Master's Thesis

Transmission Dynamics of the Relapsing Fever Spirochete – *Borrelia duttonii*

Laboratory of Molecular Ecology of Vectors and Pathogens

INSTITUTE OF PARASITOLOGY

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České Budějovice, 2018

Hain, L., 2018: Transmission Dynamics of the Relapsing Fever Spirochete – *Borrelia duttonii*. Mgr. Thesis, in English. – 59 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation: The aim of this thesis was to further our understanding of the acquisition and transmission of the relapsing fever spirochete, *B. duttonii*, by its soft tick vector *O. moubata*. Infectvity of the spirochete, infected ticks and sera was determined. The effect of prolonged in-vitro cultivation on *B. duttonii* was analysed. Furthermore, in-vitro feeding and infection with the spirochete of *O. moubata* was done.

Affirmation:

I hereby declare that I have worked on my diploma thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my master thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Abstract

Studying the acquisition and transmission of *B. duttonii,* one of the causative agents of relapsing fever in Africa, by its vector *O. moubata* was the goal of this work.

By needle inoculation of naïve mice with different dosages of *B. duttonii* we established an ID_{50} of this pathogen in mice. Furhtermore, we analysed the antibody production over the course of an infection in mice.

Acquisition timing of *B. duttonii* by *O. moubata* was determined. Like other soft ticks and relapsing fever *Borrelia*, *O. moubata* can acquire *B. duttonii* at a very high efficiency as soon as the spirochetes become detectable in the blood of infected mice.

Moreover, we assessed the capability of *O. moubata* to acquire *B. duttonii* during in vitro feeding on rabbit blood and different sera. Using pig and chicken sera gave the most promising results.

The infectivity of sera collected from infected mice and infected tick-homogenate was determined by needle-inoculation of naïve mice. We concluded that sera and post-moulting tick-homogenate contain enough spirochetes to establish an infection in a naïve host.

We compared infectivity and acquisition of the wild-type and a prolonged in-vitro passaged isolate of *B. duttonii* and identified differences in the efficiency of acquisition as well as the course of the infection in infected mice.

Antibodies against specific proteins of *B. duttonii* were raised in rabbits and their specificity for the proteins of *B. duttonii* was determined. The proteins were identified by MS-Analysis.

Interactions of *B. duttonii* with red blood cells were visualized by electron microscopy.

Various transmission experiments of *B. duttonii* from *O. moubata* to naïve hosts were conducted to establish a fully working tick-animal model.

Acknowledgment

I want to thank my supervisor Dr. Ryan Rego for his guidance, support and advice and to both him and prof. RNDr. Libor Grubhoffer, CSc., Hon. D.Sc., for the opportunity to work in the Laboratory of Molecular Ecology of Vectors and Pathogens..

Only with the help and support of the other members of the laboratory, Martin Strnad, Zuzana Vavrušková, Helena Rohackova, Maximilian Bayer and Ana Cetkovic was I able to successfully conduct my experiments. Special thanks go to Zuzka for all her help with animal work.

I want to acknowledge Mgr. Dimitry Logninov, Ph.D. and Katharina Böttinger for their help with MS-analysis and RNDR. Marie Vancová, Ph.D. for the electron microscopic part of this thesis.

I would not have been able to successfully complete my study without the support of my study colleagues and friends, Nora Hagleitner, Katharina Böttinger, Paul Strasser and Helmut Stanzl. Studying with them has motivated me more than I can tell.

Special thanks go to my family who have always supported me emotionally as well as financially. Without them studying would not have been possible.

Content

1 Introduction

1.1 Relapsing Fever *Borrelia*

Relapsing Fever (RF) *Borrelia* are the causative agents of relapsing fever. These bacteria belong to the spirochete phylum and form together with the Lyme Disease species, the *Borrelia* genus [1].

These spirochetes share a common morphology, including 25-30 µm long and 1 µm thick helical cells with an outer membrane surrounding a protoplasmic cylinder. The protoplasmic cylinder is formed by the cytoplasm, the inner cell membrane and a peptidoglycan layer. The flagellum of the bacteria is in the space between the outer membrane and the protoplasmic cylinder [1].

1.1.1 RF *Borrelia* species and Geographical Distribution

Today, there are 23 confirmed RF *Borrelia* species, which are classified depending on their vector to either be tick borne RF (TBRF) or louse borne RF (LBRF) *Borrelia*. Still new candidates for RF *Borrelia* are being identified [2–5] . The only member of the LBRF *Borrelia* group is *B. recurrentis* which is transmitted by the clothing louse *Pediculus humanus* and can be found in Eastern Africa [1].

All other RF *Borrelia* species are tick borne with most of them having a soft tick vector. *B. miyamotoi* and *B. lonestari* are the only known RF *Borrelia* which are transmitted by hard ticks [6–8]. The interaction of vector and *Borrelia* is specific, therefore the distribution of the different RF *Borrelia* species coincides with the distribution of their respective vectors [5].

RF *Borrelia* can be found world-wide and are endemic in Western United states, Southern British Columbia, Mexico, Central and South America, the Mediterranean, the Middle East, Central Asia and Africa. In the United States *B. hermsii* and *B. turicatae* are present. *B. persica* and *B. latyshewii* are found in the Middle East and Asia. Figure 1 shows the distribution of the African RF *Borrelia* strains, including *B. duttonii* in Central Africa, *B. recurrentis* in the East, *B. crocidurae* in North Africa and *B. hispanica* in Morocco [5,9].

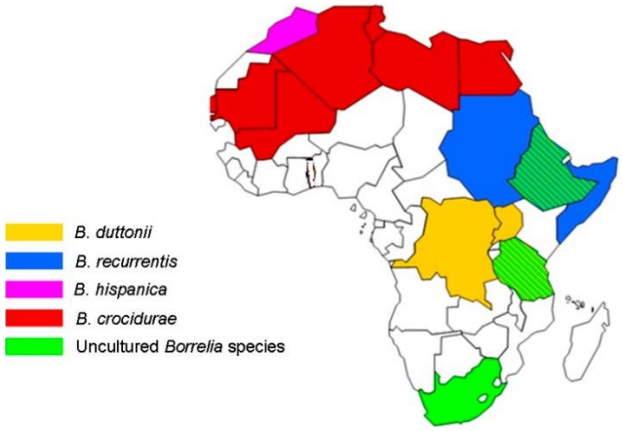

Figure 1 – Geographical Distribution of Relapsing Fever *Borrelia* in Africa, adapted from [9]

1.1.2 Relapsing Fever

Relapsing Fever is an infectious disease caused by RF *Borrelia.* The symptoms of this disease include sudden fever, chills, headache, joint and body pain and are caused by high levels of spirochetes in the blood of the patients, which can reach up to 10^7 spirochetes per mL of blood [10–12].

First symptoms normally occur between 4 to 18 days after infection in humans. LBRF caused by *B. recurrentis* often presents a more severe course of the disease compared to TBRF. Depending on the strain causing the infection the mortality rate lies between 2 and 5% [5,11]. Furthermore, certain symptoms are associated with certain strains, as *B. duttonii* is associated with stillbirth. Infection with *B. duttonii* leads to a risk of 30% of miscarriages and a fetal mortality rate of 15% [13,14].

Febrile and afebrile periods are alternating during the progression of the disease, correlating with the number of spirochetes in the blood. The correlation between the number of spirochetes in the blood and the body temperature in the case of a mouse infected by *B. duttonii* is shown in Figure 2 [15].

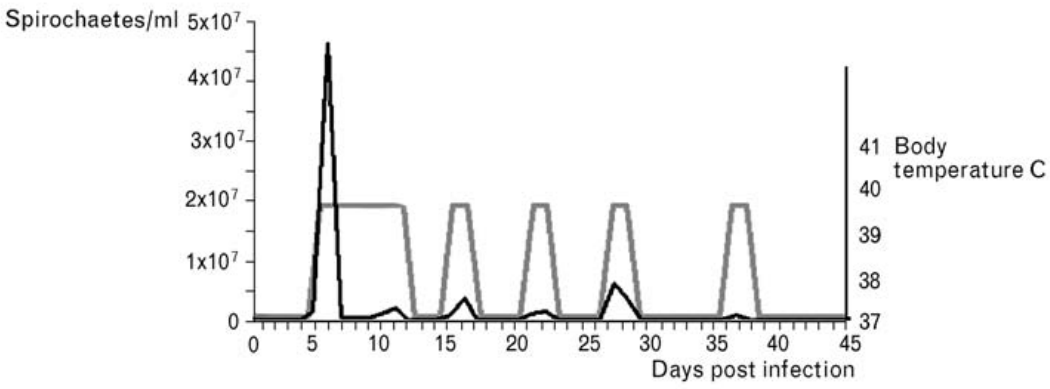

Figure 2 – Comparison of the number of spirochetes in the blood of the mouse (in spirochetes per mL) and the mouse's body temperature (in °C) over the course of an infection with *B. duttonii*, adapted from [15]

Diagnosis of RF can be challenging as the symptoms are common to other illnesses, like malaria, which are also endemic in the affected regions [16]. A simple method is checking blood smears under a dark-field microscope when spirochetemia is high [17]. Furthermore, DNA can be isolated from blood and checked with *borrelia*-specific primers [18]. In the case of a persistent infection, ELISA can be used to screen for antibodies against glpQ a protein expressed in RF *Borrelia* [19,20]. However, this test can only be used at a late stage of the disease, as an immune response for antibody production is needed. Moreover, enrichment of spirochetes in the blood by multiple centrifugation steps followed by Giemsa staining is another approach of detection of spirochetes [17]. In general, there are next to no diagnostics kits available for the detection of RF.

As TBRF and malaria share symptoms as well as geographic distribution, correct identification of RF cases is essential for correct treatment steps. Treatment of RF is usually done by administering antibiotics, such as penicillin, tetracycline or doxycycline [5].

1.1.3 Strategies of RF *Borrelia* for the Evasion of the Host's Immune System

The repeated increase and decrease of RF *Borrelia* in the blood, can be explained by different mechanisms used by the bacteria to evade the host's immune system.

One of them is antigenic variation of the RF *Borrelia's* variable major proteins (Vmps). These Vmps are divided into two classes depending on their size, first there are variable large proteins (Vlps) with a size of 36 kDa and the second group is formed by the so called variable small proteins (Vsps), which are approximately 20 kDa big. By varying the expression of these antigens, the spirochetes can escape the host's immune system, leading to another peak in spirochetemia [5,21–23].

Furthermore, RF *Borrelia* are capable of erythrocyte rosetting. In this process they bind to red blood cells and cover themselves with these cells, hiding form the host's immune response. Recent studies have shown that they bind to neolacto glycans present on human erythrocytes [24,25].

Moreover, studies conducted with TBRF *B. duttonii* and LBRF *B. recurrentis* in-vitro have shown that these two species are resistant to complement by specifically binding the host's C4b-binding protein and factor H. The C4b-binding protein plays an important role in the antibody-mediated classical complement pathway, while factor H is a regulator of the alternative complement pathway [26]. That these two *Borrelia* species are sharing this feature is not surprising, as phylogenetic analysis has shown a close relationship between the two. Suggesting that *B. recurrentis* might have evolved from *B. duttonii* [27].

1.1.4 Natural Reservoirs of RF *Borrelia*

RF *Borrelia* use their evasion techniques to increase their sustainability in their natural reservoirs which include mammals, like rodents and bats, but also birds and reptiles [5]. In rural regions of Senegal as many as 18% of small rodents are infected with *B. crocidurae* [28]. Their arthropod vectors might also be considered as reservoirs of some of the *Borrelia* as transovarial transmission has been shown for example for *B. crocidurae* in *O. erraticus* [29]. An exception to this overall trend is *B. duttonii* which has only been isolated from infected humans and no animal reservoir has yet been identified [5,30].

1.2 Soft ticks (*Argasidae)*

Most RF *Borrelia* are transmitted by members of the *Argasidae* family. The members of this family, also known as soft ticks, have a unique morphology compared to *Ixodidae* ticks (hard ticks).

The nymphal and adult stages lack a dorsal scutum, as is shown in Figure 4 and instead posses a leathery exoskeleton. Furthermore, they are nocturnal animals living in sheltered microhabitats near their hosts [31].

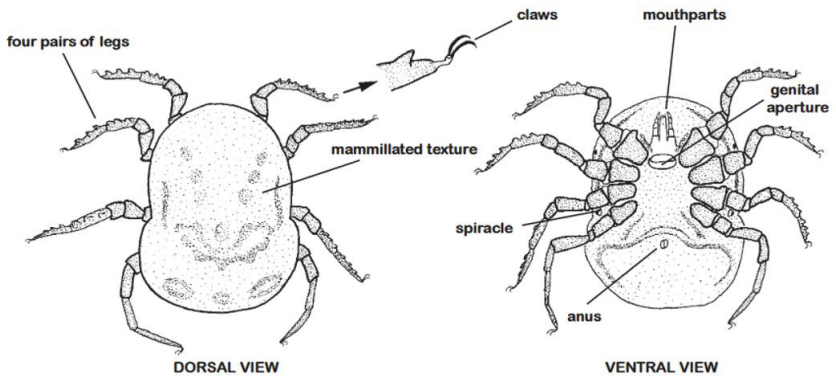

Figure 3 - Morphology of Soft ticks, adapted from [31]

Besides their morphology, also the life cycle of soft ticks is different to the one of hard ticks. First, adult female ticks feed on a host and after the blood meal, each tick lays up to 500 eggs from which 6-legged larvae are hatching. These larvae then moult to 8-legged nymphs either with or without another blood meal in between. Before the nymphal ticks moult into adults, there are up to 8 nymphal stages. Before each moult to the following nymphal or adult stage a separate blood meal is needed. Female adult ticks can lay eggs multiple times after each bloodmeal they take [31,32]. Figure 5 depicts the life cycle of *O. moubata* as a representative of the *Argasidae* ticks.

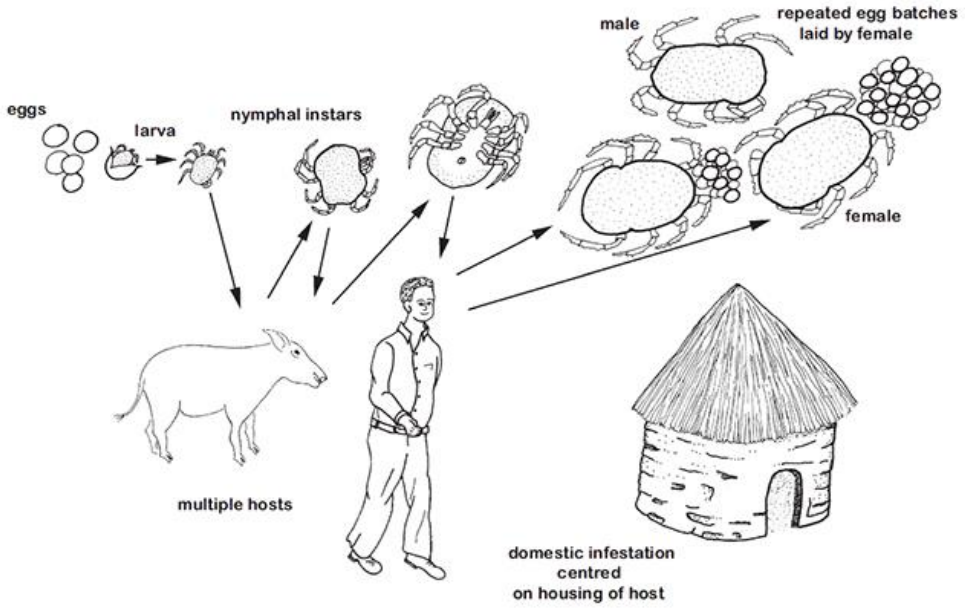

Figure 4 – Life cycle of *O. moubata*: 6-legged larval ticks are hatching from eggs and then moulting with/without a blood meal to 8-legged nymphs. After multiple nymphal stages and blood meals the adult stage is reached. In this stage, adult female *O. moubata* lay eggs after each blood meal. Adapted from [31]

Soft ticks are fast feeding animals, as they only feed for 15 to 60 minutes depending on their size and developmental stage, supporting their nidicolous life-style. Moulting normally occurs within a few weeks after feeding making these ticks ideal for scientific research as they feed and moult quickly limiting the time needed for collection and analysis [32].

