Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

Bachelor thesis in Biological Chemistry

Understanding the pathogenic lifecycle of *Borrelia duttonii*

Stefan Braunshier

Supervisor: Ryan O. M. Rego, PhD

Laboratory of Molecular Ecology of Vectors and Pathogens

Institute of Parasitology

Faculty of Science

České Budějovice, 2018

Bibliographical reference:

Braunshier, S., 2017: Understanding the pathogenic lifecycle of *Borrelia duttonii*. BSc. Thesis, in English. - 52 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Annotation: The aim of this thesis is to further the understanding of the lifecycle of *Borrelia duttonii*, to further the development of the tick-animal model and to reveal some antigenic markers that could lead to the detection of the disease in its earlier stages, as well as possible ways to prevent it altogether.

Affirmation: I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section. 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 bachelor thesis, in full form 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 defense 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.

České Budějovice, Date   Stefan Braunshier

Table of contents

1. Introduction....1

1.1. *Borrelia* and relapsing fever....1

1.2. *Borrelia duttonii* and relapsing fever....3

1.3. Comparison between soft ticks and hard ticks....4

1.4. The lifecycle of a soft tick....5

2. Aims of the thesis....7

3. Materials and Methods....7

3.1. Spirochete strain and culture preparation....7

3.2. Ticks....8

3.3. Mouse experiments....8

3.4. BSKII media preparation....8

3.5. Isolation of *Borrelia* from ticks....9

3.6. DNA isolation from ticks....9

3.7. Isolation of genomic DNA from *B. duttonii* culture....9

3.8. Isolation of RNA from *O. moubata* ticks....10

3.9. cDNA synthesis and amplification....10

3.10. Investigating differences in gene expression between naïve and infected ticks by qPCR....12

3.11. Polymerase Chain Reaction (PCR)....12

3.12. Cloning and transformation of amplified DNA into *E. coli*....13

3.13. Isolation of plasmid DNA from *E. coli*....14

3.14. Restriction digestion of plasmid DNA and sequencing of *recN* gene....14

3.15. Agarose gel electrophoresis....15

3.16. Western blotting....15

3.17. Transovarial transmission....16

3.18. Growth curve of *Borrelia* culture at 34°C and 23°C....17

3.19. Passaging of the *Borrelia* culture....17

3.20. Preparation of protein lysates....17

3.21. Comparison of passaged and wild-type protein lysates....18

3.22. Monitoring the levels of *Borrelia* in blood....18

3.23. Dependence of tick to mouse transmission of *Borrelia* to the number of ticks that fed on the mouse 18
3.24. Acquisition of *Borrelia* at different levels of spirochete density 19
3.25. Artificial infection of *O. moubata* ticks with *B. duttonii* 19
3.26. Chloroform-methanol extraction of protein lysate 20
3.27. In-solution digestion 20
3.28. 2D gel electrophoresis 20
3.29. Blue-Silver Coomassie Staining 21
3.30. Immunoblotting of 2D gel 22
3.31. In-gel digestion of cut gel pieces 23
3.32. Desalting 23
3.33. Analysis of proteins by MS 24
4. Results 25
4.1. Sequencing of *recN* gene 25
4.2. Transovarial transmission 25
4.3. *Borrelia* levels in blood 26
4.4. Comparison between passaged and wild-type *Borrelia* 27
4.5. Growth curve of *Borrelia* at 34°C 29
4.6. Dependence of tick to mouse transmission of *Borrelia* to the number of ticks that fed on the mouse 29
4.7. Acquisition of *Borrelia* at different levels of spirochete densities 32
4.8. Artificial infection of *O. moubata* ticks with *B. duttonii* 32
4.9. Investigating differences in gene expression between naïve and infected ticks by qPCR 32
4.10. Serological proteome analysis of *B. duttonii* 35
4.11. In-solution digestion of *B. duttonii* 1120K3 protein lysate 38
5. Discussion 39
6. Acknowledgements 43
7. References 44

1. Introduction

1.1. *Borrelia* and relapsing fever

Relapsing fever (RF), as implied by the name, is a disease characterized by sudden onsets of high fever and shaking chills, followed by periods of low blood pressure and defervescence. These are attributed to the high density of spirochetes of the *Borrelia* genus in the blood (Cutler, 2006). When detected by the immune system, the spirochetes change their surface antigens, which are termed variable major proteins (*vmp*), leading to immune evasion (Barbour *et al.*, 2002, Stoenner *et al.*, 1982) and repeated spirochetemia, which triggers the immune system again. As depicted in Figure 1, the process of detection, change of antigens, evasion, multiplication and detection anew is what causes the febrile response in the patients (Dworkin *et al.*, 2008).

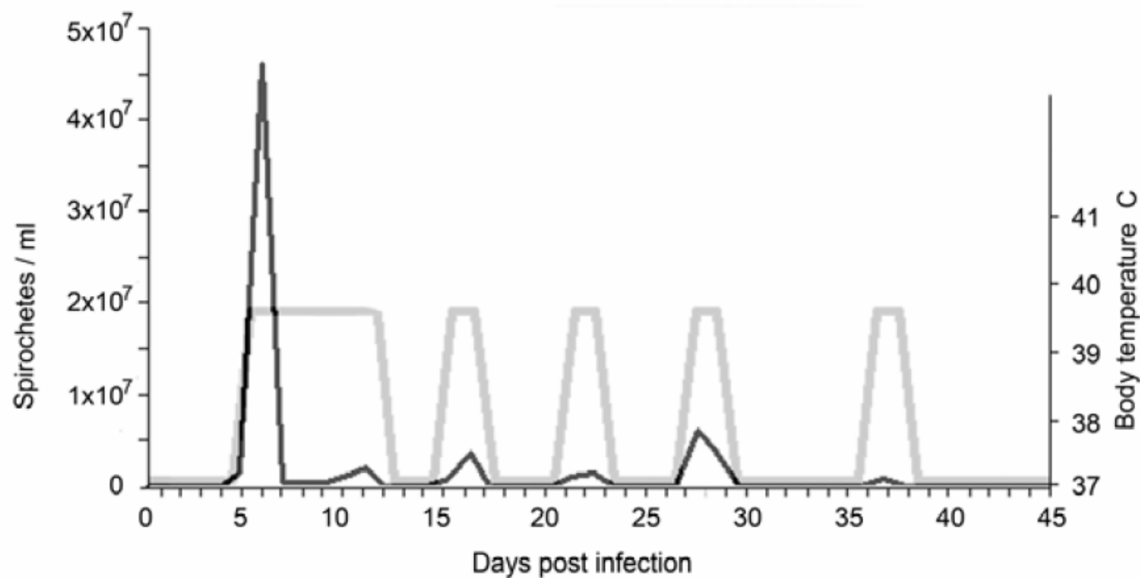

Figure 1: Typical RF spirochetemia and fever pattern (adapted from Larrson, Current issues in relapsing fever, 2009). The image is generated from a mice infected with *B. duttonii* (black line). The grey line is the usual fever pattern observed in human patients

RF is an arthropod-borne infection spread by lice (*Pediculus humanus*) and soft ticks of the *Ornithodoros* genus (Southern & Sandford, 1969; Barbour & Hayes, 1986). Therefore the disease can be split into two categories: lice-borne relapsing fever (LBRF) and tick-borne relapsing fever (TBRF).

The mortality rates of TBRF and LBRF in untreated patients are 4-10% and 10-70%, respectively (Barbour, 2006). The highest probability for death to occur is either during a febrile episode or within two hours after the start of treatment with antibiotics. The latter event, coined the Jarisch-Herxheimer reaction (J-HR), although not fully understood, may be due to the sudden release of lipoproteins from the lysed bacteria, which then cause an endotoxin shock (Bryceson *et al*., 1972).

A common problem with TBRF is its misdiagnosis as malaria (Nordstrand *et al*., 2007), which results in ineffective treatment. In a study performed in Togo, 10% of patients diagnosed with malaria were positive for TBRF. Furthermore, 4.5% of the patients were co-infected with malaria parasites and *Borrelia*. In a paper in 2009, it was determined that such co-infections lead to severe internal damage in mice, since the immune system is focused on the parasites, which makes the *Borrelia* more dangerous (Lundqvist, 2010).

While the causative agent for LBRF is only *Borrelia recurrentis*, TBRF is associated with multiple *Borrelia* species. The species can be categorized by their vector, in which case we distinguish hard tick RF (caused only by *B. miyamotoi*) and soft tick RF (e.g. *B. crocidurae*, *B. hermsii*, *B. duttonii*, etc). Another classification distinguishes "new world" TBRF caused by species present in North and South America, such as *B. hermsii*, *B. turicatae*, *B. parkeri, B. miyamotoi, B. lonestari*, etc. and "old world" TBRF with species from Asia, Africa and Europe such as *B. duttonii*, *B. recurrentis*, *B. crocidurae*, *B. hispanica*, *B persica*, etc. (Fukunaga *et al.* 1996; Ras *et al.* 1996).

Almost all TBRF *Borrelia* are maintained in enzootic cycles that involve species of small mammals and ticks. The mammals are usually small rodents (Nicolle & Anderson, 1927), but may also include pigs, goats, bats, foxes, cats, dogs, birds, etc. (Felsenfeld, 1971, Hoogstraal, 1985, Thomas *et al*., 2002, Pavlovsky, 1963).

As can be seen in Figure 2, TBRF is found throughout most of the world, with the exception of Australia, New Zealand and parts of Southwest Pacific (Flesenfeld, 1965, Southern & Sandford, 1969). It is endemic in parts of Canada, Mexico, Central and South America, central Asia, most of Africa and the Mediterranean region (Dworkin *et al*., 2008).

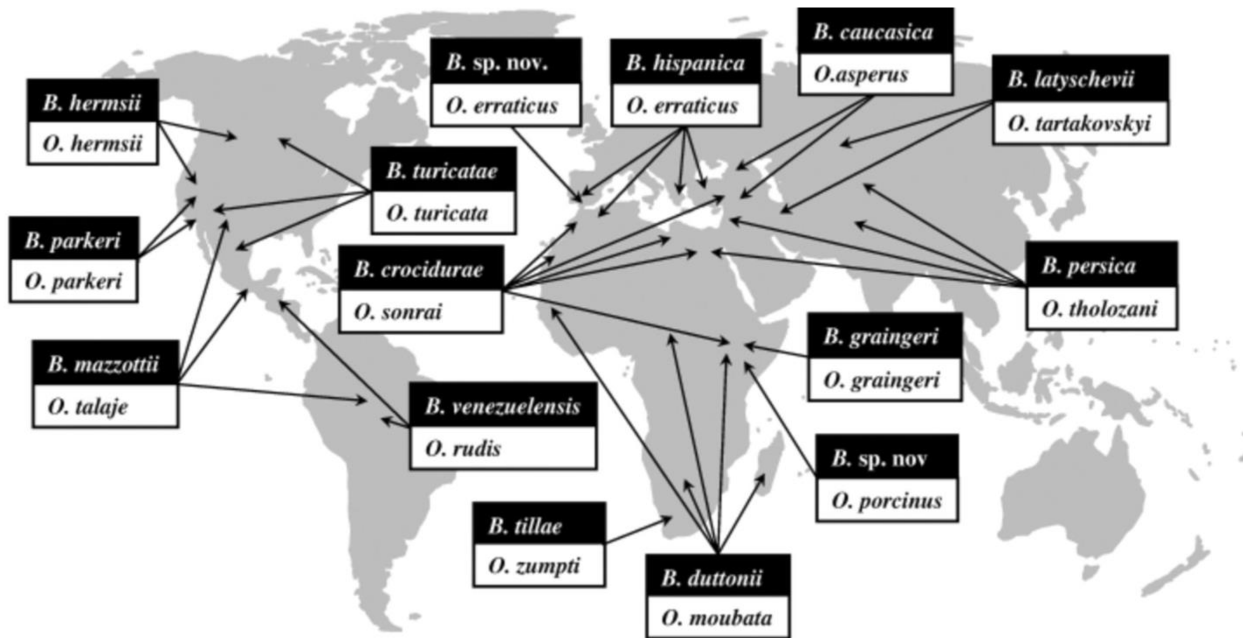

Figure 2: Geographical distribution of *Borrelia* species and their corresponding vectors (adapted from Rebaudet & Parola, Epidemiology of relapsing fever borreliosis in Europe, 2006)

1.2. *Borrelia duttonii* and TBRF

As mentioned before, most TBRF *Borrelia* species have some animal hosts as reservoirs. The supposed exception is *Borrelia duttonii*, with its vector, the tick *Ornithodoros moubata*, where the only mammalian reservoirs have appeared to be humans. However, chickens and pigs have been proposed as a possible reservoir (McCall *et al*., 2007).

Among all RF-*Borrelia* species, *B. duttonii* is associated with a high rate of TBRF. Even nowadays, it remains a significant public health problem in Africa (Cutler *et al*., 2006).

Moreover, *B. duttonii* infections have a greater incidence of neurological complications such as facial palsy, weakness and, occasionally, stupor or coma (Cadavid & Barbour, 1998). Another complication that occurs in TBRF caused by this species of *Borrelia* is erythrocyte aggregation into rosettes (Takahashi *et al*., 2000). In the case of *B. crocidurae*, erythrocyte rosetting delays the immune clearance, which might also be the case for *B. duttonii* (Burman *et al*., 1998). Finally, *B. duttonii* is associated with a high rate of pregnancy complications (Goubau & Munyangeyo, 1983, Jongen *et al*., 1997, Barclay & Coulter, 1990, Melkert, 1991).

B. duttonii, as well as, *B. recurrentis* are reported to associate with C4b-binding protein and factor H (Meri *et al*., 2006), an immune evasion method similar to that of Lyme disease pathogen, *B. burgdoferi* sensu stricto. It is also believed that *B. recurrentis* is descended from or closely related to *B. duttonii* (Lescot *et al*., 2008)

1.3. Comparison between soft ticks and hard ticks

The order Ixodida is composed of three families, two of which contain species which are vectors for *Borrelia*. The first of them, the Ixodidae family, also known as hard ticks, contains the vectors for Lyme disease *Borrelia* species. The second one, the Argasidae family, commonly known as soft ticks comprises 5 genera, out of which the TBRF transmitting *Ornithodoros* genus is the largest. The differences between the two major families are outlined in Table 1.

Table 1: Differences between hard and soft ticks (adapted from Larsson, Pathobiology of African Relapsing Fever *Borrelia*, 2007 and Sonenshine, Biology of ticks, 1997). Important differences for our study are marked in bold.

Feature	Argasidae	Ixodidae
Habitat	Sheltered: burrows, nests, caves, cracks, crevices, man-made shelters	Open: grass and bushes
Host seeking	Lives in close proximity to host	Waiting for host to pass by
Host range	Narrow	Wide
Life span	**Long**	**Short**
Size of blood meal	2-6x body weight	100x body weight
Number of blood meals	**Many**	**Few**
Feeding time	**30 minutes**	**Several days**
Mode of feeding	Secretion of excess water by coxal fluid	Regurgitation
Nymphal instars	**Many (2-8)**	**One**
Time required for *Borrelia* transmission	**Instant**	**>48 hours**
Number of eggs	**Few (200-300); multiple times**	**Many (up to 23000); only once**
Transovarial transmission of *Borrelia*	**Yes, in many species**	**No, very rare**

The appearance of soft ticks and hard ticks also differs (Figure 3). Hard ticks get their name due to the plate of chitin that covers the upper side of the body, named *scutum*. Soft ticks lack *scutum*, but instead have a leathery exoskeleton.

