The health of hedgehogs in relation to tick infestation and transmission of pathogens by the hedgehog tick, *Ixodes hexagonus in vitro*.

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The image on the front was taken by the author.

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Abstract

Background
Hedgehogs and *Ixodes hexagonus* ticks live in close contact with each other and hedgehogs play a role as reservoir for several pathogens. The hedgehog tick is a vector of a range of different pathogens. This study looks at the possibility of transmission from tick-borne diseases by an *in vitro* method.

Methods
We have submitted questionnaires to hedgehog shelters to find out what clinical symptoms hedgehogs with and without tick infestations show according to hedgehog shelter owners.
I. hexagonus will be fed *in vitro* in order to investigate the possible transmission of pathogens. During this study improvement was trying to be made to the *in vitro* feeding procedure for optimal feeding results of *I. hexagonus* ticks, using *Dermacentor reticulatus* as a control. The original feeding procedure is available at the department of Utrecht University of Tick-borne Diseases. Blood was taken from wells were ticks had fed and this blood was examined by PCR and RLB. Ticks that had fed or died during the *in vitro* feeding were also examined by RLB.

Results
A total of 45 questionnaires were received. These questionnaires showed a variety of clinical symptoms seen in hedgehogs.
Attachment rate in the *in vitro* study was low, only five *I. hexagonus* attached. Several methods were tested and it appeared to be that fresh hair from hosts of *I. hexagonus* stimulate the hedgehog tock the most to feed *in vitro*. Some pathogens were found, such as *Borrelia afzelii* and *Ehrlichia/Anaplasma* catch-all in blood samples and Rickettsia catch-all in an *I. hexagonus* tick. Unfortunately due to contamination *Ehrlichia/Anaplasma* catch-all was false positive.

Conclusions
No definite conclusions could be drawn out of the questionnaires. The questionnaires weld held shortly after hibernation, meaning hedgehogs could be more vulnerable, showing other clinical symptoms than the ones caused by ticks. Beside, questionnaires were filled in based on knowledge by experience, rather than through evidence based papers.
No conclusions could be drawn whether hedgehogs could get sick after infestations by *Ixodes hexagonus*. It is possible for *I. hexagonus* to transmit pathogens using an *in vitro* feeding system, but more research is needed to optimize the *in vitro* feeding assay for *I. hexagonus.*

Introduction

Worldwide, there are nearly 900 species of ticks known. [1] Ticks are important vectors of a wide range of pathogens. This research project focuses on the tick *Ixodes hexagonus*, the *in vitro* feeding of this tick, the pathogens this tick carries with and the possible transmission of these pathogens to hedgehogs, causing the hedgehog to get sick.

Ixodes hexagonus

Ixodes hexagonus, known as the hedgehog tick, belongs to the Arachnida. *I. hexagonus* belongs to the sub-order Ixodida, from the family Ixodidae, often called the hard ticks. With about 250 species, *Ixodes* is the largest genus in this family.

I. hexagonus primarily lives in or near nests, burrows, caves and other shelters used by its host. It is primarily a parasite of hedgehogs, but it is also found on other animals like dogs, cats, foxes and mustelids.[2, 3]

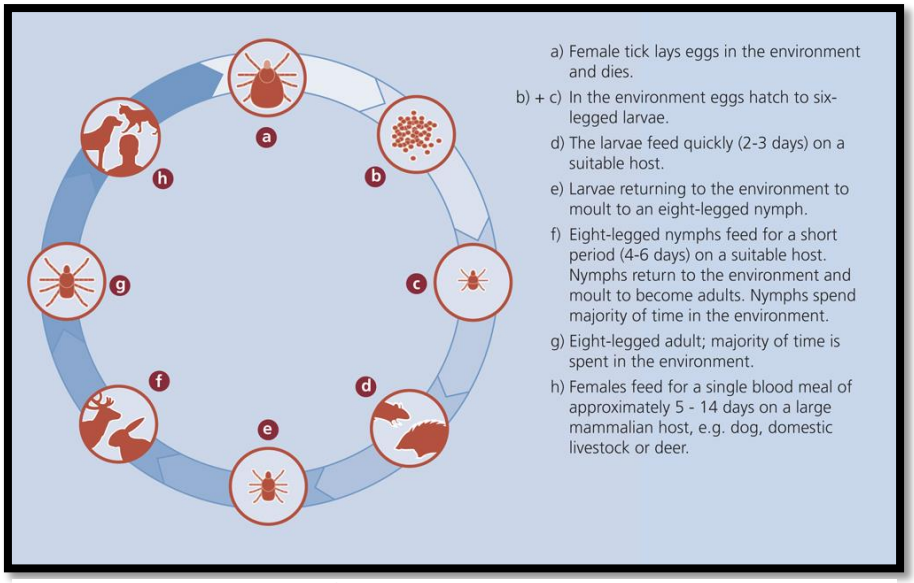

Fig. 1 Life cycle of hard tick [19]

Like all ticks, *I. hexagonus* has four life stages, an embryonic egg stage, a larval stage, a nymphal stage and an adult stage. Soon after emerging into adult stage, the ticks become sexually mature. Female adults can swell greatly during feeding, about 100 times their unfed bodyweight. After dropping on the ground adult females can produce 1000-1500 eggs over a period of 19-25 days, before they die. The hatching of the eggs begins and the emerging larvae hide in vegetation or nests to seek hosts.

I. hexagonus has a three-host life cycle, meaning they will use three hosts from larvae to adult stage. Larvae will attach and feed for 3-7 days on the first host, once fully engorged they drop off and undergoes ecdysis, where the engorged larva molts into an unfed nymph. These nymphs will feed on another host for 3-8 days and then drop off again. After dropping off these nymphs molt into the adult stage and seek a new host. The adults feed on this new host, drops off, the females will lay eggs and the cycle starts over.[4, 5]

This entire life cycle can be completed within one year in a natural environment under favorable conditions. Under laboratory conditions, this life cycle can be completed in 3 or 4 months.

In vitro feeding

To study ticks and the possible pathogen transmission, animal experiments were required. The animals can suffer from these experiments. With the introduction of *in vitro* feeding a lot of these experiments no longer are in the need of animals. From an ethic point of view it makes this method more favorable, thereby the high costs of maintaining suitable hosts are circumvented. Another advantage of this method is the possibility to study pathogen transmission easier through the controlled conditions.[6]

The first *in vitro* feeding started with soft ticks, later also hard ticks were tested. Pierce and Pierce used air cell membranes of an embryonated hen egg in 1956 to feed *Boophilus microplus* larvae.[7] In 1975, Kemp *et al*. fed the same tick species on thin slices of cattle skin.[8] The introduction of silicone membranes for hard ticks was in 1993 by Habdank and Hiepe.[9] This led to further advances as higher attachment rates, engorgement, molting into

different life stages and oviposition. On such a silicone membrane the life cycle of *Amblyomma hebraeum* was completed by Kuhnert *et al*.[10] Kröber and Guerin improved the silicone membrane, making it softer and thinner.[6] The method of Kröber and Guerin has been introduced at the Utrecht Centre for Tick-borne Diseases. This *in vitro* feeding has been successful in *Ixodes ricinus* ticks and is further optimized. [11] In the meantime adjustments were also made in the *in vitro* feeding of other ticks, such as *Dermacentor reticulatus* and *Rhipicephalus* species. In order to successfully feed the ticks, stimuli are needed to induce attachment. In this study several are tested.

Objective of this research

The main objective of this research is to determine the transmission of several pathogens by *Ixodes hexagonus*, causing the hedgehog to get sick. *In vitro* feeding assay will be used, therefore this method can also be improved to induce attachment by *I. hexagonus* on the silicone membranes.

Materials and method

The *in vitro* feeding method used in Utrecht was originally developed by Kröber and Guerin (2007).[6] Since the introduction of this method at UCTD, adjustments were made and a new protocol was developed. During this research new methods were tested due to the poor attachment result with ticks *in vitro*. These new methods are described in this paper.
To obtain information about the possible transmission of pathogens, blood was taken from the wells of a cell culture plate where ticks were attached. This blood was examined by PCR and RLB, but also the attached ticks were examined by PCR and RLB.
All protocols can be found in the appendix.

Questionnaire

Questionnaires were drafted by the author and distributed among seven randomly selected hedgehog sanctuaries in the Netherlands. From hedgehogs brought to these sanctuaries a survey was filled out by the proprietors of the sanctuaries, as these people have the most experience with hedgehogs and are therefore the most reliable source of information. The questionnaire contains general questions about the hedgehog and more specific questions about clinical symptoms. A total of eight different questions were asked and 17 different symptoms were given in which the proprietor could indicate the symptoms to be present or not present and any comments could be given. Questionnaires were filled out for hedgehogs with ticks and hedgehogs without ticks, in order to get a good picture of the symptoms between hedgehogs with and without ticks.
A total of 45 questionnaires are filled out and send to the lab. A copy of this questionnaire is added to appendix A.

Ticks

Ixodes hexagonus were send to the lab from hedgehog sanctuaries in the Netherlands. These ticks were collected from hedgehogs brought to these sanctuaries. *Dermacentor reticulatus* and *Rhipicephalus sanguineus* were derived from the collection available at UCTD.
Because of previously good results with *Dermacentor reticulatus* and due to the low number of *Ixodes hexagonus, D. reticulatus* was used as control and later also as test for improving the *in vitro* method. Only female *I.* hexagonus with a weight below 100 mg were used for *in vitro* feeding. For *in vitro* feeding a total of 93 male and 93 female *Dermacentor reticulatus*, 15 male and 77 female *Ixodes hexagonus*, 5 male and 10 female *Rhipicephalus sanguineus* were used. For odour testing 20 male and 20 female of both *Dermacentor reticulatus* and *Rhipicephalus sanguineus* were used. For reverse line blot hybridization twenty ticks collected from one hedgehog were tested and twenty-four ticks used for *in vitro* feeding were tested. From these twenty-four ticks, five ticks had been attached to the membrane and the remaining ticks had died during *in vitro* feeding.

Blood

Blood was collected weekly from cattle kept at the Department of Farm Animal Health at Utrecht University, Faculty of Veterinary medicine. Blood was collected on the day the feeding started. Two different cows were used, every two weeks the same cow, to minimize the discomfort. These two cows were not treated with any anti-parasitic drugs.
The blood was taken from the jugular vein using a sterile needle, a catheter and a sterile glass bottle. Before puncture, the skin was disinfected with alcohol twice. A total of 200-250mL was collected each time and directly after collecting, the blood was stirred with a sterile 10mL pipette for 15 minutes to defribinate manually. With stirring, a blood clot formed around the glass pipette, whereby this could be removed from the bottle.
When arrived at the laboratory 2 g/L D (+) glucose was added to the blood. Then, the blood was divided over several 50mL Falcon tubes. To reduce contamination, the bottle and the Falcon tubes were sterilized by swiftly taking them through a Bunsen burner flame. The Falcon tubes were labelled and stored at 4°C.

Membranes

A glass plate (40 x 30 cm) was taken and a layer of kitchen plastic film was spread over the plate and fixed with tape to prevent wrinkles in the film. Eight lens cleaning papers (70 x 120 mm) were divided on the plastic film and fixed with tape on the top.
A mixture was made containing 15 grams of E4 silicone glue, 4,5 grams of silicone oil, 2,9 grams of hexane (15%) and 0,15 grams of white paste. The white paste is used to enhance visibility. These amounts are sufficient for eight cleaning papers.
The mixture was spread over the cleaning papers using a hand applicator (80 mm wide). The applicator was gently moved down the papers to ensure the membranes have a constant thickness. A picture of the membranes on the glass plate and the applicator is shown in figure 2 and 3.
The membranes were then left to dry for at least 24 hours at room temperature.

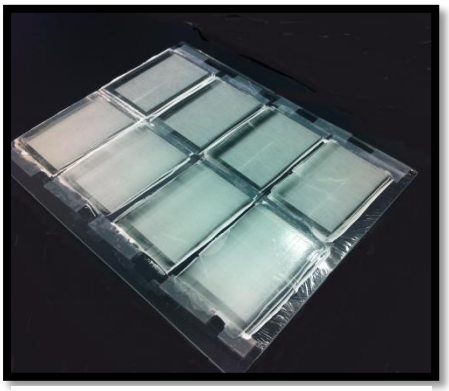
Fig. 2 Membranes on glass plate


Fig. 3 Hand applicator

The test feeding and feeding 1, 2, 3, 4 and 5 were performed with the membranes described above. Due to poor results, we developed a new membrane method. Everything remains the same until the step where the membranes were left to dry for 24 hours. Directly after spreading the silicone mixture over the cleaning papers, some hairs were gently applied on the membrane using forceps. Then the membranes were left to dry for 24 hours, so the hairs are secured onto the membranes.
The membranes with the hairs attached onto it were used in feeding 5, 6, 7, 8 and 9.

Feeding units

Feeding units consist of a Plexiglas tube with a height of 45 mm, a diameter of 26 mm and a wall thickness of 2 mm. An acrylic glass ring was constructed around it at 14 mm from one side. These units fit well in a six-well culture plate, without touching the bottom of the well.
The units were glued to the silicone membranes on the glass plate using E4 silicone glue. The side closest to the acrylic glass ring faced the membrane. To prevent glue on the inside of the unit, it was removed using a small paintbrush. The units were left to dry for at least three hours at room temperature.
After three hours, the cleaning paper with the units was cut loose from the glass plate using a scalpel. After removing the plastic film, the membranes were measured at different places using a micro calipers. The proper thickness of the membrane was between 70 and 110 µm, to ensure the hypostome of *Ixodes hexagonus* can penetrate the membrane. The thickness of the membrane was marked on the feeding unit.
Before using the units, they were checked for leakages by placing them in a petri dish with demineralized water for 15 minutes. Units which leaked were not used.
In feeding 1, 2 and 3 it was discovered all ticks preferred to crawl to the top. Due to this result the feeding method was reversed. Feeding units were shortened and now had a height of 31 mm, so that the acrylic glass ring was on the top of the unit. The membranes were glued onto to unit so that the side closest to the ring faced the membrane. Using a small paintbrush, glue was removed from the inside of the unit. These units were also left to dry for at least three hours at room temperature. Measuring the thickness of the membrane remains the same. This method was used in feeding 4 and 5.

Bonbons

To prevent the tick from escaping, the feeding units were closed with a 'bonbon'. This was made of a perforated stopper were the edges were cut off. The stopper was wrapped around with organza and this was fixed using a metal wire.
The bonbon was placed approximately 0,5 cm from the membrane, to encourage feeding and prevent the ticks from wandering.

