferase-1 (Ov-GST-1) is a house keeping gene expressed in cell for essential cellular functions and found to be not regulated in response to oxidative stress (Liebau et al., 2000). Therefore, Ov-GST-1 western blot assay was used to normalize the worm content and to determine the regulation of Ov-PPE in human nodule sample. 2µg of *O. volvulus* nodule proteins were ran on SDS-PAGE and transferred to PVDF membrane. The blot was incubated with a 1:3000 dilution of Ov-GST-1 anti-serum which detected the Ov-GST-1 protein at the predicted size of 30 kDa (Fig. 23). Ov-GST-1 anti-serum was the kind gift of Prof. Eva Liebau, Institute of Animal Physiology, University of Muenster, Germany.

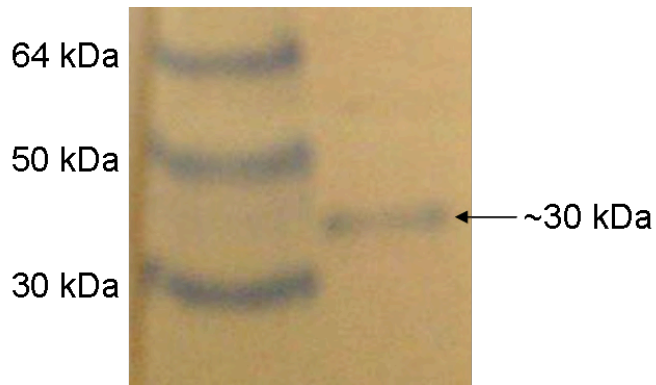


Fig. 23. Ov-GST-1 Western blot for the normalization of worm content in human *O. volvulus* nodules. 2 μ g of *O. volvulus* nodule proteins transferred to PVDF membrane. The blot was incubated with a 1:3000 dilution of anti-Ov-GST-1 serum followed by goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase. Ov-GST-1 protein of around 30 kDa was detected by incubating the blot in Alkaline Phosphatase Blue Membrane Solution (Sigma-Aldrich, Munich, Germany).

5.3.3. Ov-PPE is up-regulated in *O. volvulus* worms in nodules from doxycycline treated patients.

Onchocerciasis patients were treated 6 weeks with doxycycline and nodules were excised from patients 4 months post-treatment. 2 μ g of *O. volvulus* worm nodule proteins from control and 6 weeks doxycycline treated patients were separated on SDS-PAGE gel and then transferred to PVDF membrane. Blots were incubated with anti-Ov-GST-1 and anti-Ov-PPE sera at dilution of 1:3000 and 1:2000, respectively. With reference to Ov-GST-1 expression, there was up-regulation of Ov-PPE at the protein level from 6 week doxycycline treated worms in comparison to expression levels of Ov-PPE in control untreated worms (Fig. 24). In one untreated *O. volvulus* worm nodule, no expression of Ov-PPE was observed (Fig. 24, lane 3).

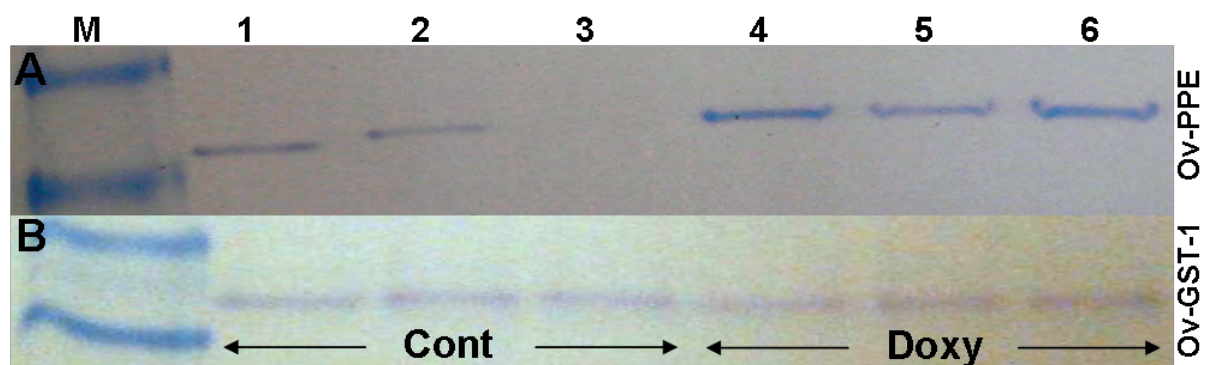


Fig. 24. Up-regulation of Ov-PPE in doxycycline treated worms. 2 μ g of *O. volvulus* worm nodule proteins were separated on SDS-PAGE and then transferred to PVDF membrane. Lane 1-3 are untreated *O. volvulus* worm

nodule extracts and Lane 4-6 are 6 weeks doxycycline treated *O. volvulus* worm nodule extracts A) Blot incubated with 1:2000 dilution of anti-Ov-PPE serum. B) Blot incubated with 1:3000 dilution of anti-Ov-GST-1 serum. Blots were incubated with goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase and specific protein was detected by incubating the blot in Alkaline Phosphatase Blue Membrane Solution (Sigma-Aldrich, Munich, Germany). Up-regulation of Ov-PPE is observed in lane 4, 5 and 6, the doxycycline treated worms whereas no change in expression levels of Ov-GST-1 is seen in control untreated and doxycycline treated worms.

5.4. Immunohistology

5.4.1. Localization of Ls-PPE and *Wolbachia* in *L. sigmodontis*.

Localization of Ls-PPE and *Wolbachia* will reveal their specific location in the worms and test the hypothesis of co-localization of Ls-PPE to the vesicles surrounding *Wolbachia*. After determining that anti-Ls-PPE serum only detects one protein of predicted MW of 53 KDa corresponding to Ls-PPE, it was used for the localization of Ls-PPE using immunohistology. Serial sections of untreated *L. sigmodontis* worms were stained with anti-Ls-PPE or anti-WSP serum for the localization of Ls-PPE and *Wolbachia* in adult worms, respectively.

Immunohistology results revealed the presence of *Wolbachia* and Ls-PPE in various worm compartments. Staining for *Wolbachia* was predominantly observed in embryos and microfilariae contained in the uterus of female worm and staining was observed to a lesser extent in the cuticle of female worm (Fig. 25 A). The staining pattern for Ls-PPE was similar to that of *Wolbachia* but staining intensity was lower. The presence of Ls-PPE was observed in embryos and very faint staining was observed in the cuticle and in microfilariae (Fig. 25 B). Hence, comparing the staining pattern of *Wolbachia* and Ls-PPE reveals co-localization of *Wolbachia* and Ls-PPE. No staining was observed in worm sections stained with rabbit pre-immune serum (negative control) (Fig. 25 C).