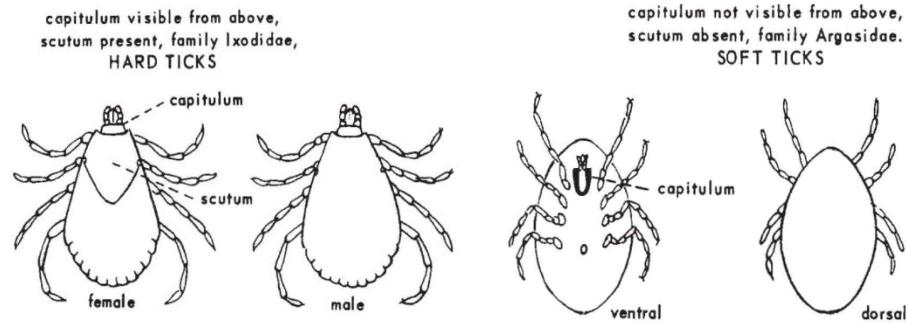

Figure 3: Differences in appearance between hard and soft ticks (adapted from Harry D. Pratt, Ticks: Key To Genera in United States)

1.4. The lifecycle of a soft tick

The lifecycle of the soft ticks (Figure 5) has some characteristics that make it more suitable for research purpose compared to hard ticks.

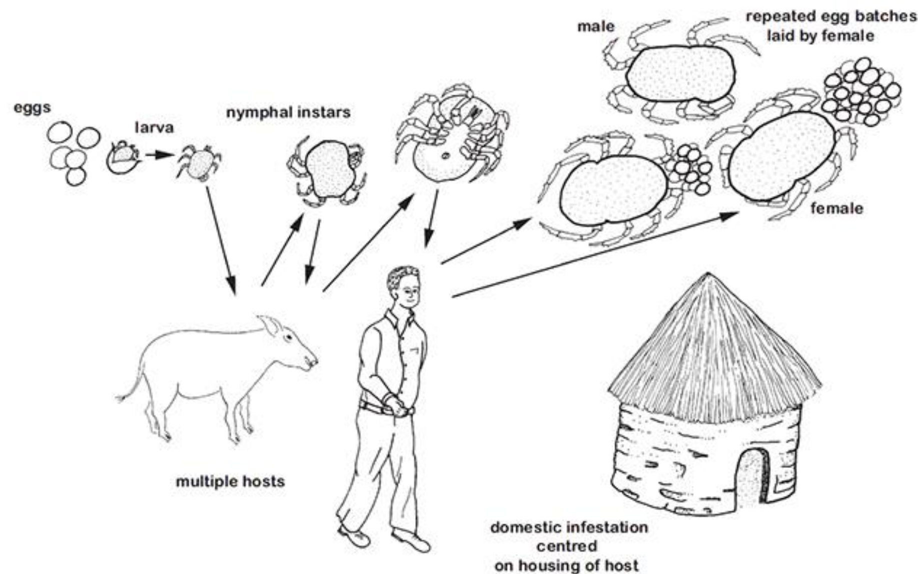

Figure 4: The lifecycle of a soft tick (adapted from Walker et al., Ticks of Domestic Animals in Africa: a Guide to Identification of Species, 2003)

Firstly, the larvae hatch and attach to their first host, usually at night. They are fully fed in around 30 minutes (hard ticks need days). When ingested into the tick gut, the spirochetes invade several organs, such as the salivary gland. As stated in Table 1, excess water is removed as coxal fluid, which may also be contaminated with the spirochetes. The larvae molts into the

first nymphal stage in a matter of weeks to a month. The infection is retained throughout molting. When the nymph feeds again, the infection is passed through saliva and/or coxal fluid contaminating the wound (Parola & Raoult, 2001).

In hard ticks, the spirochetes need to move from the midgut by penetrating the midgut wall and enter the salivary glands. The process which takes up to 48 hours, all the while no bacteria is transmitted (Ribeiro *et al*., 1987).

The soft tick nymph may then molt into a higher-stage nymph several times or molt into an adult. A female adult may then feed and, a while later, lay a relatively small batch of eggs. The eggs may become infected due to the coxal fluid (Tabuchi *et al*., 2008). The female may then feed again, laying eggs after each blood meal, while a female hard tick dies after laying a large batch of eggs. Humans may also get infected, but they are usually accidental hosts, except for *B. duttonii* where they are the only natural reservoir.

The quick feeding time makes the soft tick ideal for experimental purposes, as the ticks can be collected and analyzed on the same day, making studies such as the dissemination of the disease in mice much simpler. As soon as the tick molts it can be used for another experiment. Furthermore, obtaining adult ticks is also a process that is significantly faster for soft ticks and more efficient as the female adult can be used multiple times.

2. Aims of the thesis

For further insight into the infectious lifecycle of *Borrelia duttonii* using an established animal-tick model, the following experiments were performed:

1. Further confirmation of the identity of our strain by sequencing of the *recN* gene
2. Studying the transovarial transmission of *B. duttonii* in its vector *O. moubata*
3. Studying the transmission from tick to mouse and its dependence on the number of ticks that were fed
4. Monitoring the spirochete levels in blood during an infection
5. Studying the retention of infectivity of *B. duttonii* after prolonged *in vitro* culturing and investigating the possible differences that may occur when a loss of infectivity takes place
6. Studying the difference in growth at 23°C (the temperature inside the tick) and 34°C (the optimal growth temperature)
7. Artificial infection of the ticks by immersion in a *B. duttonii* culture
8. Investigation of the serological proteome in infected mice and detection of possible immune markers

3. Materials and Methods

3.1. Spirochete strain and culture preparation

B. duttonii strain 1120K3, provided by Dr. Sven Bergström (Universit of Umea, Sweden) , was used throughout these studies. The isolate was stored as glycerol stocks and kept at -80°C. The spirochetes were cultivated in liquid Barbour-Stoenner-Kelly (BSK) II media (Barbour, 1984), prepared in our own laboratory and supplemented with 6% rabbit serum (Sigma). Cultures were prepared by inoculating 7 mL of BSKII media with a scraping of a glycerol stock. The cultures were incubated at 34°.

3.2. Ticks

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

3.3. Mouse experiments

C3H/HeN mice were used for feeding the ticks as well as for the experiments on infectivity. The mice were bled daily by tail snips for a period of 1-2 weeks. After 3 weeks post-inoculation/tick bite the mice were bled so as to extract as much blood as possible and then euthanized. The blood was incubated at room temperature for 15 minutes and then centrifuged at 9000 rpm for 7 minutes. The sera (upper phase) was separated and stored at -20°C.

3.4. BSKII media preparation

The components and the amounts used are stated in Table 2. First, 10x CMRL, Neopeptone and BSA were mixed with 800 mL milliQ water in a 2 L conic flask. The flask was stirred for 1 hour. Afterwards, the remaining ingredients were added together with 200 mL milliQ water. The mixture was stirred for 1 more hour. Another 600 mL of milliQ water were added. The pH was adjusted to 7.6 with a 10M NaOH solution. MilliQ water was added until a final volume of 2 L. The media was then filtered and aliquoted into 250 mL bottles. A 7 mL aliquot was taken and incubated at 34°C for 3 days to check for contaminants under a dark-field microscope (20x magnification). If slight contamination was observed, a 100x solution of antibiotics was added (in a 1:100 ratio) and the aliquot was again incubated at 34°C. After a couple of days, it was rechecked. If no contamination was observed, 15 mL rabbit serum (6%) was added to each bottle, which were then stored at -20°C

Table 2: The ingredients and amounts used to prepare 2L of BSKII media

Ingredient	Amount [g]
Bovine Serum Albumin (BSA)	100
Neopeptone	10
10x CMRL	19.4
Yeastolate	4
Hepes Acid	12
Glucose	10
Sodium pyruvate	1.6
Sodium citrate	1.4
N-acetyl glucosamide	0.8
Sodium bicarbonate	4.4

3.5. Isolation of *Borrelia* from ticks

The ticks were dipped in 1 ml 3% hydrogen peroxide for 15 minutes. The peroxide is pipetted out and 1 ml 70% ethanol is added. After 15 minutes the ethanol was pipetted out and the tubes were dried in the flow box. 150 µl BSKII medium with *Borrelia* antibiotics was added and then the ticks were crushed with a sterile plastic pestle. The pestle was washed with 850 µl BSKII medium with *Borrelia* antibiotics. The tubes were then incubated at 34°C.

3.6. DNA isolation from ticks

DNA isolation was performed using DNeasy® Blood & Tissue kit (QIAGEN), according to the included protocol. 180 µl ATL buffer were added to a 1.5 mL Eppendorf tube containing the tick. The tick was crushed and 20 µl Proteinase K were added and the contents mixed by vortexing. The test tube was incubated at 56°C overnight and then vortexed for 15 seconds. 200 µl AL buffer were added, then 200 µl 70% ethanol, followed by vortexing after each step. The mixture was transferred to a DNeasy® Mini Spin Column and centrifuged at 9000 rpm for 1 minute. The flow through was discarded and the collection tube was replaced. 500 µl AW1 buffer were added to the column and the mixture was centrifuged at 9000 rpm for 1 minute. 500 µl AW2 buffer were added and the mixture was centrifuged at 14000 rpm for 3 minutes. The collection tube was replaced with a 1.5 mL Eppendorf tube. The DNA was eluted from the column with 200 µl AE buffer and centrifugation at 9000 rpm for 1 minute. The DNA was stored at 4°C.

3.7. Isolation of genomic DNA from *B. duttonii* culture

The culture was transferred to a 15 mL Falcon® tube and centrifuged at 7830 rpm for 10 minutes. The supernatant was removed and the pellet was mixed with 600 µl Nuclei Lysis Solution (Promega). The solution was transferred to 1.5 mL Eppendorf tubes and incubated first at 80°C for 5 minutes, then on ice for 2 minutes. 3 µl RNase were added, the mixture was inverted 5 times and incubated at 37°C for 30 minutes. 200 µl Protein Precipitation Solution (Promega) were added and the tube was put on ice for 5 minutes. The mixture was centrifuged at 13000 rpm for 5 minutes. The supernatant was transferred to another 1.5 mL Eppendorf tube containing 600 µl isopropanol and mixed by inverting the tube a couple of times. Afterwards,

the tube was centrifuged at 13000 rpm for 10 minutes. The supernatant was removed and the tube was dried on a paper towel. 600 µl of 70% ethanol were added and the solution was centrifuged again at 13000 rpm for 10 minutes. The ethanol was removed and the pellet was left to dry for around 5 minutes. In the end, 100 µl DNA Rehydration Solution (Promega) were added and the pellet was resuspended. The isolated DNA was stored at 4°C.

3.8. Isolation of RNA from *O. moubata* ticks

The RNA isolation was performed using the RNeasy® Mini Kit (QIAGEN), according to the included protocol: the ticks were put in 1.5 mL Eppendorf tubes and 600 µl RTL buffer were added. The ticks were crushed with a plastic rod. The tubes were centrifuged at 17500 rpm for 3 minutes. The supernatants were transferred to new 1.5 mL Eppendorf tubes and 600 µl of 70% ethanol were added. The solutions were mixed by pipetting, transferred to RNeasy® Mini Spin Columns attached to 1.5 mL collection tubes. The tubes were centrifuged at 13000 rpm for 15 seconds and the flow through was discarded. 700 µl RW1 buffer were added, followed by centrifugation at 13000rpm for 15 seconds. The centrifugation step was repeated after adding 500 µl RPE buffer. Another 500 µl RPE buffer were added and the tubes were centrifuged at 13000 rpm for 2 minutes. The columns were dried by centrifugation at 13000 rpm for 1 minute. The collection tubes were replaced with 1.5 mL Eppendorf tubes. 50 µl RNase-free water were added directly on the column. Centrifugation at 13000 rpm for 1 minute yielded the isolated RNA, which was stored at 4°C.

3.9. cDNA synthesis and amplification

cDNA synthesis and amplification were performed using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Invitrogen™). cDNA synthesis and amplification were performed in order to test the primers from Table 3. The reaction mixture composition is stated in Table 4 (A, B). The sequences needed to design the primers were obtained from the NCBI database, as well as from literature research (Rego *et al.*, 2005, 2006) The RT-PCR product was cloned and transformed into *E. coli*. Plasmid DNA isolation from white colonies was performed, followed by restriction digestion to confirm the presence of the insert. The positive colonies were sequenced. The sequences were searched in the NCBI database using BLAST, in order to make sure that the primers targeted their

corresponding genes of interest. However, if the cDNA is needed for a qPCR reaction, the reaction mixture for cDNA synthesis, but no amplification is stated in Table 5. Also, instead of a temperature program, the reaction mixture was simply incubated at 60°C for 30 minutes.

Table 3: Primers designed to be used in our qPCR experiment

Primers	Sequence
Dorin M-forward	5'-GGT GTT GGT GAT GGG TGT GC-3'
Dorin M-reverse	5'-CGC CGT CTG TCT CCA TGT CA-3'
OMFREP-forward	5'-AGC CAG CTT CAG CAC GTT TG-3'
OMFREP-reverse	5'-CGG CGA AGC TAA CGT GGA AC-3'
defA-forward	5'-TTG CTC TCG TTG TCG CCC TT-3'
defA-reverse	5'-GTT GAA CGG ACA GCC GTA GC-3'
defD-forward	5'-GTA CGA GTG TCA CGC CCA CT-3'
defD-reverse	5'-ATC GCC AGG AAG GCG ATT CT-3'
Actin-forward	5'-CGG GTG CTT CTG TGC TGT TC-3'
Actin-reverse	5'-GCA TCA TCG CCA GCG AAT CC-3'

Table 4: **A** (left) Reaction mixture and **B** (right) temperature settings for cDNA synthesis and amplification of *DorinM*, *OMFREP*, *defA*, *defD* and *Actin*

RT-PCR Master Mix 1	Volume
2x Reaction Mix	12.5 µl
RNA	2 µl
Primer F	1 µl
Primer R	1 µl
Enzyme Mix	0.5 µl
MilliQ water	8 µl
Total	25 µl

Step		t [sec]	T [°C]
cDNA synthesis		1800	60
Initial Denaturation		120	94
40 cycles	Denaturation	15	94
	Annealing	30	55
	Elongation	60	68
Final Elongation		300	68
Hold		∞	16

Table 5: Reaction mixture for cDNA synthesis

RT-PCR Master Mix 2	Volume
2x Reaction Mix	12.5 µl
RNA	2 µl
Enzyme Mix	0.5 µl
MilliQ water	10 µl
Total	25 µl

3.10. Investigating differences in gene expression between naïve and infected ticks by qPCR

qPCR was performed using FastStart Universal SYBR Green Master (Rox, Sigma-Aldrich) master mix. The reaction mixture was prepared according to Table 6 (A, B). cDNA was synthesized from 4 RNA samples isolated from infected ticks and 4 samples isolated from clean ticks; each RNA sample was isolated from 3 ticks. cDNA synthesized from the RNA was diluted 1:10 with milliQ water before use. Each sample was analyzed in triplicates. The genes to be analyzed are mentioned in Table 3. Quantification of the *Actin* gene was used as a control.

Table 6: A (left) Reaction mixture and **B** (right) temperature settings for the qPCR reactions

qPCR Master Mix	Volume
FastStart Universal SYBR Green Master (Rox)	12.5 μl
Primer F	0.375 μl
Primer R	0.375 μl
MilliQ water	9.25 μl
cDNA	2.5 μl
Total	25 μl

<table>
<tr><th colspan="2">Step</th><th>t [sec]</th><th>T [°C]</th></tr>
<tr><td colspan="2">Activation of polymerase</td><td>600</td><td>95</td></tr>
<tr><td rowspan="2">40 cycles</td><td>Denaturation</td><td>15</td><td>95</td></tr>
<tr><td>Annealing/ Elongation</td><td>60</td><td>60</td></tr>
<tr><td colspan="2">Hold</td><td>∞</td><td>16</td></tr>
</table>

3.11. Polymerase Chain Reaction (PCR)

PCR was used to amplify different genes of interest. The primers used to amplify *B. duttonii* strain 1120K3 *recN* gene were taken from Elbir *et al*. (2013) and designed using the corresponding sequence of the LY strain as template. OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (New England BioLabs) was used in all of the performed PCR reactions. Agarose gel electrophoresis was performed to check if the amplification was successful, using a 20 μl reaction mixture without DNA (9 μl milliQ water instead of 7) as negative control. The primers used in our experiment are listed in Table 7. The composition of the reaction mixture and the temperature program are listed in Table 8 (A, B).