Attachment stimuli and odour test

For retrieving an optimal attachment of the ticks to the membrane, different attachment stimuli were used. To find out which stimuli were best for *Ixodes hexagonus,* odour tests were performed. For these odour tests hair from a cow, dog and rabbit were collected. After dividing these hairs over four jars, respectively 500 mL of hexane, ethanol (70%), acetone and DCM was added. Because of the little amount of dog hair, 100 mL of each dissolvent was added.
Silicone membranes were made according to the method described above. Instead of sticking feeding units onto the membrane, the membranes were cut to use them for the odour test. For the odour test a translucent plastic (polypropylene) container was used. Two pieces of membrane were placed in the container, one on the left side and one on the right side. The pieces on the right side was the control group, this was the membrane with no perfume. The pieces on the left were sprayed with respectively cow, dog or rabbit perfume. Within these three groups four tests were done with two different tick species. Ticks were left in the container for three hours to make sure the ticks had enough time to pick a side.

Water bath

The *in vitro* feeding took place in a water bath filled with distilled water. The temperature of the water was kept at 37 degrees Celsius to mimic the body temperature of the host. Humidity was kept between 70-90%. The feeding units inside the six or twelve wells plate were placed inside the water bath and then the water bath was half closed with a triangular formed lid and covered with a black cloth to keep the ticks in dark 24 hours a day.

In vitro feeding procedure

The blood was mixed by gently swerving the Falcon tube. A sterile six wells plate was taken and the four outer wells were filled with 3,1 milliliter blood. The six wells plate was closed with a lid and then gently placed in the water bath for 15 minutes.
The ticks were placed on the membrane in the feeding unit. Four units were used per six wells plate. The units were closed slowly with the bonbon to prevent ticks to get stuck between the unit and the bonbon. If a tick got stuck, the bonbon was pulled up and then put down slowly again. The units with the ticks were placed in the pre-warmed blood, first one side and then roll off to the other side. Then the units were checked for air bubbles below the membrane. If there were bubbles the unit was removed and put back in.
The plate was placed in the water bath where it could float.

Fig. 4 The completely assembled feeding units as used in the water bath

Twice a day when the blood in the six wells plate was refreshed, the ticks can be checked for attachment and death at the same time. The procedure used for refreshing the blood is the same as described above. Before placing the units in the fresh blood, the bottom of the units are washed off with PBS (phosphate-buffered solution).

In the new method, ticks were placed into the six well plate and the units were filled with 2 mL blood. For feeding 5 a twelve well plate was used. Twice a day 1 mL blood was removed from the units and 1 mL fresh (preheated) blood was added.
When there was an attachment of an *Ixodes hexagonus*, a sample was taken from that well (1mL) and pipetted into a 1,5mL tube, labelled and stored in the freezer at -24°C. After refreshing the blood, the six wells plates were put back in the aquarium.
After one week of feeding, the ticks were removed from the units. *Ixodes hexagonus* ticks that were attached to the membrane or had been attached were labelled and stored in 70% ethanol at 4°C to examine later for possible pathogens. A total of 29 blood samples were taken and examined by means of PCR and RLB.
Protocols of the *in vitro* feeding procedure are included to appendix B.

DNA extraction

A total of six attached *Ixodes hexagonus* ticks were examined. Randomly picked ticks which were send to the lab, but did not had the proper weight to be used for *in* vitro feeding were also examined. Eighteen *I. hexagonus* had died and were therefore also examined.
Using the Nucleospin Tissue Kit (Machery-Nagel), DNA extraction was performed on these ticks. The DNA obtained was then amplified using PCR to make sure enough DNA was available for RLB hybridization.
The protocol of the Nucleospin Tissue Kit can be found in appendix B.

PCR

PCR stands for polymerase chain reaction. The origin of this method, which is used in modern techniques, was contrived by Kary Mullis in 1983. His colleagues developed this technique further to the successful application as we know it today.[12]
This technology was used to amplify specific selected strands of DNA, to make them more sensitive and specific for RLB outcome. The method contained a few steps which rely on thermal cycling, cycles of repeated heating and cooling off. At first the DNA sample was added to a mastermix, which contains a primer set water, 5x Phire reaction buffer, 10 mM dNTPs and 2 U/µL Phire hot start II DNA polymerase. This was put in an Eppendorf tube and then heated up to 95°C to split the DNA in two strands, which is called denaturation (step 1 in figure 5 below). The temperature was then set to 50-70°C, to provide the added primers to attach. The temperature was then set to 68-72°C to anneal the primers. These steps are repeated several times, resulting in many copies of the DNA sample.

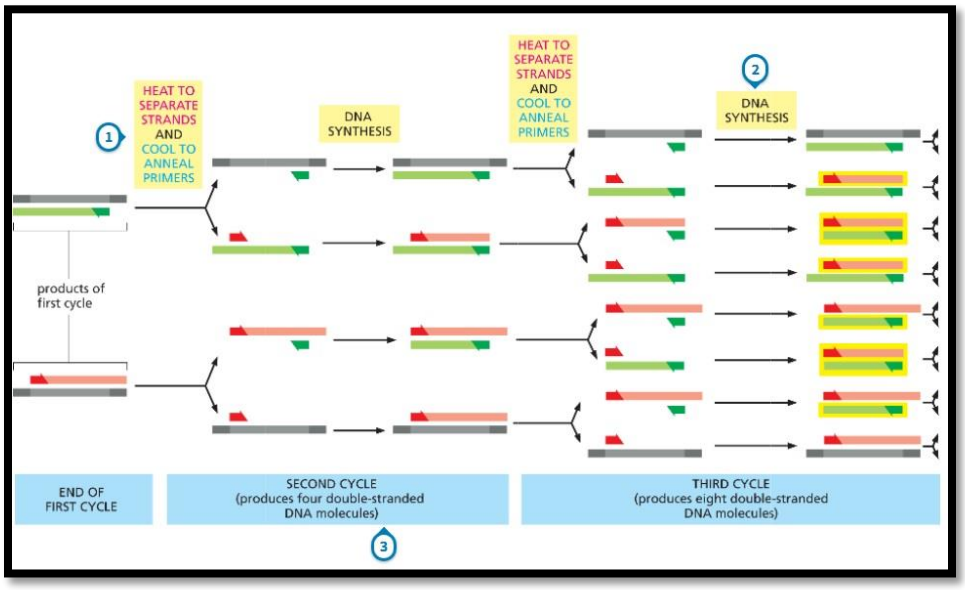

Fig. 5 Illustration of PCR[18]

Ixodes hexagonus is known as vector for many pathogens, such as *Theileria annae*, *Anaplasma phagocytophilum* and *Rickettsia helvetica*[3, 13] So primers used in this research are *Ehrlichia* spp., *Anaplasma* spp., *Theileria* spp., *Rickettsia helvetica*, *Babesia* spp. and *Borrelia* spp.

Reverse Line Blot hybridization

After performing PCR, reverse line blot hybridization was the next step. In the RLB process, PCR products were added to a membrane containing covalently bound oligonucleotide probes. These probes consist of a reverse nucleotide sequence from specific pathogens, and are bound to the membrane by the '5 terminal C6-aminolinker. Because these probes contain a reverse sequence, consequently, DNA strands with the exact opposite nucleotide sequences will attach. Therefor if the DNA strands do not match, there will be no binding. The DNA amplified from PCR is labelled with a biotin, which has the possibility to illuminate. In order to illuminate the biotin, a chemo luminescent reagent and streptavidin-peroxidase were added to the membrane. Streptavidin-peroxidase will attach itself to the biotin label with a high affinity, and the chemo luminescent reagent will cause a reaction with peroxidase. In this reaction, luminol, a substance of the chemo luminescent reagent, becomes oxidized and produces light. The membrane is placed in a lightproof cassette, containing a light sensitive film, for ten minutes so that the film can absorb enough light. After this the film is developed and printed, whereby the results can be interpreted.

Different probes are used in this research: *Ehrlichia/Anaplasma* catch-all, *Anaplasma centrale, A. marginale,* 4 types of *A. phagocytophilum, A. bovis, A. platys, Ehrlichia canis, E. chaffeenis, E. ruminantium, E. sp omatjenne, Theileria/Babesia* catch-all, 2 types of *Babesia* catch-all, *B. felis, B. divergens, B. microti, B. bigemina, B. bovis, B. rossi,* 2 types of *B. canis, B. vogeli, B. major,* 2 types of *B. caballi, B. venatorum sp EU1, Theileria equi, Theileria equi-like, Borrelia burgdorferi sensu stricto, B. garinii, B. afzelii, B. valaisiana, Rickettsia* catch-all, *Rickettsia conorii, R. helvetica, R. massiliae* and *Rickettsia raoultii.*

Protocols of the PCR and RLB methods are added to appendix B.

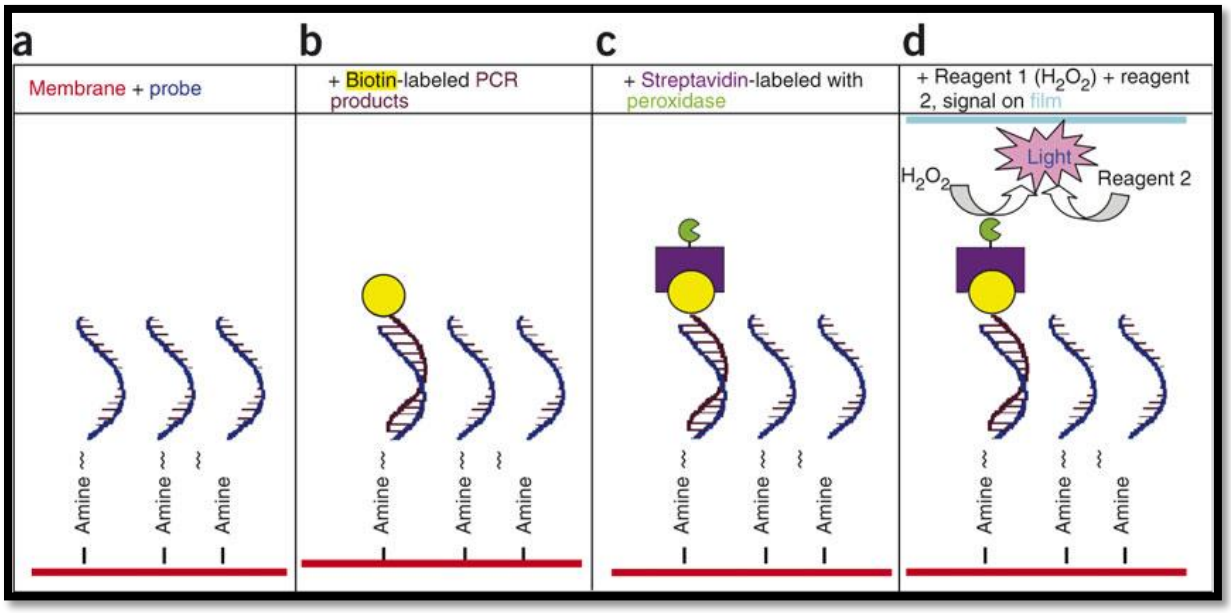

Fig. 6 Schematic illustration of RLB hybridization[14]

Results

Every week new feedings were started. For each week all results of the experiments are described below. In week 3 we also performed a perfume test, the results are described in that week.
U stands for feeding unit.

Week 1

This week a test feeding with *Dermacentor reticulatus* from the lab colony was performed. Units with a membrane thickness of 86 µm, each filled with five males and five females were used. No attachment stimuli were used. At the end there was a mortality rate of 0% and an attachment rate of 0% in all units. It appeared the ticks were too young to feed, because they produced white excrete from the tubes of Malpighi.

U1: *Dermacentor Reticulatus* (5♂ + 5♀)
U2: *Dermacentor Reticulatus* (5♂ + 5♀)
U3: *Dermacentor Reticulatus* (5♂ + 5♀)
U4: *Dermacentor Reticulatus* (5♂ + 5♀)

Test feeding Dermacentor reticulatus

Unit 1		
Time	Attachment (%)	Death (%)
17:05	0	0
23:58	0	0
41:07	0	0
47:50	0	0

Unit 2		
Time	Attachment (%)	Death (%)
17:05	0	0
23:58	0	70
41:07	0	20
47:50	0	0

Unit 3		
Time	Attachment (%)	Death (%)
17:05	0	0
23:58	0	20
41:07	0	0
47:50	0	0

Unit 4		
Time	Attachment (%)	Death (%)
17:05	0	0
23:58	0	30
41:07	0	10
47:50	0	0

Week 2

This week the first feeding with *Ixodes hexagonus* was performed. These *I. hexagonus* were derived from hedgehog sanctuaries. The first unit held one adult male and five adult female *I. hexagonus* and the other three units held five adult female *I. hexagonus.* As attachment stimuli cow hair was used in all four units. All units had a thickness of 87 µm. Glucose was not added to the blood before using it for the *in vitro* feeding, this may have affected the results.
After 66 hours the male in unit 1 died. In unit 3 one female attached after 42 hours, but she released after 66 hours. A blood sample was taken from unit 3 at 42 and 49 hours for PCR and RLB.
In unit 4, one female also attached after 17 hours and stayed attached until 66 hours. Every time blood was refreshed, and the female was attached, a blood sample was taken from this unit. After 66 hours, the blood in this unit was discolored.
Overall experience, ticks are mostly clustered and found on the bonbon instead of on the membrane.

Fig. 7 Discoloring of the blood in the well right below

U1: *Ixodes hexagonus* (1♂ + 5♀)
U2: *Ixodes hexagonus* (5♀)
U3: *Ixodes hexagonus* (5♀)
U4: *Ixodes hexagonus* (5♀)

Feeding 1 adult Ixodes hexagonus

Unit 1		
Time	Attachment (%)	Death (%)
17:24	0	0
25:09	0	0
42:14	0	0
49:18	0	0
66:07	0	17
73:47	0	17
89:51	0	17
96:20	0	17

Unit 2		
Time	Attachment (%)	Death (%)
17:24	0	0
25:09	0	0
42:14	0	0
49:18	0	0
66:07	0	0
73:47	0	0
89:51	0	0
96:20	0	0

Unit 3		
Time	Attachment (%)	Death (%)
17:24	0	0
25:09	0	0
42:14	20	20
49:18	20	0
66:07	0	20
73:47	0	0
89:51	0	20
96:20	0	20

Unit 4		
Time	Attachment (%)	Death (%)
17:24	20	0
25:09	20	0
42:14	20	0
49:18	20	0
66:07	20	0
73:47	0	0
89:51	0	0
96:20	0	0

Week 3

This week four units were used, the first two held four male and four female *D. reticulatus* which were partially fed on a cow. The last two both held five female *I. hexagonus* which were used for the feeding in week 2. All units had a membrane thickness of 102 µm and as attachment stimuli cow hair and tick feces derived from the cow where the *D. reticulatus* had fed were used.
Attachment in the *D. reticulatus* units increased a lot, after 40 hours one female in unit 1 and one female in unit 2 were attached. After 47 hours, one female and one male in unit 1 was attached, but the female in unit 2 detached. After 64 hours, two males and one female in unit 1 was attached and three males in unit 3 were attached. No attachment was seen in the *I. hexagonus* units, and again, these ticks were mostly found on the bonbon.
No mortality occurred in all units.