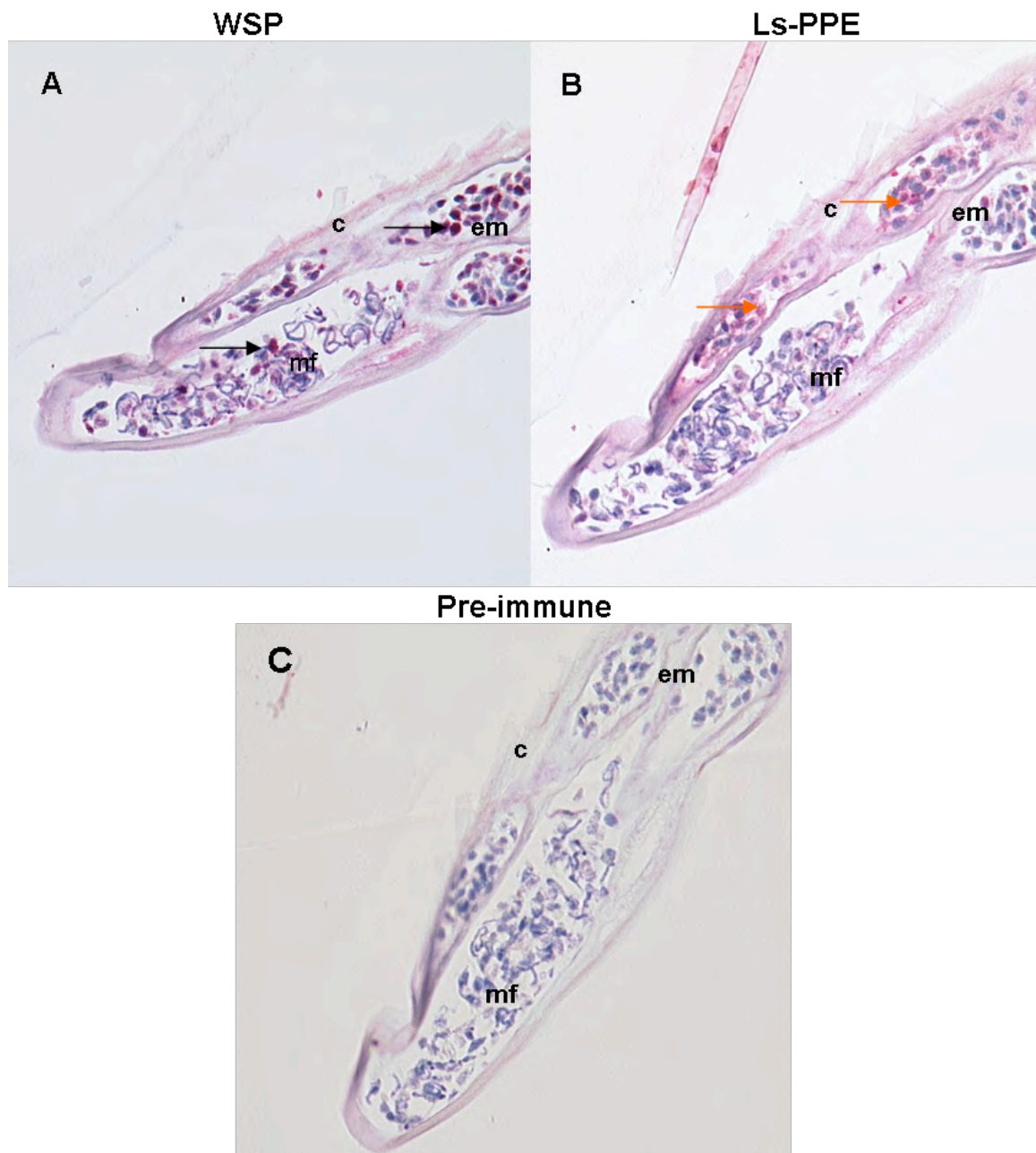


Fig. 25. Localization of Ls-PPE and WSP in *L. sigmodontis*. A) *L. sigmodontis* worm section stained with anti-WSP serum. B) *L. sigmodontis* worm section stained with anti-Ls-PPE serum and C) *L. sigmodontis* worm section stained with pre-immune serum (negative control). The bound antibodies were detected by APAAP staining (Dako Cytomation, Glostrup, Denmark) and counter stained with Mayer's hemalum solution. Black and orange arrows indicate the presence and location of *Wolbachia* and Ls-PPE in *L. sigmodontis* respectively. c, cuticle; em, embryos; mf, microfilariae.

5.4.1.1. Localization of Ls-PPE and *Wolbachia* in untreated and tetracycline treated *L. sigmodontis*

Localization of *Wolbachia* and Ls-PPE in untreated and tetracycline treated adult worms will bring an insight into potential role for Ls-PPE in nematode-*Wolbachia* endosymbiosis. To study the depletion of *Wolbachia* and regulation of Ls-PPE using immunohistology, *IL-5*-deficient BALB/c mice having patent infection with *L. sigmodontis* were treated intraperitoneally with tetracycline. Adult female worms collected from Con and Tet groups on day 36 were processed for immunohistology. The localization pattern of Ls-PPE and *Wolbachia* in serial sections of untreated and tetracycline treated *L. sigmodontis* worms was investigated using anti-WSP and anti-Ls-PPE sera, respectively.

In the Fig. 26, worm sections A1-A2, B1-B2 and C1-C2 are untreated worms and worm sections A3-A5, B3-B5 and C3-C5 are tetracycline treated worms. Worm sections labelled as A (A1-A5) were stained with anti-WSP serum, worm sections labelled as B (B1-B5) were stained with anti-Ls-PPE serum whereas worm sections labelled as C (C1-C5) were stained with pre-immune rabbit serum. In untreated worms, co-localization of *Wolbachia* and Ls-PPE was observed as clear staining for *Wolbachia* and Ls-PPE could be seen in the embryos of female worms (A1, B1) whereas no staining was observed in worms stained with pre-immune serum (C1). In contrast, in some of the untreated worm sections, no staining for Ls-PPE was observed in cuticle and microfilariae whereas staining for *Wolbachia* could be seen in cuticle and microfilariae revealing lower expression of Ls-PPE in cuticle and microfilariae of untreated worms where *Wolbachia* are in abundance (A2, B2). After tetracycline treatment, the staining pattern was reversed. Anti-WSP serum was unable to detect *Wolbachia* after tetracycline treatment revealing the depletion of *Wolbachia* (A3, A4 and A5). In contrast, higher staining for Ls-PPE was observed in the embryos of tetracycline treated worms (B3, B4 and B5). Notably, Ls-PPE staining was also observed in microfilariae of tetracycline treated worms (B5). The worm sections which were stained with rabbit pre-immune serum showed no staining (C3, C4 and C5).