Table 7: Primers used throughout our experiments

Primers	**Sequence**
recN-forward	5'-GAT GAT GTA ATT TCT AAT GAA GGA TG-3'
recN-reverse	5'-TCT TTG ACC AAA ATT CCC CTA A-3'
glpQ-forward	5'-AAG GTA TGG CTG AGG TTG C-3'
glpQ-reverse	5'-AGA CCT GTG ATT TGC CCA TTA-3'
flaB-forward	5'-AGG GTG CAC AGC AAG AAG GA-3'
flaB-reverse	5'-GCA CCA AGA TTT GCT CTT TGA TCA G-3'

Table 8: **A** (left) Reaction mixture and **B** (right) temperature settings for PCR amplification of *flaB*, *glpQ* and *recN* genes

Master Mix 1	**Amounts**
OneTaq® Quick-Load® 2X Master Mix with Standard Buffer	10 µl
DNA	2 µl
MilliQ water	7 µl
Primer F	0.5 µl
Primer R	0.5 µl
Total	20 µl

Step		**t [sec]**	**T [°C]**
Initial denaturation		300	94
40 cycles	Denaturation	30	94
	Annealing	30	55
	Elongation	60	65
Final elongation		600	94
Hold		∞	16

3.12. Cloning and transformation of amplified DNA into *E. coli*

The PCR product is ligated into the pCR™ 2.1-TOPO®Vector using the TOPO™ TA Cloning™ Kit (Invitrogen™). The reaction mixture was prepared according to Table 9, then mixed by pipetting and incubated at room temperature for 5 minutes. To 1.5 µl of the resulting solution, 100 µl competent *E. coli* cells were added, and the contents were mixed by flicking the tube. The mixture was kept on ice for 30 minutes. Afterwards, a heat shock was applied by putting the mixture in a 42°C water bath for 30 seconds, and then placing it immediately on ice. After 2 minutes, 250 µl S.O.C. media (New England BioLabs) were added and the solution was kept on a horizontal shaker at 200 rpm and 37°C for 90 minutes. In the meanwhile, LB agar plates with Carbenicillin were incubated at 37°C for an hour. Then, the plates were coated with 50 µl X-Gal (Thermo Fisher Scientific) each and incubated for 15 more minutes at 37°C. On one LB plate, 100 µl of the cell culture were added, while on the other we added 200 µl. The plates were incubated at 37°C overnight.

Table 9: TOPO reaction mixture

pCR™ 2.1-TOPO®Vector	
Reagent	**Amount**
PCR product	2 µl
Salt solution	0.5 µl
TOPO Vector	0.5 µl

3.13. Isolation of plasmid DNA from *E. coli*

White colonies were picked and dipped in a 15 mL Falcon™ tube containing 7 mL liquid LB media. The tubes were then incubated at 37 °C on a shaking incubator overnight. The following day, plasmid DNA was isolated using the NucleoSpin® Plasmid kit (Macherey-Nagel) and according to the included protocol: the tubes were centrifuged at 7830 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 250 µl buffer A1. The suspensions were transferred to 1.5 mL Eppendorf tubes. 250 µl buffer A2 were added. The tubes were inverted a couple of times and incubated at room temperature for 5 minutes. 300 µl buffer A3 were added and the tubes were inverted again. The tubes were centrifuged at 13000 rpm for 5 minutes. The supernatant was transferred to a NucleoSpin® column. The columns were centrifuged at 13000rpm for 1 minute. The flow through was removed. The columns were washed with 600 µl buffer A4 and then centrifuged at 13000 rpm for 1 minute. The flow was removed. The columns were dried by centrifugation at 13000 rpm for 2 minutes. The collection tubes were replaced by 1.5 mL Eppendorf tubes and 50 µl buffer AE were added directly on the column. Finally, centrifugation at 17500 rpm for 1 minute yielded the plasmid DNA.

3.14. Restriction digestion of plasmid DNA and sequencing of *recN* gene

Restriction digestion with EcoRI was performed on the isolated plasmid DNA. The reaction mixture was prepared according to Table 10. The solution was incubated at 37 °C for 1 hour. The samples were run on an agarose gel to check for inserts. A mixture of 2 µl of unrestricted plasmid DNA and 8 µl of milliQ water was used as a negative control. Before sending it for sequencing, 5 µl of the plasmid DNA which had the insert present were mixed with 5 µl of M13 reverse primer (TOPO™ TA Cloning™ Kit, Invitrogen™) with a concentration of 5 pmol/µl. The sequences were visualized using GENIOUS and compared to sequences in the NCBI database using the BLAST tool provided on the NCBI website.

Table 10: EcoRI digestion reaction mixture

Restriction digestion with EcoRI	
Reagent	**Amount**
DNA	3 µl
EcoRI (NEB)	1 µl
NEB buffer 2.1 (NEB)	2 µl
MilliQ water	14 µl
Total	20 µl

3.15. Agarose gel electrophoresis

Gel electrophoresis was used to check if the PCR or restriction digestion reactions were successful. For this purpose, a 1% agarose gel was prepared by weighing 1.5 g agarose powder and adding 150 mL 1x TAE buffer. The resulting suspension was microwaved until the agarose dissolved. The solution was cooled until it was slightly warm and 7.5 µl GelRed® (Biotium) were added. The liquid was poured on a caster fitted with a comb and allowed to solidify. The electrophoresis chamber was filled with 1x TAE buffer and. In one well, 10 µl of 1 kb+ DNA Ladder (Invitrogen™) were loaded. To check if a PCR reaction was successful, 15 µl of the reaction mixture were loaded. If the PCR product would be cloned and transformed, then only 5 µl of product were loaded. In the case of samples prepared by restriction digestion, 15 µl of restricted DNA were mixed with a drop of Gel Loading Dye (New England BioLabs) and then loaded into the wells. The gel was run at 100-110V for 45-60 minutes. The samples were visualized under UV light.

3.16. Western blotting

A 12% SDS-PAGE gel was prepared according to Table 11 and placed in running buffer. The protein lysates were heated at 100°C for 10 minutes. 20 µl of hot lysate were loaded in the wells of the gel as well as 10 µl of Protein Marker VI (AppliChem). The gel was run at 120V for 80 minutes. Afterwards, the gel was blotted on a nitrocellulose membrane (25V, 60 minutes). A blocking buffer consisting of 5% dehydrated milk solution in TBS-Tween 20 (0.1%) was prepared. After blotting, the membrane was incubated with 40 mL blocking buffer for 2 hours on a shaker. Meanwhile, the primary antibody solution was prepared by mixing 5 µl of mouse serum with 995 µl of blocking buffer, followed by brief vortexing. After the blocking step was finished, the membrane was cut into pieces and sealed in plastic bags together with 1 mL of the diluted serum solution. The plastic bags were then stored, in the fridge, at 4°C overnight. The following day, the plastic bags were opened and the membrane pieces were washed 3 times with TBS-Tween 20 for 15 minutes on a shaker. The secondary antibody solution was prepared by adding 5 µl of Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in rabbit (Sigma-Aldrich) to 50 mL blocking solution. The membrane pieces were incubated with the secondary antibody solution on a shaker for 60 minutes and then washed again 3 times with

TBS-Tween 20. The developing solution was prepared using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific). 1.5 mL Reagent A were mixed with 1.5 mL Reagent B, vortexed and then added to the membrane. After 5 minutes of shaking, the membrane was rebuilt and a picture was taken using the ChemiDoc™ XRS+ System and the software Image Lab™.

In later experiments, the western blots were performed using the Mini-PROTEAN II multiscreen apparatus (Bio-Rad). For that purpose, the SDS-PAGE page gel was prepared using a comb with only one small well (for the ladder) and a large well, where 150 µl of lysate were loaded. Afterwards, when incubating with the primary antibody, the 1 mL solution is pipetted in the wells of the multiscreen apparatus, avoiding bubble formation. The apparatus is then put on a shaker for 30 minutes and stored in the fridge overnight. The rest of the procedure is the same as written previously.

Table 11: Components and amounts needed to prepare a 12 % SDS-PAGE gel

12% SDS-PAGE gel preparation		
Reagent	**Separation gel - Amounts**	**Stacking gel - Amounts**
30% Acrylamide	2 mL	0.165 mL
Separation/Stacking buffer	1.25 mL	0.25 mL
MilliQ water	1.7 mL	0.575 mL
10% APS (Ammonium persulfate)	50 µl	10 µl
TEMED (N, N, N', N'-tetramethylethylenediamine)	2 µl	1 µl

3.17. Transovarial transmission

Larvae from infected adults were fed on 6 C3H mice. The larvae were allowed to molt to 1st stage nymphs. Cultures were prepared from 4 nymphs per infected mouse (in 2 tubes, each containing 2 ticks) by crushing them in media. After 9 days, the cultures were checked for *Borrelia* under a dark-field microscope (20x magnification) and then again after 2 more weeks. The mice were bled every day for a week and the spirochetes were counted under a dark-field microscope (20x magnification). The infection of the mice was also checked by a western blot. The experiment was repeated once more to confirm the results. This time, the adults were

checked for *Borrelia* by cutting them in two. Half was used for DNA isolation, while the other half was crushed in media.

3.18. Growth curve of *Borrelia* culture at 34°C and 23°C

One culture was started in 7 mL BSKII media. When the spirochete density reached 10^7 spirochetes/mL, 70µl of the culture were transferred into 6 tubes and diluted in 7 mL BSKII media resulting in six cultures each with approximately 10^5 spirochetes/mL. Three of the tubes were incubated at 34°C. The other three were kept at room temperature. In the case of the 34°C cultures, the spirochete density was determined every 2 days by dark-field microscopy using a Petroff-Hausser counting chamber (Marienfeld-Superior, 0.02 nm depth, 0.0025 nm^2 area). When the cultures reached 10^8 spirochetes/mL, protein lysates were prepared from each of them. The density of the cultures kept at room temperature was measured three weeks after preparation.

3.19. Passaging of the *Borrelia* culture

A culture was started from a glycerol stock in 7 mL BSKII media and incubated at 34°C. The spirochete density was measured every week. When it reached around 10^7 spirochetes/mL, the culture was diluted in a new tube to a final concentration of 10^5 spirochetes/mL, yielding 7 mL of passaged culture. A protein lysate was prepared from the original culture. The process was repeated with the newly passaged culture. The infectivity of the 12th passage was checked by injecting 250 µl of passaged culture (diluted to $4*10^6$ spirochetes/mL) into 3 C3H mice (mice 4-6). Another 3 C3H mice (mice 1-3) were injected with the same amount of a wild-type culture diluted to the same spirochete density to serve as positive control. Spirochetemias were quantified every day by counting the spirochetes in the blood under a dark-field microscope. Furthermore, the infectivity of the passaged culture was also checked by a western blot. A similar procedure was performed to check the infectivity of the 22nd passage.

3.20. Preparation of protein lysates

The *Borrelia* culture was centrifuged at 7000 rpm for 15 minutes. The supernatant was removed and the resulting pellet was washed by dissolving it in 1 mL cold HN buffer followed by centrifugation at 7000 rpm for 15 minutes. The washing step was repeated 2 more times and the

pellet was then lysed with 200 μl B-Per solution (Thermo Fisher Scientific). The protein concentration was determined by measuring absorption at 260 nm. Then the lysate was diluted 1:1 with 2x Laemmli sample buffer (Bio-Rad) mixed with β-mercaptoethanol (50 μl β-mercaptoethanol to 950 μl Laemmli buffer).

3.21. Comparison of passaged and wild-type protein lysates

Lysate of a wild-type culture grown to 10^7 spirochetes/mL was prepared following the procedure described above. The lysates of the wild-type and the 7th and 11th passage were then diluted to roughly the same concentration and loaded into an SDS-PAGE gel. The gel was run at 120V until the ladder reached the bottom. The gel was then washed 3 times with milliQ water and stained with Coomassie Brilliant Blue for 2 hours, followed by destaining of excess dye overnight. Some bands of interest were cut and sent for LC-MS/MS sequencing.

3.22. Monitoring the levels of *Borrelia* in blood

3 female C3H mice were inoculated with 250 μl of a *Borrelia* culture with a density of $4*10^4$ spirochetes/mL (mice 1-3). 3 more C3H mice were injected with the same amount of a culture with a density of $4*10^5$ spirochetes/mL (mice 4-6). The spirochetes in the blood obtained by tail snips were quantified by counting under a dark-field microscope (20x magnification) daily, over a period of 10 days. The infectivity was also checked by a western blot.

3.23. Dependence of tick to mouse transmission of *Borrelia* to the number of ticks that fed on the mouse

12 female C3H were used in this experiment. The ticks used were 1st stage nymphs that were fed as larvae on infected mice during the first spirochetemia and then were allowed to molt. Their infectivity was tested by crushing 4 ticks in media according to the procedure previously described. The C3H mice were split into three groups based on the number of ticks that were fed: the first group with 1 tick/mouse (mice 1-4), the second with 2 ticks/mouse (mice 5-8) and the third one with 3 ticks/mouse (mice 9-12). The mice were anaesthetized by injection with ketamine while the ticks were feeding. The mice were bled daily for 10 days and the *Borrelia* in the blood was counted under a dark-field microscope. The ticks that were fed were crushed in

media to check which were infectious. The cultures were checked for *Borrelia* under the dark-field microscope after one week and then again after three weeks. After around 6 weeks, the mice were bled and killed. A western blot was performed to check if the mice became infected. The experiment was repeated, but with 10 female C3H mice split into 2 groups: one on which 5 ticks/mouse were fed (mice 13-17) and another with 10 ticks/mouse (mice 18-22). The ticks used were from the same batch as the ones used in the previous attempt. The mice were bled daily for 8 days and the blood checked for *Borrelia*. The ticks were cut in two. One half was crushed in media. DNA was isolated from the other half. PCR was performed on the DNA samples using *Borrelia recN* gene as a target. Afterwards, a western blot using the blood from the mice was performed.

3.24. Acquisition of *Borrelia* at different levels of spirochete density

Acquisition of *Borrelia* by ticks feeding on mice was also checked during the time between the first and second spirochetemia. Only one of the infected mice from the previous experiment was used (mouse 10, on which 3 infected ticks were fed) because we were not able to observe spirochetes in the blood of the others. Therefore, we started feeding 5 naïve ticks every day on while the mouse was anaesthetized by injection with ketamine.

3.25. Artificial infection of *O. moubata* ticks with *B. duttonii*

We attempted to infect *O. moubata* ticks by immersion in a *B. duttonii* culture according to a procedure used to produce infected *O. hermsi* ticks (Policastro *et al*., 2011). Four tubes containing around 20-30 *O. moubata* larval ticks were kept in a desiccator containing 120 mL of a saturated $MnCl_2$ solution for 2 days. Afterwards, 1 ml of a *B. duttonii* culture containing 10^7 spirochetes/mL was added to each tube. The tubes were vortexed gently, and then incubated at 32°C for 90 minutes with mixing by gentle vortexing every 15 minutes. The tubes were centrifuged at 1600 rpm for 30 seconds. The culture was removed and 500 µl 1x PBS were added. The tubes were centrifuged again at 1600 rpm for 30 seconds and the liquid was removed. The ticks were washed again with 500 µl 1x PBS. The ticks were kept in a desiccator containing 300 mL of a saturated KCl solution for two weeks and then were fed on naïve C3H

mice. 5 of the ticks that survived were crushed in media. The mice were bled and killed and a western blot was performed.

3.26. Chloroform-methanol extraction of protein lysate (procedure adapted from Wessel & Flügge, 1984)

To 100 µl of lysate, 400 µl MeOH were added. The mixture was vortexed and 100 µl chloroform were added. The tube was vortexed again, 300 µl milliQ water were added and then the solution was vortexed once more. The tube was centrifuged at 15000 rpm for 1 minute. The top aqueous layer was removed, 400 µl MeOH were added and the solution was mixed by vortexing. The tube was centrifuged at 18000 rpm for 5 minutes. The supernatant was removed and the precipitate was air dried in a flow box.

