Acquisition Dynamics of *Borrelia duttonii* by *Ornithodoros moubata* over time

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Bachelor Thesis

Laboratory of Molecular Ecology of Vectors and Pathogens

INSTITUTE OF PARASITOLOGY

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Bibliography:

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Annotation:

The aim of the thesis is to identify the transmission and acquisition dynamics of *Borrelia duttonii* by *Ornithodoros moubata* under normal as well as extreme conditions. Additionally, the infectivity of *B.duttonii* from brain tissue is tested to confirm data reporting on *B.duttonii* leaving a residual infection in the brains of mice.

Affirmation:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only

České Budějovice, 14.12.2021

Signature: 

Abstract

Borrelia duttonii is one of many *Borrelia s*trains causing relapsing fever and the acquisition and transmission dynamics of the strain by *Ornithodoros moubata* is the main subject that was studied in this thesis. Furthermore, experiments were performed to confirm data which discovered the capabilities of *B.duttonii* causing residual infections in the brain of mice after the spirochetes were no longer detectable in the blood.

Experiments testing for acquisition and transmission dynamics used mice, infected by a standardized inoculation from a *B.duttonii* culture. After feeding new *O.moubata* periodically on the infectious mice, a time frame was established for the infectivity period of *B.duttonii* from a mouse reservoir. Post-inoculation, *O.moubata* can acquire the spirochetes from the host within the first month.

Further experiments tested the infection rate of *B.duttonii* by *O.moubata*, which were housed at different temperatures than the normal habitat conditions after they had acquired the spirochete from infected mice. The temperature changes did not affect the infectivity, presumably due to a high enough population of spirochetes within the vector to accommodate the possible loss of bacteria.

B.duttonii was additionally analyzed in mouse brains by homogenizing the brain and injecting the homogenate into naïve mice. The brain homogenate of some mice did appear to have Borrelia present to cause an infection in naïve mice. The occurrence was rare, and this could be due to the mouse strain used as well as the Borrelia strain .

These results provide further data in understanding the relapsing fever pathogen in its arthropod vector and the mammalian host.

Contents

1 Introduction

1.1 Relapsing Fever

Relapsing Fever (RF) is a disease caused by *Borrelia* which are gram-negative bacteria that are part of the *Spirochaetes* phylum. Some examples of known species which cause RF are *Borrelia hispanica, Borrelia recurrentis* and *Borrelia duttonii.* [1,2]

This disease is zoonotic which means that it is transmitted from a vector, such as an arthropod, to humans or other animals in a continuous cycle. Depending on the vector and the causative strain of RF, one Borrelia species can have one vector which can transmit the disease to one or multiple reservoirs in the wild. The majority of *Borrelia* species are transmitted through infected ticks, except for *B.recurrentis* which is the only species that can be acquired solely by the louse, *Pediculus humanus.* [3,4]

Due to the large number of RF Borrelia , different categories were made for tick-borne RF (TBRF) and louse-borne RF (LBRF). Additionally, TBRF is further subcategorized as soft tick-borne RF (STBRF) and hard tick-borne RF (HTBRF) depending on whether the vector is part of the *Argsidae* family (STBRF) or *Ixodidae* family (HTBRF). [4,5]

Regardless of the vector, RF shares common symptoms among all *Borrelia* species such as episodic fever, headache, hepatomegaly, myalgia and in some cases, as for an example an infection with *B.duttonii,* stillbirth can occur. In Africa, where *B.duttonii* and other *Borrelia* species are very prevalent, RF is often misdiagnosed as malaria due to the overlapping symptoms and shared geographical distribution. [1,6]

Additionally, the tools used to identify RF in patients are subpar thus further increasing the rate of misdiagnoses. Some current methods which are being used to identify RF are ELISA, Giemsa staining and analysis of blood smears under dark-field microscopy (see Figure 1). [7-9]

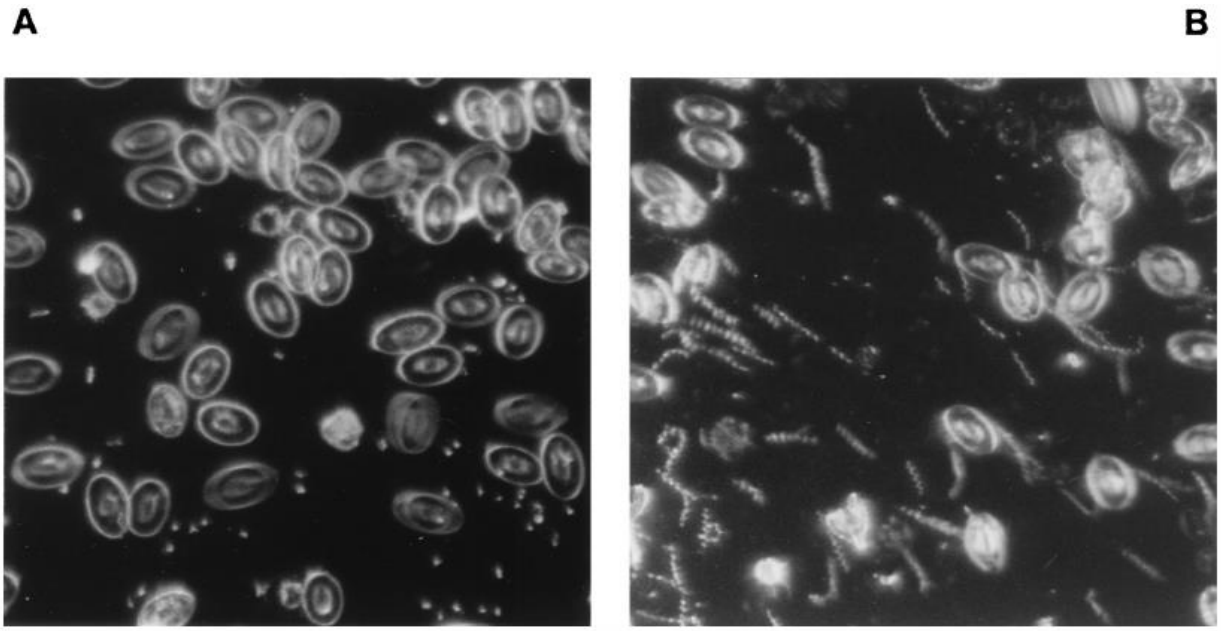

Figure 1. Picture A depicts blood cells under a dark-field microscope and B contains both blood cells and spirochetes, adapted from [10]

The limited number of diagnostics leads to mistreatment of RF with medication that is used for malaria or other diseases, thus resulting in a higher mortality rate. Due to the variety of *Borrelia* strains, the mortality rate of RF is approximated to reside between 2.3 and 5%. [1,6]
In comparison, *B.duttonii* has a mortality rate of 2.3% and since it can additionally lead to stillbirth it is even more dangerous for pregnant women, causing an increase of prenatal mortality to 44% and miscarriages to 48%. [4,1]

As of now, the disease is considered to be transient but some papers, such as Pathobiology of African relapsing fever *Borrelia* from Larsson, have found that *B.duttonii* could cause a brain infection even after the spirochetes are no longer detectable in the blood. Additionally, the hosts gene expression was identical to an uninfected animal which suggested that the hosts' immune system could not detect the *B.duttonii* spirochetes which retreated into the brain. [11]
Speculations can be made on whether *B.duttonii* could cause a new infection from within the brain but the specifics of *B.duttonii* spirochetes infectivity or its frequency of retreating into the cerebrum has not yet been researched well enough to give any well-developed theories.