Compared to the soft ticks, hard ticks moult from larvae to nymphs and then directly to adults with no additional nymphal stages, needing a blood meal before each moult. Moreover, hard ticks are feeding for multiple hours or days and the timespan between feeding is much longer [33,34].

During and after feeding soft ticks remove excess of water and ions via coxal glands, also excreting pathogens in the process. The excretion of coxal fluid after feeding, might lead to transfer of pathogens like *Borrelia* from an infected mother to their offspring [32].

Infection of soft ticks with RF *Borrelia* normally occurs by feeding on an infected host. During the next blood meal, the ticks can then transmit the pathogens to a new naïve host.

1.2.1 *Ornithodoros moubata*

O. moubata is mainly found in sub-Saharan Africa. This species is the vector for multiple pathogens, including TBRF *B. duttonii*, African swine fever virus, and West Nile Virus [35,36]. Its main hosts are pigs, poultry and humans [37].

1.3 Animal-Tick Models for RF *Borrelia*

To study the interaction between pathogen, vector and host, different tick-animal models have been developed for different RF *Borrelia* species.

The only model which had been developed for *B. duttonii* and *O. moubata* involved gerbils as hosts. It was used to study the spirochetes capability to transovarially transmit from infected adult ticks to their offspring, which was shown to not be happening for *B. duttonii* in *O. moubata* [38].

There are no tick-animal models for the study of acquisition and transmission of *B. duttonii* by *O. moubata* in other hosts available. Most of the work in recent years has been done with new-world RF species, especially the two American strains *B. turicatae* and *B. hermsii* with their vectors *O. turicatae* and *O. hermsii*, respectively [39,40].

As there is no functioning tick-animal model established for any of the old-world TBRF species, we attempted to do so in this thesis.

2 Aims of the work

- Determination of the acquisition time of *B. duttonii* required during blood feeding by the tick *O. moubata*
- Determination of the transmission time of *B. duttonii* required for successful dissemination of the pathogen into a naïve host and establishment of an infection
- Determination of the Infectious Dose 50 (ID_{50}) of *B. duttonii* by needle-inoculation of naïve mice
- Determination of the infectivity of freshly-fed and unfed tick-homogenate in naïve mice
- Determination of the effects of prolonged in-vitro cultivation on *B. duttonii*
- Successful feeding and infection with *B. duttonii* of *O. moubata* by in-vitro techniques
- Raising of antibodies against selected proteins of *B. duttonii* 1120K3 in rabbits and identification of these antigens

3 Material and Methods

3.1 Strains and *Borrelia* Culture Conditions

B. duttonii isolate 1120K3 (S. Bergström, Umeå), was used in the conducted experiments. The cultures were grown in 7 mL of BSK-H medium (complete, sterile-filtered, with 6% rabbit serum, Sigma Aldrich) at 34°C. They were either started from a frozen glycerol stock, infected mouse tissue or infected *O. moubata* ticks.
Reisolation of *Borrelia* from tissue was done in BSK-H medium containing *Borrelia* antibiotics, preventing contamination.

3.1.1 Preparation of 100x *Borrelia* Antibiotic stock solution

The composition of 100x *Borrelia* antibiotics stock solution in 20% DMSO is shown in Table 1.

Table 1 - Composition of 100x Borrelia Antibiotic stock solution

Compound	Concentration in mg/mL
Phosphomycine	2
Rifampicin	5
Amphotericin	0.25

Phosphomycine was first dissolved in milliQ H_2O, while rifampicin and amphotericin B were dissolved in DMSO. After stirring these mixtures separately for at least 2 hours, they were poured together and stirred overnight. The next day the solution was filtered through a 0.2 mm filter and aliquoted. The aliquots were stored at -20°C.

3.1.2 Reisolation of *Borrelia* from *O. moubata*

O. moubata were put individually into 1.5 mL Eppendorf tubes. The ticks were immersed in 3% H_2O_2 for 15 minutes, followed by another step in 70% ethanol. After the ethanol was pipetted out, the tubes were left open to air dry in a flow box for approximately 15 minutes. Then, 500 µL of pre-warmed BSK-H media containing *Borrelia* antibiotics were added and the ticks were crushed using a pestle. The pestle was washed with additional 1 mL BSK-H. Afterwards, the cultures were incubated at 34°C and checked after a minimum of two weeks for the presences of *Borrelia* under a dark-field microscope at 20x magnification.

3.2 Preparation of Protein Lysate

3.2.1 Preparation of Protein Lysate from a *Borrelia* culture

7 mL *Borrelia* cultures with spirochete densities of approximately 10^8 spirochetes per mL were centrifuged at 8000 rpm, 20°C for 10 minutes.

Then, the supernatant was removed and the bacterial pellet was resuspended in 1 mL cold HN-Buffer. The mixture was transferred into a fresh 1.5 mL Eppendorf tube and centrifuged again (8000 rpm, 20°C, 10 minutes). After centrifugation, the buffer was removed with a pipette. The pellet was washed a second time in 1 mL of cold HN-buffer and centrifuged (8000 rpm, 20°C, 10 minutes). The resulting pellet was resuspended in 200 µL B-PER® (Bacterial Protein Extraction Reagent, Thermo Scientific), and incubated at room temperature for 10 minutes.

The lysate was either stored for later use at -20°C or it was mixed with 190 µL 2x Laemmli Sample Buffer (BioRad) and 10 µL β-mercaptoethanol. Prior to addition of the Laemmli buffer and β-mercaptoethanol mixture the protein concentration of the lysate was determined using a Nanodrop.

3.2.2 Preparation of Protein Lysate from *O. moubata*

For the preparation of a protein lysate from *O. moubata*, a protocol for the preparation of protein lysate from *Ixodes ricinus* described by Oltean and colleagues was adapted [41].

5 unfed 3rd nymphal stage *O. moubata* and three sterile metal beads were placed into a 2 mL Eppendorf tube. Then, the tube was deep frozen in liquid nitrogen for 1 minute. The ticks were homogenized using a TissueLyserII (Qiagen) set at 30 oscillations per second for 5 minutes. Deep freezing and homogenizing was repeated a second time. Afterwards, the homogenate was resuspended in 1 mL of miliQ water. Insoluble particles were removed by centrifugation (13000 rpm, 4°C, 5 minutes) twice. The supernatant was collected, and the protein concentration was determined by Nanodrop measurement.

Prior to running the lysate on an acrylamide gel, 200 µL of lysate were mixed with 190 µL 2x Laemmli Sample Buffer (Bio-Rad) and 10 µL β-mercaptoethanol.

3.2.3 Preparation of Salivary Gland Extract from *O. moubata*

To prepare a protein extract from salivary glands a protocol for *Ixodes ricinus* was adapted [42] .

First, 25 nymphal *O. moubata* were dissected and their salivary glands were pooled together in 1x PBS (8 g NaCl, 2.68 g $Na_2PO_4.7H_2O$, 0.24 g KH_2PO_4, 0.2 g KCl in 1 L dH_2O, pH = 7.4). After washing the salivary glands with 1xPBS, they were homogenized in 500 µL 1x PBS using a glass homogenizer. The homogenate was centrifuged at 10000 rpm for 10 minutes and the supernatant was collected. Using a Nanodrop the protein concentration in the extract was determined. Prior to running it on a SDS-gel, 200 µL of the extract were mixed with 190 µL of 2x Laemmli Sample Buffer (Bio-Rad) and 10 µL β-mercaptoethanol.

3.3 SDS-Page

0.75 mm gels for SDS-Page (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were prepared with the TGX™ FastCast™ Acrylamide Kit, 12% according to the manufacturer's instructions.

Either 10-well or multichannel gels with only 2 wells (1 small well for the ladder, and 1 large well for the lysate) were prepared depending on the application. After polymerisation of the gel, it was placed in a SDS-Page cassette and filled with 1xSDS-Page Running Buffer (3.03 g Tris-Base, 14.4 g Glycine, 1 g SDS per L).

The protein lysate samples, already containing the Laemmli Sample Buffer and β-mercaptoethanol mixture, were heated at 100°C for 10 minutes prior to loading on the SDS-gel.

Besides the protein lysates, 8 µL of protein ladder (Prestained Protein Marker VI (10-245), AppliChem) were loaded to each gel.

The gels were run for approximately 80 minutes at 120 V until the entire blue colour of the Laemmli Sample Buffer passed through the gel to ensure maximum separation of the proteins.

3.4 Staining of SDS-Gels with Coomassie Brilliant Blue

After running the gel, it was removed from the glass plates and either washed directly three times for 10 minutes in distilled water or prior to washing the proteins were fixed in a Protein Fixation solution (50 % Methanol, 40% distilled water, 10 % Acetic Acid).

Then, the gel was stained in 30 mL Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad) for at least 3 hours.
Destaining of the gel was done in distilled water over night. After the first hour of destaining, the water was exchanged.
All fixing, washing, staining and destaining steps were done on a mechanical shaker.
After destaining, a picture of the gel was taken using a ChemiDoc™ MP Imaging System (Bio-Rad).

3.5 Western Blot

While running the SDS gels, according to section 3.3, two blotting paper pads and one nitrocellulose membrane (Bio-Rad, 0.2 µm) per gel blotted were soaked in 1x Blotting Buffer (3.03 g Tris-Base, 14.4 g Glycine per L).
Then, the membrane was placed on top of one of the blotting paper pads, followed by the gel in a semi-dry transfer cell (Trans-Blot® SD, Bio-Rad). The positions of the wells were marked with a pencil on the membrane. On top of the gel, the second blotting paper was placed. Using a roller, bubbles were removed. Blotting was performed for 30 minutes at 25 V.
During this time, a 5 % blocking solution was prepared by dissolving 5 g of dry milk powder in 100 mL of 1x TBS-Tween 20 (3 g Tris-Base, 8 g NaCl, 0.2 g KCl, 500 µL Tween-20 per L, pH = 7.4).
After blotting, the membrane was blocked for at least two hours in 50 mL of 5 % blocking solution on a mechanical shaker. The remaining blocking solution was stored overnight in the fridge for later use.
Primary antibody solutions were prepared by mixing 5 µL of sample sera with 995 µL 5 % blocking solution (dilution 1: 200). Serum was collected by spinning down blood samples at 9000 rpm and 7 minutes and taking the supernatant.
For 10-well gels, the membrane was cut in strips corresponding to the different protein lysate samples in the individual wells. Each strip of the membrane was sealed in a plastic bag together with the adequate primary antibody solution.
Larger numbers of samples were screened using multichannel-gels with only 2 wells, one small one for the ladder and one large one for the protein lysate, and a Mini-Protean® II Multiscreen (Bio-Rad). Instead of cutting the membrane into strips after blocking, the membrane was clamped in the multiscreen and 600 µL of the primary antibody solutions were loaded to each capillary. Then, the apparatus was wrapped in plastic foil.
Both the sealed in membrane strips and the Multiscreen were stored overnight in the fridge.

The next day, the membrane was shaken for 1 hour and then washed in 1xTBS-Tween 20 three times for 15 minutes.
Then the secondary antibody solution was prepared by diluting 5 µL of the antibody in 50 mL 5 % blocking solution (dilution 1: 10 000). Depending on the kind of sera used as primary antibody either Anti Mouse IgG (whole molecule) peroxidase antibody (SIGMA) or Goat pAb to Rabbit IgG (abcam) were used. Both antibodies were labelled with horseradish peroxidase. After washing, the membrane was shaken for at least 1 hour in the secondary antibody solution and then washed again three times for 15 minutes in 1xTBS-Tween 20.
For development Pierce® ECL Western Blotting Substrate was used. Detection Reagent 1 and 2 were mixed in a ration 1:1 and then poured over the membrane. Per membrane 2 mL of the mixture were used. The membrane was incubated for 5 minutes on a shaker and then placed between two overhead transparencies.
Using a ChemiDoc™ MP Imaging System (Bio-Rad), pictures of the membrane were taken.

3.6 Total gDNA isolation from tissue

gDNA isolation from ticks was done using the NuelcoSpin Tissue Kit from Machery Nagel.
In the case of DNA isolation from ticks, each tick was placed in a separate 1.5 mL Eppendorf Tube and after addition of the first T1 Buffer the ticks were crushed with a pestle. Then, the DNA was isolated following the manufacturer's instructions. The first lysis step at 56°C was done overnight in a heating block.

3.7 gDNA isolation from blood

DNA was isolated using NucleoSpin Blood from Macherey Nagel, following the manufacturer's instructions.

3.8 DNA precipitation

To improve the quality of DNA, some DNA samples were subjected to DNA precipitation.
First, 1/10 volume of 3 M NaOAc were added to the sample, followed by the addition of 2 volumes of 100 % ethanol. Then, the tubes were inverted five times and centrifuged at 13000 rpm for 10 minutes at 10°C. The supernatant was removed with a pipette and 1 volume of 70% ethanol was added. The mixture was centrifuged again at 13000 rpm for 10 minutes at 10°C and the supernatant was removed. Then the DNA was air dried by leaving the tube

opened for 5 minutes and ethanol droplets were removed by fine absorbent paper. In the end, the DNA was rehydrated in miliQ H_2O.

3.9 Polymerase Chain Reaction (PCR)

The nucleotide sequences of the primers used during PCR are listed in Table 2.

Table 2 - Nucleotide Sequence of primers used in PCR

Primers	**Nucleotide Sequence**
Actin – forward	5'-CGG GTG CTT CTG TGC TGT TC-3'
Actin – reverse	5'-GCA TCA TCG CCA GCG AAT CC-3'
glpQ – forward	5'-AAG GTA TGG CTG AGG TTG C-3'
glpQ – reverse	5'-AGA CCT GTG ATT TGC CCA TTA-3'
FlaB B. ans. - forward	5'-ACATATTCAGATGCAGACAGAGGT-3'
FlaB B. ans. – reverse	5'-GCAATCATAGCCATTGCAGATTGT-3'

The Actin primer pair was designed to amplify the *actin* gene of *O. moubata*. While glpQ and FlaB (B. ans.) primers were used to check for the presence of *Borrelia* DNA.

The reactions were prepared by mixing OneTaq® Hot Start Quick-Load® 2xMM (New England Biolabs), the primers, DNA and miliQ H_2O. To prevent false positive results, a negative control was done for each reaction, using water instead of template DNA. Table 3 shows the composition of the 20 µL reaction mixture.

Table 3 - Composition of the PCR reaction

Master Mix	**Volume [µL]**
OneTaq® Hot Start Quick-Load® 2xMM	10
Primer forward	1
Primer reverse	1
H_2O	6
DNA	2

For all reactions the same PCR program was used, which is shown in Table 4.