U1: *Dermacentor reticulatus* (4♂ + 4♀)
U2: *Dermacentor reticulatus* (4♂ + 4♀)
U3: *Ixodes hexagonus* (5♀)
U4: *Ixodes hexagonus* (5♀)

Feeding 2 Dermacentor reticulatus and *Ixodes hexagonus*

Unit 1		
Time	Attachment (%)	Death (%)
16:52	0	0
23:44	0	0
40:39	13	0
47:15	26	0
64:14	38	0

Unit 2		
Time	Attachment (%)	Death (%)
16:52	0	0
23:44	0	0
40:39	13	0
47:15	0	0
65:14	38	0

Unit 3		
Time	Attachment (%)	Death (%)
16:52	0	0
23:44	0	0
40:39	0	0
47:15	0	0
64:14	0	0

Unit 4		
Time	Attachment (%)	Death (%)
16:52	0	0
23:44	0	0
40:39	0	0
47:15	0	0
65:14	0	0

During this week, an odour test with *Dermacentor reticulatus* and *Rhipicephalus sanguineus* was performed, both derived from the lab. Cow perfume made out of cow hair with ethanol (70%), hexane, DCM and acetone was used. The membranes placed on the left side of the container contained perfume, membranes on the right side were used as control. The ticks were placed in the middle of the container and left in there for three hours, to make sure they had enough time to pick a side.
Unfortunately because the container consisted out of one area, the cow smell was spread over the entire space in the container, so the ticks were also spread out over the container. *D. reticulatus* was found everywhere in the container, whereas *R. sanguineus* was mostly found between the lid and on the sides of the container. The spreading of the smell made any result unreliable, so no odour tests were performed after this one.
Below are some pictures of the odour test, all pictures of this test are added to the Appendix.

Fig. 10 *D. ret* at start: 0 hours

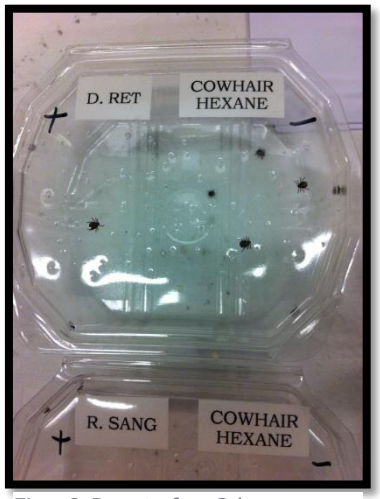

Fig. 8 *D. ret* after 3 hours

Fig. 9 *R. sang* after 3 hours

Week 4

This week four units were used. This week there was little *I. hexagonus* for *in vitro* feeding, so *Rhipicephalus sanguineus* from the lab colony was also used, this colony originally came from Greece. The first unit held five female *Ixodes hexagonus*, the second unit held fifteen male *D. reticulatus* which were partially fed on the same cow as in feeding 2. Unit 3 held fifteen female *D. reticulatus* from the lab and the fourth unit held ten female and five male *R. sanguineus.*
All membranes had a thickness of 81 µm and cow hair and cow perfume were used as attachment stimuli. After 17 hours four males in unit 2 and one female in unit 3 were attached. After 23 hours only one male in unit 2 and one female in unit 4 were attached. Unit 2 did contain a lot of feces, which was produced by the ticks. After 41 hours unit 2 had one male attached and unit 3 had 3 females attached, and after 47 hours unit 2 had two males attached, whereas unit 3 still had three females attached. No attachment was seen in unit 1 and 4, and no mortality occurred in all units.

U1: *Ixodes hexagonus* (5♀)
U2: *Dermacentor reticulatus* (15♂)
U3: *Dermacentor reticulatus* (15♀)
U4: *Rhipicephalus sanguineus* (10♀ and 5♂)

Feeding 3 Ixodes hexagonus, Dermacentor reticulatus and Rhipicephalus sanguineus.

Unit 1		
Time	Attachment (%)	Death (%)
17:07	0	0
23:23	0	0
41:04	0	0
47:07	0	0

Unit 2		
Time	Attachment (%)	Death (%)
17:07	27	0
23:23	7	0
41:04	7	0
47:07	13	0

Unit 3		
Time	Attachment (%)	Death (%)
17:07	7	0
23:23	7	0
41:04	20	0
47:07	20	0

Unit 4		
Time	Attachment (%)	Death (%)
17:07	0	0
23:23	0	0
41:04	0	0
47:07	0	0

Week 5

This week there was no *I. hexagonus* available for *in vitro* feeding, so *D. reticulatus* from the lab colony was used to test a new method. In previous feedings *Ixodes hexagonus* was mostly found on the bonbon in the feeding unit. With this in mind the system was turned upside-down, so the ticks can find blood on the top of the unit. This week the old and new method were performed parallel with *D. reticulatus.* A total of ten units were used, six of them were with the new method. In all units cow perfume (ethanol 70%) was used as attachment stimuli. Unit 1 to unit 4 were performed by the old method, unit 5 to unit 10 were performed with the new method, whereof unit 9 and 10 had a grid glued onto the membrane to make sure the ticks had support walking upside-down. Before sticking the grid onto the membrane, it was treated in a layer of cow perfume (ethanol 70%) for 5 minutes and then left to dry for about 15 minutes. This way cow smell could withdraw in the grid.

Fig. 11 System upside down, female attached to membrane, other ticks mostly on ring of the unit.

After 6 hours one female was attached in unit 9. The ticks in the remaining units from the new method seemed inactive, as they were mostly found on the bottom of the six wells plate or on the ring from the feeding unit, whereas in the units of the old method, the ticks were very active and mostly found on the bonbon and moving around. No attachment is seen in other units.
After 23 and 30 hours the female in unit 9 was still attached, but after 47 hours she detached. In the remaining units no attachment was seen in the entire week. No mortality also occurred.

U1: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 81 µm.
U2: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 82 µm.
U3: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 79 µm.
U4: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 82 µm.
U5: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 76 µm.
U6: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 92 µm.
U7: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 82 µm.
U8: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 76 µm.
U9: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 71 µm with grid.
U10: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 71 µm with grid.

Feeding 4 Dermacentor reticulatus

Unit 1		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 2		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 3		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 4		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 5		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 6		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 7		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 8		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Unit 9		
Time	Attachment (%)	Death (%)
5:59	10	0
23:24	10	0
30:17	10	0
47:13	0	0

Unit 10		
Time	Attachment (%)	Death (%)
5:59	0	0
23:24	0	0
30:17	0	0
47:13	0	0

Week 6

This week testing the new method was continued, parallel to the old method. This week some *I. hexagonus* from hedgehog sanctuaries were derived, but also *I. hexagonus* from the lab colony was used. The new membranes were tested this week, because it appeared that perfumes made from animal hair were not very successful. However when fresh animal hair was used, the ticks seem more interested in the membrane. A total of eight units were used, whereof four units were used for the old method, two of them held the new membrane and four units were used for the new method with the new membrane.
As attachment stimuli hedgehog hair and spines were used for unit 2 and 4, and the remaining unit had cow hair stuck on the membrane as attachment stimuli.
Ticks were mostly found on the ring of the feeding unit in the last feeding, so this time a twelve well plate was used. This plate has smaller wells, so the membrane, excluding the ring, covers the well. Unfortunately due to the size of the feeding unit and the size of the wells of the plate, ticks could escape out of the plate. This happened in unit 5, 6 and 8, because of the size of the ticks. Trying to fix the units onto the plate with an iron wire did not help. So after 24 hours, the units and ticks from the new method were transferred to a six well plate.
After 16 hours, one female in unit 1, two females in unit 2, two females in unit 5 and one female in unit 8 were attached. At this point unit 3 had a leak, so this unit was excluded from the feeding. Fortunately two males and one female had attached in this unit.
After 24 hours, two males in unit 1, three females in unit 2, two females and one male in unit 5 and one female in unit 6 were attached. One female in unit 7 had died.
After 40 hours, three males in unit 1, three females in unit 2 and one female in unit 5 were attached. Unit 8 had discoloring of the blood and one male had escaped. At this point the ticks and the feeding units were transferred to a six wells plate. Unit 8 had discoloring of the blood.
After 46 hours, two males attached in unit 1, two females attached in unit 2 and in both unit 5 and 6 one male had attached. In unit 1 there was discoloring of the blood.

Fig. 12 Picture of *I. hexagonus* attached to the silicone membrane

U1: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 81 µm, new membrane.
U2: *Ixodes hexagonus* from lab (5♂ + 5♀) and a membrane thickness of 87 µm.
U3: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 81 µm, new membrane.
U4: *Ixodes hexagonus* (5♀) and a membrane thickness of 88 µm.
U5: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 70 µm, new membrane.
U6: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 87 µm, new membrane.
U7: *Ixodes hexagonus* (4♀) and a membrane thickness of 87 µm, new membrane.
U8: *Ixodes hexagonus* lab (5♂ + 5♀) and a membrane thickness of 87 µm, new membrane.

Feeding 5 Ixodes hexagonus and Dermacentor reticulatus

Unit 1		
Time	Attachment (%)	Death (%)
16:35	10	0
24:02	20	0
40:32	30	0
46:00	20	0

Unit 2		
Time	Attachment (%)	Death (%)
16:35	20	0
24:02	30	0
40:32	30	0
46:00	20	0

Unit 3		
Time	Attachment (%)	Death (%)
16:35	30 Leakage!	
24:02		
40:32		
46:00		

Unit 4		
Time	Attachment (%)	Death (%)
16:35	0	0
24:02	0	0
40:32	0	0
46:00	0	0

Unit 5		
Time	Attachment (%)	Death (%)
16:35	20	0
24:02	30	0
40:32	20	0
46:00	20	0

Unit 6		
Time	Attachment (%)	Death (%)
16:35	10	0
24:02	10	0
40:32	10	0
46:00	0	0

Unit 7		
Time	Attachment (%)	Death (%)
16:35	0	25
24:02	0	25
40:32	0	25
46:00	0	0

Unit 8		
Time	Attachment (%)	Death (%)
16:35	0	0
24:02	0	0
40:32	0	0
46:00	0	0

Week 7

Due to the success of the membrane with the last feeding, this testing was continued. This week a lot of *I. hexagonus* from hedgehog sanctuaries were received, so more units with *I. hexagonus* are deployed. Eight units were used, whereof each of the first four had five female *I. hexagonus*. Unit 5 and 6 held five male and five female *I. hexagonus* derived from the lab colony and unit 7 and 8 had five male and five female *D. reticulatus.* For unit 1 to 6 hedgehog hair and spines were used as attachment stimuli, and for unit 7 and 8 the new membrane with cow hairs were used.

After 17 hours two male and one female in unit 7 and five male and three female in units 8 had attached. After 24 hours one female in unit 8 also attached, beside the other ticks that were already attached. In unit 7 nothing changed, meaning 3 ticks were still attached. After 41 hours one male attached in unit 7, beside the other ticks that were already attached and in unit 8 another female attached beside the other ticks. The blood in unit 7 was also discolored at this point. After 48 hours, three male and one female in unit 7 and 5 male and 2 female in unit 8 were attached. This stayed this way until the ending of the feeding at 70 hours.

All *I. hexagonus* were mostly found on the bonbon. The blood in unit 2, 7 and 8 was discolored at 64 hours, and after 70 hours one female died in both unit 1 and 4.

U1: *Ixodes hexagonus* (5♀) and a membrane thickness of 89 µm.
U2: *Ixodes hexagonus* (5♀) and a membrane thickness of 88 µm.
U3: *Ixodes hexagonus* (5♀) and a membrane thickness of 82 µm.
U4: *Ixodes hexagonus* (5♀) and a membrane thickness of 95 µm.
U5: *Ixodes hexagonus* lab (5♂ + 5♀) and a membrane thickness of 82 µm.
U6: *Ixodes hexagonus* lab (5♂ + 5♀) and a membrane thickness of 89 µm.
U7: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 82 µm, new membrane.
U8: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 82 µm, new membrane.

Feeding 6 Ixodes hexagonus and Dermacentor reticulatus

Unit 1		
Time	Attachment (%)	Death (%)
17:34	0	0
24:18	0	0
41:23	0	0
47:56	0	0
64:35	0	0
69:54	0	20

Unit 2		
Time	Attachment (%)	Death (%)
17:34	0	0
24:18	0	0
41:23	0	0
47:56	0	0
64:35	0	0
69:54	0	0

Unit 3		
Time	Attachment (%)	Death (%)
17:34	0	0
24:18	0	0
41:23	0	0
47:56	0	0
64:35	0	0
69:54	0	0

Unit 4		
Time	Attachment (%)	Death (%)
17:34	0	0
24:18	0	0
41:23	0	0
47:56	0	0
64:35	0	0
69:54	0	20

Unit 5		
Time	Attachment (%)	Death (%)
17:34	0	0
24:18	0	0
41:23	0	0
47:56	0	0
64:35	0	0
69:54	0	0

Unit 6		
Time	Attachment (%)	Death (%)
17:34	0	0
24:18	0	0
41:23	0	0
47:56	0	0
64:35	0	0
69:54	0	0

Unit 7		
Time	Attachment (%)	Death (%)
17:34	30	0
24:18	30	0
41:23	40	0
47:56	40	0
64:35	40	0
69:54	40	0

Unit 8		
Time	Attachment (%)	Death (%)
17:34	70	0
24:18	80	0
41:23	90	0
47:56	70	0
64:35	70	0
69:54	70	0

Week 8

This week some unfed *I. hexagonus* from a hedgehog sanctuary was received, beside some partially fed *I. hexagonus*. Due to the success of the new membrane, the same membrane was made for *I. hexagonus,* using rabbit hair. The reason rabbit hair was chosen, was because of the fact that when *I. hexagonus* of the lab colony was fed on animals, rabbits were used as host.
Eight units were used, four of them held *I. hexagonus* and the other four held *D. reticulatus*. The first two units of *I. hexagonus* held three females, the third unit held five male and five female *I. hexagonus* from the lab colony, and the fourth held the unfed *I. hexagonus,* four male and four female. Unit 5 to 8 each held five male and five female *Dermacentor reticulatus* from the lab colony.
After 18 hours there was a lot of attachment, in unit 1, 2 and 3 one female *I. hexagonus* attached and in unit 4 two female *I. hexagonus* were attached. Samples were taken from unit 1 to 4. Unit 6 and unit 7 also had one female attached. Unfortunately, unit 5 got a leak, so this unit was excluded for the rest of the week. This unit did have one female and two males attached.