1.2 *Argasidae* and *Ornithodoros moubata*

Ornithodoros moubata are soft ticks, also known as argasids which are part of the *Arthropoda* phylum, and predominantly occur in Africa. Members of the *Argasidae* family can transmit different strains of *Borrelia* depending on the species. [3,12]

An example of an *Ornithodoros* spreading more than one *Borrelia* species is the *Ornithodoros erraticus* tick. This vector can be a carrier for both *B.crocidurae* and *B.hispanica* which are distributed through the old world in areas such as western and northern Africa, as well as the Iberian Peninsula. Unlike *B.duttonii*, which only uses human reservoirs in the wild, *B.crocidurae* and *B.hispanica* transmit *Borrelia* to many different mammals like most other *Borrelia* species in the old world. The only other *Borrelia* species which relies solely on human reservoirs is *B.recurrentis* which is a louse-borne RF dispersed worldwide. [13]

Different argaside species are located in different parts of the world and are often times associated with the *Borrelia* species which they transmit predominantly, as can be seen in Figure 2. *O.moubata* is widespread in east and south Africa and is the only tick vector carrying *B.duttonii* spirochetes. [4]

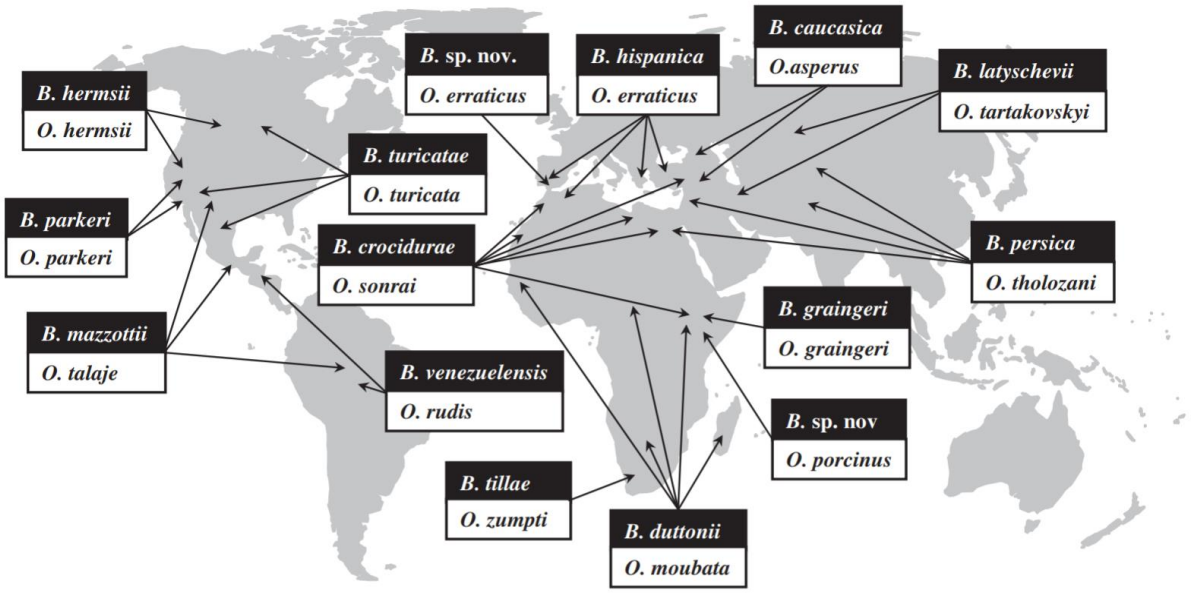

Figure 2. *Borrelia* strains and their argaside vectors across the globe, adapted from [4]

Even though argasids can be found globally, they are mainly distributed in climates that range from subtropical to arid. It is due to their unique morphology that soft ticks can survive very arid

conditions, which makes them better adapted to dryer climates than hard ticks (*Ixodidae* family). [3,12]

One of the physiological features that allows soft ticks to survive harsh environments is the integument. Unlike hard ticks, which have a hardened plate covering the dorsal area of their body, argasids have a wrinkled and leathery exoskeleton.
This unique integument will expand during a blood meal, allowing the tick to feed more rapidly and subsequently retreat quicker to a burrow. Furthermore, soft ticks can release excess water and ions via coxal glands during or after a blood meal which is used to protect the ticks' body from the environment. These physiological adaptations of the integument influenced the appearance of the mouthpiece of argasids as well. The mouthpiece, also known as the capitulum, is not visible from the posterior of an adult soft tick while it is clearly discernible in all life stages of an ixodid tick. [14,15]

Evolutionary traits, such as these, led to further changes in the life cycle of soft ticks. From one egg, a 6-legged larvae will hatch which can then moult to an 8-legged nymph regardless of whether a blood meal ensued in between. If feeding did occur, then the soft tick would withdraw into their habitats, which tend to be human shelters as well as cracks and crevices of rodent burrows. After retreating int o their territory, argaside ticks moult within a few weeks before leaving for another blood meal. [3,16]

Further on, the nymph can moult multiple times before it turns into an adult. After each moulting phase, which can either lead to a nymphal or an adult stage, a new blood meal is needed. When female nymphs grow into an adult, they can lay several eggs after each feeding session. A visualization of the life cycle can be seen in Figure 3. [16]

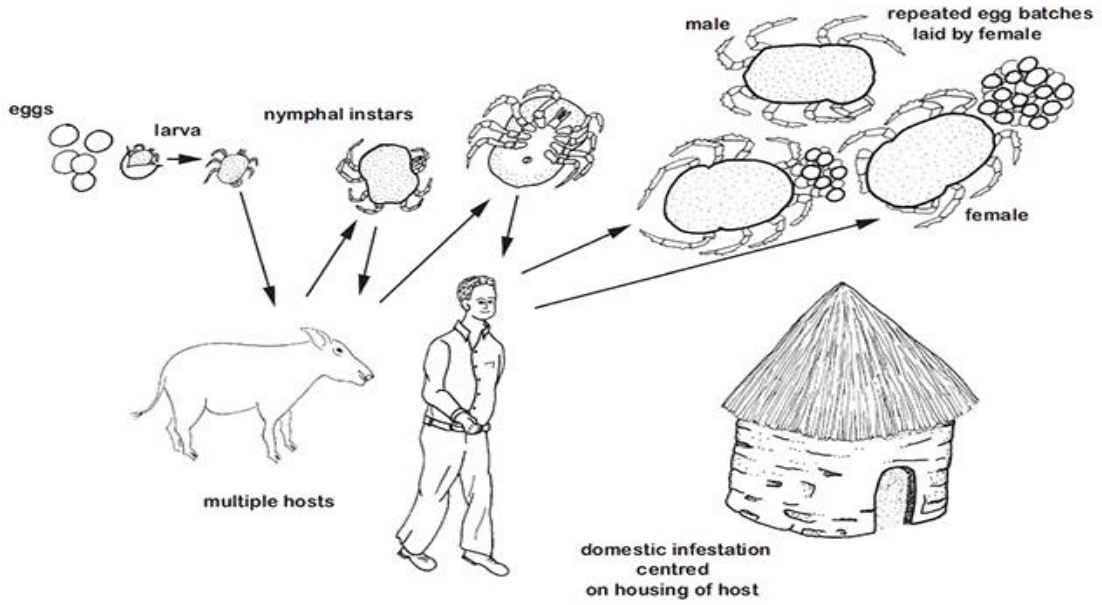

Figure 3. Life cycle of argaside ticks starting from the egg. A 6-legged larvae hatches which can feed on a host and moult into a nymph. After each blood meal the nymph can either moult into an adult or stay in the nymphal stage for a maximum of seven cycles. The female adult tick can then lay several eggs after each feeding, adapted from [17]