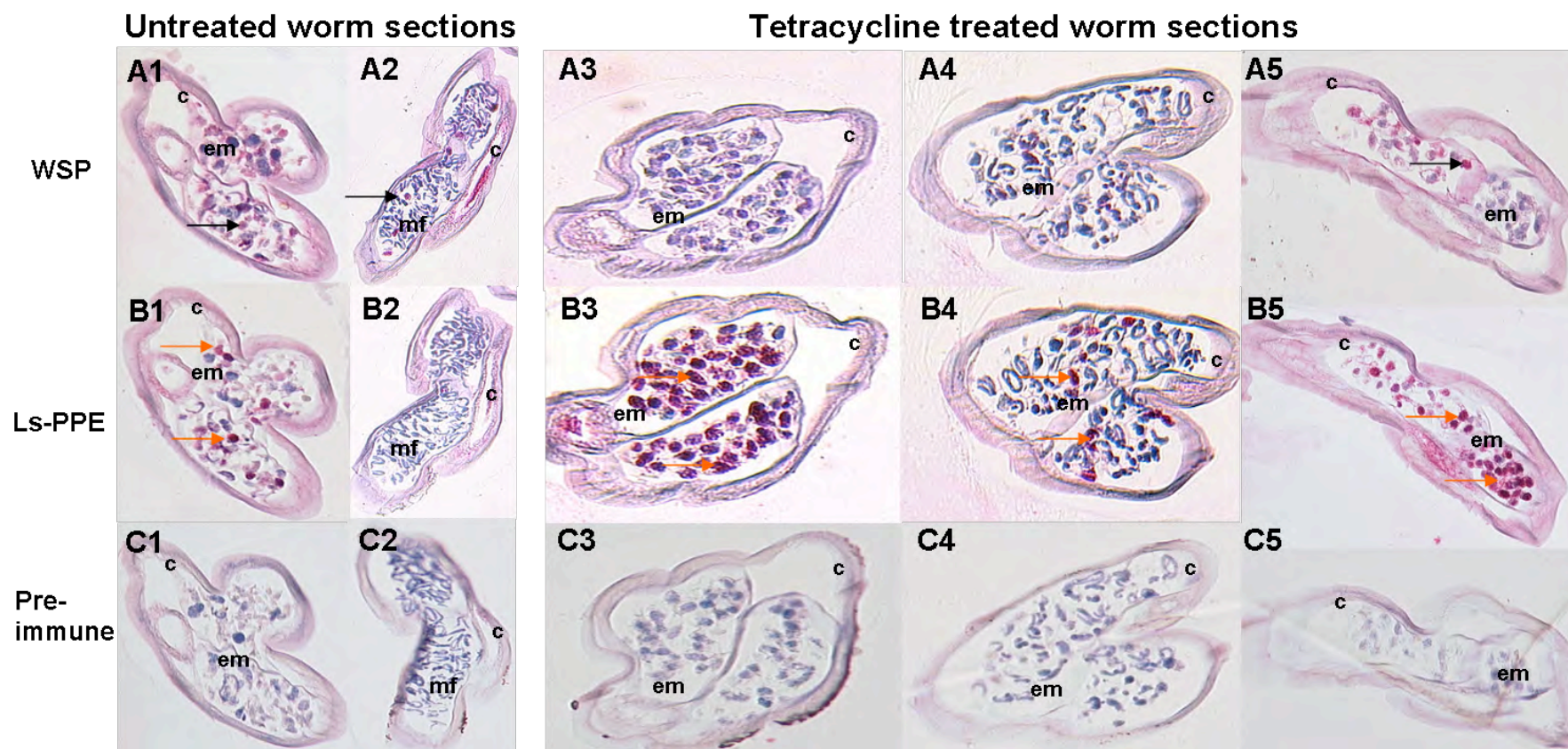


Fig. 26. Staining patterns of *Wolbachia* and Ls-PPE in untreated and tetracycline treated worm sections. A1-A2, B1-B2 and C1-C2 are untreated worms and A3-A5, B3-B5 and C3-C5 are tetracycline treated worms (Day 36). Worm sections, A1-A5 are stained with anti-WSP serum, B1-B5 are stained with anti-Ls-PPE serum and C1-C5 are stained with pre-immune serum (negative control). The bound antibodies were detected by APAAP staining (Dako Cytomation, Glostrup, Denmark) and counter stained with Mayer's hemalum solution. Black and orange arrows indicate *Wolbachia* and Ls-PPE in *L. sigmodontis*, respectively. c, cuticle; em, embryos; mf, microfilariae.

5.4.2. Localization of Ov-PPE and *Wolbachia* in untreated and doxycycline treated *O. volvulus* worm sections

Localization pattern of *Wolbachia* and *O. volvulus* phosphate permease (Ov-PPE) could provide an insight into the importance of Ov-PPE in the endosymbiosis between *Wolbachia* and human filarial nematode, *O. volvulus*. Anti-Ov-PPE serum efficient in recognizing protein of predicted MW of 52 KDa corresponding to Ov-PPE was used to localize Ov-PPE. Anti-WSP serum was used to localize *Wolbachia* on sections in untreated *O.volvulus* worm nodules and 6 weeks doxycycline treated *O.volvulus* worm nodules. Human material came from patients with onchocerciasis that received 6 weeks of daily doxycycline treatment (100 mg/day). Nodules were excised 4 months post-treatment whereas controls did not receive doxycycline.

In untreated worms, staining for *Wolbachia* could be found in hypodermis and in embryos of adult female worms whereas anti-serum to Ov-PPE produced very faint signals (Fig. 27 A1, 27 B1) revealing lower expression of Ov-PPE in untreated *O.volvulus* worms. Nodule sections incubated with rabbit pre-immune serum were completely negative (Fig. 27 C1).

In doxycycline treated worms, the staining pattern for *Wolbachia* and Ov-PPE was completely inverse to that found in untreated worms. There was a drastic reduction in WSP staining owing to depletion of *Wolbachia* in the hypodermis and in embryos of doxycycline treated worms (Fig. 27 A2). In contrast to the faint staining for Ls-PPE in untreated worms, strong staining for Ov-PPE was observed in embryos and hypodermis of doxycycline treated worms (Fig. 27 B2) whereas the worm section stained with pre-immune serum was negative (Fig. 27 C2). Hence, comparing the staining pattern of *Wolbachia* and Ov-PPE in untreated and doxycycline treated worms reveal that Ov-PPE co-localized to areas where *Wolbachia* are contained in adult worms and is up-regulated after 6 weeks doxycycline treatment *in vivo*.