Table 4 – PCR settings

Step	Temperature & Time
Initial Denaturation:	94°C for 30 sec.
30 cycles:	94°C for 30 sec. 55°C for 45 sec. 68°C for 1 min.
Final extension:	68°C for 10 min
Hold:	12°C

The success of amplification of the desired PCR product was determined by agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving agarose for DNA electrophoresis (SERVA) in 1xTAE buffer (diluted from 50x TAE buffer, MERCK chemicals). Per 100 mL gel 1.5 µL Gel Red® Nucleic Acid Stain (Biotium) were added to the mixture before it polymerized at room temperature for at least 30 minutes.
Then, 10 µL of each PCR sample, as well as 8 µL of DNA ladder were loaded to the gel. The gel was run at 110V for approximately 60 minutes. Under UV light the size of the products was determined, and a picture was taken.
For larger number of samples, a Liberty 120 High-Speed Gel System was used. This system allows a voltage of 210 V, thereby reducing the running time to 15 minutes.

3.10 Gel Extraction, Cloning and Transformation in *E. coli*

Amplified PCR products were extracted from the agarose gel using QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions.
The extracted DNA was then cloned into a pCR® 2.1-TOPO® vector and transformed in *E. coli*. The reaction mixture is given in Table 5.

Table 5 - Reaction Mixture for cloning into pCR® 2.1-TOPO®

Reagent	Amount [µL]
DNA insert	3
Salt Solution	0.5
TOPO vector	0.5
milliQ H_2O	2

After incubation of the reaction for 10 minutes at room temperature, NEB 5-alpha competent *E. coli* cells (New England Biolabs®) were transformed. First, the cells were thawed on ice for

5 minutes and then 25 µL of cells were mixed with 2 µL of the ligation reaction by flicking. The cells were incubated on ice for 30 minutes and then heat-shocked for 30 seconds at 42°C. Afterwards, the cells were allowed to recover on ice for 2 minutes before being shaken for 90 minutes at 37°C in 250 µL S.O.C.-Outgrowth media (New England Biolabs®).

First, 40 µL of X-Gal (20 mg/mL, Thermo Scientific®) were spread on prewarmed carbenicillin agar plates and then 75 µL or 150 µL of cells were plated. After 5 minutes at room temperature, the plates were incubated overnight at 37°C.

3.11 Plasmid DNA isolation and Sequencing

Blue-white screening was used to identify *E. coli* colonies containing the cloned insert. Therefore, white colonies were picked and shaken overnight in 6 mL of liquid broth (LB) media at 37°C. The next day, plasmid DNA was isolated from the *E. coli* using NucleoSpin® Plasmid DNA Purification kit (Machery Nagel) following the manufacturer's instructions. The DNA isolates were tested for the presence of the insert, by PCR with the corresponding primers as described in section 3.9, or by restriction digestion using the enzyme EcoR I (New England Biolabs).

For restriction digestion, 3 µL of DNA, 2 µL of the enzymes buffer, 0.5 µL of the enzyme and 14.5 µL milliQ H_2O were mixed and incubated at 37°C for 1 hour. Gel Electrophoresis as described in section 3.9 was used to determine the presence of the desired insert.

DNA containing the desired insert was sequenced by mixing 5 µL of plasmid DNA with 5 µL of either MT13R or T7 primers (concentration 5 pmol/µL) and then sending it to GATC Biotech Lightrun sequencing service.

3.12 Passaging of *B. duttonii* 1120K3

To see if prolonged in vitro cultivation of *B. duttonii* leads to changes in genetic content of the bacteria and protein expression, and thereafter affects infectivity of the *Borrelia*, *B. duttonii* 1120K3 isolate was grown in BSK-H media (Sigma-Aldrich). As soon as the concentration of the culture reached 10^8 spirochetes per mL, 70 µL of the old culture were passaged to 7 mL of fresh BSK-H media. This passaging was continued from the work of a previous student of the laboratory. Passages 25 to Passage 57 were done.

The number of generations corresponding to the number of passages was calculated in the following way [43]:

$$N\left(spirochetes_{passaged}\right) = 70 * 10^{-3}\ mL *\ 10^{8}\frac{sp}{mL} = 7 * 10^{6}\ spirochetes$$

$$7 * 10^{6} * 4 \sim 10^{8}$$

$$Number\ of\ Generations = Number\ of\ Passages *\ 4$$

Furthermore, protein lysates of the cultures were prepared according to the protocol described in subsection 3.2.1 and the protein expression of selected passages was compared to wild type *B. duttonii* by SDS-Page and Coomassie Brilliant Blue staining (sections 3.3 and 3.4).
Therefore, the protein concentration of protein lysate prepared from 1120K3 wild type, passage 40 and passage 51 were determined using a Nanodrop and equal amounts of proteins were loaded to compare the expression.

3.13 *Ornithodoros moubata*

3.13.1 Housing Conditions

O. moubata were kept at 27°C and 90% humidity.

3.13.2 In vivo feeding

O. moubata were fed on mice or rabbits. After capsules were placed on the animals, the ticks were allowed to feed at least 4 hours or overnight, before being removed. This was done to obtain the maximum number of fed ticks.

3.13.3 In vitro feeding and Infection of *O. moubata* by *B. duttonii*

A different way of feeding ticks is in vitro feeding via a membrane. The infection of *O. moubata* with *B. duttonii* by membrane feeding was attempted. In this set-up silica membranes or parafilm were used to cover feeding chambers. An example of a feeding chamber covered with a silica membrane and a grid is shown in Figure 5.

Figure 5 – Feeding compartment used in Membrane feeding of O. moubata

First, the membranes were checked for leakage by filling a 6 well tissue culture plate with 70 % ethanol and placing the feeding compartments on top of the liquid. Furthermore, the membranes were washed using ethanol.

Rabbit blood or different serum used for feeding was pre-warmed to 37°C in a water bath. A counting chamber was used to determine the concentration in the *B. duttonii* 1120K3 culture and the appropriate amount of the culture was added to the meal to reach the desired *Borrelia* concentration in the feeding substance. This mixture was then either incubated for 90 minutes to allow the *Borrelia* to adapt to their new environment or directly used for feeding.

For feeding, 4 mL of the mixture were pipetted into a 6 well tissue culture plate and a feeding chamber was placed on top. Then the *O. moubata* were put into the feeding compartment and the plate was put in a water bath set at 37°C. Using a cotton plug, the ticks were kept from escaping. After 4 hours the fully fed ticks were removed.

Table 6 shows the different feeding conditions and *Borrelia* concentrations used in various in-vitro feeding experiments.

Table 6 – Summary of in-vitro feeding of *O. moubata*

Experiment	**Feeding media**	**Prior infestation with *O. moubata***	**Blood collected**	**Approx. conc. of *B. duttonii***	**Prior-incubation**
A	Rabbit Blood	No	Same day	10^6	No
B	Rabbit Blood	No	5 days ago	10^6	No
C	Rabbit blood	No	2 days ago	10^5	No
	Rabbit Blood	Yes	2 days ago	10^5	No
	Rabbit blood	No	2 days ago	10^4	No
	Rabbit blood	No	2 days ago	10^3	No
D	Rabbit blood	No	11 days ago	10^5	No
E	Rabbit blood	No	1 day ago	10^6	2 hours
	Rabbit blood	No	1 day ago	10^7	2 hours
F	Rabbit Serum	-	-	10^7	No
	Goat Serum	-	-	10^7	No
	Chicken Serum	-	-	10^7	No
	Pig Serum	-	-	10^7	No
	Adult Bovine Serum	-	-	10^7	No

Sterile filtered rabbit serum (SIGMA), goat serum (Biosera), chicken serum (Biosera), pig serum (Biosera) and adult bovine serum (Capricorn Scientific) were used in Experiment F.
After moulting, the *O. moubata* were checked for infection by gDNA isolation and PCR, according to section 3.6 and section 3.9., as well as by crushing in fresh BSK-H media containing *Borrelia* antibiotics, as described in subsection 3.1.2.
The *O. moubata* fed in Experiment F were also crushed in 300 µL of BSK-H and injected into naïve mice to check for the presence of infectious *Borrelia* within the ticks after moulting. This is described in more detail in subsection 3.15.19.

3.13.4 In vitro feeding of infected *O. moubata*

In this setup 10 infected 3-4th nymphal *O. moubata* were fed on clean rabbit blood, as described in subsection 3.13.3, but without mixing in any *Borrelia*. This was done to determine secretion of *Borrelia* by the infected *O. moubata* to the rabbit blood. After feeding, 200 µL of blood were used for DNA isolation according to section 3.7 and subsequent PCR, as described in section 3.9, to determine the presence of *borrelial* DNA. Furthermore, 10 µL of blood were added to clean BSK-H media containing *Borrelia* antibiotics to reisolate *Borrelia* from the blood.

3.14 Immuno-Fluorescence Assay

To establish a different way to confirm the presence of *B.* duttonii in ticks, besides DNA isolation and PCR or reisolation from tissue, Immuno-Fluorescence Assay (IFA) was tested on *B. duttonii* cultures as well as on *O. moubata.*
First, 500 µL of a *B. duttonii* 1120K3 culture were spun down at 8000 rpm for 3 minutes. The bacterial pellet was washed twice with 1x PBS. After the last centrifugation step, the pellet was resuspended in 10 µL 1x PBS and smeared on a glass slide.
Besides *Borrelia* cultures, also *O. moubata* were analysed using IFA. Therefore, ticks were fixed on a petri dish using double faced adhesive tape and cut open. The content of the body cavity of the tick was spread together with 1xPBS on a glass slide.
To remove the possibility of using non-infected ticks, and therefore getting false negative results, the body content of some ticks was mixed with a *Borrelia* culture prior to spreading on the glass slide.
In all cases, the slides were air dried and the smears were fixed in methanol for 20 minutes. During that time the antibody solution was prepared by doing a 250x dilution of

Anti-*Borrelia* burgdorferi antibody – FITC from abcam in 1x TBS-Tween 20 (3 g Tris-Base, 8 g NaCl, 0.2 g KCl, 500 µL Tween-20 per L, pH = 7.4).
After completely air drying the slides, 1 mL of antibody solution was applied to each slide and the slides were then incubated at room temperature in a covered box for 45 minutes. Then, the slides were washed in 1x PBS for 5 to 10 minutes and again air dried. In the last step, 20 µL 50% glycerol were pipetted on the slide and a cover slide was put on. The samples were then examined under a fluorescence microscope.

3.15 Mouse Experiments

3.15.1 Mice strains

The following mice strains were used during the conducted experiments:

- Balb/c
- C3H
- GGTAL
- SCID

3.15.2 Tail-snip and 10-view counting

10-view counting was done to confirm the presence of spirochetes in the blood of mice injected with *B. duttonii* 1120K3.
With a scalpel a small incision on the tail of the mouse was made and using a pipette a few µL of blood were drawn. The blood was pipetted directly onto a microscopic slide and a cover slide was placed on top of it. The slides were checked under a dark-field microscope for the presence of *Borrelia* at 20x magnification. To assess the number of *Borrelia* present, the spirochetes were counted in 10 randomly chosen views and the numbers were summed up. Figure 6 shows a section of one view of mouse blood at a high point of spirochetemia under a dark-field microscope at 20x magnification.

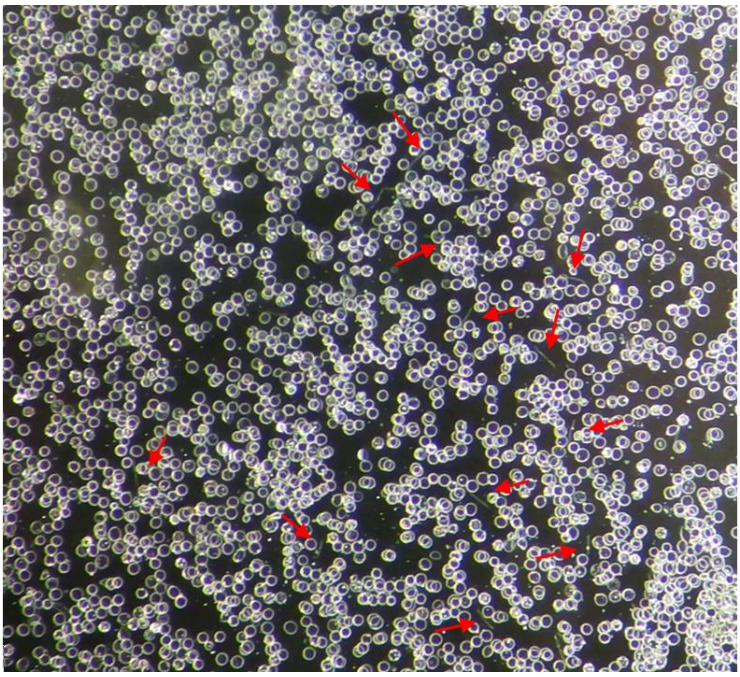

Figure 6 – Example for a section of one view on mouse blood at a high point of spirochetemia, 20x magnification, red arrows indicate the position of some of the present spirochetes

3.15.3 Determination of ID_{50} of *B. duttonii* by needle-inoculation

Cultures of *B. duttonii* 1120K3 were grown until the exponential growth phase was reached. Then the concentration of *Borrelia* in the culture was determined using a Petroff Hauser counting chamber. 10-fold dilutions in fresh BSK-H media were done to reach the desired *Borrelia* concentrations. Then 250 µL were injected into each mouse (150 µL intraperitoneal + 100 µL subcutaneous). 3 weeks post infection, blood was drawn by retro-orbital bleeding of anesthetized mice and serum was tested for the presence of antibodies against *Borrelia* to serologically confirm an infection. Additionally, the blood of some of the mice was checked microscopically for the presence of *Borrelia* under a dark-field microscope with 20x magnification, starting from 3 days post infection as described in subsection 3.15.2.
Mice strains Balb/c and C3H were used in the determination of the ID_{50}-value.

3.15.4 Determination of the onset time for Antibody production against infection by *Borrelia duttonii* in mice

To determine how soon after infection the first antibodies against *B. duttonii* are being raised, eight female naïve Balb/c mice were injected with 100 spirochetes of *B. duttonii* isolate 1120K3 in 250 µL BSK-H each (150 µL intraperitoneal + 100 µL subcutaneous). Then, the mice were divided in three groups, containing three, three and two mice respectively. Starting

at 1 day post-inoculation, approximately 50 µL of blood were collected daily by retro-orbital bleeding. The groups of mice were alternated so every fourth day the same group of mice was bled. The collected blood was then spun down at 9000 rpm for 7 minutes and the sera was checked for the presence of antibodies against *B. duttonii* 1120K3 antigens by Western Blot as described in section 3.5.

3.15.5 Infection of naïve mice by injection with infected mouse sera

The infectivity of sera collected from infected mice was determined. First, four female naïve Balb/c mice were injected with 10^4 *B. duttonii* 1120K3 in 250 µL BSK-H each (150 µL intraperitoneal + 100 µL subcutaneous). Starting from 3 days post infection tail-snips and 10-view counting was done to assess the numbers of spirochetes present in the blood, as described in subsection 3.15.2.

Four days post-infection, high spirochetemia for three of the four mice was reached, therefore as much blood as possible of the two mice with the highest spirochete counts was collected by retro-orbital bleeding and the mice were euthanized. The blood was spun down twice at 9000 rpm for 7 minutes to remove all residual red blood cells. Approximately 600 µL of sera were collected in this way and then split up and injected into three female naïve Balb/c mice, 190 µL each. 10 µL of sera was stored at -20°C to be used as a control. Three weeks post-infection, these newly injected mice were checked serologically for an infection, by Western Blot as described in section 3.5.