A stark difference between ixodid and argaside ticks becomes apparent in the duration of a blood meal. When argasids feed on a host, the time span of each blood meal is between 15 to 60 minutes depending on various factors such as the maturity and size of the tick. [18]

In contrast, hard ticks need to feed on one host for multiple days before they become fully engorged, and the time period is further influenced by whether the ticks are in the nymphal, or adult stage. Since soft ticks have a very short blood meal and moulting duration, it makes them optimal for scientific experimentation.

1.3 *Borrelia duttonii*

Borrelia duttonii is one of many *Borrelia* strains which causes RF and can be found in many parts of south Africa. Once *B.duttonii* is transmitted from the vector (*O.moubata*) to a human host, symptoms will generally appear between 4 to 18 days.

The episodic fevers are associated with the number of spirochetes circulating in the blood. During a fever, the number of *Borrelia* is higher than in the afebrile periods, which is due to the hosts' immune system combating the infection. Once the spirochetes are identified by the immune system, the infection decreases until the *Borrelia* evade the produced antibodies which

leads to another peak in spirochetemia, and a new febrile episode arises.

The spirochetes' evasive mechanism is possible due to antigenic variation of the RF *Borrelias'* variable major proteins (Vmps). The Vmps help RF spirochetes to be incompatible with the produced antibodies of the host, which is accomplished by varying the expression of their antigens. The variability of the Vmps stems from the two subcategories called variable large proteins (Vlps), and variable small proteins (Vsps). The major difference between Vlps and Vsps is the size of the proteins, and by changing the expression of these Vmps the spirochetes can hide from the hosts' immune system several times. The febrile and afebrile episodes can be seen in Figure 4 as well as a representation of how the Vmps change. [3,19-22]

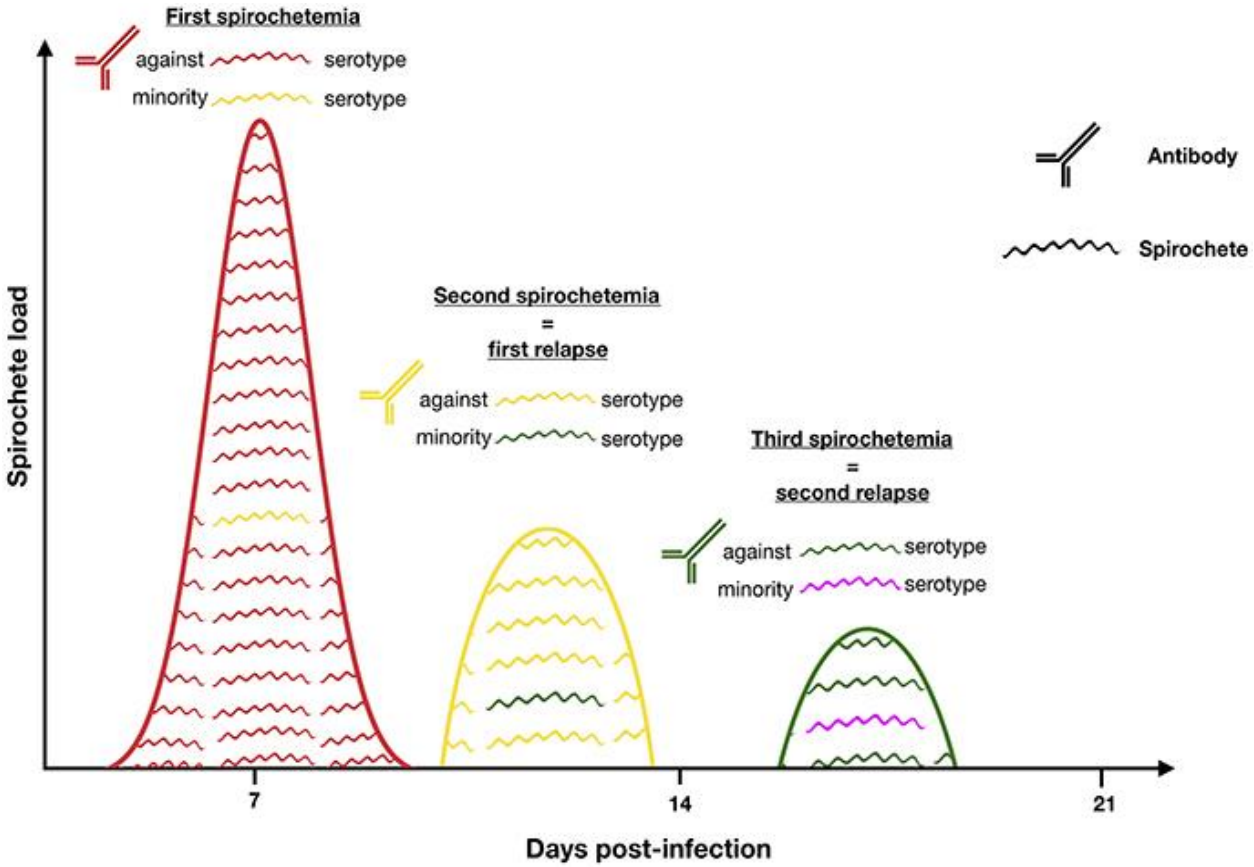

Figure 4. Graph depicting an overview of the evasive mechanism of spirochetes using Vmp expression changes, adapted from [5]

The number of times *Borrelia* can evade the hosts' immune system is termless. If the host cannot combat the infection, then the spirochetes will continue to multiply and change Vmps, as needed, until the reservoir is deceased.

