A STUDY TO DETERMINE THE VECTORIAL POTENTIAL OF THE TICK *IXODES HEXAGONUS* THROUGH IN VITRO FEEDING.

Anniek Bender
June 2016 – September 2016

A STUDY TO DETERMINE THE VECTORIAL POTENTIAL OF THE TICK *IXODES HEXAGONUS* THROUGH IN VITRO FEEDING.

Author: Anniek Bender
Location: Infectious Diseases and Immunology (I&I)
Utrecht Centre of Tick-borne Diseases (UCTD)
Supervisor: Prof. dr. F. Jongejan
Timeslot: June 2016 – September 2016

INDEX

Abstract 5

Introduction 6

Ixodes Hexagonus 6

In vitro feeding 6

Purpose of the study 7

Main research question 7

Materials and methods 7

Ticks 7

Experimental groups 7

Blood 8

In vitro feeding 8

Systems 9

Membranes 9

Bonbons 10

Attachment stimuli 10

Water bath 12

Incubator 12

Schedule 12

Check-up moments 12

Results 13

Experiment 1 – Test week (TD-system) 13

Experiment 2 – concentrated cow-extract (TD-system) 14

Experiment 3 – Concentrated dog extract (TD-system) 15

Experiment 4 – Rubbed membranes (TD-system) 16

Experiment 5 – Re-used ticks from experiment 4 (TD-system) 17

Experiment 6 - Unit + membranes 12-24 hours in odourbox (TD-system) 18

Experiment 7 – Bovine sebum extract (TD-system) 19

Experiment 8 – Membranes in odourbox, 3 to 9 days (TU- and TD-system) 21

TU-system 21

TD-system 22

Experiment 9 – Concentrated bovine sebum extract (TU- and TD-system) 23

TU-system 23

TD-system 24

Experiment 10 – Membranes in odourbox 4 to 22 days, concentrated bovine sebum extract, faeces extract (TU- and TD-system) 25

TU-system 26

TD-system 27
Experiment 11- Preconditioning, membranes in odourbox for 4 to 11 days, concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch)(TU- and TD-system) 28
TU-system 28
TD-system 29
Overview comparison different attachment stimuli 30
TU-system 30
TD-system 31
Experiment 12 – Forced feeding (TU-system) 32
Experiment field ticks 1 – concentrated bovine sebum extract (TD-system) 33
Experiment field ticks 2 - Membranes 22 days in odourbox, concentrated bovine sebum extract (TD-system) 33
Experiment force feeding field ticks 33
Experiment field tick 3 – 1 hour Preconditioning, membranes in odourbox for 11 days, concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch) (TD-system) 34
Experiment males only (TU- and TD-system) 35
Discussion 36
Attachment stimuli tests 36
Field tick tests 37
Males only test 37
Conclusion 38
References 39
Appendix 41
In Vitro Feeding – Blood preparation 42
In Vitro Feeding (Tick down system) – Unit preparation protocol 43
In Vitro Feeding(Tick down system) – Startup & Finishing up 45
In Vitro Feeding (Tick down system) – maintenance protocol 47
Graphical guide – Blood preparation 49
Graphical guide – Starting up 51
Graphical guide – Finishing up 53
Graphical guide – Maintenance 55
Graphical guide – Unit preparation (membranes + TD-system) 56
Graphical guide – Unit preparation (TD-system + Forced feeding) 59

ABSTRACT

Background

Ixodes hexagonus is one of several tick species that is found in The Netherlands. *Ixodes hexagonus* is a hard tick which belongs to the Ixodidae family. The hedgehog is its main host, but also other mammals serve as hosts. This tick is a vector for several different pathogens, but until now little is known. The aim of this study was to achieve high attachment rates of *I. hexagonus* on artificial membrane by using different attachment stimuli, and eventually use *in vitro* feeding to study its vector role. The main question in this study was: can *Ixodes hexagonus*, while being fed *in vitro*, transmit *Anaplasma* and *Borrelia* during their feeding process?