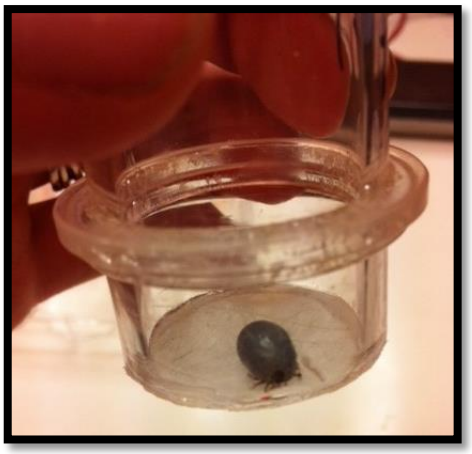

Fig. 13 *Ixodes hexagonus* attached to the new membrane with rabbit hair

After 24 hours nothing changed in unit 1 to 4, this time a blood sample was taken. The female in unit 7 detached, but in unit 6 the female was still attached and one male had attached.
After 41 hours there was still one female attached in unit 1 to 3, and in unit 4 one female detached, leaving one attached. A blood sample was taken again. The female in unit 6 detached and in unit 8 there were 2 male *D. reticulatus* attached. Blood was discolored in every unit.
After 48 hours one female was attached in unit 2 and 3, one female and one male was attached in unit 4, two females were attached in unit 7 and one male was attached in unit 6 and 8. A sample was taken from unit 2 to 4.
After 65 hours, at the end of the feeding, unit 2 and 4 had one female attached and one male and 3 female died in unit 4. A blood sample was taken and the deceased ticks were stored in alcohol to examine for possible pathogens. Unit 6 and 7 each had one male and one female *D. reticulatus* attached and unit 8 had 3 male *D. reticulatus* attached.

U1: *Ixodes hexagonus* (3♀) and a membrane thickness of 70 µm, new membrane, rabbit hair.
U2: *Ixodes hexagonus* (3♀) and a membrane thickness of 71 µm, new membrane, rabbit hair.
U3: *Ixodes hexagonus* lab (5♂+ 5♀) and a membrane thickness of 82 µm, new membrane, rabbit hair.
U4: *Ixodes hexagonus* (4♂+ 4♀) and a membrane thickness of 87 µm, new membrane, rabbit hair.
U5: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 87 µm, new membrane, cow hair.
U6: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 87 µm, new membrane, cow hair.
U7: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 91 µm, new membrane, cow hair.
U8: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 92 µm, new membrane, cow hair.

Feeding 7 Ixodes hexagonus and Dermacentor reticulatus

	Unit 1	
Time	Attachment (%)	Death (%)
17:50	33	0
24:05	33	0
41:41	33	0
47:55	0	0
65:18	0	0

	Unit 2	
Time	Attachment (%)	Death (%)
17:50	33	0
24:05	33	0
41:41	33	0
47:55	33	0
65:18	33	0

Unit 3		
Time	Attachment (%)	Death (%)
17:50	10	0
24:05	10	0
41:41	10	0
47:55	10	0
65:18	0	0

Unit 4		
Time	Attachment (%)	Death (%)
17:50	25	0
24:05	25	0
41:41	13	0
47:55	25	0
65:18	13	50

Unit 5		
Time	Attachment (%)	Death (%)
17:50	30 Leakage!	0
24:05		
41:41		
47:55		
65:18		

Unit 6		
Time	Attachment (%)	Death (%)
17:50	10	0
24:05	20	0
41:41	0	0
47:55	10	0
65:18	20	0

Unit 8		
Time	Attachment (%)	Death (%)
17:50	0	0
24:05	0	0
41:41	20	0
47:55	10	0
65:18	30	0

Unit 7		
Time	Attachment (%)	Death (%)
17:50	10	0
24:05	0	0
41:41	0	0
47:55	20	0
65:18	20	0

Week 9

This week the feeding with the new membranes continued. Again 8 units were used, unit 1 to 3 each held 4 female *I. hexagonus*, derived from hedgehog sanctuaries, but these were already used in a previous feeding, unit 4 held five male and five female *I. hexagonus* from the lab and unit 5 to 8 each held five male and five female *D. reticulatus* from the lab.

After 17 hours no ticks were attached in unit 1 to 4, one male and three females were attached in unit 5, one male was attached in unit 6 and 7 and one male and one female were attached in unit 8. The blood in unit 5 was discolored. After 24 hours nothing changed, except in unit 5, two males and two females were attached. In unit 6 the blood was discolored.

After 41 hours three males and one female were attached in unit 5 and 7, one male in unit 6 and in unit 8 three males and two females were attached. After 48 hours two females died in unit 2. In unit 5 and 8 three male and one female *D. reticulatus* was attached and in unit 6 one male attached. Unit 7 had a leak, so this unit was excluded for the rest of the week, however two males were attached in this unit.

After 65 hours the ticks in unit 2 and 3 were still dead. Nothing changed in unit 5, two males were attached in unit 6 and three males and three females were attached in unit 8. After 71 hours, nothing changed in unit 2, 3 and 6, in unit 5 four males and four females were attached and in unit 8 four males and three females were attached.

After 89 hours, the feeding was ended. In unit 2 and 3 respectively two and one female were dead. Four males and four females were attached in unit 5, one male and one female were attached in unit 6 and four males and two females were attached in unit 8.

U1: *Ixodes hexagonus* (4♀) and a membrane thickness of 79 µm, new membrane, rabbit hair.
U2: *Ixodes hexagonus* (4♀) and a membrane thickness of 90 µm, new membrane, rabbit hair.
U3: *Ixodes hexagonus* (4♀) and a membrane thickness of 82 µm, new membrane, rabbit hair.
U4: *Ixodes hexagonus* (5♂+ 5♀) and a membrane thickness of 81 µm, new membrane, rabbit hair.
U5: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 97 µm, new membrane, cow hair.
U6: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 67 µm, new membrane, cow hair.
U7: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 76 µm, new membrane, cow hair.
U8: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 94 µm, new membrane, cow hair.

Feeding 8 Ixodes hexagonus and Dermacentor reticulatus

Unit 1		
Time	Attachment (%)	Death (%)
17:00	0	0
24:15	0	0
41:13	0	0
48:11	0	0
65:20	0	0
71:29	0	0
88:45	0	0

Unit 2		
Time	Attachment (%)	Death (%)
17:00	0	0
24:15	0	0
41:13	0	0
48:11	0	50
65:20	0	50
71:29	0	50
88:45	0	50

Unit 3		
Time	Attachment (%)	Death (%)
17:00	0	0
24:15	0	0
41:13	0	0
48:11	0	0
65:20	0	25
71:29	0	25
88:45	0	25

Unit 4		
Time	Attachment (%)	Death (%)
17:00	0	0
24:15	0	0
41:13	0	0
48:11	0	0
65:20	0	0
71:29	0	0
88:45	0	0

Unit 5		
Time	Attachment (%)	Death (%)
17:00	40	0
24:15	40	0
41:13	40	0
48:11	40	0
65:20	40	0
71:29	80	0
88:45	80	0

Unit 6		
Time	Attachment (%)	Death (%)
17:00	10	0
24:15	10	0
41:13	10	0
48:11	10	0
65:20	20	0
71:29	20	0
88:45	10	0

Unit 8		
Time	Attachment (%)	Death (%)

Unit 7		
Time	Attachment (%)	Death (%)
17:00	10	0
24:15	10	0
41:13	40	0
48:11	20 Leakage!	0
65:20		
71:29		
88:45		

17:00	20	0
24:15	20	0
41:13	50	0
48:11	40	0
65:20	60	0
71:29	70	0
88:45	60	0

Week 10

This week was the last week an *in vitro* feeding was performed. Again eight units were used with *D. reticulatus* and *I. hexagonus.* Unit 1 to 3 each held 3 female *I. hexagonus*, received from hedgehog sanctuaries, the ticks from unit 2 and 3 were already used in a previous feeding, unit 4 held five male and five female *I. hexagonus* from the lab and unit 5 to 8 each held five male and five female *D. reticulatus* from the lab.
After 16 hours a lot of attachment was seen. In both unit 1 and 4 attachment was seen from one female *I. hexagonus*, in unit 5 three male and three female *D. reticulatus,* in unit 6 two male and two female *D. reticulatus*, in unit 7 two male and one female *D. reticulatus* and in unit 8 one male *D. reticulatus.*
After 23 hours nothing changed in unit 1 to 4, 7 and 8. However unit 5 now had three male and two female *D. reticulatus* attached and unit 6 had five male and two female *D. reticulatus* attached.
After 41 hours there was some mortality, one female in unit 2 and three females in unit 3. Both unit 1 and 4 still had one female attached and nothing changed either in unit 6 to 8. After 47 hours nothing changed in unit 1 to 4 and 7, unit 5 now had four male and three male *D. reticulatus* attached, unit 6 five male and three female *D. reticulatus* and unit 8 had three male and one female *D. reticulatus* attached.
After 65 hours again nothing changed in unit 1 to 4, unit 5 had four male and four female *D. reticulatus* attached, unit 6 had four male and two female *D. reticulatus* attached and unit 8 had two males attached and discolored blood. Unit 7 gained a leak, so was excluded from this feeding for the rest of the week. However, two males and one female were still attached.
After 71 hours nothing changed, except for unit 2, another female had died, so death percentage was now up to 67%. In unit 8 the blood was discolored.
After 89 hours, the feeding was ended and nothing had changed in every unit, but in unit 2 and 8 the blood was discolored.

U1: *Ixodes hexagonus* (3♀) and a membrane thickness of 85 µm, new membrane, rabbit hair.
U2: *Ixodes hexagonus* (3♀) and a membrane thickness of 95 µm, new membrane, rabbit hair.
U3: *Ixodes hexagonus* (3♀) and a membrane thickness of 72 µm, new membrane, rabbit hair.
U4: *Ixodes hexagonus* (5♂+ 5♀) and a membrane thickness of 103 µm, new membrane, rabbit hair.
U5: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 83 µm, new membrane, cow hair.
U6: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 87 µm, new membrane, cow hair.
U7: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 82 µm, new membrane, cow hair.
U8: *Dermacentor reticulatus* (5♂ + 5♀) and a membrane thickness of 104 µm, new membrane, cow hair.

Feeding 9 Ixodes hexagonus and Dermacentor reticulatus

Unit 1		
Time	Attachment (%)	Death (%)
16:22	33	0
23:46	33	0
41:25	33	0
47:29	33	0
64:57	33	0
71:39	33	0
89:03	33	0

Unit 2		
Time	Attachment (%)	Death (%)
16:22	0	0
23:46	0	0
41:25	0	33
47:29	0	33
64:57	0	33
71:39	0	67
89:03	0	67

Unit 3		
Time	Attachment (%)	Death (%)
16:22	0	0
23:46	0	0
41:25	0	100
47:29	0	100
64:57	0	100
71:39	0	100
89:03	0	100

Unit 4		
Time	Attachment (%)	Death (%)
16:22	10	0
23:46	10	0
41:25	10	0
47:29	10	0
64:57	10	0
71:39	10	0
89:03	10	0

Unit 5		
Time	Attachment (%)	Death (%)
16:22	60	0
23:46	50	0
41:25	50	0
47:29	70	0
64:57	80	0
71:39	80	0
89:03	80	0

Unit 6		
Time	Attachment (%)	Death (%)
16:22	40	0
23:46	70	0
41:25	70	0
47:29	80	0
64:57	60	0
71:39	60	0
89:03	60	0

Unit 7		
Time	Attachment (%)	Death (%)
16:22	30	0
23:46	30	0
41:25	30	0
47:29	30	0
64:57	30 Leakage!	
71:39		
89:03		

Unit 8		
Time	Attachment (%)	Death (%)
16:22	10	0
23:46	10	0
41:25	10	0
47:29	40	0
64:57	20	0
71:39	20	0
89:03	20	0

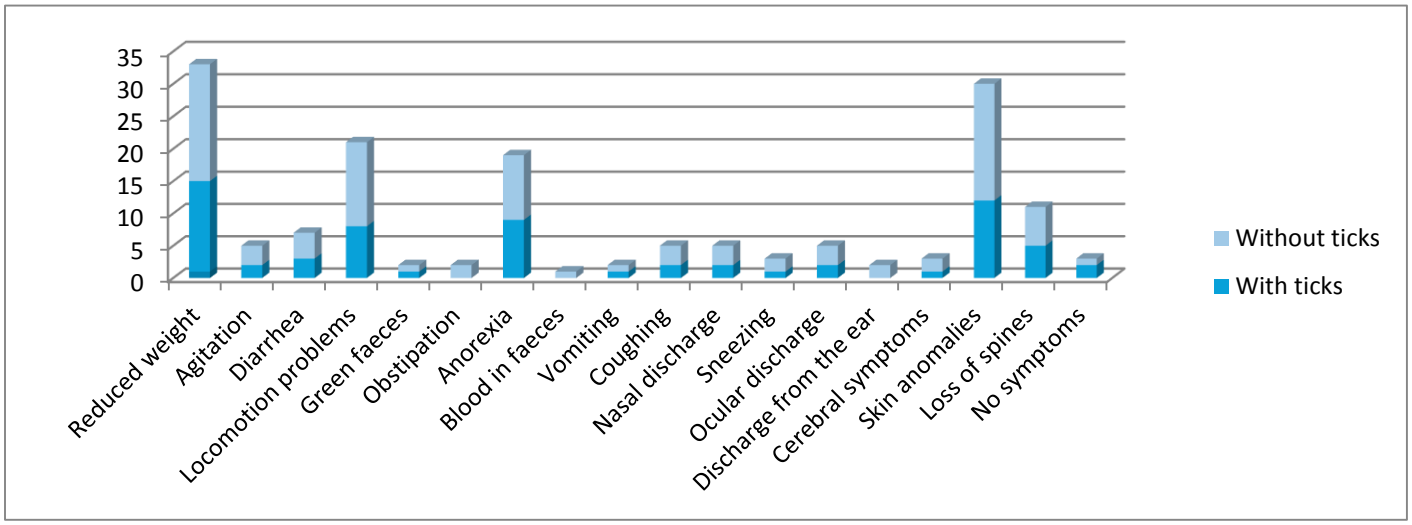

Fig. 14 Clinical symptoms and their frequency, with and without ticks

Questionnaire

A questionnaire was send to several hedgehog sanctuaries in the Netherlands. A total of 45 questionnaires were filled out and send to the lab. Table 1 and 2 down below show the results of the questionnaires. The red number indicates hedgehogs with ticks and the green number indicates hedgehogs without ticks. Most hedgehogs brought to animal sanctuaries were relatively young, up to three years, and no hedgehogs with the age between 3 and 4 years old had been brought to hedgehog sanctuaries.