As previously mentioned, *B.recurrentis* and *B.duttonii* are the only known species to exclusively use human reservoirs in the wild which could be due to the genetic similarity between the two *Borrelia* species. As can be seen in Figure 5, *B.recurrentis* and *B.duttonii* are phylogenetically related which is reflected in the Vmp sizes, G+C ratio and 16S rRNA resemblance. [4,5]

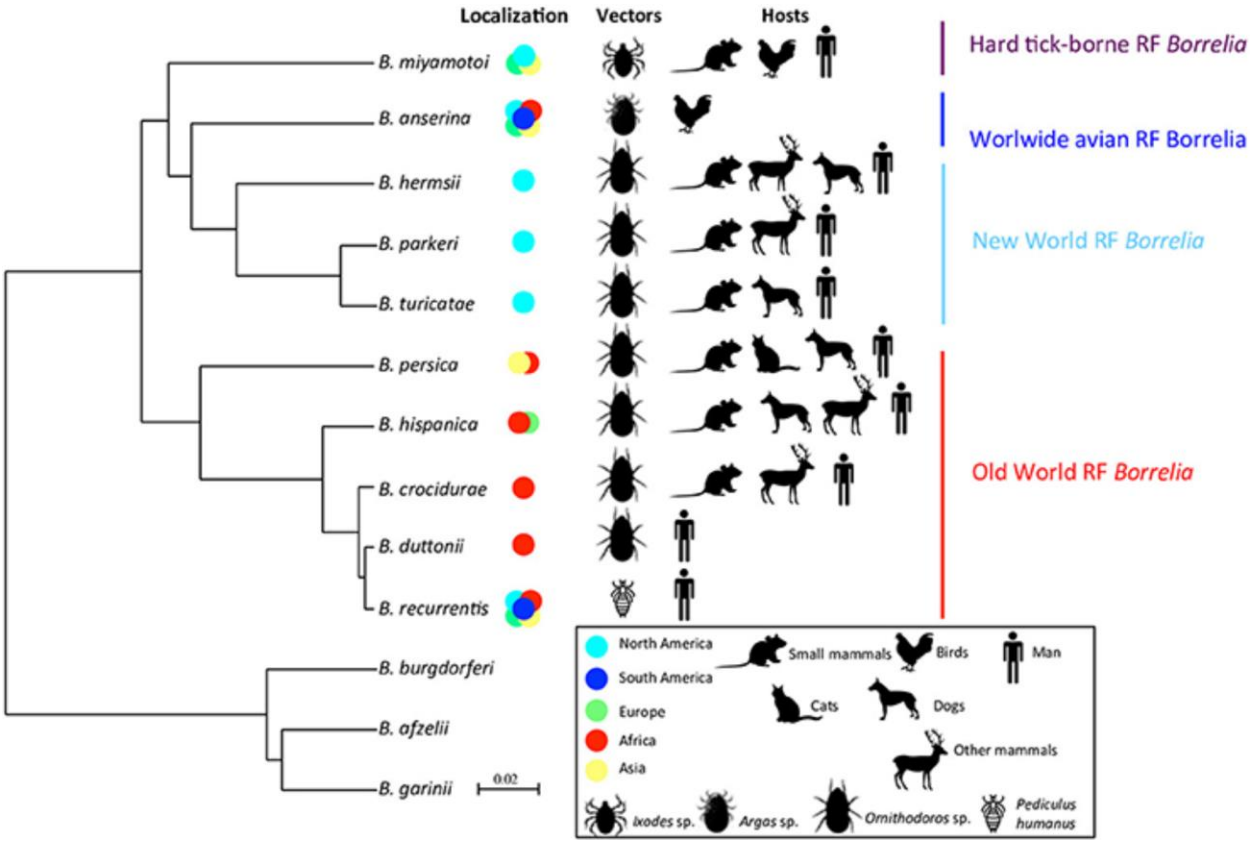

Figure 5. Depiction of *Borrelia* strains with their respective vectors and hosts as well as their global occurrence and phylogenetic correlation, adapted from [5]

Unlike other *Borrelia* species and their respective vectors, *B.duttonii* and *O.moubata* are neglected in many scientific experiments. There are a multitude of topics that still need to be analyzed as for example the acquisition and transmission dynamics of the vector. Furthermore, previously mentioned topics such as *B.duttonii* retreating into mice brain or the interchangeability of vectors between *B.recurrentis* and *B.duttonii* are yet to be further analyzed or experimented with at all.

Some earlier research has shown that *B.duttonii* can infect lab mice making it possible to experiment with this *Borrelia* species in the lab using mice models. [7]

Since mice are used as a substitute to human models, any research data acquired cannot be guaranteed to correspond completely to natural interactions like many other diseases which us substituted models for experimentation.

Previous lab experiments on acquisition dynamics of *B.duttonii* by *O.moubata* have identified the threshold of necessary spirochetes which is needed to cause an infection in the vector and subsequently in a mouse. The data has shown that once *Borrelia* are visible in the blood of the host the infectivity rate for the vectors is 100%. The observation was performed over 15 days, which leads to inquiries as to how long this infectivity rate lasts beyond the 2 weeks. [23]

Further research done on survivability of *O.moubata* infected by *B.duttonii* has shown that the infection rate of the ticks significantly decreased when the vector was housed at a temperature several degrees higher than their natural habitat climate. [24]

2 Aims

The focus of this project is to understand the transmission and acquisition dynamics of *B.duttonii* under normal and extreme circumstances, as well as the time span in which they can be acquired, and further on be transmitted via *O.moubata.* In order to achieve these objectives, the three following aims were established:

1. Acquisition and transmission period of *B.duttonii* via *O.moubata*

   Testing the time span in which *B.duttonii* can infect *O.moubata* ticks and subsequently transmit it to naïve mice by inoculation of tick homogenates.

2. *B.duttonii* survivability at different temperatures

   Identifying *B.duttonii* survivability in *O.moubata* ticks at different temperatures to analyze its' effects on the transmission of the disease.

3. *B.duttonii* retreating and causing and infection in mice brain

   Analyzing the possibility of *B.duttonii* retreating into mice brain and the spirochetes infectivity via homogenization and inoculation of old infected mice brain into naïve mice.

3 Materials and Methods

3.1 *Borrelia* strain

Borrelia strain 1120K3 was generously provided by Prof. Sven Bergstrom (University of Umea , Sweden)

3.2 Preparation of infectious mice

Two different mice strains were used during the experiments, C3H/HeN and Balb/c. Experiments were first started off by inoculating naïve mice with the *B.duttonii* 1120K3 strain from a culture. The infected mice were used once they tested positive for *Borrelia* antibodies with the exception of the experiments for the 3rd aim.

The prerequisites for mice that were used in that experiment were a previous infection with *B.duttonii* and the spirochetes were no longer visibly circulating in the blood.

3.2.1 *B.duttonii* culture growth

BSK-H, which was used for cultivating spirochetes, was stored in a fridge and preheated in a water bath (Julabo) to 32°C before use. The cultures were grown in round-bottom falcon tubes which were filled with 7 mL of BSK-H medium and 6% of the volume was comprised of rabbit serum. The media was then inoculated with the *B.duttonii* 1120K3 strain which was taken from a glycerol stock that was kept at -80°C.

For approximately 1 week, the cultures were stored in an incubator at 34°C.

3.2.2 Naïve mice inoculation

Each inoculation which used spirochetes from a culture, was standardized to contain 1000 spirochetes per 250 µL of injected media per mouse. This standard was obtained by analyzing the cultures concentration with a Petroff Hauser counting chamber (Marienfield, depth 0.02mm, 0.0025 mm^2), and subsequently preparing the dilutions for the syringes in 1.5 mL Eppendorf tubes. The mice were then inoculated intraperitoneal (150 µL) and subcutaneous (100 µL) under anesthetics.

Naïve mice which were inoculated with 250 µL of brain homogenate were intravenously

injected. The excess brain homogenate of each dissected mouse was kept in an incubator at 34°C in 1.5 mL Eppendorf tubes for further observation.

3.2.3 Blood analysis via microscopy

Preemptive blood tests were done for some of the mice, to detect visually any spirochetes before testing later for antibodies against *Borrelia*. These preemptive tests were done by taking blood samples via tail snips from a mouse 3-days post inoculation and analyzing them under a microscope.