Materials and methods

I. hexagonus was fed in vitro. Two systems were used (ticks-up and ticks-down system) in this study. To achieve higher attachment rates, several attachment stimuli were used. *I. hexagonus* from the UCTD colony were used in these experiments. Eventually, partially engorged field ticks were used to answer the main question of this study. These ticks were placed in the in vitro feeding units, which were provided with the most successful attachment stimuli.

Results

Higher attachment rates were mainly seen in the ticks-down system (TD-system). The ticks-up system (TU-system) showed lower attachment rates. The highest attachment rates among colony ticks were acquired in the TD-system with the following attachment stimuli: three days preconditioned ticks, membranes that were in the odourbox for 11 days, the concentrated bovine sebum extract (2nd batch), the faeces extract and cow hair. Among the 21 used field ticks in the TD-system, no attachment was seen. Attachment of male ticks was seen in both the TU-system and the TD-system. Only one of the ticks, used in a forced feeding experiment, survived longer than 24 hours. None of the ticks survived 48 hours or longer.

Conclusion

As shown in the results, higher attachment rates among colony ticks were achieved in the TD-system, with a specific combination of multiple attachment stimuli. The forced feeding technique used in this experiment was not useful to demonstrate transmission of *Anaplasma* and *Borrelia*, because of the low survival rate. Like female ticks, male *Ixodes hexagonus* ticks also attached to the membrane. Whether they play a role in transmission of pathogens is unclear. As a result, more research has to be done, because no conclusions can be drawn regarding transmission of *Anaplasma* and *Borrelia* during in vitro feeding.

INTRODUCTION

Worldwide, ticks are important vectors of pathogens causing diseases in animals and humans (Pfäffle et al., 2013; Pfäffle et al., 2011). In companion animals babesiosis, ehrlichiosis and borreliosis are often diagnosed (Földvári & Farkas, 2005). However, for certain tick species such as *Ixodes hexagonus*, the hedgehog tick, it is still unknown what role they play in being a vector of pathogens. During this study, the potential role of *Ixodes hexagonus* as a vector of pathogens will be further studied.

IXODES HEXAGONUS

Several tick species are found in the Netherlands. One of these species is *Ixodes hexagonus*. For instance, in the Netherlands, in 2005 and 2006, ticks on companion animals were classified as follows: *Ixodes ricinus* adults (67.6%), *Ixodes* species nymphs (12.3%), *Ixodes* species larvae (9.0%), *Ixodes hexagonus* adults (7.6%) and *Dermacentor reticulatus* (1.7%) (Nijhof et al., 2007).

Ixodes hexagonus is a hard tick from the Ixodidae family. It prefers hosts that use burrows or nests. The hedgehog is its main host, but also other mammals including dogs, cats, foxes, sheep, horses and moles serve as hosts. It is a three-host tick, which means that larvae, nymphs and adults all feed on different hosts. The feeding period takes about 8 days, while the complete life-cycle can extend to more than 3 years. The ticks are active from early spring until late autumn, but they are most active during April and May (Taylor, Coop, & Wall, 2016).
Both *I. ricinus* and *I. hexagonus* are found on European hedgehogs. In Europe, the dynamics of pathogens transmitted by *I. ricinus* have been widely investigated (Pfäffle et al., 2011). In a study of Kramczyk et al. they screened *I. hexagonus* and *I. ricinus* ticks (collected from European hedgehogs) for the presence of several pathogens. They reported *Anaplasma phagocytophilum* and *Borrelia* genospecies *B. afzelii*, *B. spielmanii*, *B. garinii*, and *B. burgdorferi sensu stricto* in both *I. hexagonus* and in *I. ricinus* (Krawczyk et al., 2015). Also other studies mentioned an association between *Borrelia* or *A. phagocytophilum* and the *I.hexagonus* (Pfäffle et al., 2011; Skuballa et al., 2007; Toutoungi & Gern, 1993).
Little is known about the transmission by *I. hexagonus* of other pathogens. One study showed the presence of *Candidatus Neoehrlichia* in *I. hexagonus* (found on Dutch hedgehogs).
However, it is still unclear which role *I. hexagonus* plays in the maintenance of several different pathogens (Pfäffle et al., 2011).