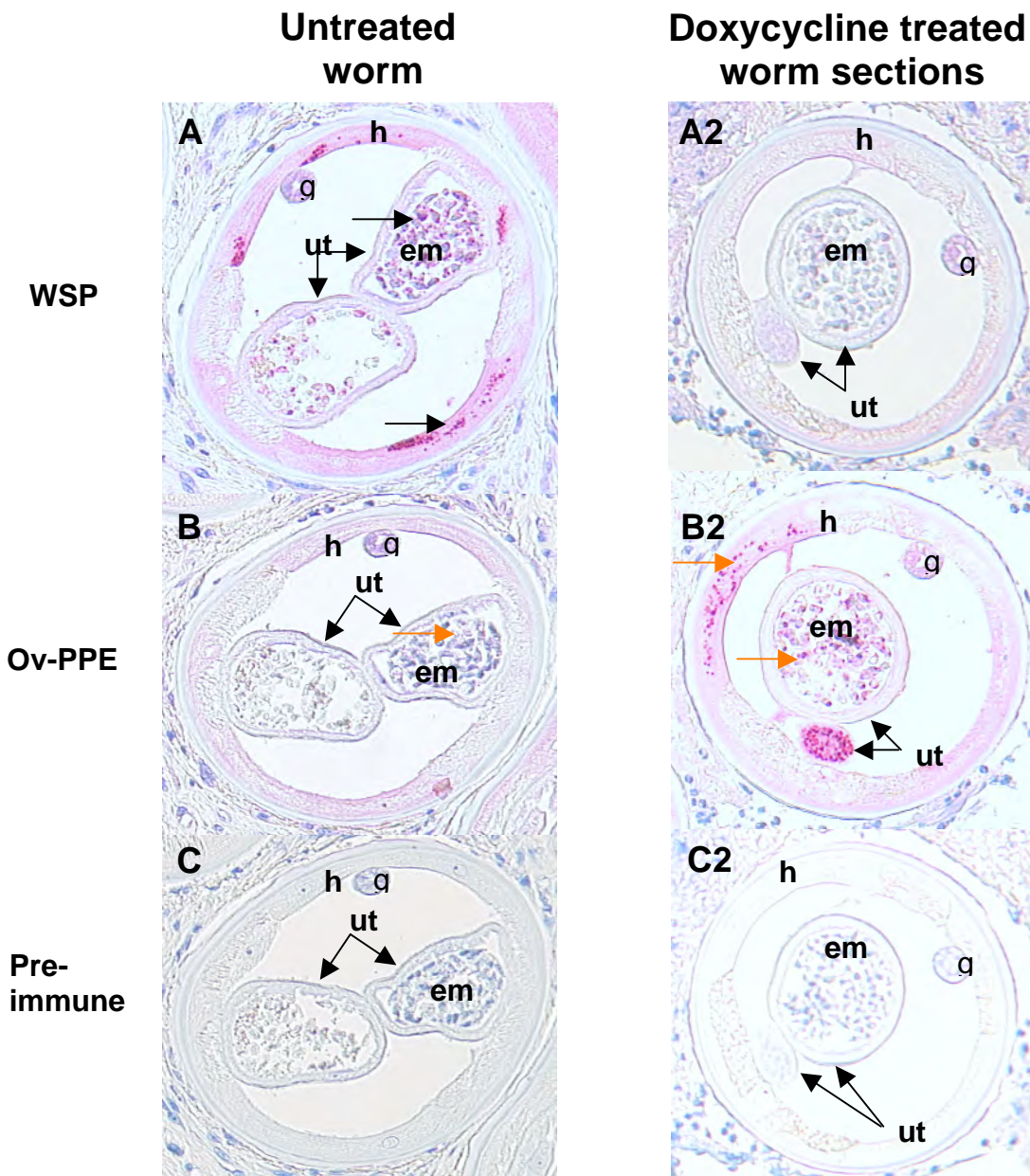


Fig. 27. Localization of *Wolbachia* and Ov-PPE in untreated and 6 weeks doxycycline treated *O. volvulus* worms. A1, B1 and C1 are untreated *O. volvulus* worm sections and A2, B2 and C2 were 6 weeks doxycycline treated *O. volvulus* worm sections. A1 and A2 were stained for *Wolbachia* using anti-WSP serum, B1 and B2 were stained for Ov-PPE with anti-Ov-PPE serum and C1 and C2 were stained with rabbit pre-immune serum. The bound antibodies were detected by APAAP staining (Dako Cytomation, Glostrup, Denmark) and counter stained with Mayer's hemalum solution. Black and orange arrows indicate the presence and location of *Wolbachia* and Ov-PPE, respectively. g, gut; h, hypodermis; em, embryos; ut, uterus.

6. Discussion

Filarial infections are endemic in several tropical countries and as many as 200 million people are infected and 1.3 billion are at risk of being infected (WHO, 2006; WHO, 2007). Filarial infections in humans can be broadly subdivided into lymphatic filariasis (LF) and onchocerciasis. LF is mainly caused by *W. bancrofti* and *Brugia* spp in India, Africa and South East Asia and is estimated to affect 44 million people (WHO, 2006). Onchocerciasis, also known as river blindness, is caused by *O. volvulus* and affects 37 million people in Africa (WHO, 2007).

Currently available drugs for control of filariasis are only efficient against microfilarial stage of filarial parasite and there are concerns on the development of resistance to these drugs. This requires development of new classes of drugs which have microfilaricidal and/or macrofilaricidal activity. For a decade, *Wolbachia* endosymbionts of filarial nematodes have earned major attention among researchers due to the profound role they have on nematode biology. Depletion of *Wolbachia* in filarial nematode in experimental animal models results in impairment of L3 to L4 larval moulting *in vitro* (Smith and Rajan, 2000; Rao et al., 2002) and *in vivo* depletion of *Wolbachia* by antibiotics causes interruption of embryogenesis leading to sterilization and death of adult worms (Hoerauf et al., 1999; Volkmann et al., 2003). Hence, *Wolbachia* are considered as potential targets for the discovery of new drugs as depletion of *Wolbachia* is macrofilaricidal and they also play an important role in the pathogenesis of filariasis (Taylor & Hoerauf, 1999; Hoerauf et al., 2001; Hoerauf et al., 2003; Taylor et al., 2005; Debrah et al., 2006; Supali et al., 2008).

The effect of tetracycline on filarial nematodes is not completely understood. In experimental animal models, tetracycline treatment leads to depletion of *Wolbachia* in nematodes which results in filarial growth retardation if the treatment is started at the onset of infection, and infertility if the treatment is started after adult worms have developed (Hoerauf et al., 1999; Volkmann et al., 2003). Oral tetracycline treatment inhibits *Brugia pahangi* development from L3 to adult worms and this effect occurs during early larval development suggesting that tetracycline treatment interferes with the moulting of larvae (Bosshardt et al., 1993; Chirgwin et al., 2003). Casiraghi et al. (2002) showed that tetracycline treatment targeting the different time points of male and female worm development (*i.e.*, treatment before/after L4 moulting) results in

a sex-ratio distortion, suggesting that *Wolbachia* may play a more active role in female than in male worms.