The tail snip was performed with a scalpel and the blood was extracted by either a pipette or by directly applying the microscope slide (ThermoFisher Scientific™) to the tail and smearing a bit of the blood onto the slide.

Two different types of slides were prepared to analyze via microscopy.
Slides that were checked with a dark-field microscope would contain a drop of blood which was topped with a cover slide (ThermoFisher Scientific™ , 24 x 60 mm).
Slides which were analyzed with a light microscope would undergo extra preparation with Giemsa staining. The blood drop, which was placed at the end of a slide, was smeared by using the edge of a second microscope slide. This was accomplished by gently dragging the width of the clean slide across the surface of the glass with the blood to create a thin blood film.

3.2.4 Giemsa staining

As described in subsection 3.1.3 the blood for the staining was acquired via tail snips and was smeared onto a microscope slide. Once the thin blood film air dried, three rectangular Coplin staining jars were filled halfway, each with a different staining solution (Labor + Technik). A specific order of the solutions was required to properly stain the blood smears. Methanol was first used as a fixation solution and then the staining was performed by eosin and lastly methylene blue. The slide was submerged for 30 seconds in each staining jar after letting the excess liquid of the previous solution drip off. Any excess solution which was still present at the end of the last staining step was washed off with water, then the slide was air dried and lastly viewed with a light microscope.

3.3 *Ornithodoros moubata* ticks

Ticks were kept in a porous and transparent container which was housed in a room with high humidity, at 27°C, until they were fed for further use.

3.3.1 In vivo feeding of *O.moubata*

The used in vivo method and the number of ticks which would feed on the infected mice would vary depending on the aim.

For aims 1 and 3, *O.moubata* were individually placed with tweezers in and around the ear region of mice, after they were subdued with anesthetics, for a maximum of 30 minutes. The sedated mice were separated into plastic containers in order to track the ticks which would fall off once they fully fed. The number of ticks which would feed in one session was between 4 to 5 per infected mouse.

When feeding 10 to 15 ticks per mouse for aim 2, capsules were taped on the back of the mice and *O.moubata* were left to feed overnight. This was done to ensure that all placed ticks were fully fed within the same time frame. Once the in vivo feeding was completed, the ticks were collected in 1.5-2 mL Eppendorf tubes.

3.3.2 Tick maintenance

All fed ticks were initially stored at 27°C with 90% air humidity in 15 mL Tubespin^{R} Bioreaktor tubes (Techno Plastic Products) containing 2-3 strips of filter paper. The majority of *O.moubata* were kept in the same conditions until they moulted.

Ticks that would be exposed to different temperatures for three weeks, were moved after one week of storage at 27°C (standard housing temperature) to different incubators.

The ticks were separated into four different groups. Each group was given the abbreviations according to the exposed temperatures of the second and third week as can be seen in Table 1.

Table 1 – Tick groups kept at different temperatures for 3 weeks

Tick groups (abbreviations used)	Week 1	Week 2	Week 3
34°C	27°C	34°C	34°C
20°C	27°C	20°C	20°C
34°C/ 27°C	27°C	34°C	27°C
CT (control group)	27°C	27°C	27°C

3.3.3 *O.moubata* homogenization and inoculation

Ticks were separated and placed into 1.5 mL Eppendorf tubes and submerged in 500 µL of 3% H_2O_2 (30% Hydrogen Peroxide from Lachner, 1:10 dilution) for 15 minutes. Subsequently, the H_2O_2 was removed via pipetting and an additional 500 µL of 70% ethanol was used for another 15 minutes. The liquid was decanted or extracted with a pipet and any residual ethanol was air dried. Subsequently, 350 µL BSK-H media was added and the ticks were crushed with a pestle, which was rinsed with an additional 150 µL BSK-H media. Once the syringes were loaded with 250 µL of the tick homogenate, the naïve mice were intraperitoneally (150 µL) and subcutaneously (100 µL) inoculated under anesthetics.

Approximately 1 mL of BSK-H media was added to the residual of the *O.moubata* homogenate followed by 80 µL of preprepared 100x *Borrelia* antibiotics stock solution (Phosphomycine 2 mg/mL, Rifampicon 5 mg/mL and Amphotericin 0.25 mg/mL in 20% DMSO solution). The Eppendorf tubes were then incubated at 34°C for further analysis via dark-field microscopy.

3.4 Preparation of serum samples

Blood was drawn from the mice 3 weeks post inoculation via retro-orbital bleeding after subduing them with anesthetics. The samples were then spun down at 9000 rpm for 7 minutes and the supernatant was pipetted into new 1.5 mL Eppendorf tubes. Subsequently, sera which was not immediately used was stored in a freezer at -20°C.

3.5 Lysate Preparation

In order to prepare a lysate, which was used to test for *Borrelia* infections via Western Blotting, a *B.duttonii* culture was grown as described in subsection 3.1.1.

After centrifuging the culture at 8000 rpm, 20°C for 10 minutes the supernatant was removed and 1 mL of cold HN buffer was added to the falcon tube. Once the pellet was resuspended, the mixture was pipetted to a 1.5 mL Eppendorf tube. The solution was centrifuged again at 8000 rpm for 10 minutes and the supernatant was discarded. Subsequently another 1 mL of cold HN buffer was added and centrifuged with identical settings as before. 200 µL B-PER (Bacterial Protein Extraction Reagent from ThermoFisher Scientific ™) were pipetted to the pellet once the last supernatant was removed. Subsequently, the pellet was resuspended and kept at room temperature for approximately 10 minutes. Finally, 190 µL of a 2x Laemmli Sample Buffer (BioRad) and 10 µL of β-mercaptoethanol were added. The lysate was then stored in a freezer at -20°C for further use.

3.6 SDS-Page

The SDS-Page (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel solutions were made according to Table 2.

Tabel 2 - Measurements for 1 gel

Reagents	**12% separation gel**	**5% stacking gel**
30% Acrylamide	2 mL	330 µL
Separation buffer	1.25 mL	-
Stacking buffer	-	500 µL
H_2O	1.7 mL	1.15 mL
10% APS	50 µL	20 µL
TEMED	2.5 µL	2 µL

The gel was polymerized between glass plates (Bio-Rad) with a thickness of 0.75 mm. First the separation gel was loaded into the cast and after 30 minutes of polymerization the stacking gel solution was added, with either a 10-well or multichannel comb (Bio-Rad), depending on the number of tested samples. The comb was removed once the stacking gel polymerized. Then, it was placed in a vertical SDS-Page electrophoresis cell (Bio-Rad) which was afterwards filled with 1x SDS-Page Running Buffer (3.03 g Tris-Base, 14.4 g Glycine and 1 g/ L SDS). The 8 µL protein ladder (PanReac AppliChem) was added to the first well once the prepared lysate, as described in subsection 3.4, was heated at 100°C for 10 minutes. Subsequently, 20 µL of lysate were added to a well for each tested sample. Afterwards, the machine was running at 120 V for 1 hour or a maximum of 90 minutes.