Six days post infection the numbers of spirochetes in the blood of one of the remaining mice has dropped. Therefore, the same procedure as before was repeated for this mouse to check for infectivity of the sera at a low point of spirochetemia. This time 350 µL of sera were collected from one mouse and split up so that two naïve mice could be injected. Again, three weeks post-infection, Western Blot was used to confirm an infection serologically according to section 3.5.

3.15.6 Acquisition Timing of *B. duttonii* by *O. moubata*

3 naïve female C3H mice were injected with 10^3 spirochetes in 250 µL BSK-H (150 µL intraperitoneal + 100 µL subcutaneous). Starting one day post-infection, every day 10 clean larval *O. moubata* from the same cohort were put on each of the mice and allowed to feed overnight. The following day the *O. moubata* were removed and fresh ticks were applied. This was done for 14 days. Furthermore, every day tail-snips and 10-view counts were done to

determine the number of spirochetes in the blood of the mice. 1 to 6 days post-infection 10 µL blood was collected for DNA isolation and qPCR for all three mice. After that time, only for one mouse the collection of blood for qPCR was continued until 12 days post-infection as the collection of blood got harder every day.

After moulting, ticks from each day and from each mouse were washed and cut in half. One half was used for DNA isolation and PCR (section 3.6 and section 3.9) to determine presence of *Borrelia* DNA, whereas the other half was used to start cultures in BSK-H as described in subsection 3.1.2.

3.15.7 Transmission Dynamics of *B. duttonii* to naïve mice by infected *O. moubata*

The transmission of *B. duttonii* to naïve mice by infected *O. moubata* was assessed in a series of experiment.

In the first experiment, the effect of the removal of the bite site directly after feeding on transmission was determined. All *O. moubata* used in the experiment, belonged to the same cohort and were infected on the same day by feeding on an infected mouse at a high point of spirochetemia, confirmed by 10-view counting (subsection 3.15.2). Infection of the ticks was confirmed by gDNA isolation and PCR (see section 3.6 and section 3.9). Naïve female C3H mice were used to conduct these experiments.

As a control, three groups each containing three nymphal infected *O. moubata* were allowed to feed till repletion on the ears of three anesthetized naïve C3H mice.

Furthermore, three infected ticks were fed on the ears of anesthetized naïve C3H mice till repletion and directly after feeding the bite sites were removed. This was done with three different naïve mice. The removed piece of ear was stored at -80°C until gDNA was isolated (see section 3.6) and checked for the presence of *Borrelia* DNA using glpQ primers according to section 3.9.

From 3 to 11 days post infection, tail-snips and 10 view counts were done to check for the presence of spirochetes microscopically as described in subsection 3.15.2. Furthermore, 3 weeks post infection blood of the mice was drawn by retro-orbital bleeding and the serum was used in a Western Blot to determine an infection of the mice serologically, according to section 3.5.

3.15.8 Infection of naïve mice by needle inoculation with freshly-fed-infected tick-homogenate

To confirm the infection of *O. moubata* used in the experiment described in subsection 3.15.7, the *O. moubata* of this experiment were allowed to feed till repletion on navie GGTAL mice (6 *O. moubata* per mouse, 3 mice). Tail-snips and 10-view counting was done 4 to 7 days post infection to check for the presence of *Borrelia* in the mouse blood as described in subsection 3.15.2.

2, 3, and 4 days after feeding, 2 of the *O. moubata* were crushed with pestles in 300 µL fresh BSK-H media and the homogenate was then injected into a naïve mouse to determine the presence of infectious *B. duttonii* within the ticks. 2 and 3 days post infection, tail-snips and 10-view counts of the blood was done to detect *Borrelia* microscopically.

Furthermore, at least three weeks post feeding/infection, the mice were bled by retro-orbital bleeding and a Western Blot was done to serologically confirm an infection of the mice according to section 3.5.

3.15.9 Determination of the effect of feeding site and number of ticks on transmission of *B. duttonii* by *O. moubata*

To determine the effect of the site of feeding, as well as the number of ticks feeding, the number of infected ticks feeding per mouse was increased compared to the experiments described in subsections 3.15.7 and 3.15.8.

Capsules were fixed on the back of 5 female naïve Balb/c mice. 7 infected nymphal *O. moubata* ticks were put into each capsule and allowed to feed overnight. The next day the *O. moubata* were collected. 3 weeks post infection blood of the mice was drawn by retro-orbital bleeding and serum was collected. A Western Blot was done to confirm an infection serologically, according to section 3.5.

3.15.10 Transmission of *B. duttonii* to SCID mice by *O. moubata*

To assess the ticks' transmission capability, infected *O. moubata* were fed on severe-combined immunodeficient (SCID) mice. On two mice five *O. moubata* in their 3rd nymphal stage and on one mouse ten *O. moubata* in their 1st nymphal stage were fed till repletion. Feeding was done on the belly of the mice, as shown in Figure 7. Therefore, the mice were injected intraperitoneally with 80 µL of an anaesthetic mixture containing 4% Narokomon (5%, Spofa)

and 1% Rometar (2%, Spofa) in 1x PBS. After the mice fell asleep, the *O. moubata* were placed on the mice and allowed to fully feed.

Figure 7 - *O. moubata* feeding on the belly of an anesthetized Balb/c mouse

To determine an infection in these immuno compromised mice, tail-snips and 10-view counts (subsection 3.15.2) of the blood were done 3, 7, 11 and 18 days post-infection. For 7, 11, and 18 days post infection, a few µL of blood were pipetted directly in fresh BSK-H media containing *Borrelia* antibiotics to reisolate *B. duttonii*.

To ensure the infection of the *O. moubata* used in this experiment, some of the *O. moubata* of each group were crushed and injected into naïve Blab/c mice after moulting. From the 2nd nymphal stage ticks, two out of ten *O. moubata* were crushed in 300 µL BSK-H and 250 µL were injected, while from the 4th nymphal stage ticks, one tick per group were crushed in 300 µL BSK-H and 250 µL were injected. In total 3 mice were injected. 3 weeks post infection the blood of these mice was drawn by retro-orbital bleeding and a Western Blot was done to confirm an infection serologically (section 3.5).

3.15.11 Reisolation of *B. duttonii* from coxal fluid of infected *O. moubata*

Directly after feeding, the infected *O. moubata* used in the experiment described in subsection 3.15.103.15.11 were put on their back in a petri dish. The secreted coxal fluid was collected using a pipette and put directly into 1.5 mL Eppendorf tubes filled with BSK-H media containing *Borrelia* antibiotics to check for the presence of *Borrelia* in the secretion. Furthermore, part of the coxal fluid was subjected to DNA isolation and PCR as described in subsections 3.6 and 3.9.

3.15.12 Infection of naïve mice by needle inoculation with infected post-moulting tick homogenate

O. moubata were infected by feeding on infected mice on a high point of spirochetemia and then allowed to moult. Their infection was determined by DNA isolation and PCR, as described in sections 3.6 and 3.9.

After 28 days, five of these *O. moubata* were crushed individually in 300 µL fresh BSK-H media and 10-fold dilutions of this homogenate were prepared. To determine an infection threshold for in vivo-grown *Borrelia*, 250 µL of the original, the 1:100 and the 1:10 000 dilutions were injected into naïve Balb/c mice (150 µL intraperitoneal + 100 µL subcutaneous). Three weeks post-infection a Western Blot, as described in section 3.5, was done to determine an infection serologically.

Furthermore, 4 months and 8 days after last feeding of these *O. moubata*, five more ticks were crushed in 300 µL BSK-H and again 250 µL of this homogenate were injected into another set of naïve mice to see if the bacteria within the ticks are still infectious. 6 days post-infection the blood of the mice was checked for the presence of spirochetes under the microscope as described in subsection 3.15.2. An infection was then confirmed serologically by a Western Blot, 3 weeks post infection.

3.15.13 Determination of dissemination of *B. duttonii* 1120K3 to different Mouse tissue during infection

After serological confirmation of an infection, mice injected with *B. duttonii* 1120K3 wildtype and passage 40 were dissected 4 and 5 weeks post infection, respectively, and their bladders were removed. The bladders were incubated in fresh BSK-H media containing *Borrelia* antibiotics to check for reisolation of the bacteria from this tissue.

Furthermore, four wild-type infected mice, two Balb/c and two C3H, were bled by retro-orbital bleeding and euthanized 3 months and 28 days post infection. 10 µL of blood were pipetted directly to 7 mL fresh BSK-H media containing *Borrelia* antibiotics. Furthermore, the skulls of the mice were opened, and the brains were washed with autoclaved 1x PBS to remove residual blood. Part of the brain was used for DNA isolation (section 3.6), however the majority was homogenized using a pestle and put into 7 mL BSK-H media containing *Borrelia* antibiotics to reisolate *Borrelia* from the brain tissue. The isolated DNA was subjected to PCR (section 3.9) to determine the presence of *Borrelia* DNA.

3.15.14 Acquisition of *B. duttonii* by *O. moubata* 3 months post-infection

Clean larval *O. moubata* were fed on serologically confirmed infected C3H and Balb/c mice 3 months and 14 days post infection. After moulting of the *O. moubata*, part of them were crushed in fresh BSK-H media, following the protocol in subsection 3.1.2 and others were used for DNA isolation and subsequent PCR as described in sections 3.6 and 3.9 to confirm the presence of *Borrelia* within the ticks.

3.15.15 Comparison of Infectivity of *B. duttonii* 1120K3 and Passages

To determine the infectivity of the passaged 1120K3 *Borrelia*, Passage 40 and Passage 51 were injected into naïve Balb/c or C3H mice. The number of spirochetes in the cultures was determined using a Petroff Hauser counting chamber and 10^5 and 10^3 spirochetes in 250 µL (150 µL intraperitoneal + 100 µL subcutaneous) BSK-H media were injected into each mouse. Per dilution and passage 3 mice were injected as is shown in Table 7.

Table 7 - Strain and number of mice injected with different amounts of passaged *B. duttonii*

Passage	Mouse strain	Number of mice	Number of spirochetes injected
40	Balb/c	3	10^5
		3	10^3
51	C3H	3	10^5
		3	10^3

Starting 3 days post infection, 10-view counts were done for Passage 40 infected mice and for mice injected with Passage 51 for the higher dose, until the first spirochetes in the blood were detected.

Four weeks post injection, an infection was confirmed serologically by Western Blot as described in section 3.5.

3.15.16 Acquisition Dynamics of *Borrelia duttonii* Passage 51

To determine if *O. moubata* are still able to acquire prolonged in-vitro grown *B. duttonii* Passage 51, clean larval ticks were fed on mice infected with Passage 51, 5 days p.i. As the numbers of spirochetes determined by 10-view counting were much lower than expected, feeding of clean ticks was repeated on the 8th day post infection as the spirochete numbers for one of the mice were higher on that day.

The moulted ticks were checked for infection by DNA isolation and PCR (sections 3.6 and 3.9) and by reisolation of *Borrelia* in BSK-H media, as described in subsection 3.1.2.

3.15.17 Comparison of in-vivo grown *Borrelia duttonii* Wild-type and Passage 51

1st stage nymphal *O. moubata,* that were infected either with *B. duttonii* 1120K3 wild-type or Passage 51, were crushed in clean BSK-H media containing *Borrelia* Antibiotics and injected into naïve mice.

For each injection, 3 *O. moubata* were crushed in 300 µL media using a pestle and 250 µL (150 µL intraperitoneal + 100 µL subcutaneous) were injected into naïve female Balb/c mice. 3 mice were injected with the wild-type infected and 3 mice with the passage infected *O. moubata* homogenate.

For 3 to 9 days post infection, tail-snips and 10-view counts were done according to subsection 3.15.2 to compare the number of spirochetes in the blood of the mice injected with the wild-type and the passage.

3 weeks post injection, an infection of the mice was confirmed serologically by Western Blot as described in section 3.5.

3.15.18 Visualization of Erythrocyte Rosetting in infected mouse blood by Electron Microscopic Analysis

Naïve female C3H and male Balb/c mice were injected with different amounts of *B. duttonii* isolate 1120K3 to be able to collect blood of the mice at a high point of spirochetemia. Therefore, the spirochete load in the mouse blood was determined 4 days post infection by tail-snips and 10-view counting (section 3.15.2). Table 8 gives an overview of the mice injected with *B. duttonii* 1120K3 wildtype to collect blood samples for analysis with electron microscopy.

Table 8 - Mice injected with different amounts of *B. duttonii* for blood collection for EM analysis

Mouse strain	**Number of mice**	**Number of spirochetes injected**
Female C3H	2	10^6
	5	10^5
	2	10^4
Male Balb/c	2	10^6
	2	10^5

One drop of blood of the selected mice was collected from the tail and frozen by high pressure freezing (LEICA, EM Pact2). Then freeze substitution (LEICA AFS) in the presence of 2% OsO_4 and emedding into resin Polybed 812 (EMS) was done.
Serial sectioning with a section thickness of 140 nm on the surface of a silicon wafer using a diamond knife was performed and after staining with uranyl acetate and lead citrate the wafer was carbon coated. Back-scaterred electrons were used in a scanning electron microscope (JEOL 7401F) for observation and the individual section images were processed by Amira software to prepare a 3D-model.

3.15.19 Determination of the infectivity of in-vitro infected *O. moubata*

To determine the infectivity of *O. moubata* infected by membrane feeding on different sera in experiment E as described in subsection 3.13.3, two ticks per sera were crushed in 300 µL BSK-H media containing *Borrelia* antibiotics. 250 µL (150 µL intraperitoneal + 100 µL subcutaneous) of this homogenate were then injected into a naïve female Balb/c mouse. As 5 different sera were tested, 5 mice were injected.
3 weeks post infection, blood of the mice was drawn by retro-orbital bleeding and a Western Blot was done to confirm an infection serologically (section 3.5).

3.16 Rabbit Experiments

3.16.1 Raising Antibodies against specific proteins of *Borrelia duttonii*

As described in sections 3.2 and 3.3 protein lysate of *B. duttonii* 1120K3 wild-type was separated according to size and stained with Coomassie brilliant blue stain, according to section 3.4 without protein fixation. The most prominent bands were cut out of the gel using a scalpel and homogenized in 500 µL 1x PBS by a Tissue LyserII (Qiagen). Then the homogenate was injected intraperitoneally to a naïve rabbit. This immunization step was repeated two more times every 10 – 14 days. The first immunization was done with Freund's adjuvant complete, while the others were a mixture of the protein with Freund's adjuvant incomplete. The dilution of protein to adjuvant was in all steps 1:1. After the third immunization, blood of the rabbit was drawn and the serum was checked for the presence of antibodies against the injected protein by Western Blot (described in section 3.5). If necessary, a fourth immunization was done.

Furthermore, the specificity of the antibodies raised was assessed by Western Blot. The sera were run against the protein lysate of *B. duttonii* 1120K3, *B. duttonii* Ly, *B. burgdorferi* sensu stricto (s.s) B31, and *B. afzelii* CB43.

Following this assessment, the bands used for immunization as well as bands recognized by the antibodies of B31 lysate were subjected to MS analysis for identification as described in section 3.17.

3.16.2 Raising Antibodies against saliva of *Ornithodoros moubata*

To raise antibodies against the saliva of *O. moubata*, late stage nymphal (Day 1 – Day 16) and adult (Day 17) ticks were fed on naïve rabbits overnight. The next day, the fully-fed ticks were removed. The feeding schedule is shown in Table 9.