3.27. In-solution digestion

The protein pellet obtained by chloroform-methanol extraction of 100 µl lysate was redissolved in 20 µl of a 6M urea in 50mM NH_4CO_3 solution. 5 µl of 25mM tris-(2-carboxyethyl)-phosphine were added and the mixture was incubated for 45 minutes at room temperature. 5 µl of a 330mM Indole-3-Acetic Acid (IAA) solution was added. The tube was incubated for 30 minutes at room temperature in the dark. 170 µl of 50mM NH_4CO_3 and 5 µl of 330mM Dithiothretiol (DDT) were added. The solution was incubated for 45 minutes at 56°C. 3.2 µl of a 12.5 ng/µl trypsin solution was added to achieve a 1:50 trypsin:protein ratio. The mixture was incubated at 37°C for 2 hours. Lastly, 5 µl of a 5% formic acid (FA) solution were added. The mixture was analyzed by LC-MS/MS.

3.28. 2D gel electrophoresis

The 2D electrophoresis was performed using the PROTEAN® i12™ IEF System (BioRad). A pellet obtain from chloroform-methanol extraction of 100 µl of lysate was dissolved in 125 µl Rehydration/Sample Buffer (BioRad). The sample solution was poured in the i12™ 7 cm rehydration/equilibration tray (BioRad), together with the ReadyStrip™ IPG strips (7 cm, pH 3-10), placed gel-side down. 4 mL of mineral oil (BioRad) were added on top of the strip and left overnight. The following day, the strip was washed with distilled water (dH_2O) and dried on a

fibreless towel. Two paper wicks were wetted with milliQ water. The IPG strip was placed in the i12™ 7 cm focusing tray with the paper wicks on both ends of the strip. Mineral oil was added so that it completely covers the strip. The tray was placed in the PROTEAN® i12™ IEF cell and then the protocol described in Table 12 was run. The strip was washed, dried and stored at -70°C overnight. A polyacrylamide separation gel was prepared. The following day, the strip was placed in the i12™ 7 cm rehydration/equilibration tray (BioRad) with 2.5 mL equilibration buffer (1x BioLyte® 3/10 Ampholyte, BioRad) containing 2% DTT and incubated for 10 minutes on a shaker. The liquid was decanted, the strip washed, dried and transferred to a new tray, where 2.5 mL equilibration buffer containing 2.5% IAA. The strip in buffer was incubated for 10 minutes on a shaker. In the meantime, overlay agarose (Tab. 13) was melted. The strips were washed and dried. 4 µl of protein ladder (Pierce™ Prestained Protein MW Marker, Thermo Fisher Scientific) were poured over a piece of filter paper. Overlay agarose was added onto the previously prepared separation gel. The IPG strip was placed on top of the stacking gel, together with the filter paper containing the ladder. Once the agarose solidified, running buffer was added and the gel was run at 80V for around 2 hours. Two gels were prepared simultaneously. One gel was stained using Blue-Silver Coomassie staining. An immunoblot was performed on the other gel. The procedure was repeated with a more concentrated protein pellet that was prepared from 200 µl lysate.

Table 12: Isoelectric focusing program used for running the IPG strip

Step	Voltage (V)	Ramp	Time	Units
1	250	Rapid	0:20	HH:MM
2	8000	Gradual	1:00	HH:MM
3	8000	Rapid	26000	Volt Hr
4	1500	Hold		

Table 13: Preparation of overlay agarose for 2D SDS-PAGE

Component	Conc.	Amounts
Running buffer		50 mL
Low melting agarose	0.5%	0.25 g
Bromphenol blue (1%)	0.003%	150 µl

3.29. Blue-Silver Coomassie Staining

The gel was washed with milliQ water for 30 minutes. The water was removed and Blue-Silver Coomassie staining solution was added. The gel was put on a shaker for around 1 hour.

Afterwards, the staining solution as removed and the gel was rinsed a couple of times with milliQ water, until the excess stain was removed. A picture of the gel was taken and bands of interest were cut, based on the results obtained from the immunoblotting of the other gel. The bands were kept in a storing solution containing 10% acetic acid and 40% MeOH until further processing.

3.30. Immunoblotting of 2D gel

The gel was washed a couple of times with milliQ water, then incubated with blotting buffer for 5 minutes. The gel was blotted on a PVDF membrane, which was activated by incubation in MeOH for 5 minutes. The transfer was run at 25V for 1 hour. In the meantime, a blocking solution was prepared by dissolving 15 g of dry milk in 300 mL TBS-Tween 20. After blotting, the membrane was incubated with the blocking solution on a shaker for 2 hours. A primary antibody solution was prepared by mixing 50 mL blocking solution with 250 µl serum from infected mice. The membrane was incubated with the primary antibody solution in the fridge overnight. The following day, the gel was washed 3 times for 15 minutes with TBS-Tween 20. The secondary antibody solution was prepared by mixing 5 µl Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in rabbit (Sigma-Aldrich) with 50 mL blocking solution. The membrane was incubated with the secondary antibody solution for 1 hour. Afterwards, the gel was washed again 3 times for 15 minutes with TBS-Tween 20. A developing solution was prepared using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific). 1.5 mL Reagent A were mixed with 1.5 mL Reagent B, vortexed and then added to the membrane, and then shaken for 5 minutes. A picture was taken using the ChemiDoc™ XRS+ System and the software Image Lab™. Two gels were immunoblotted using this procedure. In one case serum from mice infected by needle-inoculation was used as primary antibody. In the second case, serum from mice infected by tick-bite was used. The immunoblot was used to select spots of interest to be cut from the gel that underwent Blue-Silver Coomassie staining.

3.31. In-gel digestion of cut gel pieces (procedure adapted from Shevchenko *et al.*, 2007)

The storage solution was removed and the gel pieces were washed with 50 µl milliQ water and the vortexed. The milliQ water was removed and 50 µl acetonitrile were added and the mixture incubated for 10 minutes at room temperature. The supernatant is removed and 50 µl 10mM DDT in 100mM NH_4CO_3 were added, followed by incubation at 56°C for 45 minutes. The liquid was then removed and 50 µl acetonitrile were added. The mixture was incubated at room temperature for 10 minutes. 50 µl 55mM IAA in 100mM NH_4CO_3 were added, followed by incubation in the dark for 25 minutes. 50 µl acetonitrile were added and the solution was incubated for 10 minutes at room temperature. The acetonitrile is removed and 15 µl of a 12.5 ng/µl trypsin solution were added. The tubes were incubated for 1 hour in the fridge. The trypsin is removed and 25 µl 25mM NH_4CO_3 were added and the tubes were incubated at 37°C overnight. The following day, the liquid was transferred to 0.6 mL Eppendorf tubes and 100 µl extraction buffer (5% FA, ACN:H_2O 1:2) were added. The mixture was incubated at 37°C for 15 minutes. The Eppendorf were centrifuged under vacuum until all the liquid dried out. 50 µl 5% FA were added and the tubes were vortexed for 10-15 minutes.

3.32. Desalting (procedure adapted from Rappsilber *et al.*, 2007)

Columns were prepared and inserted into Eppendorf tubes. The columns were conditioned by adding 50 µl isopropanol to the column, followed by centrifugation at 3100 g for 2 minutes. This step was repeated once. 50 µl 5% FA were added to the column and the tubes were centrifuged at 1500 g for 2 minutes. This step was repeated. The samples containing the extracted proteins were sonicated for 1 minute and then briefly centrifuged. 50 µl of sample were added to the column. The tubes were centrifuged at 600 g for 5 minutes. 50 µl 5% FA were added and the tubes were centrifuged at 1500 g for 2 minutes. The Eppendorf tube containing the flow through was replaced. Fractional separation was performed. The first fraction was obtained by adding 50 µl of a solution containing 2.5% FA and 8% MeOH to the column, followed by centrifugation at 700 g for 5 minutes. The Eppendorf tube was replaced. The 2nd fraction was obtained by adding 50 µl of a solution containing 2.5% FA and 16% MeOH and repeating the previous steps. For the 3rd fraction, 50 µl 2.5% FA and 32% MeOH were used,

while, for the 4th fraction, 50 µl 2.5% FA and 50% MeOH were added. The contents obtained from each fraction were transferred to PCR tubes and dried by centrifuging under vacuum.

3.33. Analysis of proteins by MS

The dry fractions obtained earlier were redissolved in 5 µl 0.1% FA. 1 µl was spotted on the MALDI plate. Once the drop dried, 1 µl α-cyano-4-methoxycinnamic acid amide (CMCA) was added over the sample. Once the CMCA dried, the plate was analyzed by MS. The remaining sample was transferred to a LC vial and then vacuum dried. 10 µl of a solution containing 0.1% FA and 36% acetonitrile were added and the samples were analyzed by LC-MS/MS. The search parameters are described in Table 14.

Table 14: Parameters for database search after LC-MS/MS analysis

Database	ncbi_borrelia	**Enzyme**	Trypsin
Var. Modifications	Acetyl (protein N-term) Deamidated (NQ) Oxidation (M)	**Ions Score Threshold for Significant Peptide IDs**	35
Fixed Modification	Carbamidomethyl (C)	**Peptide Tolerance**	50 ppm
Instrument Type CID	ESI-QUAD-TOF	**Peptide Charge**	+1, +2 and +3
Ions Score Cut-off	15	**Peptide rank cut-off**	10
Mass	Monoisotopic	**MS/MS Tolerance**	0.5 Da
Significance Threshold	0.05	**Min. Peptide Length**	6
Missed Cleavages	2		

4. Results

4.1. Sequencing of *recN* gene

The PCR reaction was successful (Fig. 5). The negative control was clean, whereas the PCR product showed one band each at ~100 bp. The transformation and plating also worked, as proven by the restriction digestion showing the vector and the DNA insert (Fig. 6).Three of the plasmids with the insert present were sent for sequencing. The BLAST search revealed that our sequence was indeed part of the *recN* gene of *B. duttonii*. Also, there were no identical matches and the sequence with the highest identity score was that from the LY strain, which implies that our sequence is that of the 1120K3 strain.

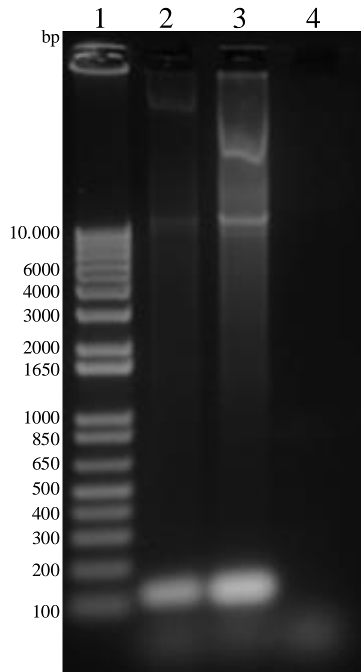

Figure 5: PCR reaction with *RecN* gene:
1 – 1 kb plus DNA ladder (Invitrogen)
2 – *B. duttonii* 1120K3 *RecN*
3 – *B. duttonii* LY *RecN*
4 – Negative control

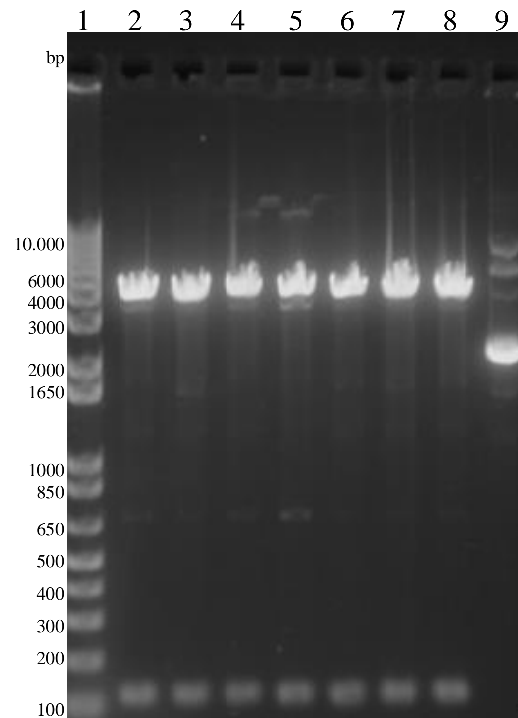

Figure 6: EcoRI restriction digestion:
1 – 1 kb plus DNA ladder (Invitrogen)
2-5 – 1120K3 *RecN* mini prep
6-8 – LY *RecN* mini prep
9 – Negative control

4.2. Transovarial transmission

No *Borrelia* were detected in the blood smears. Non-infection was confirmed by western blot, which was negative for all mouse samples. Upon inspection of the crushed ticks in culture media, no spirochetes were found. This suggests that transovarial transmission is not possible in *O. moubata* and *B. duttonii*.

4.3. *Borrelia* levels in blood

A comparison of the levels of *Borrelia* in mouse blood over time after needle-inoculation with different amounts of *B. duttonii* is shown in Figure 7. The western blot shows that all mice were infected (Fig. 8). The mice injected with less *Borrelia* show fewer bands than the others. Due to a shortage of protein ladder, a positive control was used for comparison of the bands.

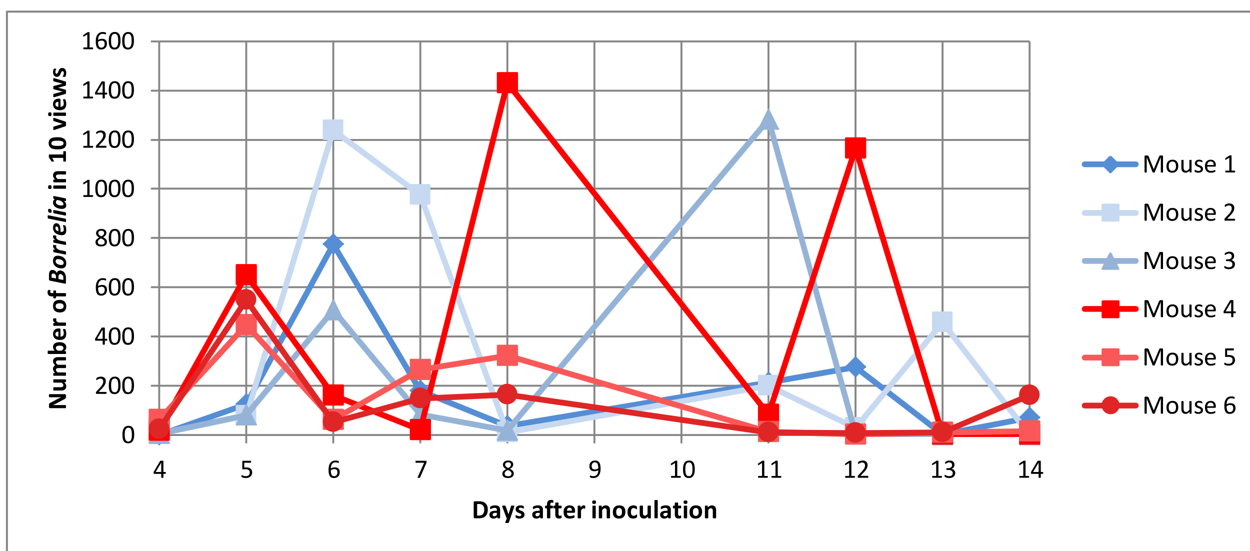

Figure 7: A graph showing the evolution of *Borrelia* after the mice were infected by needle-inoculation with different amounts of *B. duttonii* strain 1120K3. Mice 1, 3 and 3 were inoculated with 10^4 *Borrelia*, while mice 4, 5 and 6 were inoculated with 10^5 *Borrelia*.