However, the results described above may also have raised from a direct effect of tetracycline on the larvae rather than the depletion of *Wolbachia* leading to reduced larval development. This is supported by the shorter treatment times compared to the 3-4 weeks needed to sterilize adult worms (Hoerauf, et al., 2003; Debrah, et al., 2006; Turner et al., 2006). Additionally, a modified tetracycline with no antimicrobial activity also inhibited the L3-L4 molt, suggesting possible direct pharmacological action of tetracycline on the L3 independent of its anti-wolbachial activity (Smith and Rajan, 2000). Hence, it is still unclear whether loss of *Wolbachia* inhibits moulting and development or rather it is a direct anti-filarial effect of tetracycline on nematodes, which affects the development. The first part of the thesis aims to answer the above question where MF were depleted of *Wolbachia* and gerbils were then infected with mites carrying infective L3 larvae that developed from *Wolbachia*-depleted MF to study the role of *Wolbachia* in the outcome of male and female worm development.

Molecular mechanisms governing the endosymbiosis between *Wolbachia* and their filarial nematode hosts still remain to be elucidated. The annotated genome of *Wolbachia* bacteria of *B. malayi* (*wBm*) provides information regarding the basic biochemistry and potential targets of *wBm*, which could be targeted for chemotherapy. It is hypothesized that *wBm* provide riboflavin, flavin adenine dinucleotide, heme and nucleotides to their nematode host- *B. malayi* and in return the nematode host probably provides amino acids essential for growth of *Wolbachia* (Foster et al., 2004). Drugs inhibiting the biochemical pathways of the above biomolecules of *wBm* could lead to sterilization and death of adult worms (Pfarr and Hoerauf, 2005). One way to identify key biochemical pathways and biomolecules involved in the *Wolbachia*-nematode endosymbiosis would be to study the expression of nematode genes in response to *Wolbachia* depletion. RNA differential display was used to identify up-regulated filarial genes in *L. sigmodontis* in response to *Wolbachia* depletion. Several genes were found to be up-regulated in response to *Wolbachia* depletion and one of the up-regulated genes had similarity to the phosphate permease family of proteins with orthologues in *Caenorhabditis elegans*, *A. viteae* and *B. malayi* (Heider et al., 2006). The second part of thesis further defines a potential role for phosphate permease in the endosymbiosis between *Wolbachia* and their filarial hosts.

6.1. Sex-ratio distortion study

This study was executed to monitor the influence of *Wolbachia* endosymbionts on the development of *L. sigmodontis* in Mongolian gerbils. The first aim of the work was to study the effect of loss of *Wolbachia*, achieved by treatment with tetracycline, on MF development to L3 larvae in the intermediate host - mites. Secondly, to study the development of *Wolbachia*-depleted L3 larvae in a definitive host - Mongolian gerbils. Earlier reports on susceptibility to tetracycline have shown that tetracycline treatment was stage dependent; hence tetracycline treatment initiated before or after the L3-L4 molt affects male and female worm development differently (Casiraghi et al., 2002). In these earlier experiments, effects on the development of worms in the treated groups could have been due to an anti-parasitic activity of tetracycline rather than its anti-wolbachial activity. Similar results with *in vitro* nematode studies have shown that tetracycline is capable of arresting the L3-L4 molt, with no measurable change in levels of *Wolbachia* DNA detected in treated nematodes (Smith and Rajan, 2000). Treating worms in culture with a tetracycline modified to have no antibacterial activity also inhibited larval moulting, suggesting that part of the action of tetracycline could be a direct pharmacological effect on the nematode independent of *Wolbachia* (Smith and Rajan, 2000).

To determine either a direct effect of tetracycline or the absence of *Wolbachia* on moulting and development of *L. sigmodontis*, gerbils were infected with L3 larvae that had developed from *Wolbachia*-depleted MF. By this method, only the L1 larvae would be exposed to tetracycline, which has been shown to have no apparent harmful effects on MF (Chirgwin, et al., 2003; Hoerauf et al., 2003; Debrah, et al., 2006).

6.1.1. Lower *Wolbachia* levels in MF (L1) impairs the development of L1 to L3 in intermediate mite hosts

Tetracycline treatment significantly reduced *Wolbachia* levels in MF from 1° Tet gerbils in comparison to that of 1° Con and greatly affected development of MF to L3 larvae in the intermediate host. Mites infected with *Wolbachia*-depleted MF had a lower prevalence of L3 larvae (9%) in comparison to Con mites (54%). Because the microfilaremia of the animals used

to infect these mites was the same, the low prevalence of L3 larvae in Tet mites probably occurred because these mites had fed on *Wolbachia*-depleted MF. This suggests that a reduction in initial levels of *Wolbachia* in the MF affects the development of L1 to L3 in the intermediate host. Also, *Wolbachia* levels of L3 larvae that developed in 1° Tet gerbils were significantly lower than the *Wolbachia* levels of L3 larvae that developed in 1° Con gerbils. A similar impairment of larval development in the intermediate host was seen in two previous studies. In one study, tetracycline treatment of *Mastomys coucha* infected with *B. malayi* resulted in interference of subsequent development of L3 larvae in the mosquito vector (Srivastava and Misra-Bhattacharya, 2003), while in the other tetracycline treatment of *B. pahangi* infected *Aedes togoi* mosquitoes, the intermediate host, resulted in lower recovery of L3 (Sucharit et al., 1978). However, in the first study, *Wolbachia* levels were not determined and it is unknown if the *B. malayi* L3 were able to develop into adult worms. In the second study, tetracycline treatment of infected *Aedes togoi* resulted in lower L3 recovery, the same effect was also seen in our study, but without tetracycline treatment of the intermediate host. This later fact supports the conclusion that *Wolbachia* depletion alone causes the impairment of larval development and is not a direct effect of tetracycline on the larvae.

6.1.2. Sex-ratio distortion caused by *Wolbachia* depleted L3 larvae

Infection of naive gerbils with *Wolbachia*-depleted L3 larvae resulted in drastic reduction in the development of female worms, but did not affect the development of male worms. This male biased sex-ratio distortion in Tet gerbils caused by *Wolbachia*-depleted L3 larvae suggests that *Wolbachia* have more influence on the development of female than male worms. This is supported by the fact that male worms with significantly fewer *Wolbachia* could be selected by tetracycline treatment. However, female worms with few *Wolbachia* could not be selected by tetracycline treatment. The few female worms that developed from *Wolbachia*-depleted MF had endobacteria levels equivalent to control worms. We postulate that these worms developed from MF that originally had *Wolbachia* levels above the median level, which were then depleted by the tetracycline treatment to a level that still allowed for successful development. Because the ranges of *Wolbachia* content of the control MF are so large, sometimes nearing that of the high range of *Wolbachia*-depleted MF, it was not possible to determine the threshold of *Wolbachia* needed for female worms to properly develop. An experiment that stops tetracycline treatment of

the gerbils at various points of time might provide enough statistical power to be able to calculate this threshold.