IN VITRO FEEDING

The use of animals in experiments raises various ethical questions. Besides that, the maintenance costs of these animals is high (Kröber & Guerin, 2007).
To reduce the number of experimental animals, in vitro feeding may be a suitable alternative.
There is a long history regarding the in vitro feeding technique, while using different materials and methods (Kröber & Guerin, 2007).
Several materials were used, like animal skin, mammalian gut tissue and bat wings (Mango & Galun, 1977; Osborne & Mellor, 1985).
A method of feeding ticks with capillary tubes placed over the hypostome was also used, but did not provide viable offspring. To produce offspring, the ticks first had to be partially fed on living animals (Osborne & Mellor, 1985; Purnell, 1973).
In 1990, glue impregnated membranes were introduced by Wallade et al. (Waladde et al., 1991). In 1993, the first silicone membranes were presented by Habedank and Hiepe (Kröber & Guerin, 2007). Over the years this silicone membranes method has been further developed.

Silicone-impregnated membranes were used for attachment of the hard tick *Ixodes ricinus* in earlier studies. Unfortunately, little is known about in vitro feeding of *I. hexagonus*. However, Kröber and Guerin write that there are common principles to achieve good attachment to artificial membranes. Only minor modifications are required regarding the attractant on the membrane to feed different tick species in different life stages. Several attachment stimuli were used in different studies, for instance: host hair, tick faeces, hair extracts, pheromone mixtures, mosquito netting, and a plastic cross (Andrade, Xu, & Rich, 2014; Fourie et al., 2013; F Kuhnert, Diehl, & Guerin, 1995; Frank Kuhnert, 1996; Oliver et al., 2016).

Whether a tick does attach or not, depends on a variety of chemical and physical stimuli (Kröber & Guerin, 2007).

PURPOSE OF THE STUDY

The aim of this study was to achieve a high attachment rate of *I. hexagonus* on artificial membranes by using several different attachment stimuli, and eventually use in vitro feeding to study its vector role.

MAIN RESEARCH QUESTION

Can *Ixodes hexagonus*, while being fed in vitro, transmit *Anaplasma* and *Borrelia* during their feeding process?

MATERIALS AND METHODS

TICKS

Only *I. hexagonus* ticks were used for the experiments. In the department of Utrecht Centre for Tick-borne Diseases, a large number of uninfected *I. hexagonus* colony ticks are available. The colony provided both males and females in different life stages (adult, larvae and nymphs). Only the adult ticks were used in the experiments with the purpose to provide a better attachment rate.

In the experiments regarding the potential transmission of *Anaplasma* and *Borrelia* infected ticks were necessary. Field ticks were used for these experiments.
Due to the fact that partially engorged ticks can withdraw their mouthparts and reattach somewhere else (Kröber & Guerin, 2007), field ticks were manually detached from host animals. After which they were put on a silicone membrane to give them the possibility to reattach again.
These were collected by 15 different hedgehog shelters in the Netherlands and Belgium. When the hedgehogs came into the hedgehog shelters, they were given a total check-up, including a check to see if they carried any ticks with them. When they found one or more ticks on the hedgehog they would send them (according to the written regulations) to the UCTD. In the UCTD the ticks were sorted by species, life stage and gender.
Only the partially engorged adult *I. hexagonus* ticks were used in the experiments. The duration of the attachment on the hedgehog was unknown.
It was also unknown whether or not the ticks were infected. After the experiments, the ticks were examined on the presence of *Anaplasma* and *Borrelia* by DNA extraction, PCR and RLB.