	0 to 1 year	*1 to 2 years*	*2 to 3 years*	*3 to 4 years*	*4 to 5 years*	*5 to 6 years*	*6 to 7 years*	*7 to 8 years*	*8 to 9 years*	***Total***
Locomotion problems	1-3	3-1	2-3		1-1	2	1-1	2		**21**
Agitation		1	1-2			1				**5**
Diarrhea	1-3	1	1					1		**7**
Blood in faeces								1		**1**
Green faeces			1					1		**2**
Obstipation			1				1			**2**
Anorexia	2-2	2-2	4-2		1	1	1-1	1		**19**
Reduced weight	4-3	4-3	3-4			4	1-2	2-2		**32**
Vomiting	1	1								**2**
Coughing	1	1	1-2							**5**
Nasal discharge	1	1					1	2		**5**
Sneezing	1		1					1		**3**
Ocular discharge	1		1		1-1			1		**5**
Discharge from ear	1					1				**2**
Cerebral symptoms	1	1			1-1					**4**
Skin anomalies	4-3	5-3	2-2			5	1-1	3	1	**30**
Loss of spines	1-1	2	1-2			1	1-1	1		**11**
No symptoms		1	1				1			**3**

Table 1 Result of questionnaire: frequency of clinical symptoms with and without ticks

Symptoms frequently seen were locomotion problems, reduced weight, anorexia and skin anomalies. Within skin anomalies, sarcoptes was the most frequent cause, seen in table 2. The frequency of these symptoms seen could be due to hibernation, since this questionnaire is taken shortly after winter period.

As seen in figure 13 and table 1, there is little to no difference between the symptoms of hedgehogs with and without ticks. Due to this, it is hard to tell whether these symptoms are caused by the ticks or environmental influences.

	0 to 1 year	*1 to 2 years*	*2 to 3 years*	*3 to 4 years*	*4 to 5 years*	*5 to 6 years*	*6 to 7 years*	*7 to 8 years*	*8 to 9 years*
Dry skin	1	2-1							
Sarcoptes	2	2-1	1-2			3	1-1	3	
Fungus	1								
Remaining	2-1	1-1	1			2			1
Total	**7**	**8**	**4**			**5**	**2**	**3**	**1**

Table 2 Skin anomalies: different clinical symptoms and their frequency

RLB

Reverse Line Blot hybridization was also performed on 20 female *I. hexagonus* derived from hedgehogs. These ticks could not be used for *in vitro* feeding due to their high weight. Some females had already laid eggs, so these eggs were examined too. The result of this RLB showed that two out of twenty ticks are positive for *Theileria* catch-all and *Ehrlichia/Anaplasma* catch-all, the *Ehrlichia* present is *Ehrlichia canis*. But the negative control group also showed *Ehrlichia/Anaplasma* catch-all. In one tick *Borrelia valaisiana* was present.
Attached *I. hexagonus* and the ticks that died during the *in vitro* feeding were also examined. A total of five attached *I. hexagonus* and 19 dead *I. hexagonus* were examined by means of RLB. Pathogens were only found in one tick, this one was not attached to the membrane, but has died during the feeding. The RLB showed a *Rickettsia* catch-all for a male *I. hexagonus* from feeding 7 unit 4. This male was received from a hedgehog shelter together with other males and females, which were unfed.
Blood samples were taken every day after attachment of an *I. hexagonus*. A total of 29 samples were taken and examined by means of RLB. Some of these samples were positive for *Ehrlichia/Anaplasma* catch-all. These samples were from feeding 7 and feeding 9, respectively one taken from unit 1 day 2 in the morning and two taken from unit 1 day 3 and day 4 in the evening. Two samples were also positive for *Borrelia afzelii*, originated from a sample from feeding 7, unit 1 day 3 in the morning, and from feeding 9 unit 1 also day 3 in the morning. All results of the RLB are added to appendix D. In this appendix a table can be found connecting the ticks to the blood samples.

Discussion

Test feeding

Mortality was high and attachment rate was very low in this feeding. Only *D. reticulatus* was used and no attachment stimuli. After ending the feeding it appeared that the ticks were too young, because of the white excrete from the tubes of Malpighi.[15] Due to this discovery, this could be the reason the ticks did not attach.

Feeding 1

This feeding glucose was forgotten to be added to the blood. Glucose is necessary to stabilize the erythrocytes.[6] After a while the blood was discolored, this could be due to lack of glucose. Despite glucose was forgotten, two ticks were attached, but also two ticks died. Whether this is due to unfavorable conditions or viability of the ticks is hard to say.
Cow hair as attachment stimuli was used in this feeding. Attachment rate was low, compared to previous *in vitro* studies with hard ticks. Whether this was due to the attachment stimuli or the tick itself is hard to tell.

Feeding 2

This week *I. hexagonus* and *D. reticulatus* were used parallel. Again cow hair as attachment stimuli was used, this time tick feces from *D. reticulatus* were added. The *D. reticulatus* used was partially fed on a cow. Attachment rate of *D. reticulatus* increased a bit and in all units no mortality was seen, but also no *I. hexagonus* was attached. Maybe these attachment stimuli do not work for *I. hexagonus*, because this tick has different life styles and hosts then *D. reticulatus*.

Feeding 3

This week three different kinds of ticks were used, *I. hexagonus, D. reticulatus* and *R. sanguineus*. Cow hair and cow perfume were used as attachment stimuli. Low attachment rates were only seen in the units with *D. reticulatus*. No mortality occurred, so the poor attachment could not be because of the viability of the ticks. It appears to be that cow stimuli do not work for both *I. hexagonus* and *R. sanguineus.*

Feeding 4

This week no *I. hexagonus* was available for *in vitro* feeding, so only *D. reticulatus* was used. A new method was developed due to the results of previous weeks. Cow perfume was used as attachment stimuli and only one tick out of 100 was attached. No mortality occurred, so the low attachment rate could not be because of the viability of the ticks. Because only cow perfume is used, this could be the reason of the low attachment rate. The perfumes seem to have a different smell compared to an animal itself or his hairs.

Feeding 5

Testing was continued using the new method this week, parallel with the old method. Hedgehog hair and spines were used for two *I. hexagonus* units, because hedgehogs seemed to be the main host of these ticks. Unfortunately it was not very successful. Only a few ticks from the lab colony attached in the old method. This could be because of the low amount of hairs and spines used, due to the limited supply.
Luckily attachment was seen in every *D. reticulatus* unit, in both the old and new method. It appeared to be that cow hairs are more attractive than cow perfume. However a leakage occurred in unit 3, an unit from the old method with the new membrane. Sticking hairs onto the membrane before gluing the unit, could leave gaps between the membrane and the feeding unit, so leakages are more likely to occur.
The new method also does not work optimally, due to the escaping of the ticks from the twelve wells plate. This method needs more development to work optimally.

Feeding 6

This feeding the old method with the old membranes was used, parallel with the new membranes. Attachment in *I. hexagonus* units was disappointingly low, despite the use of hedgehog hairs and spines. Apparently, using

these materials does not stimulate the ticks at all to feed, again too little hair and spines were used. Hedgehogs are protected species in The Netherlands, so it is difficult to obtain enough of this material.
Fortunately the new membranes were very successful with *D. reticulatus*. Attachment rates up to 90% were seen. A disadvantage of this new membrane is the fact that the blood discolors more frequently after refreshing. Because of the little gaps between the feeding unit and the membrane, blood remains after washing with PCB solution, so contamination is inevitable. Beside the hairs stuck on the membrane comes into contact with the blood, which also contaminate the blood.

Feeding 7, 8 and 9

These feeding were performed according the old method, with the new membranes. The attachment rates of *D. reticulatus* were very positive, so it seemed this method could be used successfully for this tick. Disadvantage of this method is the high possibility of contamination and leakages due to the gaps and the hairs sticking out.
For *I. hexagonus* rabbit hairs were used, and this seems to be successful in feeding 7. However feeding 8 and 9 had low attachment rates of *I. hexagonus*. So after stabilization of the method, attachment rates fluctuate too much. Maybe the hairs used for the membranes were too old, so the smell has faded, so ticks had lost their interest to feed. Another explanation could be the amount of ticks used in one feeding unit. Feeding 7 and 9 held three female *I. hexagonus* in one unit and there was attachment. Feeding 8 held four *I. hexagonus* in one unit and no attachment was seen.
In feeding 9 the mortality rate in one unit was very high. This could be because of the viability of the ticks, because ticks from previous feedings were used.

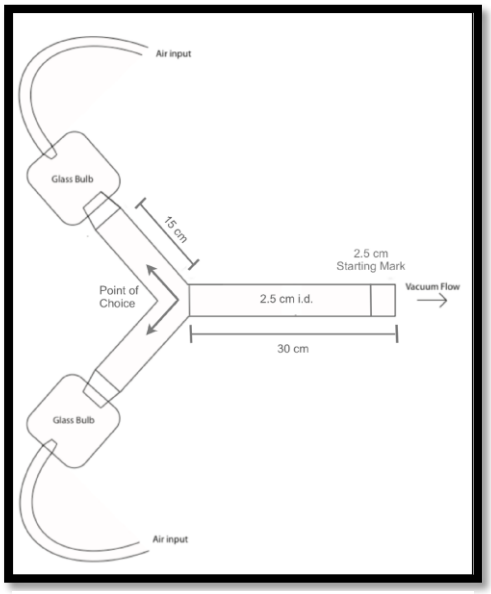

Fig. 15 Diagram of Y-tube olfactometer

Odour test

The odour test used in week 3, was not very successful. Because the container consisted out of one area, the smell could spread very easily. After three hours, the ticks were spread throughout the entire container, so any results were unreliable.
A better way to test what odour ticks like the most is by means of a special assays called Y-tube olfactometer assay or tick climbing assay. The Y-tube olfactometer consists out of a glass Y-tube, a vacuum pump, a flow meter and two glass bulbs (see figure 13). The vacuum flow makes sure the air inside the Y-tube is clean from odours. Then in one of the glass bulbs an odour is added, like cow perfume on a filter paper. The other glass bulb is the control group with the solvent of the perfume, like ethanol, on a filter paper. These filter papers are put in the glass bulb using forceps. Then the ticks were put at the beginning of the tube and the testing can begin.[16]

The other assay, the tick climbing assay, a platform with two aluminum rod with a glass tube over it, is placed in a tray with shallow water, figure 14. On top of these aluminum rods a strip of filter paper is stapled to provide the source of the test odours. One filter paper is treated with the test odour and the odour with a control, like the solvent of the test odour. The glass tube is placed around the aluminum rods from 3 cm above the base platform. Ticks were set up on both sides of the platform and can pick a side.[17]

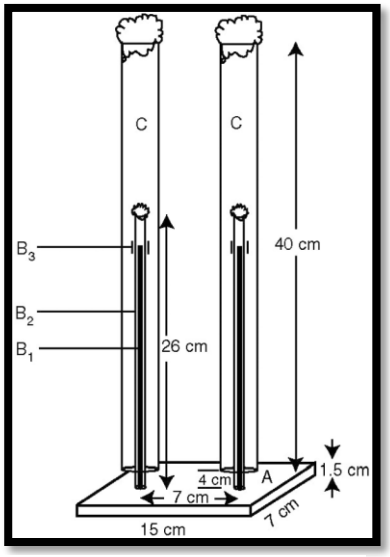

Fig. 16 Tick climbing bioassay apparatus

Questionnaire

The results obtained through the questionnaires were based on knowledge by experience rather than through reading evidence based papers. When filling out the questionnaire, people may not have recognized all clinical symptoms as such. An untrained eye may not distinguish some neurological symptoms from locomotion problems. Because this questionnaire was held briefly after hibernation of the hedgehogs, a lot of hedgehogs brought to the sanctuaries have reduced weight. Due to this great amount of weight loss, hedgehogs could be more vulnerable, so they show more clinical symptoms where ticks are not the cause of this. Distinction between clinical symptoms due to weakness after hibernation or due to infection by ticks cannot be made. Beside, little is known about the symptoms caused by pathogens transmitted by ticks. Therefor no definite conclusions can be made out of this questionnaire.

RLB

The result of the RLB showed in every RLB performed on ticks and blood samples, some ticks or samples are positive for *Ehrlichia/Anaplasma* catch-all. The negative control group also showed *Ehrlichia/Anaplasma* catch-all, in the RLB performed on ticks derived from the hedgehog, this could be because of the use of *Ehrlichia/Anaplasma* as positive control. So it was contaminated, showing a false positive result in some RLB tests. To get an accurate result, the RLB should be performed again, to exclude the possibility of a false positive result.

Conclusion

It appears to be very difficult to feed *Ixodes hexagonus in vitro.* Several methods were performed, and many were not very successful. In the beginning *Dermacentor reticulatus*, the control group, had also low attachment rates. It appears that perfume made from animal materials is not very attractive for ticks. More testing, with perhaps an olfactometer is needed.
Hair as attachment stimuli seemed to be successful for both *I. hexagonus* and *D. reticulatus*. Attachment of *D. reticulatus* increased a lot after the use of cow hair. Attachment of *I. hexagonus* fluctuated a lot after the use of rabbit hair, so more testing is needed. Perhaps hair of more different animals, like cats and dogs, perhaps even hedgehogs, foxes and mustelids can be tested for *in vitro* feeding, since *I. hexagonus* is also found on these hosts.[2, 3] In this paper, hedgehog hairs and spines were already used in unit 5 and 6, but not very successful, maybe due to the little amount of hair and spines used. Hedgehogs are protected species in The Netherlands, so it is difficult to obtain enough of this material.
The “hair membranes” were very successful, especially with *D. reticulatus,* but there is a high contamination risk and high chance of leakages. But even after discoloring of the blood, attached ticks stayed attached until the end of the feeding, so it seemed that discolored blood is not repellent for these ticks.
The “upside down” method could be successful, but this method needs more testing. Ticks escaped or were only found on the outer ring of the feeding unit, this way ticks were not interested in feeding. A new designed feeding unit could be helpful to improve this method. But also with this method, more testing is needed.
Little was found in the blood samples, meaning ticks can transmit pathogens to the blood, but due to the low number of ticks attached and little amount of pathogens found in blood, no conclusions can be made whether hedgehogs can get sick after infestations by *I. hexagonus* ticks. For such conclusions, hedgehogs should also be examined, maybe also by means of RLB on a blood sample from these animals.