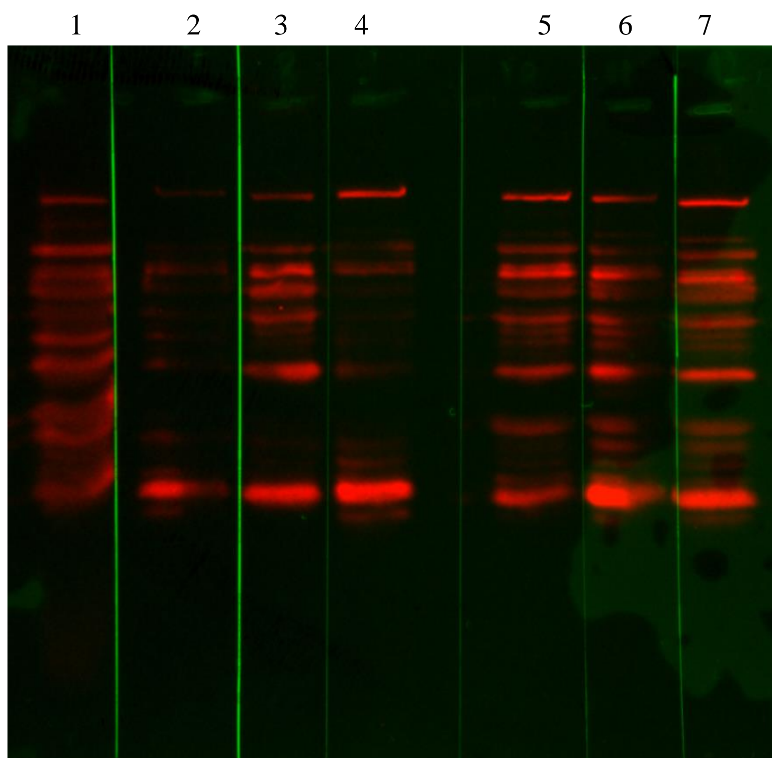

Figure 8: Western blot of mice 1- 6:
1 – Positive control (used instead of ladder)
2-4 – Mice 1, 2 and 3 (injected with 10^4 *Borrelia*)
5-7 – Mice 4, 5 and 6 (injected with 10^5 *Borrelia*)

4.4. Comparison between passaged and wild-type *Borrelia*

After checking the blood smears under the dark-filed microscope and counting the *Borrelia* in 5 views for 8 days in a row, the results were compiled in a graph. The measurements were started 5 days after inoculation. The mice were inoculated with 10^6 *Borrelia* each. The first experiment, which compares a wild-type culture with the 12th passage, is shown in Figure 9. The mice were sacrificed after we considered that we gathered enough data and a western blot was performed. The results prove that the passage retained infectivity. The experiment where the 22nd passage was used proved to be inconclusive. A western blot (not shown) revealed that the passage was not infectious. However, when the 22nd passage was inspected a couple of days later, there were no spirochetes swimming in the media. Furthermore, the 23rd passage also showed no *Borrelia* growth. As a result, we concluded that the 22nd passage did not contain active *Borrelia* and we decided that the experiment will have to be repeated. After the repetition, we determined that the 22nd passage retained infectivity. An SDS-PAGE comparing the wild-type protein lysate with the 7th and 11th passage was also performed. Some bands of interest (Fig. 10) were cut and analyzed by MALDI-MS and LC-MS/MS. The results are listed in Table 15.

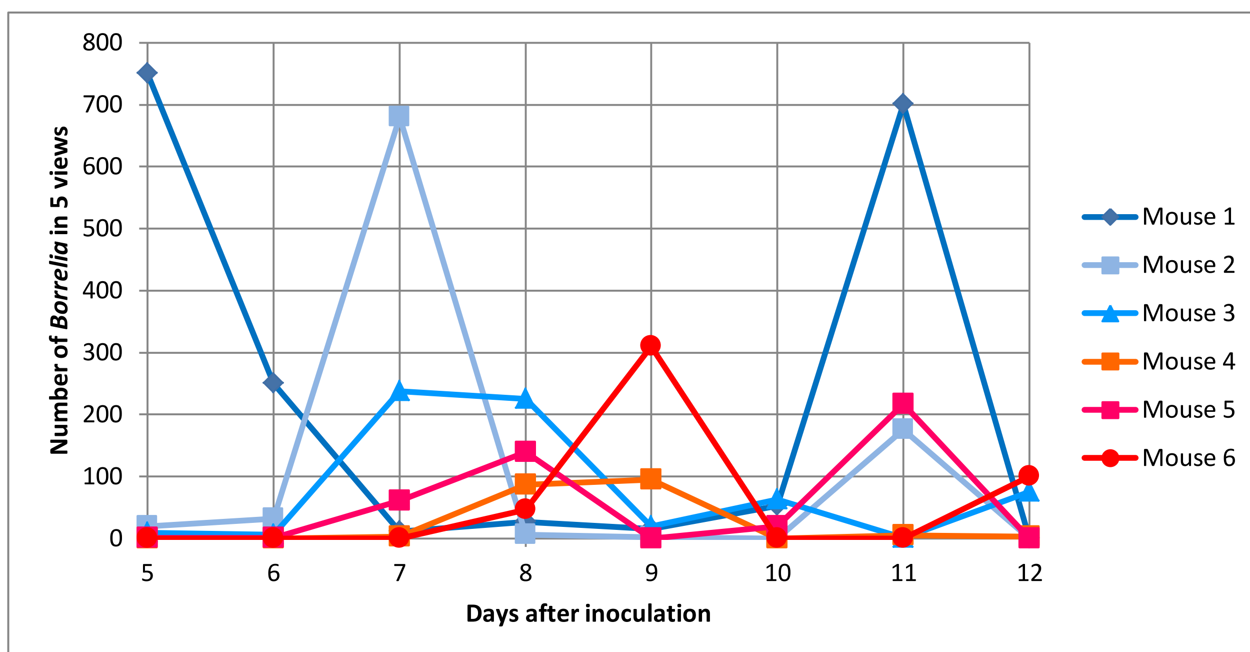

Figure 9: A graph showing the presence of *Borrelia* in blood after needle-inoculation with 250 µl (10^6 *Borrelia*) of a culture of *B. duttonii* strain 1120K3. Mice 1, 2 and 3 were inoculated with a wild-type culture, while mice 4, 5 and 6 were inoculated with the same amount of the 12th passage.

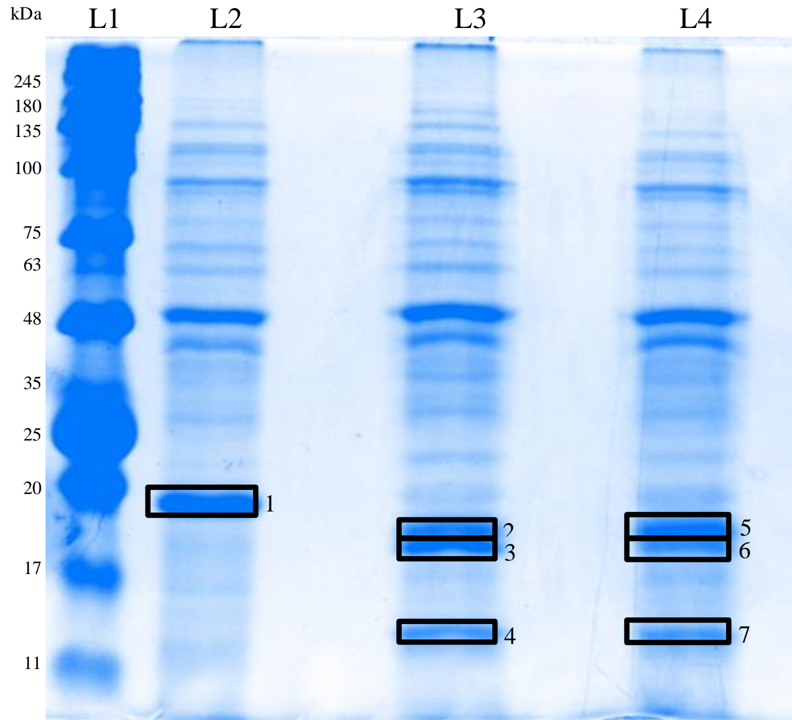

Figure 10: SDS-PAGE comparing passaged and wild-type cultures.
L1 – Protein Marker VI (AppliChem) L2 – Protein lysate from wild-type culture
L3 – Protein lysate from 11th passage L4 – Protein lysate from 7th passage

Table 15: Results from LC-MS/MS. The spots and fractions that are not mentioned in the table yielded either low scores or no results upon analysis

Spot no.	Fraction no.	Identified protein	No. of peptides	Score
1	2	Vsp1 [*B.crocidurae*]	1	113.44
2	1	Vsp protein [*B.duttonii*]	5	236.18
		Vsp protein [*B.recurrentis*]	2	151.56
	2	Vsp1 [*B.crocidurae*]	3	160.95
	3	Vsp1 [*B.crocidurae*]	2	119.57
		Vsp3 [*B.crocidurae*]	2	114.32
3	1	Vsp1 [*B.crocidurae*]	2	99.24
	4	Neuropeptide-like 2 [*Drosophila melanogaster*]	3	191.64
4	1	hypothetical protein [*B. duttonii*]	3	153.28
	2	arthropod-associated lipoprotein [*B.miyamotoi*]	2	100.71
		hypothetical protein [*B.duttonii*]	2	100.16
5	1	hypothetical protein [*B.crocidurae*]	4	191.88
	2	Vsp1 [*B.crocidurae*]	4	227.42
		hypothetical protein [*B.crocidurae*]	5	226.02
		vsp protein (plasmid) [*B.recurrentis* A1]	4	217.68
		vsp protein (plasmid) [*B.duttonii* Ly]	3	165.81
6	1	FlaB [*B.hispanica*]	3	146.39
		Vsp1 [*B.crocidurae*]	3	127.03
	2	Vsp1 [*B.crocidurae*]	2	129.49

4.5. Growth curve of *Borrelia* at 34°C

The logarithmic growth curve is depicted in Figure 11. It was computed using the average of the spirochete densities of the three cultures. The curve clearly shows the exponential growth expected with bacterial strains. We observed a doubling time of 2 days. Therefore, we measured the spirochete density every two days. We decided that the end point should be when the density reaches 10^8 spirochetes/mL. As such, the last measurement was done on the 15th day after the culture was started. Unfortunately, the 23°C culture was contaminated. We attempted to restart it, though unsuccessfully. Therefore, no comparison between growth rates and expressed proteins could be made.

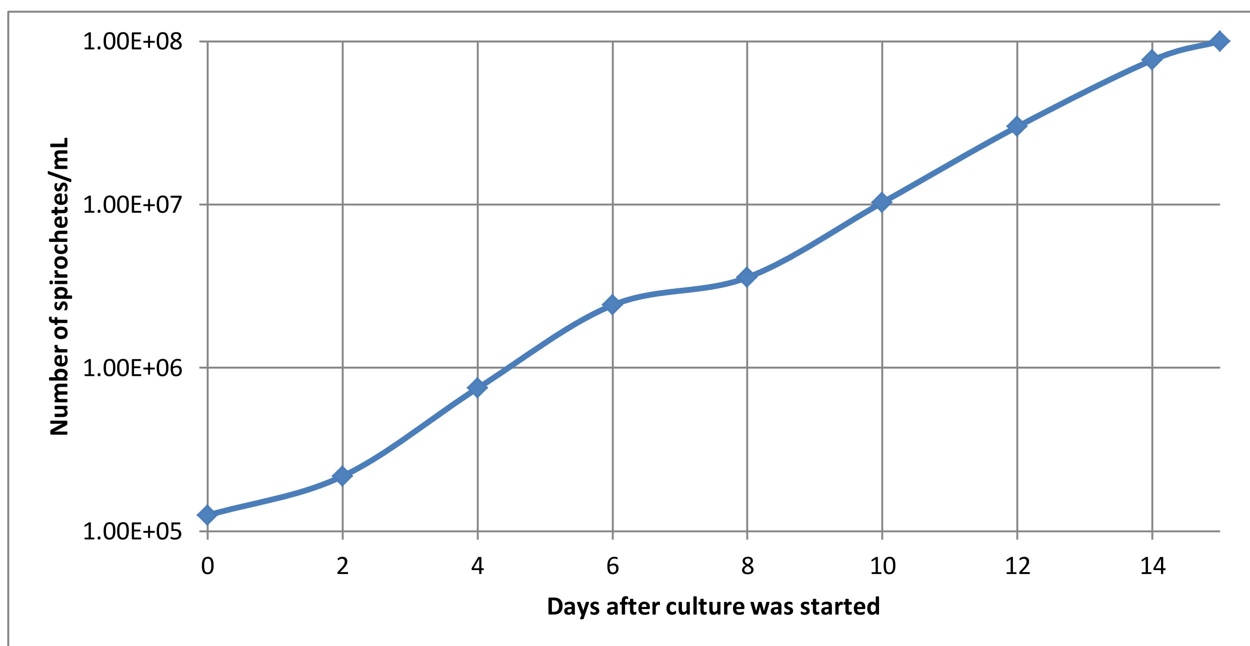

Figure 11: A graph showing the growth of a wild-type *B. duttonii* strain 1120K3 culture at 34°C. The linearity proves the exponential growth rate. The doubling time was observed to be 2 days.

4.6. Dependence of tick to mouse transmission of *Borrelia* to the number of ticks that fed on the mouse

The first experiment proved to be unsuccessful as none of the mice on which 1 and 2 ticks fed and only 2 out of 4 mice, on which 3 ticks fed were infected. The results of the western blot are shown in Figure 12. When inspecting the ticks that were crushed in media, we observed that most of them were infected. The results are listed in Table 16.

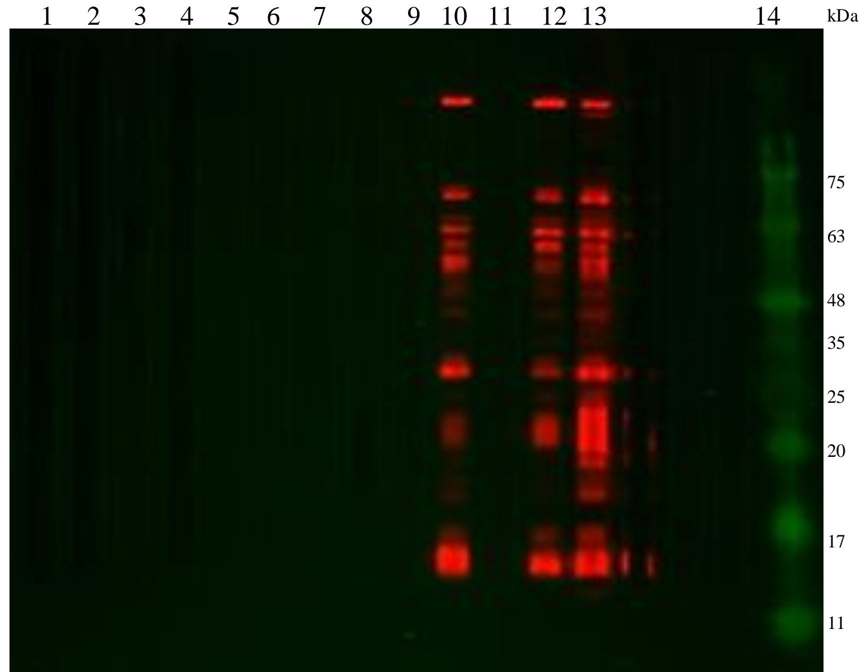

Figure 12: Western blot of mice 1-12
1-4 – Mice 1-4, on which 1 tick/mouse fed
5-8 – Mice 5-8, on which 2 ticks/mouse fed
9-12 – Mice 9-12, on which 3 ticks/mouse fed
13 – Positive control
14 – Protein Marker VI (AppliChem)

Table 16: Results obtained from checking the ticks crushed in media. The entries marked with Y are infected, the entries marked with N are not and the entries marked with N/A have been contaminated; therefore, we could not tell whether they were infected or not.