3.7 Western Blot

Approximately 10 minutes before the end of the electrophoresis, as described in section 3.4, the required number of blotting paper and nitrocellulose membrane (Bio-Rad) were prepared. For one gel, two blotting papers and one nitrocellulose membrane were submerged and soaked in 1 x Blotting Buffer which was a mix of 3.03 g Tris-Base and 14.4 g/L Glycine. After the electrophoresis finished, the blotting sandwich was prepared on top of the anode of the blotting machine (Bio-Rad, Trans-Blot Semi-Dry Transfer Cell) by first putting down a blotting paper followed by the membrane and the gel. The wells of the gel were then marked onto the membrane with a pencil. Then, the gel was covered by the second blotting paper and any air bubbles were removed by using a roller. Finally, the cathode was placed on top, and the machine was running at 25 V for 30 minutes.

Subsequently the sera were prepared into primary antibody samples (1:200 dilution) by adding 5 µL of the tested mouse serum to 995 µL of a 5 % blocking solution mix (5g dehydrated milk per 200 mL of 1x TBS-Tween 20). Once the blotting ended, the membrane was submerged in the blocking solution and left on a mechanical shaker for 2 hours. Depending on whether a 10-well of 15-well comb was used for the gel, the membrane was prepared in the following ways.

When using 10-well gels, the membrane was cut into strips depending on the individually marked wells. Each strip was then added to a plastic bag with one primary antibody solution to which it would correspond. The bag was then sealed and stored in the fridge until the next day.

The membrane was not cut for multichannel gels which consisted of one well for the ladder and one large well for the protein lysate. After the nitrocellulose membrane was placed onto the Mini-Protein II Multiscreen (Bio-Rad), all air was removed by clamping down the top part. Each capillary corresponded to one sample which was loaded with a pipet from the bottom hole until the entire capillary was filled. The apparatus was stored in the fridge overnight after enveloping it in saran warp.

On the following day, a new 50 mL of a 5% blocking solution was prepared containing 5 µL Anti-Mouse antibody IgG (Sigma-Aldrich). Subsequently, the nitrocellulose membrane was washed three times with 1x TBS-Tween 20 (3 g Tris-Base, 8 g NaCl, 0.2 g KCl, 500 µL Tween-20 per L, pH = 7.4) for 15 minutes each wash. For approximately 1 hour, the membrane was left to shake in the secondary antibody solution and then washed once more three times for 15 minutes in 1 x TBS-Tween 20.

Finally, the membrane was covered for 5 minutes with the Pierce™ ECL Western Blot Substrate (ThermoFisher Scientific™) for which the substrates, Peroxide solution and Enhancer solution, were mixed in a 1:1 ratio. For each membrane 2 mL of the mixture was used to cover it. Subsequently, the membrane was placed between two overhead transparencies (Xeroxs) and the picture was developed using a Chemi-Doc™ MP Imaging System (Bio-Rad).

4 Results

4.1 Acquisition and transmission period of *B.duttonii* via *O.moubata*

In order to determine the time frame in which *O.moubata* ticks could acquire an infection from a mouse, C3H/HeN mice were inoculated with a standardized injection of 1000 spirochetes per 250 µL of injected media. The blood from the tail snip of the mice was analyzed via dark field microscopy. Some of the mouse blood was additionally viewed with a light microscope when Giemsa staining was performed on the samples. The pictures seen in Figure 6 are the first time our lab has visualized a successful Giemsa staining of *B.duttonii*.

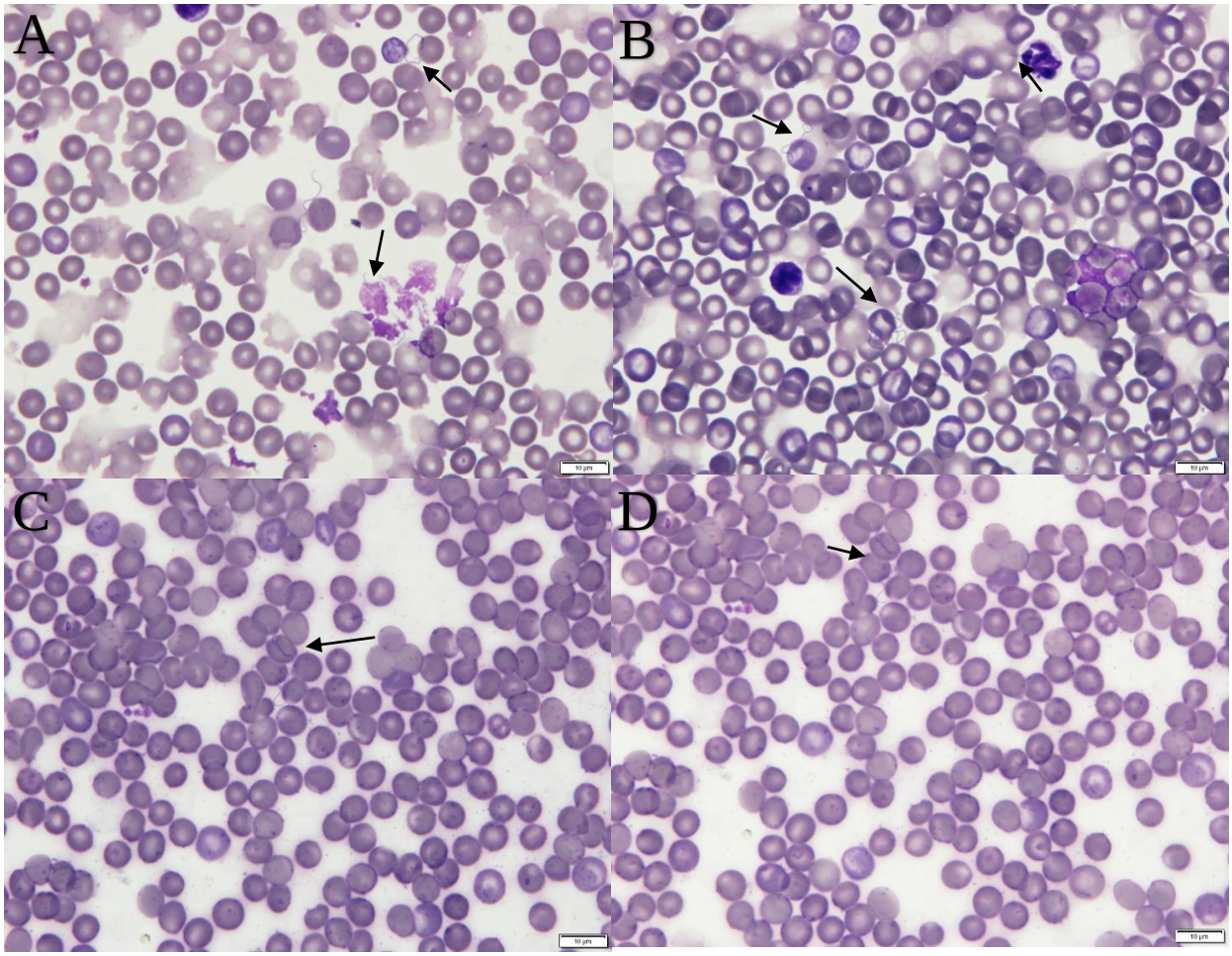

Figure 6. Pictures (A-D) of *B.duttonii* and hemoglobin stained with Giemsa and captured with a light microscope. A 10 µm reference is in the bottom right corner of each picture

Once an infection was confirmed serologically via Western Blots, ticks were fed monthly on the infected mice. After moulting for a maximum of 3 week, the ticks were homogenized individually and injected into naïve mice. The infection rate of the ticks for each month was confirmed by testing the blood of the tick inoculated mice via a Western Blot. The experiment was performed twice to obtain significant data for each month. The results for this aim can be seen in Table 3.