Another possibility that a few female worms with equivalent numbers of *Wolbachia* as controls were able to develop from *Wolbachia*-depleted L1s is that their endobacteria were resistant to tetracycline. However, there is compelling evidence that this is not the case. Firstly is that microfilaria levels in the 1^o Tet gerbils never recovered after treatment, indicating that the antibiotic treatment had successfully blocked embryogenesis. Secondly, from annotation analysis of the genome of the filarial endosymbionts, no evidence for extra-chromosomal DNA (i.e. a resistance plasmid) has been found (Foster et al., 2005); and, in contrast to *Wolbachia* of arthropods, the endobacteria in filarial worms appear to be under very little selective pressure, probably due to their mutualistic symbiosis. Finally, no evidence for recombination, another method by which a resistant gene might be acquired, has been identified in filarial *Wolbachia* (Casiraghi et al., 2003).

6.1.3. Importance of *Wolbachia* in worm development

These results support that not only are *Wolbachia* necessary for oogenesis and embryogenesis (Hoerauf et al., 1999; Hoerauf et al., 2000; Hoerauf et al., 2001; Hoerauf et al., 2003), but they are also needed for proper development of female worms, maybe as a source of heme for enzymes participating in biosynthetic pathways of hormones or other metabolites needed during moulting (Foster et al., 2005, Pfarr and Hoerauf, 2006). Insect hormones such as ecdysone and 20-hydroxyecdysone affect the moulting of *Dirofilaria immitis* and *B. pahangi* larvae *in vitro*, indicating that ecdysone, or a similar molecule, may play a role in moulting (Barker et al., 1991; Warbrick et al., 1993). Insect ecdysone induces transcription factors, which carry out moulting. A putative *D. immitis* orthologue of the *Drosophila* ecdysone response early gene E78 has been identified and is expressed in adult females but not males (Crossgrove et al., 2002). *Wolbachia* could serve as a source of heme, since *B. malayi* apparently lack the necessary genes (Foster et al., 2005; Ghedin et al., 2007). These enzymes and hormones are especially necessary for female worm development; hence they define a more active role of *Wolbachia* in the development of female worms. In addition, *Wolbachia* possess all genes for enzymes involved in riboflavin and flavin adenine dinucleotide biosynthesis and contain genes for complete *de novo* synthesis of

purines and pyrimidines. The *B. malayi* genome lacks genes for purine biosynthesis and hence *B. malayi* worms may be dependent on nucleotides provided by *Wolbachia* as nucleotides play a vital role in the embryogenesis (Foster et al., 2005; Pfarr and Hoerauf, 2006; Ghedin et al., 2007).

6.2. *L. sigmodontis* phosphate permease (Ls-PPE) and *O. volvulus* phosphate permease (Ov-PPE) regulation in response to *Wolbachia* depletion

Mutualist symbiotic *Wolbachia* endobacteria found in most filarial nematodes, are essential for embryogenesis and for larval development into adults, and thus represent a new target for anti-filarial drug development. Tetracyclines, rifampicin and chloramphenicol, are efficient in inhibiting larval moulting (from L3 to L4) and their development *in vitro* (Smith and Rajan, 2000; Rao et al., 2002). Several studies have shown detrimental effects of antibiotics on the growth and reproduction of filarial nematodes in experimental animal models. Antibiotic treatment leads to blockage in embryogenesis which results in reduction in the number of MF released to the blood which occurs after depletion of *Wolbachia* (Bosshardt et al., 1993; Rao et al., 2002; Bandi et al., 1999; Hoerauf et al., 1999; Townson et al., 2000). In *Onchocerca ochengi*, a filarial nematode infecting cattles, tetracycline treatment kills the adult worms (Langworthy et al., 2000). Doxycycline, a modified tetracycline, is effective in eliminating *Wolbachia* in human filarial nematodes which reduces filarial infections. Doxycycline treatment of patients infected with onchocerciasis resulted in depletion of *Wolbachia* in adult worms leading to severe degeneration of embryos (Hoerauf et al., 2000; Hoerauf et al., 2001; Hoerauf et al., 2003). Doxycycline treatment is efficient in killing adult *W. bancrofti* (Taylor et al., 2005; Debrah et al., 2007). Anti-*Wolbachia* therapy also leads to amelioration of suprastesticular dilated lymphatic vessels and with an improvement of pathology in lymphatic filariasis patients (Debrah et al., 2006). At present a combination of doxycycline and anti-filarial drugs such as ivermectin (onchocerciasis) and diethylcarbamazin (lymphatic filariasis) has been implemented for better microfilaricidal and macrofilaricidal activity (Taylor et al., 2005; Debrah et al., 2007; Supali et al., 2008). Still little is known about the molecular mechanisms of the symbiotic interaction between *Wolbachia* and their filarial nematodes. Nematode genes that respond to anti-*Wolbachia* antibiotic treatment may play important roles in the symbiosis. Differential display PCR was

used to detect and characterize several candidate genes that are up-regulated after 6, 15 and 36 days of tetracycline treatment (Heider et al., 2006).

One of these genes, *Ls-ppe-1*, was similar to a family of phosphate permeases with putative orthologues in *O. volvulus*, *B. malayi*, *C. elegans* and *A. viteae* was found to be up-regulated during *Wolbachia* depletion. *Ls-ppe-1* steady-state mRNA levels were elevated by day 3–6 of treatment, and remained elevated through to 70 days post-treatment. In comparison to untreated worms, there was a threefold up-regulation of *Ls-ppe-1* in tetracycline treated worms (Heider et al., 2006).

6.2.1. Up-regulation of *L. sigmodontis* phosphate permease (Ls-PPE) at the protein level

To study the up-regulation of *Ls-ppe-1* at the protein level, anti-Ls-PPE serum was used in a Western blot assay which detected a protein band of 53 KDa, the predicted molecular weight of Ls-PPE. Ls-PPE was found to be up-regulated at the protein level on days 15 and 36 of tetracycline treatment. In addition, on day 36 of tetracycline treatment, there was significant reduction of *Wolbachia*. There was no up-regulation of Ls-PPE at the protein level observed when filarial worms were treated with gentamycin, a drug ineffective in killing *Wolbachia*. This suggests that Ls-PPE is up-regulated in response to *Wolbachia* depletion, and antibiotic treatment itself does not cause an increase in expression of the nematode phosphate permease in this species. Thus, the up-regulation of Ls-PPE at the protein level shows that this nematode phosphate permease is transcriptionally up-regulated and then translated in response to the loss of *Wolbachia* from the host cells. Ls-PPE could have functional role in the nematode biology and *Wolbachia*-nematode endosymbiosis because in *C. elegans*, an orthologue of *Ls-ppe-1* named CeC48A72 phosphate permease is expressed in larval and adult stages of the nematode and knockdown of this gene by RNA interference (RNAi) results in a phenotype characterized by embryonic lethality and sterility of adult nematodes (Maeda et al., 2001) which are also characteristic phenotype occurring as result of *Wolbachia* depletion from filarial nematodes (Hoerauf et al., 2003). Therefore, considering the RNAi data from *C. elegans*, it appears that *Ls-ppe-1* could have a functional role in the endosymbiosis. Hence, the above findings suggest that Ls-PPE is up-regulated both at the mRNA and protein levels in response to *Wolbachia* depletion

which is probably not a direct effect of the antibiotic on the parasite and Ls-PPE could have functional role in the nematode biology and *Wolbachia*-nematode endosymbiosis.