Mouse no.	Infected	Mouse no.	Infectious	Mouse no.	Infectious	Mouse no.	Infectious
1	Y	2	Y	3	Y	4	Y
5	Y	5	Y	6	Y	6	N
7	Y	7	N	8	N	8	N
9	N	9	N	9	N	10	Y
10	N/A	10	N/A	11	N	11	Y
11	N	12	N	12	Y	12	N/A

The experiment was repeated, this time feeding 5 ticks/mouse on 5 mice (13-17) and 10 ticks/mouse on 5 other mice (18-22). We started collecting blood and inspecting it under the dark-field microscope 3 days after infecting the mice. Upon monitoring the blood levels of Borrelia for 12 days, we concluded that the mice were not infected. This conclusion was confirmed by a western blot. When we started processing the ticks, we noticed that a large number of them had died – only 49 out of 75 had survived. The ticks were crushed in half and one part was crushed in media, while the other part was used for DNA isolation and PCR. The

results from the tick cultures showed that only 11 ticks produced live Borrelia (1 tick from mouse 15, 16 and 17; 2 ticks from mice 14 and 20 and 4 ticks from mouse 18). The results from the PCR (Fig. 13) show that most of the ticks were infected.

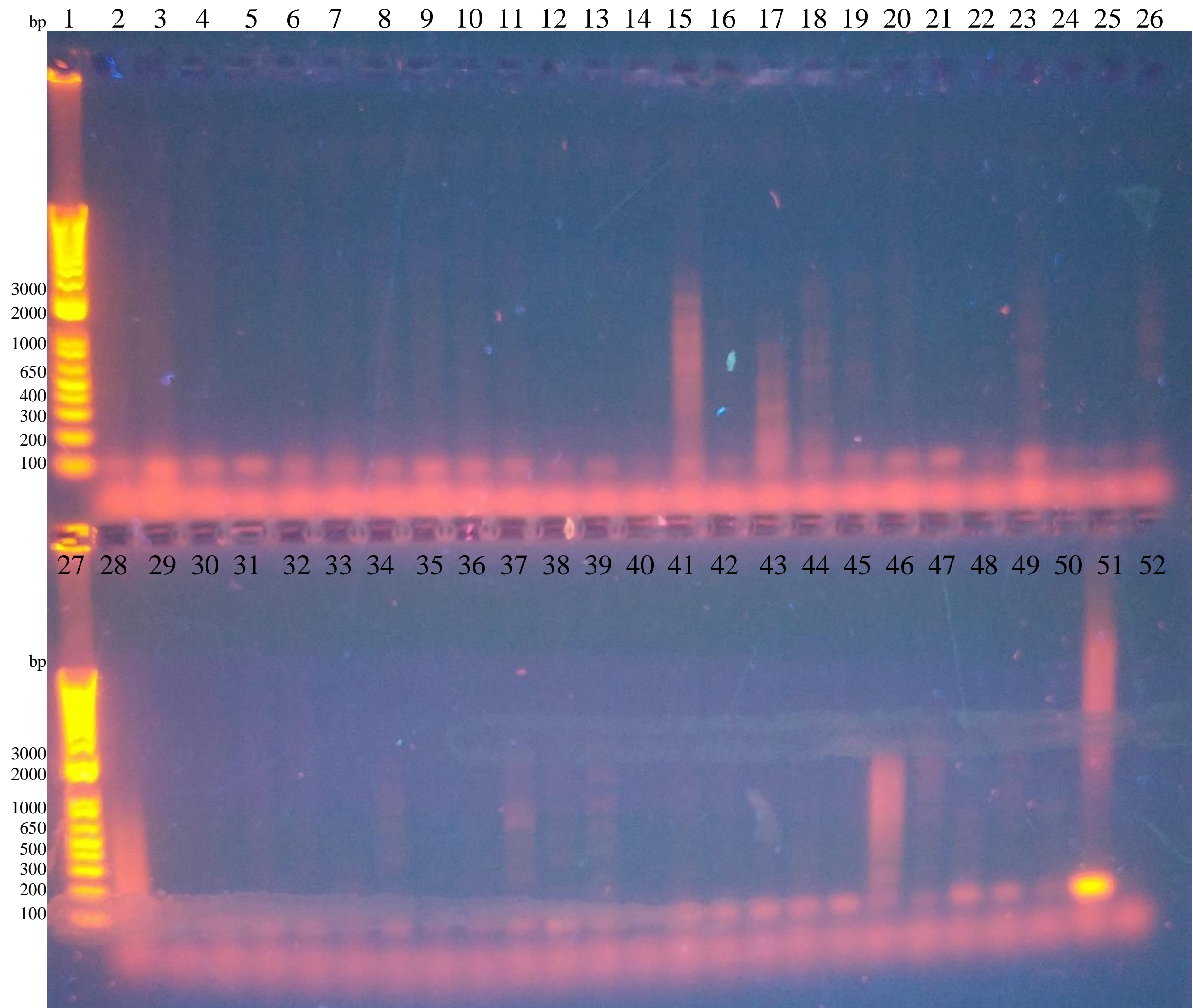

Figure 15: PCR of ticks from the 2nd transmission experiment
1, 27 – 1 kb plus DNA ladder (Invitrogen)

2-6 – Ticks from mouse 13	7-10 – Ticks from mouse 14	11-14 – Ticks from mouse 15
15-19 – Ticks from mouse 16	20-22 – Ticks from mouse 17	23-26, 28-29 – Ticks from mouse 18
30-36 – Ticks from mouse 19	37-42 – Ticks from mouse 20	43-49 – Ticks from mouse 21
50 – Tick from mouse 22*	51 – Positive control	52 – Negative control

*The PCR product of the other 2 ticks from mouse 22 were ran on a different gel and came up negative for *Borrelia*

4.7. Acquisition of *Borrelia* at different levels of spirochete densities

The ticks that were fed on mouse 10 were allowed to molt. However, despite a period of 3 months having passed, none of the ticks had molted. Therefore, the analysis of the ticks was not performed.

4.8. Artificial infection of *O. moubata* ticks with *B. duttonii*

The tick were washed in a *B. duttonii* 1120K3 culture. The ticks that survived were split and fed on 2 mice. The ticks were allowed to molt and 5 of them were crushed in media. After checking the tick cultures under a dark-field microscope 4 weeks later, no *Borrelia* was found. The mice were bled and a western blot was performed, showing that the mice were not infected. The experiment has to be continued by allowing the ticks to feed again on naïve mice, followed by a western blot.

4.9. Investigating differences in gene expression between naïve and infected ticks by qPCR

The primers were tested by RT-PCR, followed by transformation into *E. coli* cells, plating with blue-white screening, picking positive colonies from the plates, isolation of plasmid DNA from the grown colonies, restriction digestion of the isolated DNA with EcoRI and sequencing. As can be seen from Figure 14 the PCR reaction was successful. One band at ~100 bp is present where we expected our target gene. We assigned the faint band in the negative control to excess nucleotides.

The transformation and plating was also successful as we obtained some white colonies. 4 colonies from each primer pair were picked and grown in LB media. The restriction digestion that was performed from the isolated DNA (Fig. 15) proves that most of the colonies contained an insert. Three of the plasmid DNA samples that contained the insert were sent for sequencing. The results show that the *Defensin D* primer pair was the only one that did not work at all. The *actin* and *OMFREP* primers also provided unsatisfactory results (only 1 out of 3 colonies contained our target fragment).

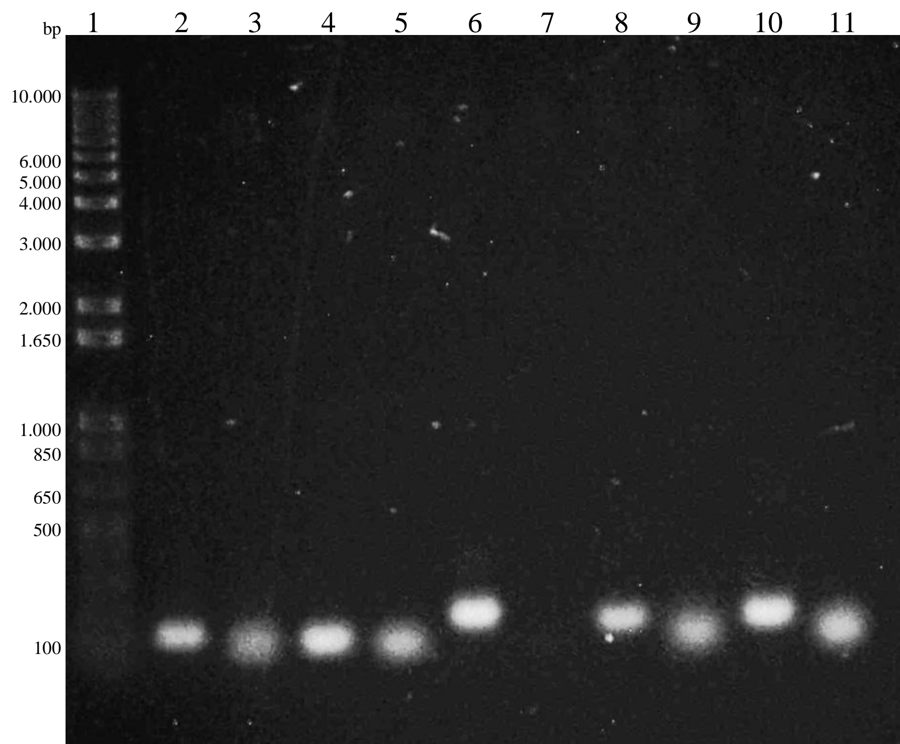

Figure 14: Products of the RT-PCR reaction used to test the primers
1 – 1 kb plus DNA ladder (Invitrogen) 2 – DorinM 4 – Defensin A 6 – Defensin D
8 – OMFREP 10 – Actin 3, 5, 7, 9, 11 – Negative controls

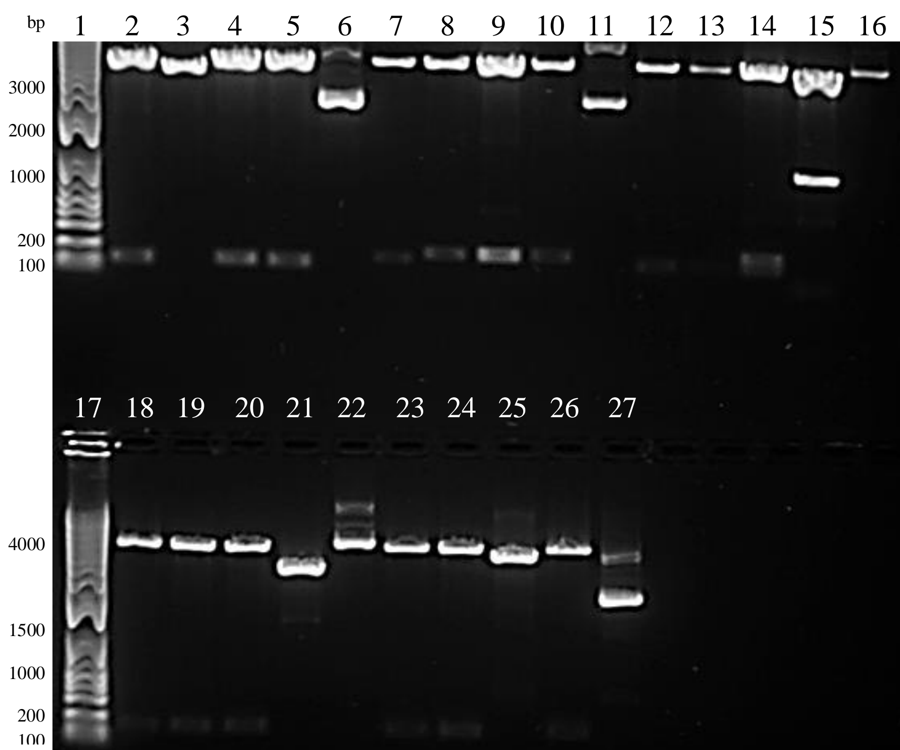

Figure 15: Restriction digestion of plasmid DNA with EcoRI
1, 17 – 1 kb plus DNA Ladder (Invitrogen)
2-5 – Defensin A 7-10 – Defensin D 12-15 – DorinM
18-21 – OMFREP 23-26 – Actin 6, 11, 16, 22, 27 – Negative controls

As a consequence, we decided to pick 5 more colonies from the *Actin* and *OMFREP* plates. The procedure was repeated as stated above. The restriction digestion is shown in Figure 16. One isolate from *Actin* (no. 4) and 2 isolates from *OMFREP* (no. 3 and 4) were sent for sequencing.

bp 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

5000 4000 3000 2000 1650 1000 850 650 500 400 300 200 100

Figure 16: The second restriction digestion of plasmid DNA with EcoRI
1 – 1 kb plus DNA Ladder (Invitrogen) 2, 8 – Positive controls
3-7 – Defensin A 9-14 – Defensin D 8, 15 – Negative controls

While the Actin samples still did not contain the target gene, the *OMFREP* samples did. Despite, the poor performance from the *Actin* primers, we decided to continue with the experiment. Therefore we isolated RNA from 4 infected and 4 naïve ticks, and we performed a qPCR using those samples (each in triplicates) and our primers (except *defensin D*, due to the primers not working, and *defensin A*, due to time restrictions). The qPCR was, unfortunately, unsuccessful and could not be repeated due to time restrictions. When the concentrations of the

RNA samples were measured using a NanoDrop™ (Thermo Fisher Scientific), we noticed their low values: 20-40 ng/µl for the RNA isolated from infected ticks, 7-9 ng/µl for 2 RNA samples isolated from clean ticks and 60-130 ng/µl for the 2 remaining RNA samples isolated from clean ticks before the previous 6.

4.10. Serological proteome analysis of *B. duttonii*

This experiment was performed according to a paper where a similar analysis of *B. burgodferi* membrane-associated proteins was performed (Nowalk *et al.*, 2006). For that purpose, 2D electrophoresis was performed. First 2 gels were prepared. One gel was immunoblotted using sera from mice infected by needle-inoculation (Fig. 17) and the other was stained with Blue-Silver Coomassie (Fig. 18). The bright spots obtained from the immunoblot were marked and cut from the stained gel, then processed and analyzed by MALDI-MS and/or LC-MS/MS. The results are listed in Table 17.