EXPERIMENTAL GROUPS

The size of the experimental groups was variable during this research.
The experiments to improve the attachment rate started with 10 ticks per unit (5 males and 5 females). Since experiment 4 the group size was enlarged to 15 ticks per unit (mostly 5 males and 10 females). The amount of units per experiment was also variable.

The group size and the amount of units used with the field ticks was also variable, because the number of ticks used in the experiments totally depended on the number of ticks that were sent in.

Every experiment that is described in the results section, is provided with a description of the used experimental group and the number of units that were used.

BLOOD

Blood was collected from cattle kept at the farm animal department of the Utrecht University. The same two cows were used for the acquisition of blood during the whole research. They made sure that those two cows were not treated with systemic anti-parasitic drugs.
Every Monday new blood was taken from these cows from the jugular vein. During the research two methods of anti-coagulants were used. During the first three experiments anti-coagulated blood was obtained by the de stirring-technique. Directly after the collection of the blood in an Erlenmeyer, the blood was stirred for 15 minutes with a pipette, to remove all agglutination factors from the blood. After 15 minutes of stirring the pipette with the cloth of agglutination factors was thrown away.
It was the purpose to mimic the real situation as optimally as possibly, in order to maximize the chances of proper attachment of the ticks. By taking away the agglutination factors, the composition of the blood changes. That is why the decision was made (after the third experiment) to leave the agglutination factors in the blood, and add heparine (1 ml/250 ml blood) instead.
After obtaining the blood, glucose (2g/L) was added to stabilize the erythrocytes (Kröber & Guerin, 2007). The blood was divided in several sterile 50 ml tubes and stored in a refrigerator (4°C).
The blood in the units was refreshed daily. The blood was taken out of the refrigerator (4°C) and ATP was added as a general tick-feeding stimulus (Kröber & Guerin, 2007). During the first three experiments the blood turned black after 1 or two days after starting the experiments. After making a blood smear, contamination was confirmed. After confirming the contamination, it was decided to add an antibiotic in the blood that was used for the daily refreshing. Gentamicin was the chosen antibiotic, because *Anaplasma* and *Borrelia* are resistant towards this antibiotic and these bacteria will not be impeded in growth.
Before exposure of the new blood to the ticks, the blood was heated in the incubator (37°C).

IN VITRO FEEDING

UTCD has worked for several years with different types and versions of in vitro systems. The in vitro feeding system that was used for this research was originally developed by Kröber and Guerin (Kröber & Guerin, 2007)(figure 1). During the years, the UTCD made a lot of adjustments towards the in vitro feeding system developed by Kröber and Guerin, which resulted in their own protocols. During this study the methods were adjusted as well, resulting in an updated protocol (see appendix).

In this study, two types of systems were used: The Ticks-up system and the Ticks-down system.
The materials used for the production of both systems were similar, only the design was different.

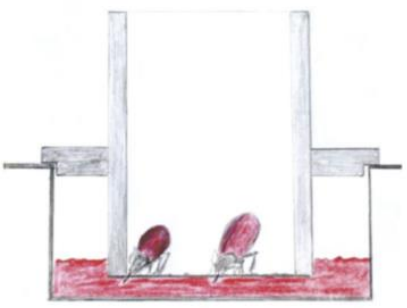


Figure 1. in vitro feeding system by Kröber and Guerin.

SYSTEMS

TICKS-DOWN SYSTEM (TD-SYSTEM)

The TD-system was the first system that was used in this research. The system contains a six-well plate (blood compartment), a unit with a silicone membrane (tick compartment), which has to be placed in the blood compartment and a bonbon (which prevent the ticks from escaping and provides the unit from fresh air) which has to be placed in the tick compartment.
When the ticks want to take a blood meal, they have to crawl down to penetrate the membrane.