6.2.2. Up-regulation of *O. volvulus* phosphate permease (Ov-PPE) at the protein level

O. volvulus have an orthologue of Ls-PPE and anti-*Wolbachia* therapy has been implemented in the treatment of onchocerciasis where depletion of *Wolbachia* leads to detrimental effect on worm viability and fertility (Hoerauf et al., 2000; Hoerauf et al., 2001; Hoerauf et al., 2003; Heider et al., 2006; Debrah et al., 2007; Supali et al., 2008). Hence investigating the regulation of *O. volvulus* phosphate permease (Ov-PPE) during *Wolbachia* would be interesting as Ov-PPE could be considered a possible drug target. Anti-Ov-PPE serum was able to recognize a protein band of 52 KDa, predicted molecular weight of Ov-PPE in a Western blot assay in 6 weeks doxycycline treated worms. Phosphate permease was also up-regulated during *Wolbachia* depletion in human filarial nematode *O. volvulus*. The data suggest that Ov-PPE could play a pivotal role in worm biology as its expression is up-regulated during *Wolbachia* depletion with *Wolbachia* being essential for nematode embryogenesis.

6.2.3. Localization of phosphate permease and *Wolbachia* in *L. sigmodontis* and *O. volvulus* worm sections

Investigating the location of phosphate permease will test the hypothesis of co-localization of the protein with *Wolbachia*. To test this hypothesis, anti-Ls-PPE and anti-Ov-PPE sera were used to localize phosphate permease in *L. sigmodontis* and *O. volvulus* worm sections, respectively. Anti-WSP serum was used to localize *Wolbachia* in *L. sigmodontis* and *O. volvulus* worm sections. Interestingly, both in *L. sigmodontis* and *O. volvulus*, phosphate permease was found in worm compartments where *Wolbachia* are found. Both in *L. sigmodontis* and *O. volvulus*, the localization pattern of phosphate permease and *Wolbachia* in untreated and antibiotic treated worms was similar. In untreated worms, there was an abundance of *Wolbachia* in the hypodermis, in embryos and in microfilariae, whereas there was a very low expression level of phosphate permease in the same tissues of the worm. After antibiotic treatment of *L. sigmodontis* and *O. volvulus*, the localization pattern of *Wolbachia* and phosphate permease was completely reversed. No staining for *Wolbachia* could be observed in antibiotic treated worms, whereas a

stronger expression of phosphate permease was observed in antibiotic treated *L. sigmodontis* and *O. volvulus* worms. In *L. sigmodontis*, the localization of Ls-PPE was observed in cuticle, in embryos and in microfilariae of tetracycline treated worms, places where *Wolbachia* are abundant in untreated worms, suggesting that the depletion of *Wolbachia* cause up-regulation of Ls-PPE in these tissues. Similarly, staining for Ov-PPE in 6 weeks doxycycline treated worms was found in the hypodermis and in embryos inside the uteri of female worms where *Wolbachia* was in abundance in untreated *O. volvulus* worms. These results strongly suggest that phosphate permease and *Wolbachia* might be located in close proximity in filarial worms, a requirement if Ls-PPE/Ov-PPE is supporting phosphate needs of the endosymbionts. In addition, both in *L. sigmodontis* and *O. volvulus*, there was increased staining of phosphate permease in antibiotic treated worms which had no or very few *Wolbachia*, hence strengthening the finding that phosphate permease is up-regulated at the protein level during *Wolbachia* depletion by antibiotic treatment.

6.2.4. Importance of phosphate permease in *Wolbachia*-nematode endosymbiosis

The importance of phosphate permease in the symbiosis may relate to a possible involvement in nucleotide metabolism. The genome of the *Wolbachia* bacteria from the filarial nematode *B. malayi* (wBm) provides broad spectrum of information regarding genes which could play an important role in the endosymbiosis (Foster et al., 2005). In contrast to most endosymbiotic bacteria, wBm is able to make only one amino acid *de novo*, but has retained all genes necessary for the *de novo* synthesis of nucleotides. It is postulated that wBm may provide nucleotides or nucleotide precursors to its host for processes such as embryogenesis (Foster et al., 2005; Pfarr and Hoerauf, 2005). *Ls-ppe-1* has a predicted secretory signal peptide and the deduced amino acid sequence of *Ls-ppe-1* has 12 transmembrane helices suggesting that this protein is a membrane associated protein. As phosphate is an essential molecule for nucleotide synthesis, one can hypothesize that phosphate permease could transport phosphate to *Wolbachia* for the *de novo* nucleotide biosynthesis. From Western blot and immunohistological results in this study, we now know that phosphate permease expression is increased at the protein level during *Wolbachia* depletion and co-localizes with *Wolbachia*. *Wolbachia* depletion after tetracycline treatment would lead to disturbance in the homeostasis of nucleotide levels and the worm cell might then attempt to compensate for the lack of nucleotides by increasing the expression of Ls-PPE,

thereby providing more phosphate to the vesicle containing the *Wolbachia* for nucleotide synthesis (Fig. 28).