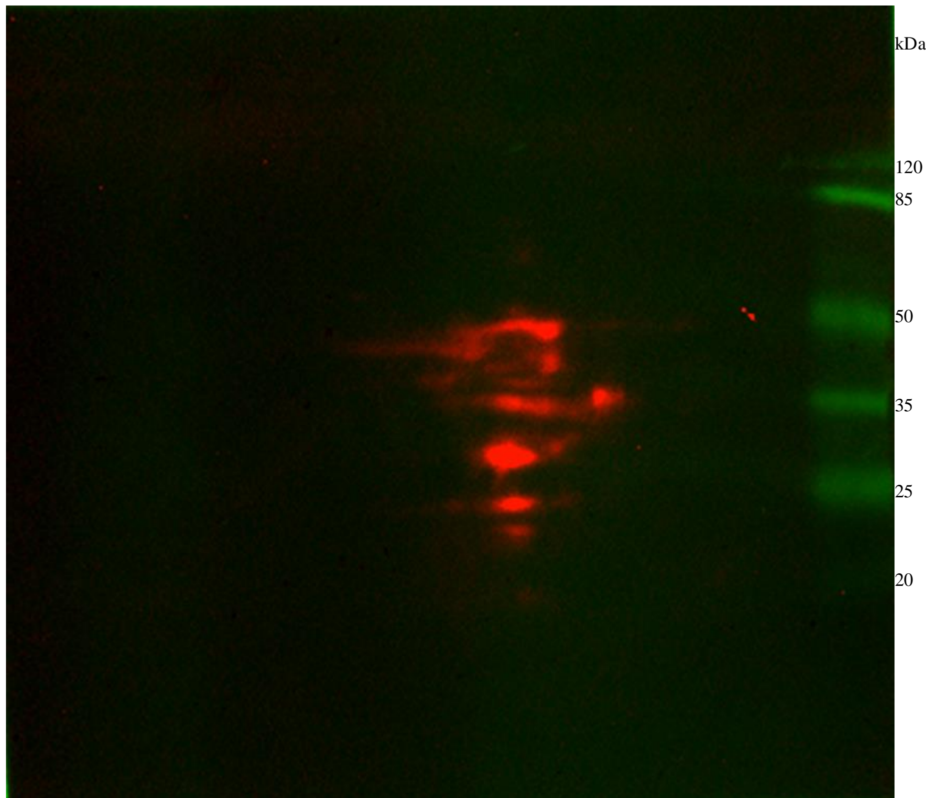

Figure 17: Immunoblot with sera from mice infected by needle inoculation. The ladder used is Pierce™ Prestained Protein MW Marker (Thermo Fisher Scientific)

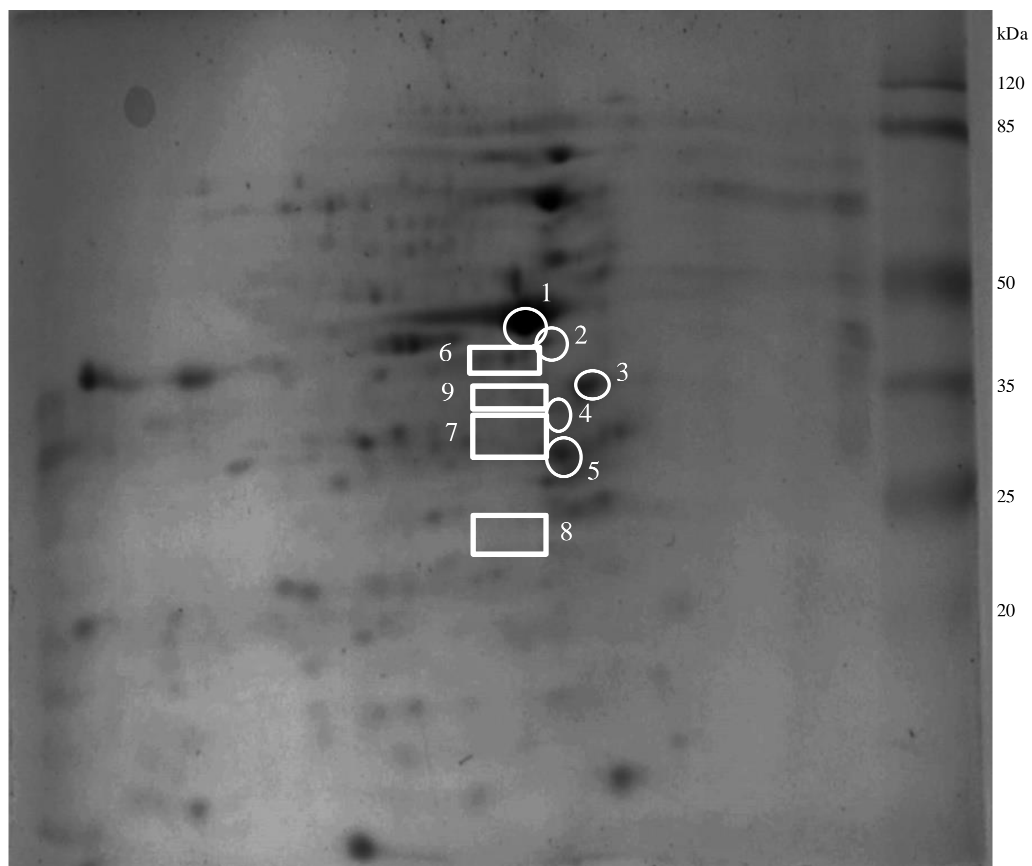

Figure 18: Coomassie Blue Silver stain of first 2D gel. The ladder used is Pierce™ Prestained Protein MW Marker (Thermo Fisher Scientific)

Table 17: LC-MS/MS results from screening against sera from mice infected by needle inoculation. Only the spots mentioned in the table yielded any results

Spot no.	Fraction no.	Identified protein	No. of peptides	Score
1	1	Flagellin [*B.miyamotoi*]	4	179.19
	2	FlaB [*B.hispanica*]	5	349.30
	3	FlaB [*B.hispanica*]	3	156.95
6	2	FlaB [*B.hispanica*]	3	137.42
		Lactate dehydrogenase [*B. crocidurae*]	2	123.78
	3	FlaB [*B.hispanica*]	2	95.76

Similarly, 2D electrophoresis was performed with another set of gels. One gel was immunoblotted with sera from mice infected by tick bite (Fig. 19), while the other was stained with Blue-Silver Coomassie (Fig. 20). Again, spots were cut according to the results from the immunoblot and analyzed by MALDI-MS (no LC-MS/MS due to time concerns). The results are listed in Table 18. For running each gel from this set, 200 µl of lysate (no dilution with Laemmli buffer) were used. This explains the larger number of spots that appeared on the second stained gel when compared to the first one.

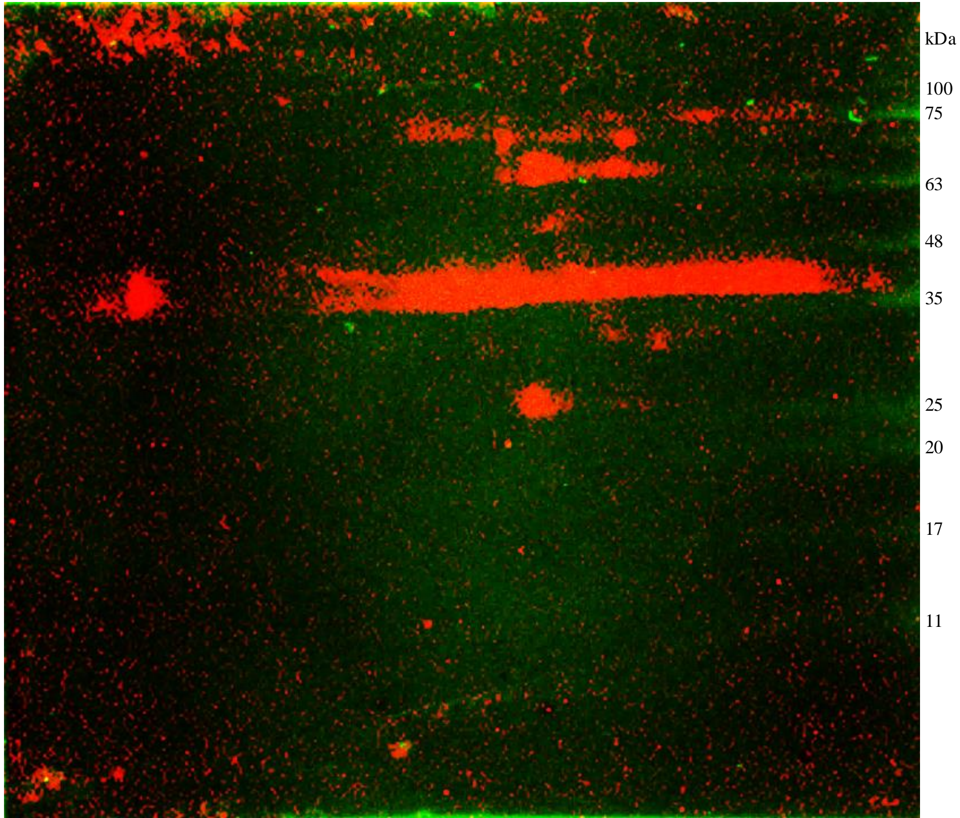

Figure 19: Immunoblot with sera from mice infected by tick bite. The ladder used was Protein Marker VI (AppliChem)

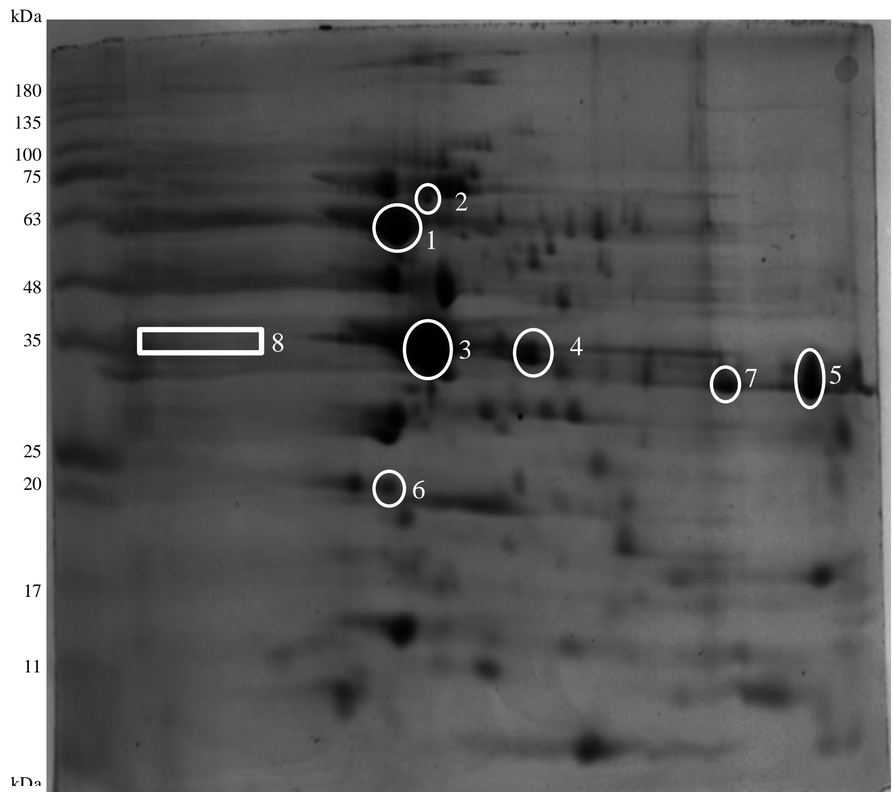

Figure 20: Coomassie Blue Silver stain of second 2D gel. The ladder used was Protein Marker VI (AppliChem)

Table 18: LC-MS/MS results from screening against sera from mice infected by tick bite. Only the spots mentioned in the table yielded any results

Spot no.	Identified protein	No. of peptides	Score
3	Flagellin, partial [*B. microti*]	3	218.7
5	Glyceraldehyde-3-phosphate dehydrogenase [*B. duttonii* LY]	9	516.7

4.11. In-solution digestion of *B. duttonii* 1120K3 protein lysate

100 µl of a protein lysate (after dilution with Laemmli buffer) of *B. duttonii* strain 1120K3 was analyzed by in-solution digestion followed by LC-MS/MS to see which proteins are expressed in *Borrelia* grown in culture. The obtained proteins are listed in Table 19.

Table 19: LC-MS/MS results from in-solution digestion of *B. duttonii* 1120K3 lysate. Separation in fractions was not performed.

Protein no.	Identified protein	No. of peptides	Score
1	Flagellin, partial [*B. duttonii*]	4	193.58
2	Glyceraldehyde-3-phosphate dehydrogenase [*B. duttonii* LY]	4	163.50
3	Vsp protein (plasmid) [*B. duttonii* LY]	4	155.95
4	Putative lipoprotein [*B. recurrentis* A1]	3	128.18

5. Discussion

The aim of this thesis was to investigate the infectious lifecycle of *B. duttonii* and further development of a mouse-tick model. Furthermore, we also wanted to understand the difference in the proteins expressed by *B. duttonii* upon infection by needle-inoculation and tick-bite through serological proteome analysis. While doing that, we also tried to find possible protein markers that could be used for diagnosis.

The discovery of *B. duttonii* is credited to J. Everett Dutton in 1904. (Dutton *et al*., 1905) also proving that its vector was the tick species *O. moubata*. This was also confirmed by Koch (Koch, 1905, 1906).

Since its discovery, TBRF *Borrelia* has mostly been classified as a less important field of study compared to its sister group, Lyme *Borrelia*. Among RF species, those categorized as "Old World" species, such as *B. duttonii* have been neglected. Therefore, relatively little research on the pathogenic lifecycle has been done.

In our study, we used the 1120K3 strain of *B. duttonii* instead of the LY strain, because the LY strain was not capable to complete the infectious cycle from mouse to tick and then back to mouse (Ringhofer, 2016, unpublished). In order to confirm the identity of the strain as 1120K3, the *glpQ* and *flaB* genes were sequenced during Brian Ringhofer's work, while we sequenced the *recN* gene. Therefore, we have proved three times that the strains we used were indeed 1120K3 and LY, respectively.

The logarithmic growth curve at 34°C helps with future work, such as when the *Borrelia* cells need to be grown in larger volumes and with the estimating the amount needed to start a culture that would have a particular density based on doubling time.

In 2008, Tabuchi *et al*. reported on the lack of transovarial transmission of *B. duttonii* (Tabuchi *et al*., 2008). As noted in his paper, those results are in contradiction to those obtained by a Swiss group in 1958 (Aeschlimannn, 1958), who reported a 90% rate of transmission from mother to progeny, with 60% of them being capable further transmit the spirochetes upon reaching maturity. In our wok, we were not able to observe any transovarial tranmsmission, thus confirming the conclusion of Tabuchi and colleagues. However, our experiments were

performed using ticks from the same mother. In the future we could investigate a larger pool of larvae coming from different mothers. Also, we did not perform PCR on the larvae as we considered that if an infection was present in the tick, it would show by crushing the tick media. As it can be seen from some of our other experiments, that does not always apply.

B. hermsii and *B. turicatae*, causative agents of TBRF belonging to the New World category, were shown to retain infectivity after one year of continuous passaging (Lopez *et al*., 2008). After 22 weeks of passaging, our *B. duttonii* culture also retained infectivity, although it was noticeably reduced compared to the control culture. When inspecting the SDS-PAGE gel, we noticed that some changes in protein expression occurred. The MS analysis shows that some *vsp* proteins were expressed more in the passaged culture than in the wild-type culture. This could explain the loss in infectivity, since the immune evasion mechanisms loses some of its ability to properly control the variability in outer surface proteins, making the bacteria more likely to be detected by the immune system and neutralized. Another protein whose level seems to have increased is an arthropod-associated lipoprotein. This increase makes the bacteria more likely to be detected by the mouse in the early stages of the infection, resulting a decreased bacterial population and lifetime in the mouse. A similar analysis should be performed to the 22nd passage to check whether any major changes in protein expression occurred.

In 2014, Boyle *et al*. published a paper in which the transmission of *B. turicatae* from its arthropod vector, *O. turicata*, to mice was investigated. They determined that 3 ticks are sufficient to infect all animals (Boyle *et al*., 2014). We performed a similar experiment for our species, but as shown by our results, we only had a maximum of 2 infected ticks feeding on a mouse. The mice that became infected were the mice from the group where 3 ticks/mouse were fed. However, in that group only a maximum of 1-2 ticks out of the 3 that fed were actually infected. Despite that, 50% of the mice in that group were infected.

Upon repeating the experiment, we noticed that even 10 ticks were not able to provide a large enough dose to infect a mouse. Despite some of the ticks being from a different colony than the ones that were used earlier, their infectivity was confirmed by crushing some of ticks and inspecting them by dark-field microscopy. As can be seen by our results, the ticks were infected, but they were not able to pass the infection. That may be due to the presence of a bottleneck in transmission of *Borrelia* that was incubated in ticks for a longer period of time (our ticks were

infected for roughly 4 months after use). Therefore, the tick-mouse model may only work with fresh infections, thus possibly making the model unstable with time. A similar experiment was performed by Tabuchi *et al*. (2008), where infected ticks were fed on naïve gerbils at a ratio of 5 ticks to 2 animals. They noticed efficient transmission of spirochetes. However, the ticks that were used in their experiments were infected only 3 weeks prior. In order to further analyze our theory, we planned to crush freshly infected ticks in media and, if spirochetes were observed, to inject the culture into mice. This would reveal whether there is a bottleneck due to the tick or the mouse.

B. hermsii was reported to be acquired by *O. hermsi* ticks from infected mice even at low levels of spirochete density (Lopez *et al*., 2011). We performed a similar experiment to test whether this also applies to our *Borrelia* species; however, I was not able to complete the work. There seem to be no reports of the time required for soft ticks to molt after a blood meal, therefore we do not know if such a delay is normal or should be investigated further.