Four and a half weeks after the first ticks were allowed to feed on the rabbits, the blood of the rabbits was collected, and the rabbits were euthanized. The blood was spun down at 9000 rpm for 7 minutes and the serum was removed using a pipette. The sera samples were stored at -20 °C.

Table 9 - Feeding schedule for the raising of antibodies against tick saliva

Rabbit	**Number of *O. moubata* applied**								
	Day 1	Day 6	Day 8	Day 9	Day 13	Day 14	Day 15	Day 16	Day 17
1	8	8	8	8	7	7	7	7	4
2	8	8	8	8	7	7	7	7	4

By Western Blot, as described in section 3.5, the sera were checked for the presence of antibodies against tick saliva by running it against protein lysate prepared from whole ticks and against lysate prepared from dissected tick salivary glands, as described in subsections 3.2.2 and 3.2.3, respectively.

3.17 Mass Spectrometric Analysis of selected protein bands

Proteins of *B. duttonii* 1120K3 and *B. burgdorferi* s.s. B31 against which antibodies have been raised in rabbits, as described in subsection 3.16.1, were identified by MS analysis. First the protein samples were prepared, by separating the proteins according to size by SDS-page and Coomassie Brilliant Blue staining (sections 3.3 and 3.4) which a protein fixation time of 25 minutes. After destaining of the gel overnight, the selected protein bands were cut out in a

flow box to prevent contamination and tryptic in-gel digestion was performed as previously described [44]. The peptides were dried and re-dissolved in 5 µL 5% FA. Then, C18 micro-gradient purification and fractionation followed: the process included conditioning with 0.1% TFA, sample loading and washing in one step. Afterwards, the peptides were directly gradient-eluted in 0.5 µl steps onto the MSP AnchorChip^{TM} Target (Bruker Daltonik) using the following gradient: 1, 3, 3, 2, 2, 1 µl of 2, 8, 16, 24, 32, 50%ACN/0.1%TFA. The mass spectrometric measurements were done on an Autoflex Speed MALDI-TOF/TOF (Bruker Daltonik). The automatic measurement was started using WarpLC 1.3 (Bruker Daltonics) and the acquired MS spectra were processed using ProteinScape 3.1 (Bruker Daltonik). Proteins were identified by submitting data to search engines against a *Borrelia* in-house prepared protein database, which was generated by downloading protein sequences from *Borrelia burgdorferi* or *Borrelia turicatae* and *B. hermsii* on NCBI database (version 20180515). The used search engine was Mascot (Matrix Science).

3.18 Artificial Infection of *O. moubata* by *B. duttonii* 1120K3

A protocol used for the artificial infection of *O. hermsii* with *B. hermsii* was adapted [45]. To infect *O. moubata* with *B. duttonii*, the ticks were dried in a desiccator containing 250 mL of saturated K_2CO_3 solution. In total, 80 larval *O. moubata* from the same cohort were split into four tubes with a screw-cap and left in the desiccator for 48 hours. The caps of the tubes were punctured with a pin.

After 48 hours the concentration of a *B. duttonii* 1120K3 wild type culture was determined using a Petroff Hauser counting chamber. Then the 7 mL culture was spun down at 4000 rpm, 20°C for 20 minutes. The resulting bacterial pellet was resuspended in 4 mL BSK-H media, thereby concentrating the *Borrelia*. 1 mL of this concentrated culture was then added to each of the screw cap tubes, which were sealed with parafilm. The ticks were incubated for 90 minutes in the culture at 32°C. Every 15 minutes the solution was mixed by gentle vortexing. After this incubation step, the tubes were centrifuged at 1600 rpm for 30 seconds and the culture was removed. Then the ticks were washed twice in 500 µL 1x PBS (1600 rpm, 30 seconds).

The parafilm sealing the tubes was removed and the ticks were put back into the desiccator now containing 300 mL saturated KCl solution for 19 days to recover.

Then, the ticks were fed till repletion on a naïve Balb/c mouse. After moulting of the ticks, they were checked for an infection with *B. duttonii* by reisolation in BSK-H media, as described in subsection 3.1.2.

4 Results

4.1 Infectious Dose 50 (ID_{50}) of *B. duttonii* by needle inoculation

To determine the ID_{50} of *B. duttonii* isolate 1120K3, multiple mice in the C3H and Balb/c background were injected with different amounts of spirochetes. Table 10 and Table 11 show the number of mice infected and uninfected for each mouse strain as well as for the different amounts of *Borrelia* injected. Infection of the mice was first determined by 10-view counting and later confirmed serologically by Western Blot.

Table 10 - Number of mice with a C3H background injected with *B. duttonii* for the determination of ID_{50}

Strain	Dose	number of infected mice	number of uninfected mice	Total		Percentage infected
				Uninfected	Infected	
C3H	10^6	5	0	0	23	100 %
	10^5	8	0	0	18	100 %
	10^4	5	0	0	10	100 %
	10^3	5	0	0	5	100 %

Table 11 - Number of mice with a Balb/c background injected with *B. duttonii* for the determination of ID_{50}

Strain	Dose	number of infected mice	number of uninfected mice	Total		Percentage infected
				Uninfected	Infected	
Balb/c	10^6	6	0	0	30	100 %
	10^5	6	0	0	24	100 %
	10^4	-	-	-	-	-
	10^3	9	0	0	18	100 %
	10^2	5	1	1	9	90 %
	10	3	3	4	4	**50 %**
	1	1	4	8	1	**11.11 %**

The numbers of the mice with a Balb/c background were used for the calculation of ID_{50} [46]:

$$\frac{50 - 11.11}{50 - 11.11} = 1 \qquad log\ 1 = 0$$

$$\boldsymbol{ID_{50}} = 10^{1+0} = \mathbf{10}\ \boldsymbol{spirochetes}$$

The ID_{50} with *B. duttonii* by needle inoculation in Balb/c mice was determined to be 10 spirochetes.

4.2 Detection of Antibodies against *Borrelia duttonii*

Antibody production against *Borrelia duttonii* during the course of infection for 6 to 21 days post infection is shown in Figure 8. For days 1 to 6 post infection, no bands were visible in the Western Blot. At 7 days post-infection the first bands were detected. Furthermore, starting at 10 days post infection multiple and more intense bands were detected. Between 12 and 21 days post infection the intensity and the number of bands increases even more. This suggests that after 7 days infection the first immune response is at a detectable level.

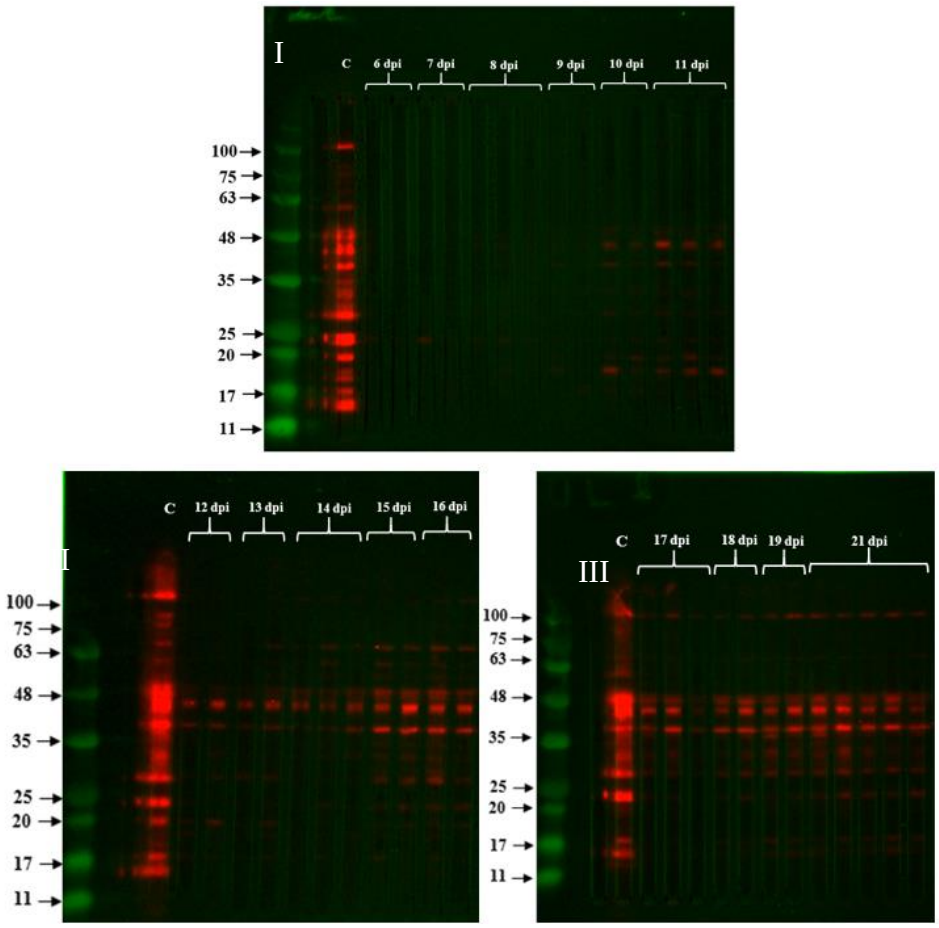

Figure 8 - Pictures of Membranes showing the continuous raising of antibodies against an infection of *B. duttonii* in mice; I: 6-11 days post infection, II: 12 – 16 days post infection and II: 17 – 21 days post infection; C: control serum (collected 6 weeks and 4 days post infection)

4.3 Infectivity of mouse sera collected at different points of spirochetemia

A Western Blot confirmed the infection of all 5 mice injected with sera collected from mice at high as well as at low points of spirochetemia, as shown in Table 12. Even though there were no spirochetes detectable by microscopy in the blood of mouse 3 at 6 days post-infection, the sera collected from it on that day was shown to be infectious in a naïve mouse.

In the part of the sera which was kept and was not injected, no antibodies were detected against the *B. duttonii*, so the antibodies formed in the mice injected with sera, resulted from an immune response against the bacteria, confirming an infection.

This means that even after centrifuging twice at 9000 rpm for 7 minutes the number of spirochetes remaining in the sera is above the ID_{50} of 10 spirochetes.

Table 12 - 10 -view counts for the mice from which sera was collected for injection to naïve mice

	Number of spirochetes in 10 views		**Naïve mice injected with sera**	
	4 days p.i.	**6 days p.i.**	**Total**	**Positive**
Mouse 1	1950	N.A.	3	3
Mouse 2	2096	N.A.		
Mouse 3	1598	0	2	2
Mouse 4	1	804	N.A.	N.A.

4.4 Acquisition Timing of *B. duttonii* by *O. moubata*

To determine a threshold for acquisition of *B. duttonii* by *O. moubata,* three mice were infected with *B. duttonii* and clean *O. moubata* were fed on these mice over 14 days.

Figure 9 – 11 show the number of spirochetes detected on each day by 10 view-counting as well as the percentage of *O. moubata* capable of picking up the infection for the three injected mice respectively.

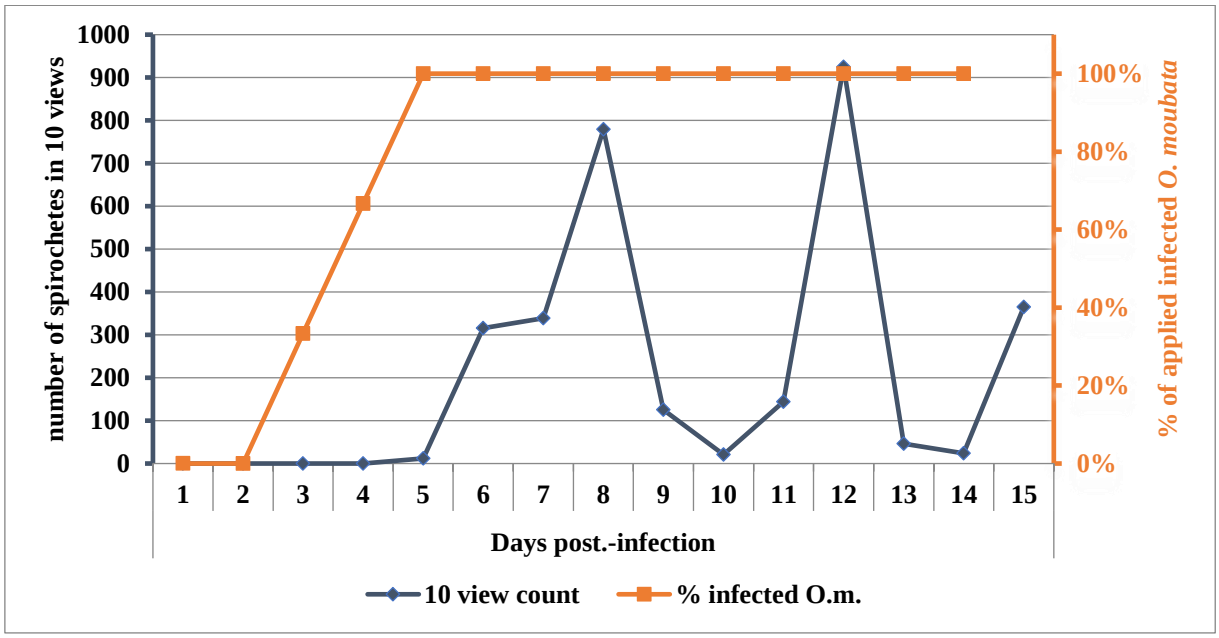

Figure 9 - Acquisition efficiency of *B. duttonii* by *O. moubata* compared to number of spirochetes visible in the blood for Mouse 1

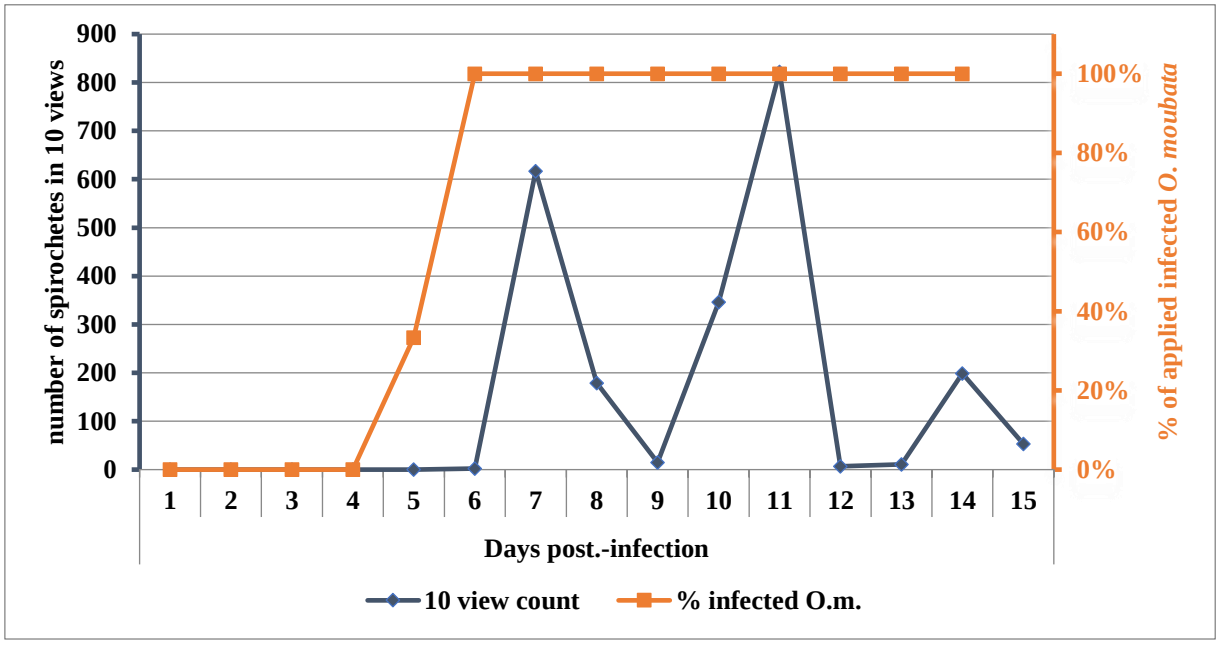

Figure 10 - Acquisition efficiency of *B. duttonii* by *O. moubata* compared to number of spirochetes visible in the blood for Mouse 2