So all together, more tests are needed to optimize the *in vitro* feeding method for *Ixodes hexagonus*.

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Appendix

Appendix A

Enquête opvangcentra

Gericht op individuele egel

Algemeen:

Nummer/naam van egel?
Wat is het geslacht van de egel?
Wat is de (geschatte) leeftijd van de egel?
Hoe is de egel in de opvang gekomen?

Teken:

Heeft de egel teken?
Hoeveel teken zijn er van de egel ingezameld?
Welke stadia zijn gevonden op de egel? (larven, nimfen, volwassen)
Waar zijn de teken op het lichaam gevonden? (snuit, lies etc.)

Ziekteverschijnselen:

Vertoont de egel ziekteverschijnselen? Aankruisen, eventueel ernst bij zetten.

	Ja	Nee	Ernst van verschijnsel	
Moeite met lopen				
Onrust				
Diarree				
Bloed in ontlasting				
Groene ontlasting				
Obstipatie				
Niet willen eten				
Vermageren				
Braken				
Hoesten				
Neusuitvloeiing				
Niezen				
Ooguitvloeiing				
Ooruitvloeiing				
Hersenverschijnselen				Kop scheef, draaien met ogen, zwalken
Huidaandoeningen				Wondjes, schimmel, schurft
Verlies van stekels				

Algemeen ziekte:

Is er verschil in ziekteverschijnselen tussen egels met teken en egels zonder teken?
Is er verschil in ziekteverschijnselen tussen egels met veel teken vergeleken met egels met weinig teken? Wat zijn deze verschillen?

Appendix B

DNA EXTRACTION FROM TICKS

Room	
Number of samples	
Sample description	

Water bath ID	
Sonification bath ID	
TissueLyser LT ID	
Heating block ID	
Centrifuge ID	

Wear gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with sodium hypochloride.	
2	Turn on a water bath at 56°C.	
3	Take the proteinase K solution from the freezer and store at 4°C.	
4	Wash the ticks in a sonofication bath with demineralized water for up to 30 seconds.	
5	Put the ticks, with cleaned forceps, in 1.5ml tubes with 70% ethanol and vortex for several seconds.	
6	Wash the forceps in 70% ethanol followed by washing in demineralized water after each tick.	
7	Take the ticks from the tubes and let it dry on a clean tissue paper and place the dried ticks in a sterile 2ml tube with 180µl T1 lysis buffer.	
8	Freeze the samples at -80°C for 15 minutes.	
9	Add a 5 or 7mm (depending on tick size) metal bead to the frozen samples.	
10	Disrupt the ticks in the TissueLyser LT at 50 oscillations per second for 3 minutes.	
11	Briefly spin down the tubes. **1000x g maximum!**	
12	Add 25µl proteinase K and vortex.	
13	Prelyse the samples at 56°C in a water bath for 3 hours and vortex every hour.	
14	**During the incubation**; empty and clean the sonification bath.	
15	**During the last incubation hour** ; turn on the heating block at 70°C and preheat the BE buffer.	
16	Briefly spin down the tubes. **1000x g maximum!**	
17	Add 200µl B3 buffer and vortex.	

18	Incubate the tubes at 70°C for 15 minutes.	
19	Briefly spin down the tubes. **1000x g maximum!**	
20	Add 210µl 96% ethanol, vortex and briefly spin down the tubes. **1000x g maximum!**	
21	Transfer the supernatant to new sterile 1.5ml tubes. (Tick parts are allowed to be transferred.)	
22	Centrifuge the tubes at 11,000x g for 2 minutes.	
23	Transfer the supernatant to spin columns. Avoid pipetting tick parts, as it can block the spin column.	
24	Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
25	Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
26	Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
27	Centrifuge the columns at 11,000x g for 1 minute.	
28	Place the spin columns in sterile 1.5ml tubes. **Label the tubes accordingly.**	
29	Add 100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute.	
30	Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns.	
31	Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation.	
32	Turn off all equipment and clean working space with sodium hypochloride.	

DNA extraction done:

by_____ on _____ _____

Signature

Comments:

DNA EXTRACTION FROM BLOOD PROCEDURE

Room	
Number of samples	
Sample description	

Heating block ID	
Centrifuge ID	

Wear gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with sodium hypochloride.	
2	Take the proteinase K solution from the freezer and store at 4°C.	
3	Turn on the heating block at 70°C and preheat the BE buffer.	
4	Vortex the blood samples and add 200µl of each to sterile 1.5ml tubes.	
5	Add 25µl proteinase K.	
6	Add 200µl B3 buffer and vortex vigorously (10-20 seconds).	
7	Incubate the tubes at room temperature for 5 minutes.	
8	Incubate the tubes at 70°C for 15 minutes. (The samples should turn brown/black. If this does not happen, extend incubation time up to 30 minutes and vortex vigorously several times.)	
9	Briefly spin down the tubes.	
10	Add 210µl 96% ethanol, vortex and briefly spin down the tubes.	
11	Transfer the supernatant to spin columns.	
12	Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
13	Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
14	Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
15	Centrifuge the columns at 11,000x g for 1 minute.	
16	Place the spin columns in sterile 1.5ml tubes. **Label the tubes accordingly**.	
17	Add 100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute.	

30	Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns.	
31	Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation.	
32	Turn off all equipment and clean working space with sodium hypochloride.	

DNA extraction done:

by_____ on _____ _____

Signature

Comments:

PCR RLB PROCEDURE

Rooms		
Number of samples		
Sample description		

Workstation 1 ID	
Workstation 2 ID	
PCR machine ID	

Wear (green) gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

Primers:	*Anaplasma Ehrlichia*	*Babesia Theileria*	*Borrelia*	*Rickettsia*	Other:

Reagent	1x	Number of samples + 10%	

PCR grade H_2O	15.875µl	
5x Phire reaction buffer	5.0µl	
10mM dNTPs	0.5µl	
Forward primer (20pmol/µl)	0.5µl	
Reverse primer (20pmol/µl)	0.5µl	
2U/µl Phire Hot Start II DNA polymerase	0.125µl	

		Done	
1	Put DNA samples a (few) day(s) before the PCR at 4°C.		
2	Turn on the DNA workstations in the clean room and the dirty room.		
3	Clean workspace in both DNA workstations with sodium hypochloride.		
4	Label the PCR and Eppendorf tubes and put them in the DNA workstation in the clean room		
5	Turn on the UV-light in both DNA workstations for 20 minutes.		
6	**During the UV-light**; thaw the PCR reagents at room temperature, except the polymerase.		

7	Prepare the PCR mix in the Eppendorf tube(s). Multiply the reagent volumes by the number of samples plus 10% of the number of samples: 40 DNA samples + 1 PCR control = 41 + 10% = 45 samples.	
8	Pipet the master mix gently up and down to mix well.	
9	Pipet 22,5µl master mix to each PCR tube and add the leftover mix to an additional tube which will be the negative PCR control.	
10	Close the PCR tubes and remove them from the workstation, clean the workspace with sodium hypochloride and turn on the UV-light for 20 minutes.	
11	Take the closed PCR tubes to the dirty room and place them in the workstation.	
12	Vortex the DNA samples, spin them down briefly at 11,000x g and place them in the workstation.	
13	Add 2.5µl DNA sample to the corresponding PCR tube.	
14	Add 2.5µl of the positive control (, corresponding to the PCR to be performed,) to the positive PCR control tube.	
15	Vortex and spin down briefly.	
16	Clean the workstation with sodium hypochloide and turn on the UV-light for 20 minutes.	
17	Run the corresponding PCR program.	
18	Store the PCR products at 4°C for use within the next few days or store at -20°C for long term preservation.	
19	Turn off both DNA workstations after the UV-light is switched off.	

PCR done:

by_____ on _____ _____

Signature

Comments:

REVERSE LINE BLOT HYBRIDIZATION PROCEDURE

Room	
Number of samples	
Sample description	

Heating block ID	
Water bath ID	
Hybridization oven ID	
Shaker ID	
Membrane ID	
Blotter ID	

Wear gloves and use non-filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with 70% ethanol.	
2	Turn on a heating block at 100°C.	
3	Turn on the hybridization oven at 42°C en preheat 50ml 2x SSPE/0.5% SDS solution.	
4	Turn on the water bath at 50°C en preheat the bottle with 2x SSPE/0.5% SDS solution.	
5	Combine and dilute the PCR products per DNA sample in a 1.5ml tube. Take 10µl of every PCR product and add 2x SSPE/0.1% SDS to a final volume of 160µl. (10µl *Anaplasma/Ehrlichia* PCR + 10µl *Babesia*/*Theileria* PCR + 140µl 2x SSPE/0.1% SDS.)	
6	Take 10µl of the RLB positive controls and add 150µl 2x SSPE/0.1% SDS to a 1.5ml tube,	
7	Denature the diluted PCR samples and controls at 100°C for 10 minutes.	
8	**During the denaturation step**; wash the membrane at room temperature with 2X 2SSPE/0.1% SDS for 5 minutes under gentle shaking and fill a bucket with ice.	
9	Immediately transfer the samples in order on ice after the denaturation.	
10	Prepare the miniblotter by placing the membrane on the lanes, with the line pattern of the membrane perpendicular to the lanes of the blotter. Place de support cushion on the membrane followed by the other half of the blotter. Turn the blotter right-side up without moving the membrane and turn the screws hand-tight,	
11	Remove residual fluid in the slots by aspiration.	
12	Briefly spin down the tubes at 4°C and place them back on ice in order.	
13	Fill the slots with the samples (150µl) and fill the first, last and other empty slots with 2x SSPE/0.1% SDS. **Avoid air bubbles.**	
14	Hybridize the blotter at 42°C for 60 minutes in the hybridization oven without shaking.	
15	Remove the samples by aspiration.	

16	Dissemble the blotter and remove the membrane from the blotter.		
17	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS at 50°C for 10 minutes under gentle shaking.		
18	**During the washing step**; clean the blotter and the support cushion.		
19	Incubate the membrane with 50ml 2x SSPE/0.5% SDS + 5µl streptavidin at 42°C for 30 minutes in the hybridization oven under gentle shaking. **Discard the streptavidin solution in a tube and into the bio-waste bin. Do not pour it in the sink.**		
20	**During the streptavidin hybridization**; change the water bath temperature to 42°C and preheat the bottle with 2x SSPE/0.5% SDS solution. Keep the lid open.		
21	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS solution at 42°C for 10 minutes under gentle shaking.		
22	Change the water bath temperature to 80°C and preheat the bottle with 1% SDS solution.		
23	Wash the membrane **twice** with 2x SSPE at room temperature for 5 minutes, under gentle shaking.		
24	**During the washing step**; prepare the foil and film cassette and check if the developing machine is on (5^{th} floor).		
25	Add 10ml ECL (5ml ECL1 + 5ml ECL2) to the membrane and gently shake by hand until the whole membrane is covered. **Discard the ECL in a tube and into the bio-waste bin. Do not pour it in the sink.**		
26	Cover the membrane in foil and place it in the film cassette. **Avoid air bubbles.**		
27	Go to the dark room and expose a film to the membrane for 10 minutes.		
28	Develop the film with the developing machine.		
29	Strip the membrane or store it in a seal bag with 20mM EDTA at 4°C until stripping.		
30	Turn off all equipment and clean workspace.		

RLB hybridization done:

by_____ on _____ _____

Signature

Comments:

IN VITRO FEEDING UNIT PREPARATION PROCEDURE

Room		Number of units made	

Wear gloves!

	Day 1	Done
1	Clean workspace with 70% ethanol.	
2	Clean the glass plate(s) with 70% ethanol and cover it with foil. Tighten the foil with tape.	
3	Tape the top of 8 lens papers on the foil of equidistant per glass plate.	
4	Prepare the silicon mix inside the fume hood.	
5	Apply the silicon mix onto a piece of carton.	
6	Cover the lens papers with an equal layer the silicon mix with clean hand applicator at a 45° angle, while applying light pressure.	
7	Dry the membrane for 24 hours on a bench or overnight in a closed environment with 97% humidity.	
8	Dispose all silicone waste in a seal bag into the biological waste bin.	
9	Clean workspace and used tools with 70% ethanol.	

Silicon mix	**Weight (g) (For 8 lens papers)**	**Weighted (g)**
White color paste	0.15	
Silicon glue	15.00	
Silicon oil	4.50	
Hexane	2.90	

	Day 2	Done
1	Clean workspace with 70% ethanol.	
2	Clean whole Plexiglas tubules with 70% ethanol. When re-using tubules, remove all glue residues (use 70% ethanol and rinse immediately with demi-water) and check for cracks.	
3	Apply some silicone glue onto a plastic petri dish and smooth it to a thin layer.	
4	Place a tubule with a rotating movement in the glue and remove it from the glue with a rotating movement. Check if the ring is completely covered in glue. Excess glue can be remove with a spatula.	
5	Place the tubule on the membrane and slightly rotate while applying pressure. Once on the membrane, don't move the tubules. Excess glue on the inside of the unit can be removed with a small brush.	
6	Continue with the next tubules until every usable piece of the membranes is used.	

7	Dry the units for 3 hours at room temperature.	
8	Cut the membranes with the tubules and plastic foil from the glass plate with a scalpel. Be careful not the loosen the tubules from the membrane.	
9	Remove the foil without damaging the membranes.	
10	Measure the membrane with the micro calipers at different spots around each tubule. The layer should be between 70 and 100µm thick. Don't use the unit If 1 or more of the measurements deviates too much from the other measurements. Write down the thickness on the unit.	
11	Cut the membranes precisely around each unit with a scalpel.	
12	Tie a piece of white organza around a lid and fasten it. Make sure there is enough voile on top of the lid to hold it.	
13	Place a lid on each unit.	
14	Store the units in a closed box.	
15	Clean the used glass plate(s) with water and soap.	
16	Clean workspace with 70% ethanol.	