Tabel 3 – Serum samples of mice showing infection rate of ticks feeding on *B.duttonii* inoculated mice over the span of 3 months

	Month 1	**Month 2**	**Month 3**
1st attempt (Positive mice/total mice)	6/9	0/6	Not tested
2nd attempt (Positive mice/total mice)	5/5	0/5	Not tested

The experiments have shown that ticks will most probably be infected if they feed on a *B.duttonii* infected mouse within the first month. Ticks that were collected in the 3rd month were no longer tested since the results for the 2nd month for two separate experiments were negative.

4.2 *B.duttonii* survivability at different temperatures

With the aim of testing for *B.duttonii* survivability in diverse conditions, infectious *O.moubata* were housed at different temperatures for 3 weeks and inoculated via homogenization into naïve mice.

Once the ticks were fed, they were divided into four different groups. The abbreviation for each group and conditions in which they were maintained can be read in subsection 3.2.2.

After three weeks under different housing conditions the ticks were homogenized and inoculated into naïve mice. An example of a Western Blot result can be seen in Figure 7 which shows the serologically tested mice from group 37°C and 37°C/27°C. The last sample in Figure 7 belongs to a mouse which was inoculated with *O.moubata* from the 20°C group.

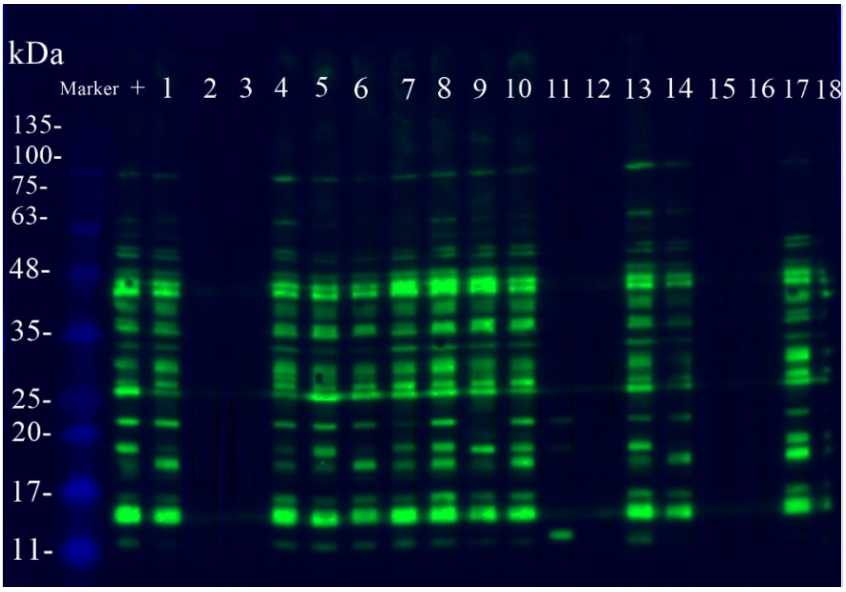

Figure 7. Western Blot testing the infectivity of tick groups 37°C, 37°C/27°C and one 20°C sample; Marker: protein ladder, +: positive control, 1-9: Tick group 37°C, 10-17: Tick group 37°C/27°C, 18: Tick group 20°C

O.moubata which were kept at 37°C for two weeks had a high infection rate with only two samples being negative. The ticks from the 37°C/27°C group returned four out of eight samples positive with the ninth sample being placed on the consecutively performed Western Blot. A summary of all the results can be seen in Tabel 4.

Tabel 4. Infection rate of ticks housed at different temperatures

Groups	**37°C**	**37°C/27°C**	**20°C**	**CT**
Positive mice/total mice	7/9	4/9	8/9	1/9

The results depict an increased infectivity for tick groups housed at higher and lower temperatures. The group which was changing between 37°C and 27°C has less than a 50% infection rate and the control group has turned out predominantly negative. Since ticks were kept at 27°C in every other experiment as a control group and have repeatedly produced positive results, it was determined that this experiment would need to be repeated.

4.3 *B.duttonii* retreating and causing an infection in mice brain

In order to test whether *B.duttonii* retreats into a mouse brain, previously infected mice were dissected and their brains were homogenized. The homogenate was then inoculated into naïve mice and the infection rate was tested serologically via Western Blots. The results can be seen in Figure 8.

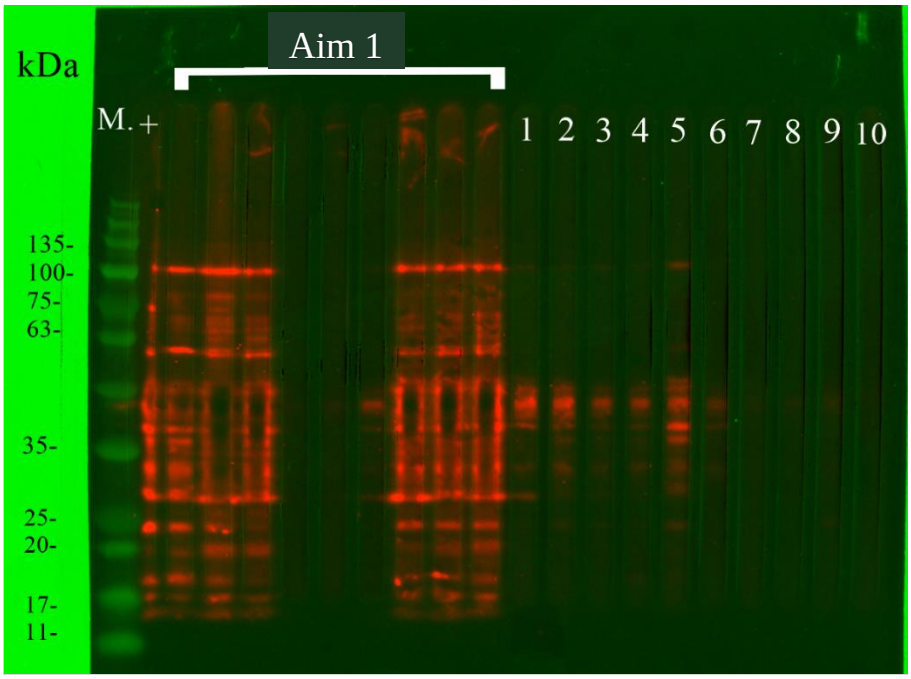

Figure 8. Western Blot containing sera results from aim 1 (left half) and aim 3 (right half). Aim 3 depicting sera results of mice inoculated with brain homogenate of previously old, infected mice; M.: protein ladder, 1-10: sera of mice infected with brain homogenate

The sera samples from mice 1-6 returned an ambiguous result and 7-10 were definitively negative. Since more than half of the results were possibly positive the experiment was repeated with new mice and new brain homogenates to increase the data set and get more precise results. The mice from the 2nd attempt were inoculated with the sera of the mice from the 1st attempt while the brains of the same 1st mice were homogenized and used for the 3rd set of mice. The 4th and final naïve mouse group was inoculated with new brain homogenate from a different positively infected mouse experiment. The overall infection rate can be seen in Table 5.