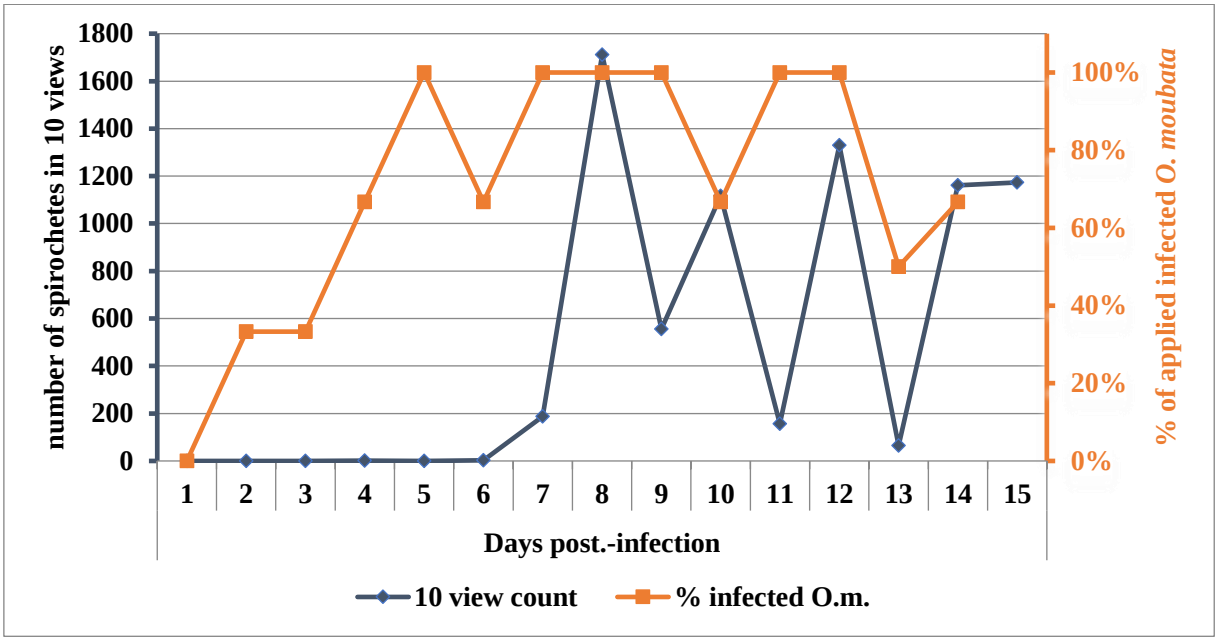

Figure 11 - Acquisition efficiency of *B. duttonii* by *O. moubata* compared to number of spirochetes visible in the blood for Mouse 3

For mouse 1 and 2, the efficiency of acquisition of *B. duttonii* 1120K3 by the *O. moubata* ticks stayed at 100 % as soon as spirochetes became detectable in the blood of the mice by microscopy and continued irrespective of the low numbers below the level of microscopic detection.

Also ticks feeding on mouse 3 acquired the bacteria before the first spirochetes were detected in the blood. The efficiency of acquisition is not linearly increasing to 100 % and staying at this level but is dropping to 50 % on 13 days post infection. Over the course of the experiment, mouse 3 developed a tail infection. This infection is a possible explanation for the deviating data obtained from this mouse.

4.5 Transmission of *B. duttonii* to naïve mice by *O. moubata*

The transmission of *B. duttonii* to naïve mice by *O. moubata* was unsuccessful. None of the mice on which three infected *O. moubata* fed on the ears were positive by microscopy or serologically, confirming that transmission did not happen. Furthermore, PCR done on the DNA isolated from the ear pieces cut off in one experiments was negative as well.

Also increasing the number of fed ticks to six did not yield a different result. However, the presence of infectious *Borrelia* within these ticks was confirmed by crushing and injecting the tick homogenate into naïve mice. Already the ticks crushed and injected 2 days after feeding

caused an infection in the mice. Microscopically, the first spirochetes were detected 2 days post-inoculation in all these tick-homogenate injected mice, as shown in Table 13.

Table 13 - 10 view counts for mice injected with tick homogenate, 2, 3 and 4 days after feeding of the ticks

Mouse strain	**Days post-feeding before being crushed**	**10 view counts**	
		2 days p.i.	**3 days p.i.**
GGTAL	2	1	94
	3	12	89
	4	2	46

Further increase of the number of ticks feeding per mouse to seven and changing the position of feeding to the back still gave the same results, and all mice remained uninfected.

Moreover, no spirochetes were detected in the blood of the SCID mice, on which different stage nymphal *O. moubata* fed, by microscopy. Reisolation of *Borrelia* from the blood at different time points after infection was unsuccessful.

The *O. moubata* fed on the SCID mice were confirmed infected by crushing and injecting some of the ticks into naïve mice, causing an infection.

Table 14 summarizes all the transmission experiments conducted.

Table 14 - Summary of Transmission Experiments conducted

Mouse type	**Number of mice**	***O. moubata* stage**	**Number of ticks applied**	**Feeding time**	**Tick feeding: Capsule / mouse belly**	**Comment**
C3H	3	1st nymphal	3	Fully fed	Belly	
	3	1st nymphal	3	Fully fed	Belly	Bite site removed
GGTAL	3	1st nymphal	6	Fully fed	Capsule (Back)	
Balb/c	5	1st nymphal	7	Fully fed	Capsule (Back)	
SCID	2	3rd nymphal	5	Fully fed	Belly	
	1	1st nymphal	10	Fully fed	Belly	

4.6 Reisolation of *B. duttonii* from coxal fluid

After six weeks in the incubator, the cultures started from coxal fluid excreted by infected *O. moubata* directly after feeding remained negative for the presence of *Borrelia,* suggesting that the *O. moubata* are not excreting the bacteria in the fluid.

4.7 Infectivity of post-moulting-tick homogenate

For the mice injected with crushed tick homogenate 28 days after feeding and post moulting, all mice injected with the original tick homogenate were confirmed infected by Western Blot, while the rest of the mice injected with dilutions 1:100 and 1:10 000 of the crushed tick homogenate remained uninfected. The results are presented in Figure 12. After an additional two weeks, still no antibodies were produced by any of the mice injected with the dilutions, suggesting that the number of spirochetes present in the ticks is so low that upon dilution an infection cannot be established. As the ID_{50} of *B. duttonii* in mice was determined to be 10 spirochetes, the number of *Borrelia* present in the ticks may possible be in this range as well.

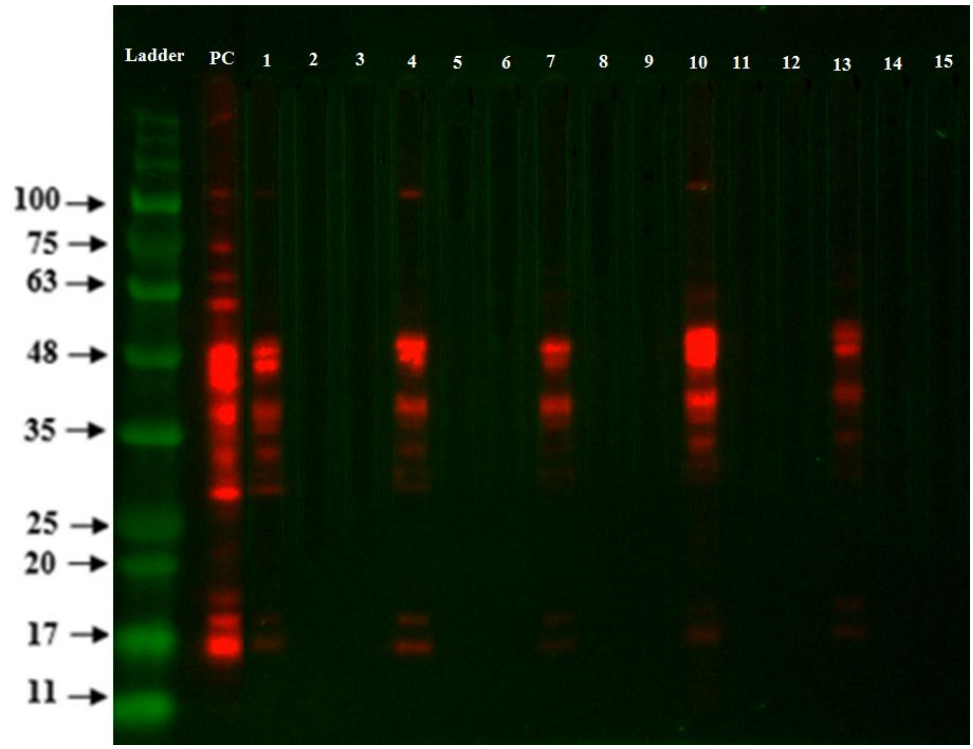

Figure 12- Results of the Western Blot with sera collected from mice injected with different dilutions of tick homogenate (after moulting) against *B. duttonii* protein lysate: PC – control, 1-3: Tick 1 (1: original, 2: 1:100, 3: 1:10 000), 4-6: Tick 2 (4: original, 5: 1:100, 6: 1:10 000), 7-9: Tick 3 (7: original, 8: 1:100, 9: 1:10 000), 10-12: Tick 4 (10: original, 11: 1:100, 12: 1:10 000), 13-15: Tick 5 (13: original, 14: 1:100, 15: 1:10 000)

Furthermore, the ticks of the same group which were crushed more than 4 months after their last blood meal caused an infection in naïve mice. 6 days post infection spirochetes were

detected microscopically in 4 of the 5 mice injected with the tick homogenate suggesting an infection. The infection in all five mice was confirmed serologically by a Western Blot.

4.8 Dissemination of *B. duttonii* to different mouse tissue

Reioslation of *B. duttonii* from the bladder of mice infected with *B. duttonii* 1120K3 wild-type and passage 40 was unsuccessful, as shown in Table 15. Even after 8 or 9 weeks in the incubator no spirochetes were detected under the dark-field microscope.

Table 15 - Reisolation of *B. duttonii* from the bladders of infected mice

Mouse strain	***Borrelia***	**Number of mice dissected**	**Weeks in incubator**	**Number of cultures**	
				Positive	**negative**
Balb/c	Wild-type	7	9	0	9
	Passage 40	6	8	0	8

The same is true for the cultures started from the brains of mice dissected almost 4 months post infection with wild-type *B. duttonii* 1120K3. No reisolation of *Borrelia* from the brains was possible, as shown in Table 16.

Table 16 - Reisolation of *B. duttonii* from the brain of mice (almost 4 months p.i.)

Mouse strain	**Number of mice dissected**	**Weeks in incubator**	**Number of cultures**	
			Positive	**negative**
Balb/c	2	8	0	2
C3H	2	8	0	2

To determine the presence of *Borrelia* in the brain of the mice a little less than 4 months after infection, the DNA isolated from the brain was checked for *Borrelial* DNA with *glpQ* and *FlaB* primers. For both PCRs, multiple bands were amplified in some of the samples, and one of these bands had the expected size. However, cloning, transforming and sequencing of this piece of DNA were unsuccessful, so we were not able to confirm the presence of *Borrelia* DNA in the brains.

4.9 Acquisition of *B. duttonii* by *O. moubata* 3 months post infection

Clean *O. moubata* were allowed to feed on infected mice 3 months post infection. None of the ticks were positive for *Borrelia*, neither by reisolation in BSK-H media, nor by gDNA isolation and PCR. Suggesting that acquisition is not possible after a long period of infection.

4.10 Effect of prolonged in-vitro cultivation on *B. duttonii* 1120K3

How prolonged in-vitro cultivation effects *B. duttonii* 1120K3 in respect to protein expression and infectivity was analysed. Figure 13 shows the comparison of protein lysate of 1120K3 wild-type, passage 40 and passage 51 protein lysate.

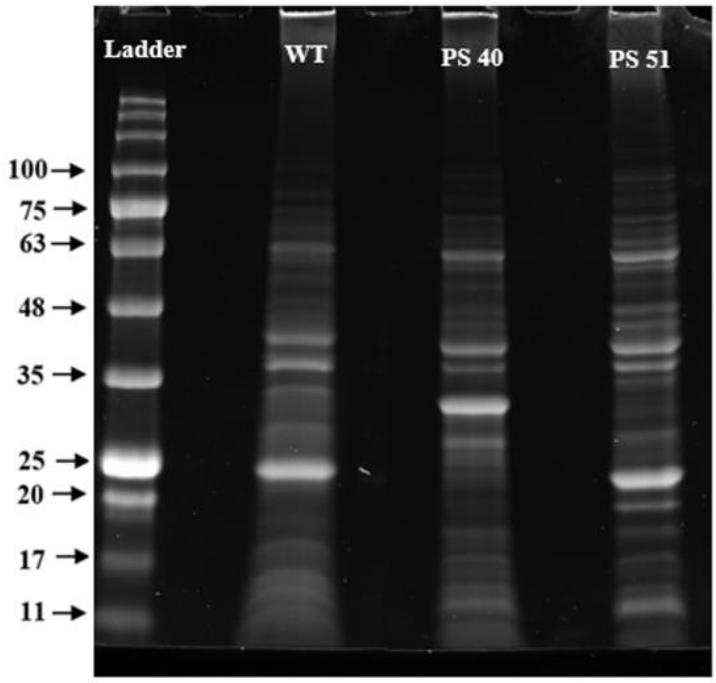

Figure 13 - Coomassie Brilliant Blue stained acrylamide gel comparing the protein lysate of *B. duttonii* 1120K3 wild-type and passages: WT - wild-type,, PS 40 - Passage 40, PS 51 - Passage 51

The protein bands present in the wild-type lysate are also all present in the different passages, even though the concentration of some proteins is different. Some are more expressed in the wild-type compared to the passages and vice-versa.

Furthermore, the infectivity of the passages was determined by injection into naïve mice. As passage 40 was still infectious, passage 51 was injected into naïve mice to check if there was a change in infectivity next to passage 40. Table 17 shows that both Passage 40 and Passage 51 are infectious to mice via needle-inoculation.

Table 17 - Infectivity of *B. duttonii* Passage 40 and Passage 51

Passage	Mouse strain	Number of mice	Number of spirochetes injected	% of mice infected
40	Balb/c	3	10^5	100
		3	10^3	100
51	C3H	3	10^5	100
		3	10^3	100

Furthermore, the ability of the passage 51 spirochetes to be acquired by *O. moubata* ticks was assessed, by allowing clean larval ticks feed on the passage infected mice at different levels of spirochetemia.

The success rate of acquisition was determined by crushing ticks in media. The number of positive cultures is shown in Table 18.