Unit preparation day 1 done:

by_____ on _____ _____

Signature

Unit preparation day 2 done:

by_____ on _____ _____

Signature

Comments:

IN VITRO FEEDING BLOOD PREPARATION PROCEDURE

Room	

Cow number	
Blood volume	
Glucose weight	

	Meal preparation	Done
1	Take a sterile 250ml Erlenmeyer, 2 10ml pipettes, a piece of parafilm and a pen.	
2	Go to the department of Farm Animals (LHD), dress appropriately.	
3	Go the office of Thijmen Struik (room 230) to pick up the welfare diary.	
4	Ask an animal care taker for help. The care taker will collect the blood from the cow.	
5	Use a tube to direct the blood into the Erlenmeyer. Collect 200-250ml blood.	
6	Let the animal care taker fill in and sign the welfare diary.	
7	Stir 15 minutes with the 10ml pipet to remove all agglutination factors from the blood.	
8	Remove the pipette gently, dispose it in the yellow bio-waste bin and seal the Erlenmeyer with parafilm	
9	Bring the welfare diary back to the office of Thijmen Struik.	
10	Take the Erlenmeyer to the UCTD, add glucose to a concentration of 2g/L and swerve the jar.	
11	Pour the blood in the sterile 50ml tubes. Sterilize the edge of the Erlenmeyer and the tubes by putting it through a blue flame before pouring.	
12	Turn off the Bunsen burner.	
13	Label the tubes accordingly and store at 4°C. (The blood can be stored for 1 week.)	
14	Clean workspace with 70% ethanol.	

In vitro feeding meal preparation done:

by_____on _____ _____

Signature

Comments:

IN VITRO FEEDING SETTING UP PROCEDURE

Room	
Number of units	
Number of ticks	

Tick species	
Tick strain	
Blood	
Smell/hair/perfume	

Wear gloves

		done
1	Clean the flow cabinet with 70% ethanol.	
2	Disinfect a 50ml Falcon tube with 70% ethanol and place it in the flow cabinet.	
3	Turn on the UV-light for 15 minutes.	
4	Turn on a water bath at 37°C.	
5	If needed, fill the outer water bath with distilled water until the upper line.	
6	Turn on the tick race track in the acaridarium.	
7	Disinfect a tube with blood with 70% ethanol and place it in the flow cabinet together with (a) sterile 6-wells plate(s).	
8	Pipette 3.1ml blood to the 4 outer wells of the 6-wells plate(s). Cover the other wells, when pipetting.	
9	Cover the plate with the lid and incubate it in the water bath at 37°C for 15 minutes.	
10	Clean the flow cabinet with 70% ethanol.	
11	Place the ticks from the incubator into the units on the tick race track.	
12	Place the bonbon onto the unit until 0.5cm above the membrane. Be aware that no ticks are stuck between the bonbon and the side of the unit.	
13	Turn off the race track and place the units in the flow cabinet.	
14	Take the 6-wells plate(s) from the water bath and dry the outside before placing it in the flow cabinet.	
15	Place the units side-ways onto the blood. **Avoid air bubbles between the membrane the blood**.	
16	Place the 6-wells plate(s) with the units in the water bath.	
17	Close the water bath and cover it with a black cloth.	

18	Clean the flow cabinet with 70% ethanol.	
19	Turn on the UV-light for 15 minutes	
20	Turn off the flow cabinet.	

In vitro feeding setting up done:

by_____on _____ _____

Signature

Comments:

IN VITRO FEEDING MAINTENANCE PROCEDURE

Wear gloves

	Maintenance and sampling
1	Clean the flow cabinet with 70% ethanol.
2	Place a 50ml Falcon tube, 6-wells plate(s) and 500µl tubes (only for sampling) in the flow cabinet.
3	Turn on the UV-light for 15 minutes.
4	If needed, fill the water bath with distilled water until the upper line.

5	Disinfect a tube with blood with 70% ethanol and place it in the flow cabinet.
6	Pipette the blood up and down to homogenize.
7	Pipette 3.1ml blood to the 4 outer wells of the 6-wells plate(s). Cover the other wells when pipetting.
8	Cover the plate(s) with the lid and incubate it in a water bath at 37°C for 15 minutes.

9	Take the 6-wells plate(s) with the fresh blood from the water bath and dry the outside.
10	Disinfect the outside of all 6-wells plates and units with 70% ethanol before placing them in the flow cabinet.
11	Rinse the membranes with PBS above a glass petri dish.
12	Check if any ticks are attached or seem dead in each unit. Write the numbers down.
13	Place the units side-ways onto the fresh blood. **Avoid air bubbles between the membrane the blood**.
14	For sampling: pipette the old blood up and down and take a sample of 500µl each into sterile cryotubes. **Label the tubes accordingly**.
15	Remove the leftover blood from the used 6-wells plate(s) by pipetting. Dispose it in the 50ml Falcon tube.

16	Place the 6-wells plate(s) with the units in the water bath.
17	Close the water bath and cover it with a black cloth.
18	Check the temperature of the water and fill in the logbook (date, time, name, activity, number of dead and attached ticks per unit, temperature and humidity water bath).
19	Store the blood samples at 4°C for usage within the next few days or at -20°C for long term storage.

20	Empty the glass petri dish and clean with 70% ethanol.
21	Clean the flow cabinet with 70% ethanol.
22	Turn on the UV-light for 15 minutes.
23	Turn off the flow cabinet.

IN VITRO FEEDING FINISHING PROCEDURE

Room	
Number of units	
Number of ticks	

Wear gloves!

Tick species		
Tick strain		
Blood ID		
Smell/perfume		
Hair		
Water bath ID		
Flow cabinet ID		
Tick track ID		

	Maintenance	Done
1	Clean the flow cabinet with 70% ethanol.	
2	Disinfect a 50ml Falcon tube with 70% ethanol and place it in the flow cabinet.	
3	Turn on the UV-light for 15 minutes.	
4	Turn on the tick track in the acaridarium.	
5	Take the 6-wells plate(s) with the units from the water bath and dry the outside before placing it in the flow cabinet.	
6	For sampling: Pipette the blood from the 6-wells with the old blood up and down and take a sample of 500µl each into sterile cryo tubes. **Label the tubes accordingly**.	
7	Rinse the membranes of the units with PBS above the glass petri dish..	
8	Place the units in a new 6-wells plate**.**	
9	Remove the blood from the old 6-wells plate(s) by pipetting and dispose it in the 50ml Falcon tube..	
10	Take the 6-wells plate with the ticks to the acaridarium.	
11	Clean the flow cabinet with 70% ethanol.	
12	Turn on the UV-light for 15 minutes	
13	Turn off the flow cabinet.	
14	Remove the bonbons and take pictures for documentation.	
15	Put the ticks that are attached in a sterile 1.5ml tube with 70% ethanol, store them at room temperature. Label the tubes accordingly.	
16	Put dead ticks in a jar with 70% ethanol and place it in the fridge.	

17	Inform what has to be done with the rest of the ticks.	
18	Turn off tick track.	

In vitro feeding finishing done:

by_____on _____ _____

Signature

Comments:

Appendix C

Odour test

Dermacentor reticulatus

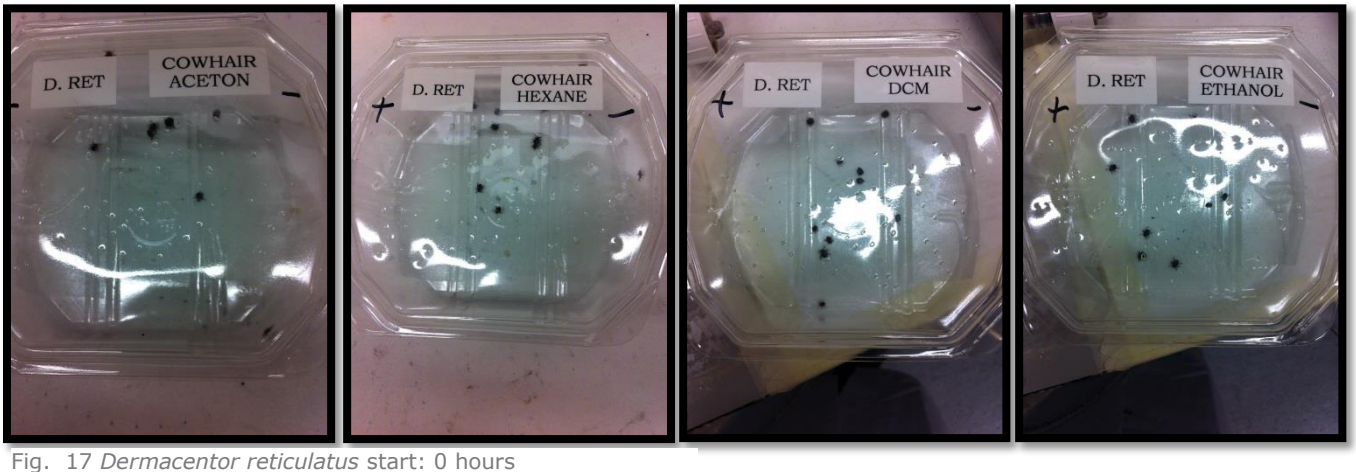


Fig. 17 *Dermacentor reticulatus* start: 0 hours

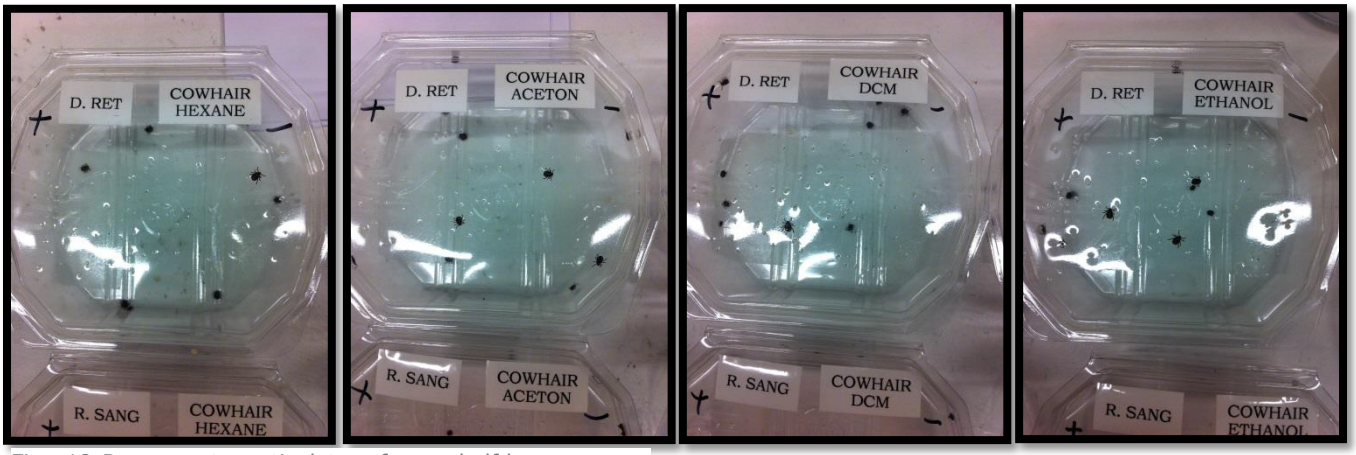


Fig. 19 *Dermacentor reticulatus* after an half hour

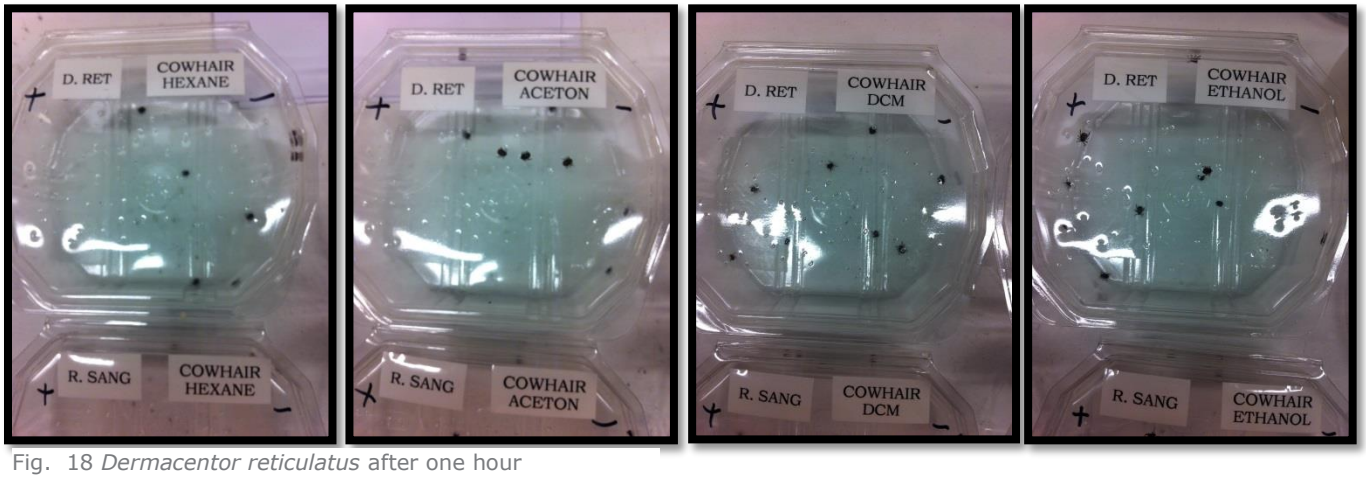


Fig. 18 *Dermacentor reticulatus* after one hour

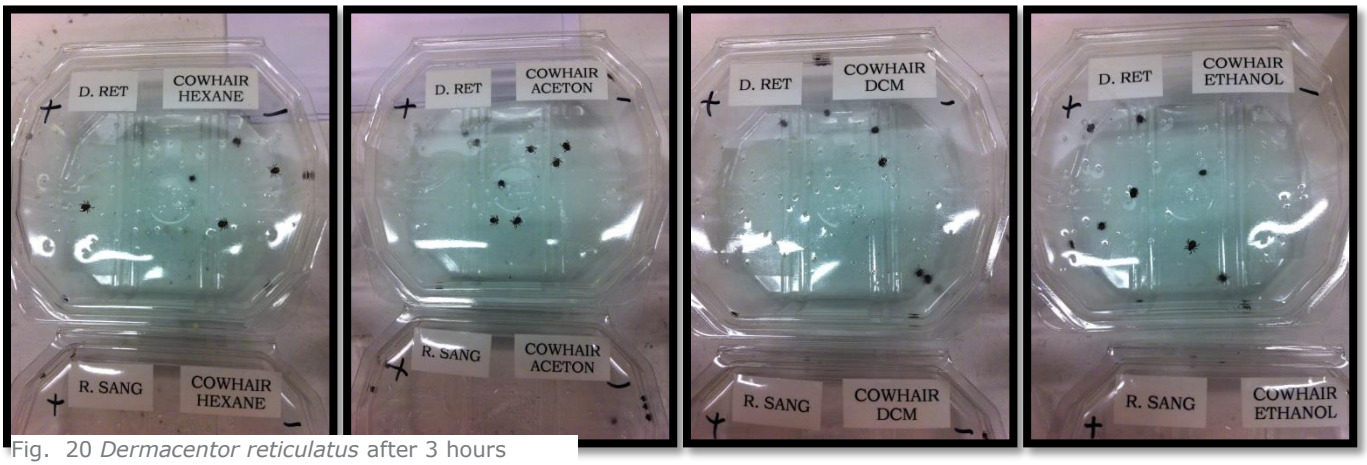


Fig. 20 *Dermacentor reticulatus* after 3 hours

Rhipicephalus sanguineus

Fig. 22 *Rhipicephalus sanguineus* start: 0 hours

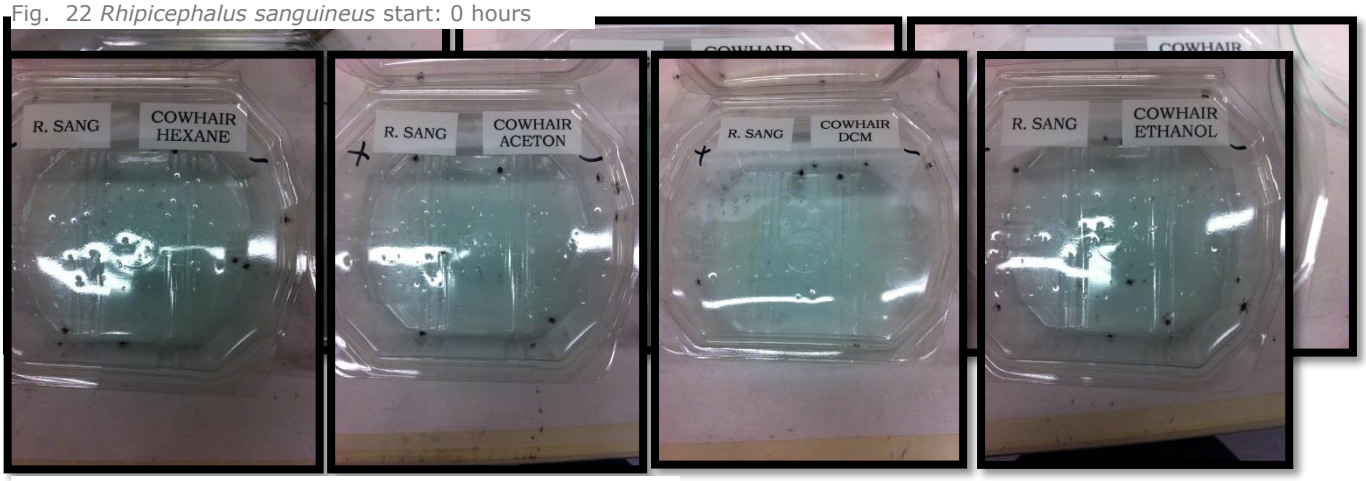


Fig. 21 *Rhipicephalus sanguineus* after an half hour

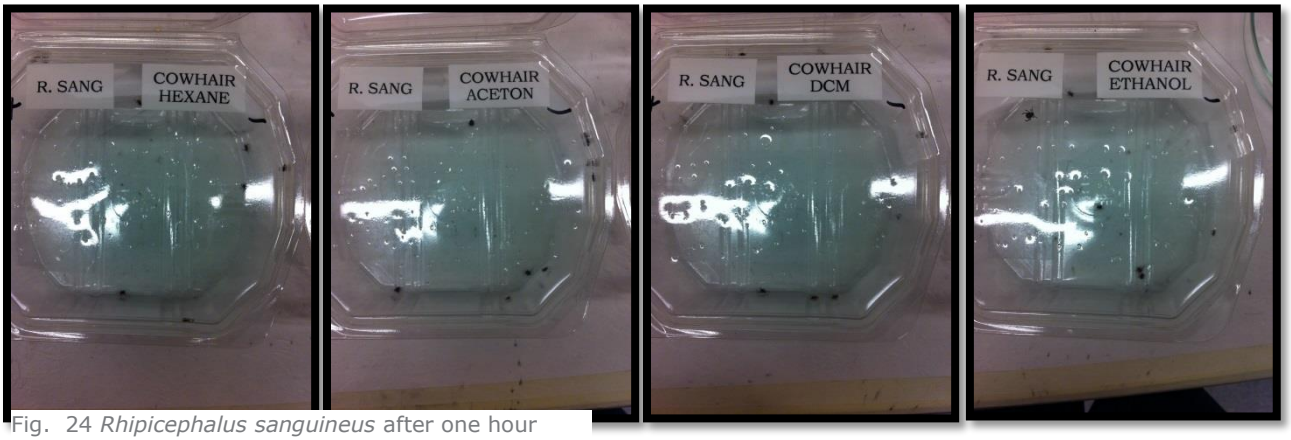


Fig. 24 *Rhipicephalus sanguineus* after one hour

Fig. 23 *Rhipicephalus sanguineus* after 3 hours

Appendix D

RLB results tick from hedgehog

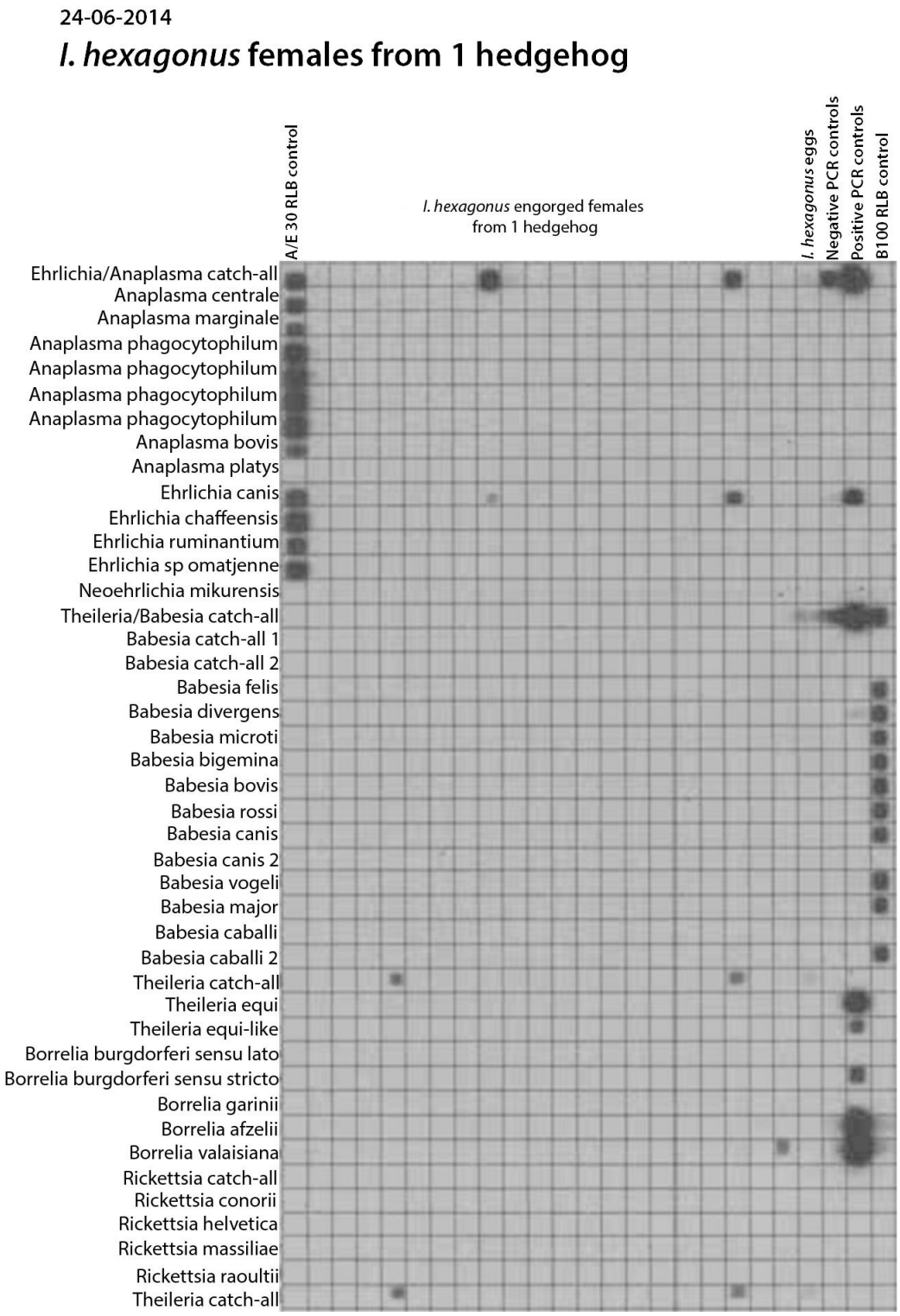
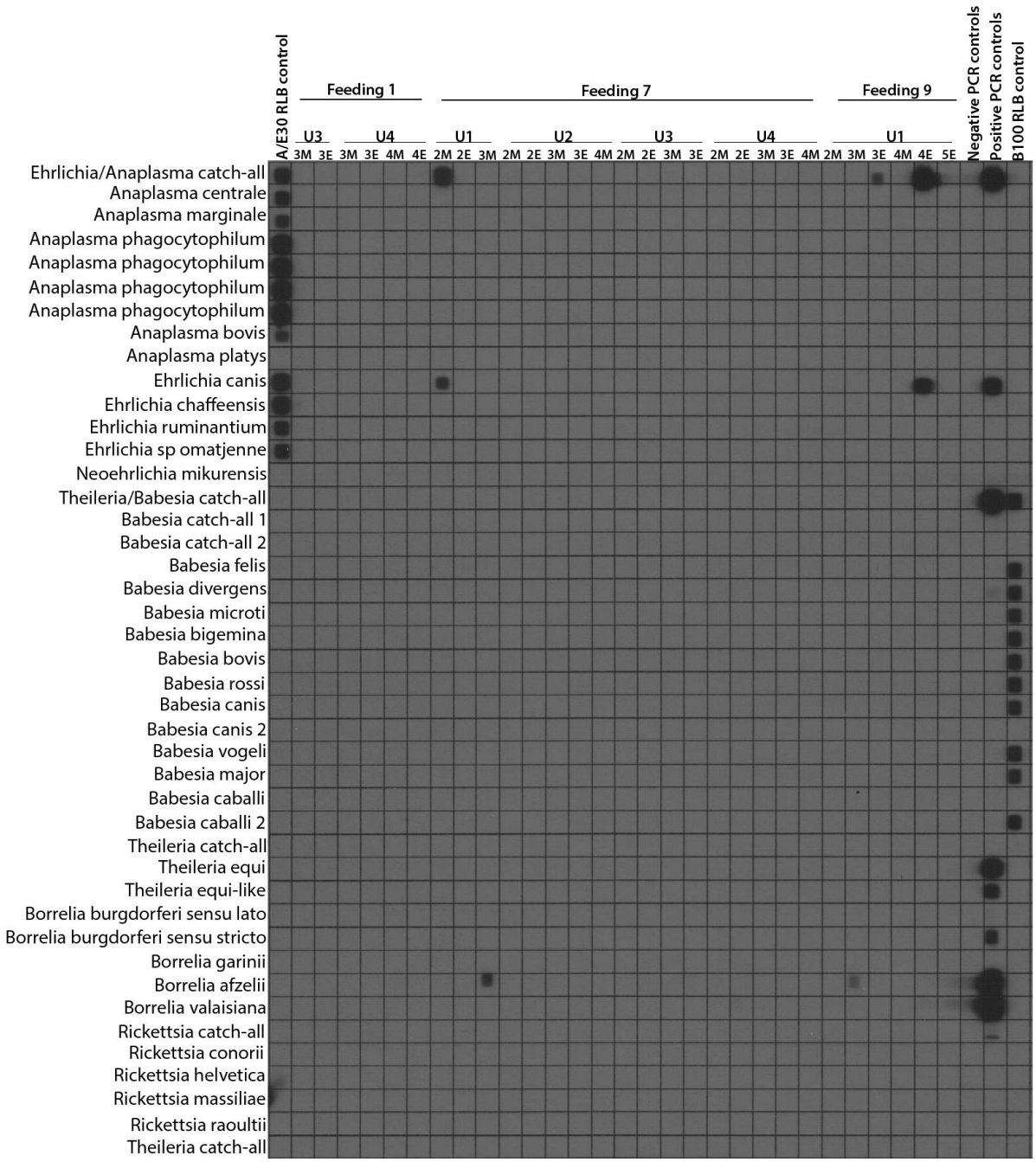


Table of samples and ticks from *in vitro* feeding

RLB ticks in vitro feeding		RLB feeding samples	
A	F1 U4, fed ♀	**1**	Feeding 1, day 3M U3
B	F1 U3, dead ♀	**2**	Feeding 1, day 3E U3
C	F1 U1, dead ♂	**3**	Feeding 1, day 3M U4
D	F6 U1/4, dead ♀	**4**	Feeding 1, day 3E U4
E	F6 U1/4, dead ♀	**5**	Feeding 1, day 4M U4
F	F7 U1, fed ♀	**6**	Feeding 1, day 4E U4
G	F7 U3, dead ♀	**7**	Feeding 7, day 2M U1
H	F7 U2, fed ♀	**8**	Feeding 7, day 2E U1
I	F7 U4, dead ♀	**9**	Feeding 7, day 3M U1
J	F7 U4, dead ♀	**10**	Feeding 7, day 2M U2
K	F7 U4, dead ♀	**11**	Feeding 7, day 2E U2
L	F7 U4, fed ♀	**12**	Feeding 7, day 3M U2
M	F7 U4, dead ♂	**13**	Feeding 7, day 3E U2
N	F8 U3, dead ♀	**14**	Feeding 7, day 4M U2
O	F8 U2, dead ♀	**15**	Feeding 7, day 2M U3
P	F8 U2, dead ♀	**16**	Feeding 7, day 2E U3
Q	F8 U2, dead ♀	**17**	Feeding 7, day 3M U3
R	F8 U1, dead ♀	**18**	Feeding 7, day 3E U3
S	F9 U1, fed ♀	**19**	Feeding 7, day 2M U4
T	F9 U2/3, dead ♀	**20**	Feeding 7, day 2E U4
U	F9 U2/3, dead ♀	**21**	Feeding 7, day 3M U4
V	F9 U2/3, dead ♀	**22**	Feeding 7, day 3E U4
W	F9 U2/3, dead ♀	**23**	Feeding 7, day 4M U4
X	F9 U2/3, dead ♀	**24**	Feeding 9, day 2M U1
		25	Feeding 9, day 3M U1
		26	Feeding 9, day 3E U1
		27	Feeding 9, day 4M U1
		28	Feeding 9, day 4E U1
		29	Feeding 9, day 5M U1

In vitro feeding samples

09-07-2014



RLB results attached *Ixodes hexagonus*

Attached *Ixodes hexagonus*
09-07-2014