Table 5. Infection rate of mice inoculated with either brain homogenate or sera samples, from mice with inoculated brain homogenate

	Positive mice	**Ambiguous positive mice**	**Negative mice**	**Mice total**
1st attempt (brain)	0	6	4	10
2nd attempt (sera)	1	0	4	5
3rd attempt (brain)	3	0	5	8
4th attempt (brain)	0	0	13	13

While experiments 1-3 have returned some positive results or ambiguous positive results, the 4th attempt was entirely negative. The 2nd and 3rd mouse group used samples from the 1st mouse group, which leads to the conclusion that some of the ambiguous results were proven to be positive.

5 Discussion

The old world RF caused by *B.duttonii* is a continuously growing disease in Africa and it has been strongly neglected in research due to the lack of a proper working model. [1,5]
Until now, besides humans, *B. duttonii* has not been identified in any other mammalian reservoirs in nature, so it becomes increasingly important to identify acquisition and transmission dynamics in order to establish the right safety measurements and diagnostics against this STBRF disease. The studies in this work have laid an important groundwork for further research in this area as it sets a framework which shows in what ways, and for how long an infection can be acquired and transmitted by *B.duttonii* and its' vector *O.moubata*.
The time frame in which the naïve vector can still acquire and be infected with *B.duttonii* spirochetes from an infected mouse to cause an infection in the argaside tick, was determined to be approximately one month post inoculation of the rodent. There were no mice which build antibodies against *B.duttonii* after being injected with ticks, that fed on a mouse two months post inoculation. Therefore, a tick which feeds on a host 30 days since it was infected can no longer become infected from the ingested blood. Previous research from our lab, performed by Lisa Hain, has analyzed the starting time span in which the vector can acquire an infection from an inoculated mouse. The earliest viewed infection via a microscope was determined to be between 2-4 days after which an almost 100% infection rate persisted until the 15^{th} day. [23]
By combining both of these data sets from our lab we can establish that *O.moubata* can acquire an infection from an inoculated mouse starting earliest from the second day until approximately the 30^{th} day post infection.
A research paper, which has tested the infection compatibility of gerbils with *B.duttonii*, has observed that after a successful inoculation the spirochetemia episodes persist no longer than 15 days in the animal and could only reach a total of three cycles. [25]
Since symptoms of an infection with *B.duttonii* in humans can appear between 4-18 days it's most likely that the mouse model data translates more accurately to a human, than the research which used gerbil models. Nonetheless having a bigger variety of models, which are compatible with *B.duttonii*, is important for expanding the research areas that can be achieved with this *Borrelia* species.
This study has furthermore confirmed that *B.duttonii* can be more easily detected/counted with a

light microscope when blood samples are prepared via Giemsa staining. In order to detect whether the mice in this study were infected, a serological test was performed which is not always the best diagnostic for testing a patient which might have contracted RF. A Western Blot requires the patient to have built up antibodies against *B.duttonii* to return a positive result. This can take up to fourteen days for the body's immune system to complete. A Giemsa stain would solve this problem as it directly visualizes the spirochetes in the blood of the patient after processing a sample.

Additionally, previous research work from our lab has analyzed the infectivity rate of *O.moubata,* which have fed on inoculated mice after they were housed at different temperatures. The research in that experiment used the same temperatures as the second aim in this study but the results were an inverse of the data that was obtained from the newly acquired information. Ticks which were housed at a higher temperature than the control group were shown to have a lower infectivity rate than ticks which switched from a high temperature back to 27°C in the previously conducted study. [24]

The work performed in this study has shown that ticks which were kept at higher and lower temperatures than the control and the 37°C/27°C group had a higher infectivity rate than the rest. The difference in the research data is due to the discrepancy in the housing time before the ticks were placed at separate temperatures. The previous work, performed by Maximilian Bayer, kept infectious ticks for several months at 27°C before changing the environment for the argasids to different temperatures. These few extra months of housing were sufficient to change the infectivity rate of ticks at higher and lower temperatures.

Since the ticks in this newly performed experiment were kept at 27°C post-feeding for only 1 week before changing housing temperature, the *Borrelia* presumably did not have enough time to stabilize within the tick. This could mean that when the ticks in the new study were placed at a different temperature, the number of spirochetes circulating within the tick were higher than the experiment performed by Maximilian Bayer. The loss of spirochetes during the extreme high and low temperatures were not detrimental to the infection process of the tick if it has fed within 1 week prior to the environmental change. The number of spirochetes in a tick is highest after feeding on an infected mouse, then it will decrease and remain stagnant once the tick has moulted.

The control group in this study was determined to be predominantly negative due to an error

during homogenization of the *O.moubata*, which could have also occurred during the work with the 37°C/27°C group since the infectivity rate is relatively low when compared to group 37°C and 20°C.

If both experiments resulted in accurate data, it can be concluded that the infection process of *B.duttonii* is not affected by temperature changes if the population of the spirochetes are high enough to accommodate the loss of bacteria in the new climate. Once the spirochetes stabilized in the vector, a temperature change would cause a severe amount of loss which would decrease the infectivity rate of the previously contagious ticks. The experiments have shown that *B.duttonii* are very persistent bacteria. This is also reflected in a different research project published by Christer Larsson. [11]

In the experiment, described in Pathobiology of African Relapsing Fever Borrelia, a *B.duttonii* infection was detected in the brain of C57BL/6 mice after the spirochetes were cleared from the blood.

The possibility of infectious *Borrelia* persisting in the mouse brain was investigated in this study to determine whether *B.duttonii* retreated into the cerebrum of any mouse strain such as C3H/HeN and Balb/c or if it was a behaviour, specific related to the C57BL/6 mice. While the experiments did return some positive results, the number of mice negative for infection suggested that it was a rare occurrence for *B.duttonii* spirochetes to leave residual infections in C3H/HeN and Balb/c mouse brains. It is also possible that the requirements for *B.duttonii* to retreat into the cerebrum are not dependent on the mouse strain but rather on other factors that need to be further investigated. Since *B.duttonii* does not have a proper working model it is difficult to translate such results to a natural behaviour of these spirochetes in human bodies.

6 Conclusion

The experiments from our lab have determined that *B.duttonii* can be acquired from a host by a vector for approximately one month following the infection of the reservoir. The exact starting time in which the host is contagious can be as early as two days after an infectious vector transmitted the *B.duttonii* spirochetes to the reservoir.

When an *O.moubata* tick feeds on an infectious reservoir the number of spirochetes ingested are higher than after the vector completes its' moulting process. The temperature experiments have shown that the amount of spirochetes post feeding are sufficient enough to cause an infection in the tick, even after undergoing temperature changes for two weeks. In contrast to those results, the data from Maximilian Bayer has shown that within the time span in which a tick moults, the spirochete population decreases and stabilizes to a lower number which could not survive a strong change in temperature and reverses the infection in the vector.
Previous experiments, from Christer Larsson, suggesting that *B.duttonii* could cause residual infections in the cerebrum of mice were confirmed by some of the experiments performed in our lab, but it seemed to be a rare occurrence. Since *B.duttonii* does not have a working model yet, and its' only natural reservoirs are humans, it is difficult to interpret such results into the natural process of the zoonotic cycle of this spirochete species.

7 Literature

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