Table 18 - Acquisition Efficiency of *B. duttonii* Passage 51 by *O. moubata*

Number of spirochetes in 10 views	***O. moubata* positive**	***O. moubata* negative**	**Acquisition efficiency [%]**
11	1	2	33.3
18	1	2	33.3
7	0	0	0
133	1	2	33.3

Moreover, the comparison of the course of an infection established by injecting naïve mice by a homogenate generated by crushing wild-type and passage 51 infected ticks is shown in Table 19 and Figure 14.

Table 19 - 10-view counts for mice infected with in-vivo grown *B. duttonii* wildtype and Passage 51

Mouse strain	***B. duttonii***	**Mouse number**	**10 view counts – days post infection**						
			3	**4**	**5**	**6**	**7**	**8**	**9**
Balb/c	Wild-type	1	0	5	442	1695	0	14	36
		2	0	9	880	38	0	0	221
		3	9	332	2310	1	61	1082	361
	Passage 51	4	0	0	0	0	0	0	0
		5	0	0	0	0	0	0	0
		6	0	0	0	6	1	5	5

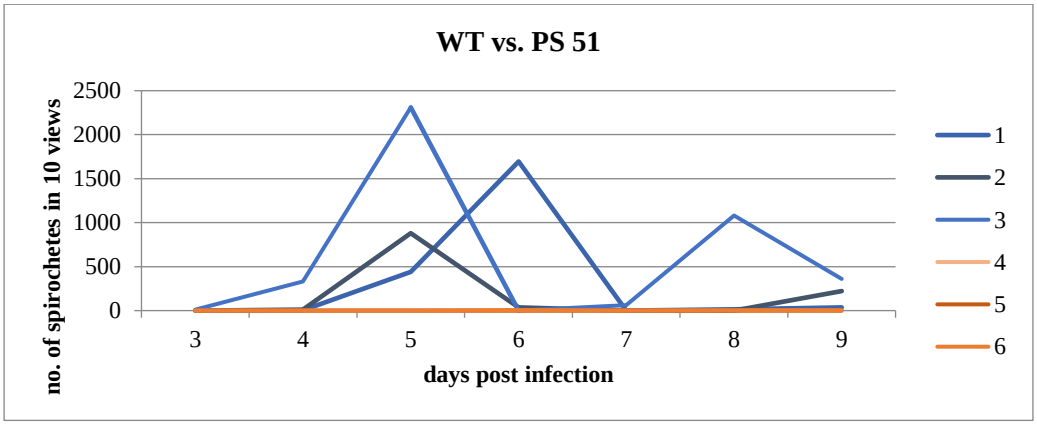

Figure 14 - Comparison of the number of spirochetes in the blood of wild-type and passage 51 infected mice: 1-3 - WT infected, 4-6 - PS 51 infected

There is a clear difference between the mice injected with the wild-type and passaged spirochetes. The *O. moubata* crushed and injected were all 1st nymphal stage and infected by feeding on infected mice.

The number of spirochetes in the blood of the mouse from which the *O. moubata* acquired *B. duttonii* 1120K3 passage 51 was 133. This number of spirochetes led to an acquisition efficiency with the wild-type isolate of 100%. Therefore, we assumed that also all *O. moubata* which fed on the passage infected mouse at this point of spirochetemia were able to acquire. This seems not to be true and only one third of the *O. moubata* acquired and sustained spirochetes during moulting, as only one of the three mice injected with crushed *O. moubata* got infected. Furthermore, the spirochete numbers of the passage infected mouse were much lower and the first spirochetes were detected only 6 days post infection, while in the wild-type infected mice already 3 days post infection the first mouse was positive by microscopy. Also the number of spirochetes in the wild-type infected mice reached much higher levels than the passage, showing a clear difference and effect on the spirochetes by prolonged in-vitro cultivation.

Even though the efficiency of acquisition is lower for passage 51, acquisition is still happening after 204 generations grown in-vitro.

4.11 Antibodies against specific proteins of *B. duttonii*

Figure 15 shows the protein bands of *B. duttonii*1120K3 lysate used for immunization of rabbits to produce specific antibodies against these proteins of *B. duttonii* 1120K3 isolate.

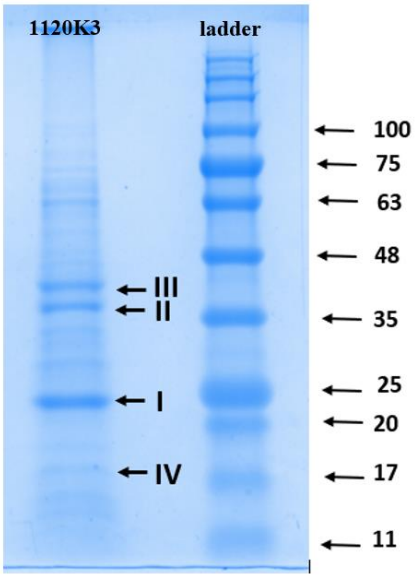

Figure 15 - Coomassie Brilliant Blue stained acrylamide gel with the protein lysate of *B. duttonii* 1120K3, the marked protein bands were used for injection

The sera collected from the rabbits was then further tested by Western Blot for presence of antibodies against these proteins and as well as for specificity against *B. duttonii* 1120K3 protein lysate. No antibodies were raised against Band III and the antibodies rose for Band I, II, and IV were nonspecific for *B. duttonii* Ly isolate and *B. afzelii* CB43. However, when using the sera against the protein lysate of *B. burgdorferi* s.s. B31 there was strong binding to some proteins which was not specific to the sizes to which the antibodies were generated.
The protein bands I-IV of the *B. duttonii* 1120K3 lysate as well as the most prominent bands which were hit by the antibodies in the sera of the protein lysate of *B. burgdorferi* s.s. B31 were subjected to MS analysis. Table 20 lists the samples' labels as well as the approximate sizes of the proteins.

Table 20 - Protein Bands subjected to MS analysis for identification - label and approximate sizes

Sample	Sample Label	Approx. size [kDa]
B. duttonii	Band I	23
	Band II	36
	Band III	40
	Band IV	17
B. burgdorferi s.s.	A	30
	B	12
	C	11

Table 21 to Table 24 show the results of analysis for samples Band I, II, III and sample A in the expected size range.

Table 21 - Results of MS Analysis for Band I

Protein	**Species**	**Accession number**	**Score**	**Size [kDa]**	**Number of peptide**	**Sequence coverage [%]**	**pI**
variable small protein 1	*B. hispanica*	WP_024655201.1	420.48	21	4	16.1	8.98
variable small protein 27 (plasmid)	*B. hermsii*	AMR76050.1	225.86	22.5	2	9.7	8.81
superoxide dismutase	*B. crocidurae*	AFI30934.1	175.69	23.7	4	27.6	6.17
chaperon protein GrpE	*B. duttonii* Ly	ACH93462.1	118.72	21.5	3	18.7	5.00
variable small protein 1	*B. crocidurae*	WP_040131982.1	111.24	22.2	2	8.7	8.89

Table 22 - Results of MS Analysis for Band II

Protein	Species	Accession number	Score	Size [kDa]	Number of peptide	Sequence coverage [%]	pI
flagellin, partial	*B. microti*	AEC46979.1	303.55	25.1	3	20.9	5.38
flagellin, partial	*B. burgdorferi*	AGL93161.1	234.52	14.4	2	23.9	4.54
flagellin	*B. hermsii*	ADD63784.1	126.66	35.1	1	5.1	5.62

Table 23 - Results of MS Analysis for Band III

Protein	Species	Accession number	Score	Size [kDa]	Number of peptide	Sequence coverage [%]	pI
glyceraldehyde 3-phosphate dehydrogenase	*B. duttonii* Ly	ACH93017.1	201.51	36.2	4	14.0	8.29
glyceraldehyde 3-phosphate dehydrogenase	*B. hermsii* DAH	AAX16579.1	114.83	3.2	2	7.2	8.31

Table 24 - Results of MS Analysis for A

Protein	Species	Accession number	Score	Size [kDa]	Number of peptide	Sequence coverage [%]	pI
Outer surface protein A	*B. burgdorferi*	Q45040	1968.18	29.3	25	68.9	8.77
Outer surface protein A	*B. sp.* LV5	O54323	1607.40	29.4	20	60.4	8.78

Mass Spectrometric analysis identified Band I as a variable small protein, Band II as Flagellin and Band III as glyceraldehyde 3-phosphatedehydrogenase. Sample A of *B. burgdorferi* B31 lysate was identified as outer surface protein A. No results were obtained during analysis of Band IV and no conclusive results were obtained for Sample B and Sample C, therefore this has to be repeated.

4.12 In-vitro feeding of *Ornithodoros moubata*

4.12.1 Acquisition of *B. duttonii* by *O. moubata* via in-vitro feeding

First, *O. moubata* were fed on rabbit blood with a *B. duttonii* concentration of 10^6 spirochetes per mL. The ticks fed on the fresh and the 5 day old rabbit blood. Feeding and subsequent moulting of the ticks was successful, however in none of the ticks *B. duttonii*, could be detected. Figure 16 shows a top view on the feeding compartment with fully fed nymphal *O. moubata.*

Figure 16 - In-vitro feeding of *O. moubata*: top view of a feeding compartment with fully fed *O. moubata*

In the following feeding experiment, *O. moubata* were again fed on rabbit blood, however this time two different types of blood were used. On one of the rabbits, *O. moubata* were allowed to feed prior to bleeding and euthanizing.

The percentage of ticks feeding on blood from rabbits prior infected with *O. moubata* (groups A and B) and the once feeding on clean rabbit blood (groups C and D) is shown in Table 25. Furthermore, the percentage of fed ticks, which then moulted to the next stage, was determined.

Table 25 - Membrane Feeding experiment determining the effect of prior infestation on feeding and moulting behaviour of the ticks

Group	Prior infestation	% of ticks fed	% of fed ticks moulted
A	+	93.33%	100.00%
B	+	74.19%	91.30%
C	-	96.77%	100.00%
D	-	86.67%	92.31%

No trend in feeding and moulting behaviour was detected, suggesting that the prior infestation of rabbit with *O. moubata* does not affect the feeding behaviour of the ticks.

O. moubata also fed on 11 day old rabbit blood which was stored in the fridge. Even though the percentage of ticks fed was almost 100%, all of the *O. moubata* died after a few days without moulting. Fresh blood is essential for successful in-vitro feeding of these ticks.

None of the ticks which were fed on rabbit blood containing different concentrations (10^3, 10^4. 10^5, 10^6 and 10^7 spirochetes per mL) of *B. duttonii* were able to acquire and maintain the bacteria after feeding/moulting as shown by DNA isolation and PCR and unsuccessful

reisolation from crushed ticks. Also, prior incubation of the *B. duttonii* within the blood to allow some adaption did not yield a different result. Reioslation of *B. duttonii* from the blood used during feeding was successful in some of the experiments conducted.

In the next set of experiment, we switched to different sera as feeding medium. However, the number of ticks feeding, dropped when comparing it to rabbit blood, as shown in Table 26.

Table 26 – Membrane feeding experiment comparing the effect of different sera on feeding and moulting behaviour

Serum	% of ticks fully fed	% of fed ticks moulted
Rabbit	52.94%	11.11%
Goat	66.67%	50.00%
Chicken	40.00%	16.67%
Pig	66.67%	60.00%
Adult Bovine	53.33%	62.50%

Figure 17 shows the difference in feeding behaviour depending on the meal provided during in-vitro feeding. The percentage of fully-fed ticks when feeding on rabbit blood is higher than the values for the different sera.

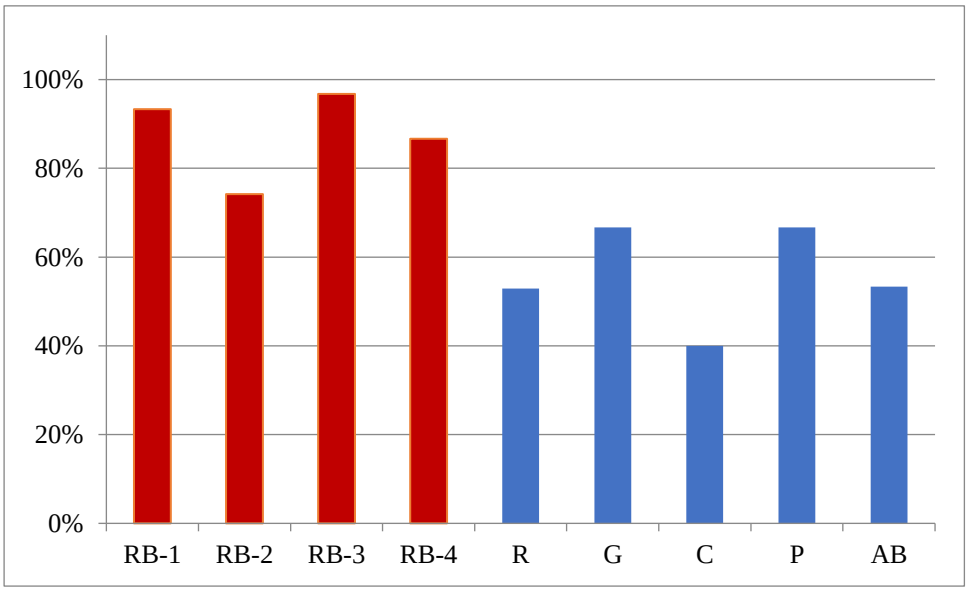

Figure 17 - Percentage of fully fed *O. moubata* after in-vitro feeding on different meals: Red (Rabbit Blood – RB1-4), Blue (different Sera: R = Rabbit, G = Goat, C = Chicken, P = Pig, AB = Adult Bovine)

The difference is even more prominent, when comparing moulting efficiencies of the fully-fed ticks, as shown in Figure 18.

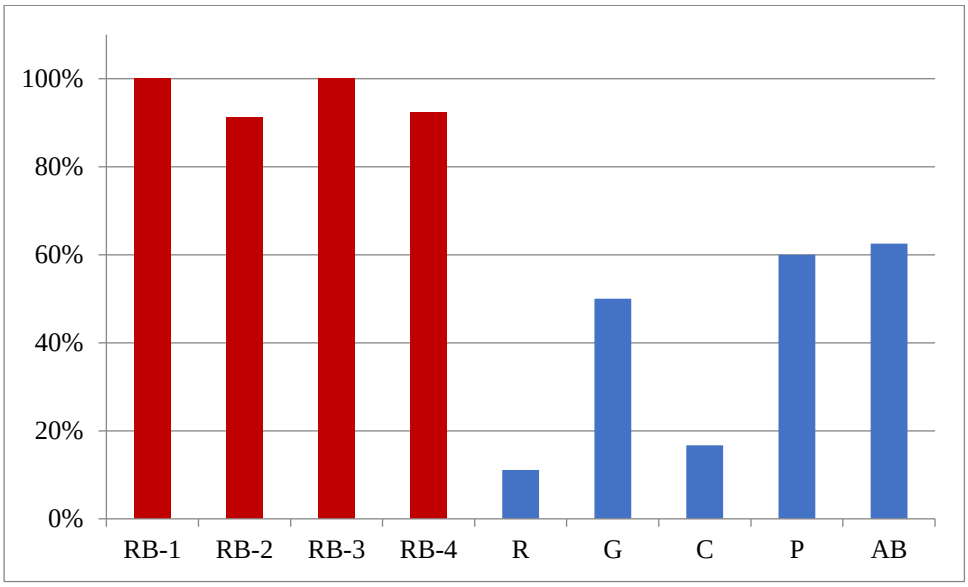

Figure 18 - Percentage of moulted *O. moubata* after in-vitro feeding (relative to the number of fully fed ticks): Red (Rabbit Blood – RB1-4), Blue (different Sera: R = Rabbit, G = Goat, C = Chicken, P = Pig, AB = Adult Bovine)

Reisolation of *Borrelia* from the sera used for feeding was successful for chicken, rabbit and pig serum. Furthermore, the ticks which fed on chicken and pig serum caused an infection in naïve Balb/c mice when being crushed and injected. This confirms the uptake and presence of infectious *Borrelia* by *O. moubata* via in-vitro feeding on these two types of sera.