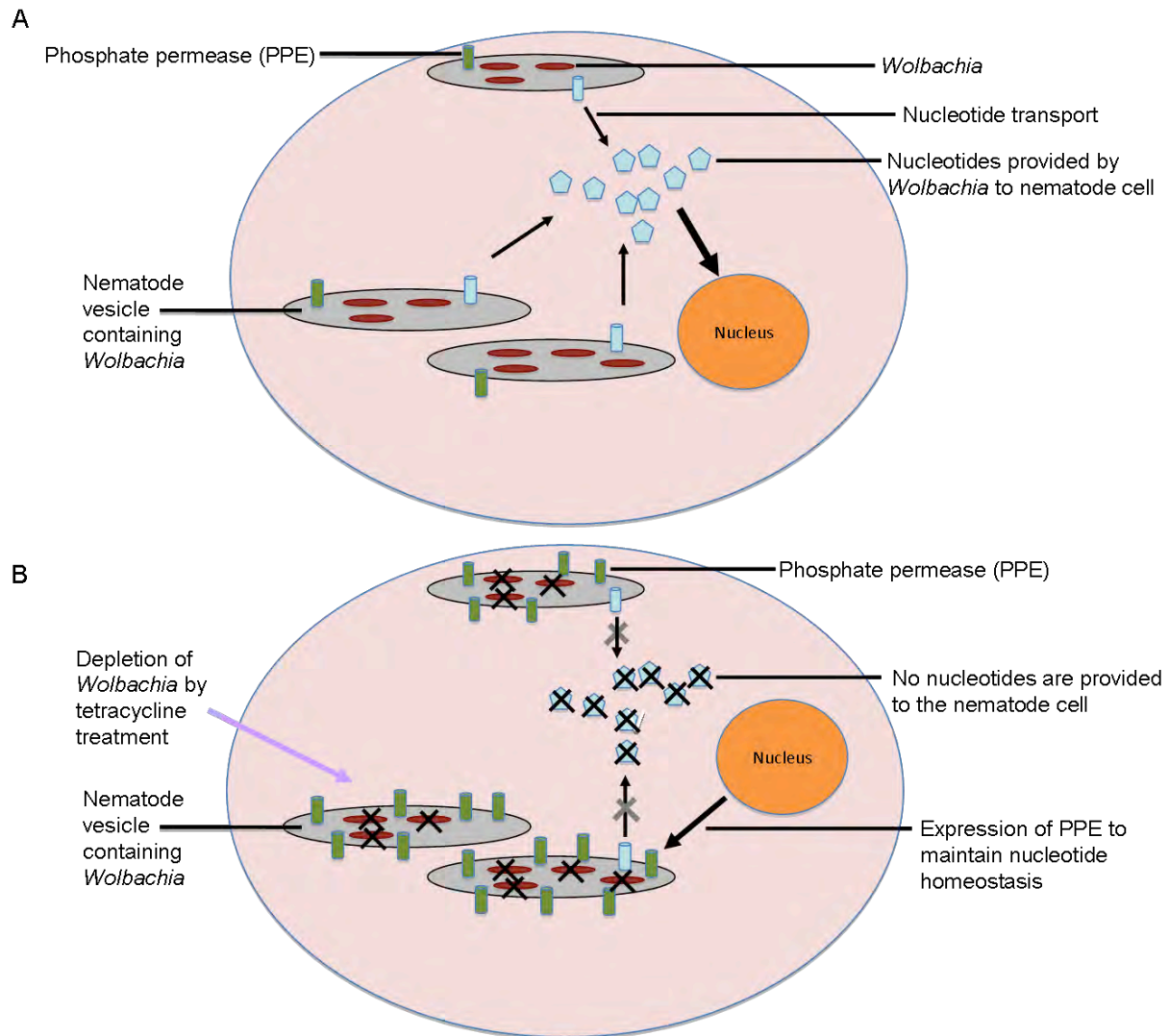


Fig. 28. Schematic representation of the role of phosphate permease (PPE) in the *Wolbachia*-nematode endosymbiosis. A) *Wolbachia* contained in nematode vesicles provides nucleotides to the host cell. B) Depletion of *Wolbachia* by tetracycline treatment leads to lack of nucleotides in nematode cell and the nucleotide homeostasis is disturbed. To compensate for this, the nematode cell up-regulates the expression of phosphate permease.

Should future experiments further support a direct role of Ls-PPE in the symbiosis between *Wolbachia* and *L. sigmodontis*, phosphate permease could be a potential nematode drug target. Data from comparative genomics of *B. malayi* with *C. elegans* reveal that out of 11771 predicted gene products in the data of *B. malayi* genome, 7435 have an ortholog in *C. elegans* and of these,

3059 were mapped to the RNAi positive map in *C. elegans*, serving as a predicted essential *B. malayi* genome. The majority of these essential genes from *B. malayi* have close human orthologues and hence can't be considered as drug targets. The remainder of essential *B. malayi* genes which do not have human orthologues constitute a set of 589 genes which can be considered as candidate drug targets. Interestingly, of these 589 genes, phosphate transport/permease genes are included (Kumar et al., 2007). Hence, nematode phosphate permease not only plays an important role in the *Wolbachia*-nematode endosymbiosis but also could be a potential candidate drug target to combat filariasis.

In conclusion, contributing to the better understanding of the biology of the *Wolbachia*-nematode symbiosis, this is the first report, which shows that depletion of *Wolbachia* alone causes inhibition of moulting and development of the filarial nematode *L. sigmodontis*, and this effect is not an anti-parasitic activity of the tetracycline antibiotic used. This is also the first report to show the up-regulation of phosphate permease from *L. sigmodontis* and *O. volvulus* (Ls-PPE and Ov-PPE) at the protein level in response to *Wolbachia* depletion. Localization of Ls-PPE, Ov-PPE and WSP in filarial worms shows that Ls-PPE and Ov-PPE are confined to areas in worms where *Wolbachia* are found and up-regulation of Ls-PPE and Ov-PPE permease in response to *Wolbachia* depletion is seen in the same tissues, further supporting a direct role of phosphate permease in the biology of the *Wolbachia*-nematode endosymbiosis. The importance of phosphate permease is further indicated since in *C. elegans*, knockdown of an orthologous phosphate permease results in embryonic lethality, a phenotype seen when filarial nematodes are depleted of *Wolbachia*. Thus, phosphate permease could be considered as a potential drug target for the control of filariasis. Further investigations such as electron microscopic ultrastructural localization of PPE and *Wolbachia* and RNAi mediated knockdown of *Ls-ppe-1* in *L. sigmodontis* will elucidate the exact function of PPE in the *Wolbachia*-nematode symbiosis.

7. References

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8. CURRICULUM VITAE

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- Ph.D Microbiology from the University of Bonn (2004-2009).
- B.Sc. Microbiology from the University of Madras (1996-1999).
- M.Sc. Biotechnology from the University of Madras (1999-2001).

RESEARCH EXPERIENCE, TRAINING AND CERTIFICATION

- Sep 08 – till date: Scientific co-worker at the Institute for Medical Microbiology, Immunology and Parasitology, University Clinic Bonn, Germany 53105.
- Oct 2004 – Jun 2008: DAAD Exchange Programme PhD Research Scholar at the Institute for Medical Microbiology, Immunology and Parasitology, University Clinic Bonn, Germany 53105.
- Sep 2002 - Jul 2003: Senior Research fellow (SRF) at the Vector Control Research Centre (VCRC) - Indian Council of Medical Research (ICMR) – Pondicherry, India.
- Nov 2001 - Jun 2002, Chemist at the Qualpro Diagnostics (Tulip group of companies), Goa.
- Nov 2000- Apr 2001: M.Sc project trainee at the Vector Control Research Centre (VCRC) - Indian Council of Medical Research (ICMR) – Pondicherry, India.

PUBLICATIONS

1. Hoti, S.L., **Sridhar, A.**, Das, P.K. (2003). Presence of *Wolbachia* Endosymbionts in Microfilariae of *Wuchereria bancrofti* (*Spirurida: Onchocercidae*) from Different Geographical Regions in India. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, Vol. 98(8): 1017-1019
2. **Sridhar, A** and Pradeep Kumar, N. (2003). Report on *Wolbachia* Endosymbionts in *Culex quinquefasciatus* Mosquito Population from Pondicherry and Tamil Nadu. *Tropical Biomedicine* 20, no. 2: 185–88.

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