O. hermsi ticks can be artificially infected by exposing them to reduced-humidity conditions followed by immersion in a *B. hermsii* culture (Policastro *et al*., 2011). The artificial infection is costly on the tick population, as almost half of the ticks that were used in our experiment have died. At first, it seems that the artificial infection was not successful, with the ticks not being able to acquire the spirochetes. However, due to the tick-pathogen biology, an incubation time may be needed for the pathogen to adapt to the environment inside the tick. Therefore the ticks may be infectious after molting. To confirm this, the experiment will have to be repeated.

The *in solution* digestion and MS analysis of whole *Borrelia* lysate shows that the proteins which are expressed the most in culture-grown spirochetes either have metabolic roles (GAPDH), aid in mobility (flagellin) or are part of or attached to the membrane (lipoproteins, *vsp*).

In 2006, Nowalk and colleagues published a paper where they investigated the serological proteome of *B. burgdoferi* (a causative agent of Lyme disease) membrane-associated proteins (Nowalk *et al*., 2006). We investigated the serological proteome of a whole lysate of *B. duttonii* against sera from mice infected by tick bite and needle-inoculation. We identified a common immunogenic protein, flagellin (Dressler *et al*., 1993) as well as a more interesting candidate

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was reported as an immunogenic protein in both Lyme and TBRF *Borrelia* (Lopez *et al*., 2009). GAPDH was found only in serum from mice infected by tick bite. This could be due to differences in the concentrations of the lysates used, as well as limitations in our procedure. Another protein was lactate dehydrogenase, an enzyme required in the bacteria's metabolism, which is also an antigen used to detect malaria. To check the significance of our results, the experiment would have to be repeated with a balanced protein concentration. Another modification that could be done is taking serum from mice at different time points after infection and checking whether the previously recorded proteins are persistent.

In conclusion, this project contributed to the better understanding of the pathogenic lifecycle of *B. duttonii* and provided the starting point for the search of immune-diagnostic markers as well as possible vaccine candidates against TBRF caused by *B. duttonii*.

6. Acknowledgements

First of all, I would like to thank my supervisor, Ryan O. M. Rego, Ph.D., for allowing me to work in his lab and for calmly listening to all the things that went wrong. Secondly, I would also like to thank our lab technician, Zuzana Vavrušková, for helping me with all the mice-related experiments and making sure I did not harm any mouse with my rough handling and also Martin Strnad, for always telling us that our gels look fine (even when they did not) and for providing us with only the best music while we performed our experiments. Special thanks go to Dmitry Loginov, Ph.D. for helping me with the proteomics work performed later (western blots were starting to get boring after a while so it was a nice change) and sharing his collection of Game of Thrones eBooks with me (half a book done, many more to go). Many thanks go to my colleagues who worked in the same lab or building for sharing their mistakes and laughing at ours (what better way to learn from them?). Finally, I would like to thank prof. RNDr. Libor Grubhoffer, CSc., for guiding us through our year abroad and for the encouraging words.

7. References

1. Aeschlimann, A. Développement embryonnaire d'*Ornithodorus moubata* (Murray) et transmission transovarienne de *Borrelia duttoni*, *Acta Tropica*, **15**, 15–64 (1958)
2. Barbour, A.G. Isolation and cultivation of Lyme disease spirochetes, *Yale J. Biol. Med.*, **57**(4), 521–525 (1984)
3. Barbour, A.G**.** Antigenic Variation by Relapsing Fever *Borrelia* Species and Other Bacterial Pathogens, Craig N, Craigie R, Gellert M, Lambowitz A (ed), *Mobile DNA II*, ASM Press, Washington, DC, 972-994 (2002)
4. Barbour, A.G. Relapsing fever and other Borrelia diseases, *Tropical Infectious Diseases Principles, Pathogens, & Practice*, 3rd Ed., Guerrant, R.L., Walker, D.H. and Weller P.F., eds. 499–510 (2006)
5. Barbour, A.G., & Hayes, S.F. Biology of *Borrelia* species, *Microbiol. Rev.*, **50**, 381–400 (1986)
6. Barclay, A. J., Coulter, J.B**.** Tick-borne relapsing fever in central Tanzania, *Trans. R. Soc. Trop. Med. Hyg.*, **84**(6), 852-856 (1990)
7. Boyle, W.K., Wilder, H.K., Lawrence, A.M., & Lopez, J.E. Transmission Dynamics of *Borrelia turicatae* from the Arthropod Vector. *PLoS Negl. Trop. Dis.*, **8**(4), e2767 (2014)
8. Bryceson, A.D., Cooper, K.E., Warrell, D.A., Perine, P.L., & Parry, E.H. Studies of the mechanism of the Jarisch-Herxheimer reaction in louse-borne relapsing fever: evidence for the presence of circulating *Borrelia* endotoxin, *Clin. Sci.*, **43**, 343–354 (1972)
9. Burman, N., Shamaei-Tousi, A., & Bergström, S. The Spirochete *Borrelia crocidurae* Causes Erythrocyte Rosetting during Relapsing Fever. *Infect. Immun.*, **66**(2), 815–819 (1998)
10. Cadavid, D., & Barbour, A.G. Neuroborreliosis during relapsing fever: review of the clinical manifestations, pathology, and treatment of infections in humans and experimental animals, *Clin. Infect Dis.*, **26**(1), 151-64 (1998)
11. Cutler, S.J. Possibilities for Relapsing Fever Reemergence, *Emerg. Infect. Dis.*, **12**(3), 369–374 (2006)

12. Cutler, S.J., Akintunde, C.O., Moss, J., Fukunaga, M., Kurtenbach, K., Talbert, A., Zhang, H., Wright, D.J., & Warrell, D.A., Successful in vitro cultivation of *Borrelia duttonii* and its comparison with *Borrelia recurrentis*, *Int. J. Syst. Bacteriol.*, **49**(Pt 4), 1793-1799 (1999)
13. Dressler, F., Whalen, J., Reinhardt, B.,& Steere, A. Western Blotting in the Serodiagnosis of Lyme Disease, *J. Infect. Dis.*, **167**(2), 392-400 (1993)
14. Dutton, J.E., & Todd, J.L., The nature of tick fever in the eastern part of the Congo Free State, *Br. Med. J.*, **2,** 1259-1260 (1905)
15. Dworkin, M.S., Schwan, T.G., Anderson, D.E., & Borchardt, S.M. Tick-Borne Relapsing Fever, *Infect. Dis. Clin. North Am.*, **22**(3), 449–viii (2008)
16. Elbir, H., Henry, M., Diatta, G., Mediannikov, O., Sokhna, C., Tall, A., *et al*. Multiplex Real-Time PCR Diagnostic of Relapsing Fevers in Africa, *PLoS Negl. Trop. Dis.*, **7**(1), e2042 (2013)
17. Felsenfeld, O. Borreliae, human relapsing fever, and parasite–vector–host relationships, *Bacteriol. Rev*., **29**(1), 46–74 (1965)
18. Felsenfeld, O. *Borrelia*: Strains, Vectors, Human and Animal Borreliosis, St. Louis, MO, Warren H. Green (1971)
19. Fukunaga, M., Okada, K., Nakao, M., Konishi, T., & Sato, Y. Phylogenetic analysis of *Borrelia* species based on flagellin gene sequences and its application for molecular typing of Lyme disease borreliae, *Int. J. Syst. Bacteriol.*, **46**(4), 898-905 (1996)
20. Goubau, P.F., & Munyangeyo, C. Fièvre récurrente à tiques et grossesse. Une étude clinique au Rwanda, *Ann. Soc. Belg. Med. Trop*., **63**(4), 367-369 (1983)
21. Hoogstraal, H. Argasid and Nuttalliellid ticks as parasites and vectors, *Adv. Parasitol.*, **24**, 135–238 (1985)
22. Jongen, V.H., van Roosmalen, J., Tiems, J., van Holten, J., & Wetsteyn, J.C. Tick-borne relapsing fever and pregnancy outcome in rural Tanzania, *Acta Obstet. Gynecol. Scand.*, **76**(9), 834-838 (2009)
23. Koch, R. Vorlaufige Mitteilungen uber die Ergebnisse einer Forchungreise nach Ostafrika, *Deutsche Medizinishce Wochenschrift*, **31**, 1865–1869 (1905)
24. Koch, R. Ueber afrikanischen Recurrens, *Berliner Klinische Wochenschrift*, **43**, 185–194 (1906)

25. Larrson, C., Pathobiology of African relapsing fever *Borrelia*, Umeå University Medical Dissertations, Sweden (2007)
26. Larsson, C., Andersson, M., & Bergström, S. Current issues in relapsing fever, *Curr. Opin. Infect. Dis.*, **22**(5), 443-449 (2009)
27. Larsson, C., Andersson, M., Guo, B. P., Nordstrand, A., Hägerstrand, I., Carlsson, S., & Bergström, S. Complications of Pregnancy and Transplacental Transmission of Relapsing-Fever Borreliosis, *J. Infect. Dis.*, **194**(10), 1367–1374 (2006)
28. Lescot, M., Audic, S., Robert, C., Nguyen, T.T., Blanc, G., Cutler, S.J., *et al.*, The Genome of *Borrelia recurrentis*, the Agent of Deadly Louse-Borne Relapsing Fever, Is a Degraded Subset of Tick-Borne *Borrelia duttonii*, *PLoS Genet.*, **4**(9), e1000185 (2008)
29. Lopez, J.E., McCoy, B.N., Krajacich, B.J., Schwan, T.G. Acquisition and Subsequent Transmission of *Borrelia hermsii* by the Soft Tick *Ornithodoros hermsi*, *J. Med. Entomol.*, **48**(4), 891–895 (2011)
30. Lopez, J.E., Porcella, S.F., Schrumpf, M.E., Raffel, S.J., Hammer, C.H., Zhao, M., Robinson, M.A., & Schwan, T.G. Identification of conserved antigens for early serodiagnosis of relapsing fever *Borrelia*, *Microbiology*, **155**(Pt 8), 2641–2651 (2009)
31. Lopez, J.E., Schrumpf, M.E., Raffel, S.J., Policastro, P.F., Porcella, S.F., & Schwan, T.G. Relapsing Fever Spirochetes Retain Infectivity After Prolonged *in vitro* Cultivation, *Vector Borne and Zoonotic Dis.*, **8**(6), 813–820 (2008)
32. Lundqvist, J., Larsson, C., Nelson, M., Andersson, M., Bergström, S., & Persson, C. Concomitant Infection Decreases the Malaria Burden but Escalates Relapsing Fever Borreliosis, *Infect. Immun.*, **78**(5), 1924–1930 (2010)
33. Meri, T., Cutler, S.J., Blom, A.M., Meri, S., & Jokiranta, T.S. Relapsing Fever Spirochetes *Borrelia recurrentis* and *B. duttonii* Acquire Complement Regulators C4b-Binding Protein and Factor H, *Infect. Immun.*, **74**(7), 4157–4163 (2006)
34. McCall, P.J., Hume, J.C., Motshegwa, K., Pignatelli, P., Talbert, A., & Kisinza, W. Does tick-borne relapsing fever have an animal reservoir in East Africa?, *Vector Borne Zoonotic Dis.,* **7**(4), 659-666 (2007)
35. Melkert, P.W., & Stel H.V. Neonatal *Borrelia* infections (relapsing fever): report of 5 cases and review of the literature, *East. Afr.Med. J.*, **68**(12), 999-1005 (1991)

36. Nordstrand, A., Bunikis, I., Larsson, C., Tsogbe, K., Schwan, T.G., Nilsson, M., & Bergström, S. Tickborne Relapsing Fever Diagnosis Obscured by Malaria, Togo, *Emerg. Infect. Dis.*, **13**(1), 117-123 (2007)
37. Nowalk, A.J., Gilmore, R.D., & Carroll, J.A. Serologic Proteome Analysis of *Borrelia burgdorferi* Membrane-Associated Proteins, *Infect. Immun.*, **74**(7), 3864–3873 (2006)
38. Parola, P., Raoult, D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat, *Clin. Infect.Dis.*, **32**(6), 897-928 (2001)
39. Pavlovsky, Y.N. Human Disease with Natural Foci, Moscow, Foreign Languages Publishing House, 138–184 (1963)
40. Policastro, P.F., Raffel, S.J., & Schwan, T.G. Cotransmission of Divergent Relapsing Fever Spirochetes by Artificially Infected *Ornithodoros hermsi*, *Appl. Environ. Microbiol.*, **77**(24), 8494–8499 (2011)
41. Rappsilber, J., Mann, M., & Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, *Nat. Protoc.*, **2**(8), 1896-1906 (2007)
42. Ras, N.M., Lascola, B., Postic, D., Cutler, S.J., Rodhain, F., Baranton, G., & Raoult, D. Phylogenesis of relapsing fever *Borrelia* spp., *Int. J. Syst. Bacteriol.*, **46**(4), 859-865 (1996)
43. Rebaudet, S., & Parola, P. Epidemiology of relapsing fever borreliosis in Europe, *FEMS Immunol. Med. Microbiol.*, **48**(1), 11–15 (2006)
44. Rego, O.R., Hajdušek, O., Kovář, V., Kopáček, P., Grubhoffer, L.,& Hypša, V. Molecular cloning and comparative analysis of fibrinogen-related proteins from the soft tick *Ornithodoros moubata* and the hard tick *Ixodes ricinus*, *Insect Biochem. Mol. Biol.*, **35**(9), 991-1004 (2005)
45. Rego, O.R., Kovář, V., Kopáček, P., Weise, C., Man, P., Šauman, I., & Grubhoffer, L. The tick plasma lectin, Dorin M, is a fibrinogen-related molecule, *Insect Biochem. Mol. Biol.*, **36**(4), 291-299 (2006)
46. Ribeiro, J. M., Mather, T.N., Piesman, J., & Spielman A. Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae), *J. Med. Entomol.*, **24**(2), 201-205 (1987)
47. Samuels, D.S., & Radolf, J.D. *Borrelia*: Molecular Biology, Host Interactions and Pathogenesis, Caister Academic Press, 1st Ed. (2010)

48. Shevchenko, A., Tomas, H., Havli, J., Olsen, J.V., & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes, *Nat. Protoc.*, **1**(6), 2856-2860 (2007)
49. Sonenshine, D.E. Biology of Ticks, Oxford University Press (1997)
50. Southern, P., & Sanford, J. Relapsing fever: A clinical and microbiological review, *Medicine*, **48**, 129-149 (1969)
51. Stoenner, H.G., Dodd, T., & Larsen, C. Antigenic variation of Borrelia hermsii, *J. Exp. Med*, **156**, 1297–1311 (1982)
52. Tabuchi, N., Kataoka-Ushijima, Y., Talbert, A., Mitani, H., & Fukunaga, M. Absence of transovarial transmission of *Borrelia duttonii*, a tick-borne relapsing fever agent, by the vector tick *Ornithodoros moubata*, *Vector Borne Zoonotic Dis.*, **8**(5), 607-613 (2008)
53. Takahashi, Y., Cutler, S.J., & Fukunaga, M. Size Conversion of a Linear Plasmid in the Relapsing Fever Agent *Borrelia duttonii*, *Microbiol. Immunol.*, **44**(12), 1071-1074 (2000)
54. Thomas, N.J., Bunikis, J., Barbour, A.G., and Wolcott, M.J. Fatal spirochetosis due to a relapsing fever-like *Borrelia* sp. in a northern spotted owl, *J. Wildl. Dis.*, **38**(1), 187–193 (2002)
55. Walker, A.R., Bouattour, A., Camicas, J.-L., Estrada-Peña, A., Horak, I.G., Latif, A.A., Pegram R.G., & Preston P.M. Ticks of Domestic Animals in Africa: a Guide to Identification of Species, Bioscience Reports, Edinburgh (2014)
56. Wessel, D., & Flügge, U.I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids, *Anal. Biochem.*, **138**(1), 141-143 (1984)