4.12.2 Transmission of *B. duttonii* by *O. moubata* to rabbit blood

The DNA isolated form the rabbit blood on which infected *O. moubata* fed was negative for *borrelial* DNA by PCR. Also the reisolation of the *Borrelia* from the blood was unsuccessful, suggesting that no *Borrelia* got transmitted through the membrane to the blood meal during feeding.

4.13 Immuno-Fluorescence Assay

We tested the immuno-fluorescence assay (IFA) as a new way besides gDNA isolation followed by PCR and reisolation by crushing in media to determine the presence of *B. duttonii* in *O. moubata*. An assessment of this method was done by analysing pure culture, as well as a mixture of tick gut content with the culture and *O. moubata* which were expected to be infected, as they fed on a mouse at a high point of spirochetemia.

Even though the antibodies used in the experiment were against the Lyme disease spirochete *B. burgdorferi* sensu stricto (s.s.), *B. duttonii* were detected by IFA, when analysing the pure culture. However, upon mixing the culture with the cavity content of *O. moubata* and for

supposedly infected ticks, the method does not yield the desired results and we were unable to determine *B. duttonii* within ticks by IFA.

4.14 Artificial Infection of *O. moubata* by *B. duttonii*

After 3 weeks in the incubator, in none of the cultures started from crushed artificially infected *O. moubata* spirochetes, were detectable, suggesting that artificial infection of *O. moubata* by *B. duttonii* is not possible.

4.15 Visualizing the interaction between red blood cells and *B. duttonii* by Electron Microscopy

The mice with the highest number of spirochetes in their blood were selected and their blood was collected for analysis by electron microscopy. Table 27 lists the number of spirochetes in the blood of the mice selected for analysis.

Table 27 - Number of spirochetes in 10-views of infected mice for EM blood collection

Mouse		**Number of spirochetes in 10 views**
Balb/c	1	2207
	2	1035

High pressure freezing, sectioning and scanning electorn microscopy was done to determine interactions between the spirochetes and red blood cells. Figure 19 shows an 2D-picture of the 3D model constructed by cryo-sectioning showing spirochetes and red blood cells. Due to the low quality of the image, retaking pictures at higher quality will be done in the future.

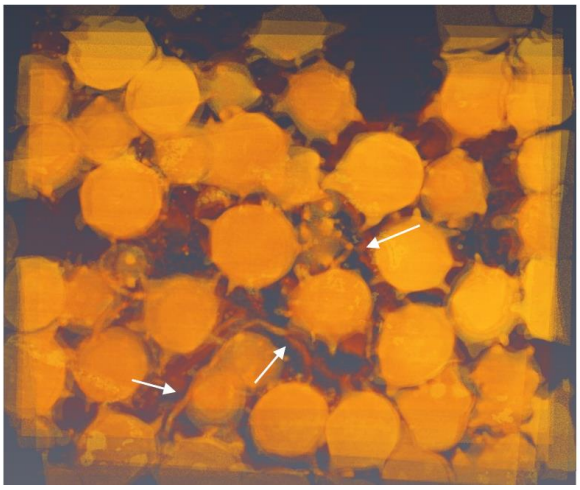

Figure 19 - 2D-picture of a 3D-model of spirochetes and red blood cells taken by cryo-electron microscopy, arrows indicate the position of *B. duttonii*

5 Discussion

B. duttonii, a representative of TBRF *Borrelia* in Central and Eastern Africa and its vector *O. moubata* have long been neglected when it comes to research in the past years. The serious symptoms of the RF caused by this bacterium, such as miscarriages and the difficulty of diagnosis of RF make this vector-bacteria combination an important subject for acquisition, transmission and infectivity studies as conducted in this work. For the first time, we are showing data about infectivity and the interplay of *B. duttonii* and *O. mouabata*, as a representative for old world RF.

The ID_{50} for an infection with *B. duttonii* in naïve mice by needle inoculation was determined to be 10 spirochetes. It has been reported before that for other RF *Borrelia,* like *B. hermsii,* one single spirochete is enough to cause an infection in a naïve host [47]. In our study, also one of 5 mice injected with one *B. duttonii* bacterium became infected. In recent studies the number of bacteria used for infection of naïve mice lies at 10^5 spirochetes which is 10^4-fold over the ID_{50} determined [48,49]. Far less *Borrelia* can be used to establish infections in new hosts. Furthermore, injection of naïve mice with possibly infected tissue samples is a very sensitive method for detection of *B. duttonii* as numbers needed for an infection are that low. Such mouse infectivity tests have been done before, to determine dissemination of *B. duttonii* to different mouse tissue during an infection [49]. A reason for the need of such high infectivity of these spirochetes might be their vector, *O. moubata* which is a fast feeding soft tick. Therefore, the transmission time of the spirochetes to a new host is very limited which might explain the low number of bacteria needed to establish an infection.

During an infection with *B. duttonii* in mice, 7 days after inoculation antibodies are detectable. Meaning only after this time the infection becomes detectable by serological tools. However, after that period, the first peaks of spirochetemia have already happened which always include the most severe symptoms. As successful diagnosis is essential for the right treatment, testing for antibodies might be too late in the course of the disease [16].

As *B. duttonii* can be detected easily in the blood and centrifugation of blood samples followed by staining is a novel approach of detection of these bacteria. We determined that sera collected at high and low points of spirochetemia is infectious when injected into a naïve host. For the sera collected after the first peak, no more spirochetes were detectable microscopically in the blood of the mice, which is still a common method for detection of RF *Borrelia* [17]. However, the number of spirochetes in the sera was still high enough to establish an infection in a naïve host. As no *Borrelia* are detectable by microscopy, no antibodies have yet been formed, and symptoms of an infection normally also disappear when spirochete numbers are

dropping, the possibility exists that infectious blood and sera is used for transfusions, thereby transmitting the infection to healthy individuals. In future experiments, the infectivity of sera which was collected from infected mice weeks after inoculation and was then stored at -20°C will be used to further asses the risk of infectious sera. New preventative measures should be taken as has been done before for other vector-borne disease, like West Nile, Dengue Chikungunya to limit the risk of transmission by transfusion [50–53].

Studying the acquisition dynamics of *B. duttonii* by *O. moubata* gave similar results as has been seen before with *B. hermsii* and its vector *O. hermsii*. *O. moubata* can acquire *B. duttonii* from infected mice even before the spirochetes in the blood reach microscopically detectable numbers. And as soon as spirochetes are detectable the acquisition reaches 100% efficiency. Such a threshold is also found for *B. hermsii* and *O. hermsii*. As soon as the spirochetes in the blood reach a density of 15 spirochetes per µL leading to an acquisition efficiency of 50%. By a further increase of spirochete density the efficiency increases to 100% [40]. This suggests that for both *Borrelia* species and their vectors there is a rather low threshold of spirochetes needed for successful acquisition.

Tick-animal models with successful acquisition and transmission have beenestablished in mice for the new world RF species, *B. hermsii* and *B. turicatae*, with their vectors *O. hermsii* and *O. turicatae* respectively [39,40]. None of the transmission experiments conducted here were successful. Neither increasing numbers of ticks, changing the feeding position or varying the stage of infected ticks led to successful transfer of *B. duttonii* to naïve mice. Also allowing infected ticks to feed on severe combined immunodeficient (SCID) mice yield no different result. Moreover, detection of *B. duttonii* in the coxal fluid secreted by infected *O. moubata* directly after feeding was unsuccessful. Reisolation of the *Borrelia* from coxal fluid secreted from infected ticks after feeding was not possible. Our findings contradict the assumption that *B. duttonii* is excreted in the coxal fluid [54]. We determined that infectious *B. duttonii* were present within the ticks after feeding on the SCID mice and the number of spirochetes needed to establish an infection in a new host is quiet low. Therefore, no spirochetes at all are transmitted during feeding of *O. moubata*. This hypothesis is also strengthened by the fact that we were unable to detect spirochetes in rabbit blood on which infected *O. moubata* were membrane-fed.

Previous studies conducted in the laboratory, have shown that transmission of *B. duttonii* to naïve mice by *O. moubata* is possible. Repeating this, needs further work. Using *B. duttonii* reisolates from ticks obtained during those experiments might give a different result.

Infectious *B. duttonii* are sustained in *O. moubata* for multiple months without a blood meal, as shown by injecting post-moulting-tick homogenate to naïve mice. RF *Borrelia* are expressing two kinds of proteins, the variable major protein (Vmp) in a mammalian host and the variable tick protein (Vtp) in the tick host. Studies with *B. hermsii* have shown that the switch between the expression of these genes can be induced by a temperature shift. While Vmp is important for the establishment of an infection in a naïve host, Vtp plays a crucial role in transmission from the ticks to a new host. Decrease of temperature and so mimicking the tick environment leads to the expression of *vtp*. Upon transmission from an infected tick to a new host, *vtp* is downregulated and *vmp* upregulated, resulting in the establishment of an infection [55–57]. *B. duttonii* is also able to perform this switch to rapidly adapt to a new environment.

The number of spirochetes within the ticks needs to lay in the range of ID_{50} of 10 spirochetes, as a dilution by 10^2-fold, decreases the number of *B. duttonii* in such an extent that an infection cannot be established. To determine the number of spirochetes present in *O. moubata*, qPCR or plating of the ticks should be done. Plating of ticks has been shown to give reliable results for various species of the *B. burgdorferi* s.l. complex. Plating of RF *Borrelia* is much harder and just in recent years a protocol for *B. hermsii* and *B. turicatae* has been developed [58]. Adapting this protocol for *B. duttonii* could greatly enhance research possibilities of this spirochete, as the number of living *Borrelia* could be determined as well as spirochete population with identical genetic content could be isolated.

Reisolation of *B. duttonii* from mouse bladders and brains was unsuccessful. We could not confirm previous reports that *B. duttonii* crosses into the brain and is persistent there [49]. Repetition of the experiment with an injection of the tissue to naïve mice instead of DNA isolation or reisolation of bacteria in media, might give different results, as this seems to be a more precise method of detection [49].

After 204 generations of *B. duttonii* grown in-vitro, the bacteria are still infectious by needle inoculation. The same is the case for *B. hermsii*, another RF *Borrelia* species that maintained its infectivity after 520 generations [43]. However, a phenotype in *B. duttonii* was observed by comparing the acquisition efficiency between wild-type and passage 51 as well as when checking the course of infection in mice. Acquisition of the spirochetes by *O. moubata* is still happening even though the efficiency decreased. Furthermore, the time between inoculation and the first peak increased when comparing it with the wild-type isolate and the passaged spirochetes do not reach as high concentrations in the blood of the host as the wild-type. This may suggest a loss of genetic information (plasmid loss) or change of expression due to the

prolonged in-vitro passaging, as was shown in the Lyme Disease spirochete *B. burgdorferi* s.s. [59].

The proteins of *B. duttonii* against which antibodies were raised in rabbits, were identified as variable small protein, flagellin and glyceraldehyde 3- phosphate dehydrogenase. The variable small protein plays a crucial role in the evasion of the host's immune response to an infection with the *Borrelia* while flagellin enables the movement of the spirochetes [60]. These proteins had different sizes compared to the ones of *B. burgdorferi* s.s., which are also bound by the antibodies. One of these proteins is the outer surface protein A. Furhter work to analyse this result is needed.

In-vitro feeding of *O. moubata* through parafilm membrane with fetal bovine serum has been shown before [61,62]. However, we wanted to generate ticks infected with *B. duttonii*. Clearly feeding on rabbit blood is preferred by *O. moubata*, however, when using this type of meal, the ticks are not able to maintain the *Borrelia*. Only ticks fed on chicken and pig serum mixed with a *B. duttonii* culture successfully acquired the spirochetes. As pigs and poultry are known hosts of *O. moubata* these two sera appear to be the most promising candidates. In-vitro feeding and infecting ticks is a good alternative to feeding on infected hosts, as the number of experimental animals can be reduced. Feeding of other vectors of RF *Borrelia*, like *B. turicatae*, has been shown before, but we were the first to also study infection of ticks by this method [63].

Furthermore, infection of *O. moubata* by immersion in a *B. duttonii* culture was attempted. This procedure has been proven to be effective for the infection of *O. hermsii* with *B. hermsii* [45]. However, *O. moubata* seem to be resistant to the drying and further immersion into the culture, as no *Borrelia* could be reisolated from the ticks. Maybe the numbers of spirochetes present within the ticks was so low that reisolation was unsuccessful and testing by crushing and injecting into naïve mice would give different results.

Moreover, we attempted to simplify and accelerate the determination of an infection in *O. moubata*. IFA has been shown before to be a capable method for this purpose. However, we were unable to reliable identify spirochetes in the ticks. The fact that we used the whole content of the tick's body cavity might explain the failure of this technique. As the antibody which was originally raised against *B. burgdorferi* has been proven to work for *B. duttonii* as well. Others have shown that IFA can be used to detect RF *Borrelia* in the salivary glands of the ticks [55]. This might be a possibility to enhance the sensitivity of this assay. In the future we would like to dissect *O. moubata* and use only the salivary glands of the ticks in identification.

An immune system evasion strategy employed by some of the RF *Borrelia* is erythrocyte rosetting [64]. *B. duttonii* interacts with red blood cells, which we attempted to visualize using electron microscopy. Even though our pictures are of low quality, interactions between the erythrocytes and the spirochetes are visible. Future enhancement of the quality of the pictures is needed to determine the extent of these interactions.

6 Conclusion

Our experiments confirmed that low doses of in vitro grown *B. duttonii* are enough to establish an infection in naïve hosts. Due to their presence in the blood of their hosts and the shown infectivity of sera before any antibodies against the disease have been generated or *Borrelia* are detectable in the blood, transmission by blood transfusion may be a serious problem in endemic regions of the disease.

Acquisition of the bacteria has a 100% efficiency as soon as *B. duttonii* is detectable in the blood by microscopy and before detection colonization of ticks is happening to some extent. Besides acquisition by feeding on infected animals, *O. moubata* can also acquire *B. duttonii* during in-vitro feeding on pig or chicken serum mixed with the bacteria, allowing a reduction of used laboratory animals. Furthermore, we have shown that the *O. moubata* can sustain infectious *B. duttonii* for multiple months.

Prolonged in-vitro cultivation of *B. duttonii* lead to a decrease in acquisition efficiency as well as the course of the infection is changed. The onset of disease is later compared to wild-type infected mice and the spirochete load in the blood is also reduced, suggesting a loss of genetic information due to the passaging.

Furthermore, we were able to raise antibodies against specific proteins of *B. duttonii* which can be used during the development of a diagnostic kit for this disease.

Physical interacitons between various cells in the blood including red blood cells and *B. duttonii* are occurring as we have visualized them by electron microscopy.

Transmission of *B. duttonii* to naïve mice by their vector seems not to be possible in the experiments conducted during this work, so our animal-tick model is not yet complete. Further revision needs to be done to complete the circle.

7 References

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