TICKS-UP SYSTEM (TU-SYSTEM)

The TU-system (figure 2) is a system that is developed based on the fact that most of the ticks were on the bonbon during the check-moments and crawl up when the bonbon was taken away. During the study the ticks showed the same pattern, so after experiment 7, the TU-system was add into the experiments.
The TU-system is a system that contains several parts: a blood compartment with the silicone membrane (figure 3), a tick compartment (figure 4), a lid to cover the blood (figure 5) and a bonbon (figure 6) to prevent the ticks from escaping and provides the unit of fresh air.
The assembled units had to be placed in an empty six-wells plate and covered with six-holes for stability.

Figure 2. The TU-system.

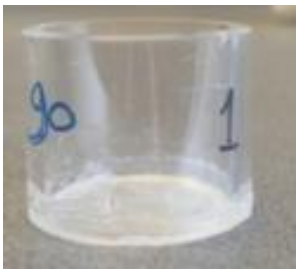


Figure 3.
Blood compartment with the silicone membrane.

Figure 4.
Tick compartement.

Figure 5.
A cap.

Figure 6.
The bonbon.

MEMBRANES

The silicone membranes were all produced by hand. A glass plate was covered with plastic film and fastened with tape on the back and front of the plate. The air underneath the foil, had to be removed with a lens cleaning paper, to prevent imperfections on the membranes. Eight lens cleaning papers were divided on the surface, and secured with tape on the top. A silicone mix was produced according to the protocol, and applied with an applicator onto the lens cleaning paper. The thickness of the membranes can be varied by changing the pressure on-, or the angle of the applicator.
The membranes had to dry for at least 24 hours at room temperature.

When the membranes were dry, the units (Tick compartment for the TD-system, blood compartment for the TU-system) would be glued on the membrane with silicone-glue. It was essential that the glue was divided equally on the edge of the unit. If not, this would become a weak spot, which could potentially lead to a leakage. The units glued on the membranes would have to dry for at least 3 hours, at room temperature.

When the glue was dry, the units could be cut out with a scalpel. It was important to make sure that the foil was cut out as well, and stayed on the membrane while taking it of the glass plate. After cutting all the units out of one lens cleaning paper, the thickness of the membranes (without the attached foil) was measured with a micro meter (Mitutoyo).
Only the membranes between 70 and 120µm were used. Membranes thinner than 70µm gave a higher risk of leakage. Membranes thicker than 120µm were difficult for the ticks to penetrate.
(See the appendix for the complete protocols and a graphical guide.)

BONBONS

The bonbons prevented the ticks from crawling out of the units and also provided a place for air ventilation. The bonbon contained a perforated piece of plastic with organza tied around it, secured with a metal wire. The piece of plastic could be placed in the organza in two ways, with the rounded edge directed towards the ticks or with the straight edge directed towards the ticks.
The *I. hexagonus* ticks crawled in all corners that they could find. During the study, some of the bonbons were not secured tightly in the unit, because the units were made in several batches, and the size of the inner circle varied in micrometres to millimetres, especially in the TU-system. Because the ticks wanted to crawl in edges and corners, some of them managed to crawl along the surface of the bonbon and escaped out of the unit (figure7). In order to make the edges less attractive for the ticks, the pieces of plastic in the organza were placed with the straight edge directed towards the ticks. A disadvantage of this method was that it was more difficult to place the bonbons in the units.
To make the bonbon fit better in the wider units, the bonbons were provided with an additional layer of organza.

Figure 7. Ticks crawl along the bonbon surface

ATTACHMENT STIMULI

During this study, a variety of attachment stimuli has been produced and tested, to provide a better attachment rate.

HAIR

Hair is a basic attachment stimuli, and has been successfully used in different other studies (Andrade et al., 2014; Kröber & Guerin, 2007; F Kuhnert et al., 1995; Frank Kuhnert, 1996). So it was used as a basic attachment stimulus in all the experiments in this study.
The source of the hair can vary, because for *I. hexagonus*, different animals can serve as a host animal. Cow hair was chosen for practical considerations. The Farm Animal Department from the Utrecht University has a large amount of farm animals (including cows) at its disposal. A few calves, which had not been treated with anti-parasitic drugs were selected and shaved to provide the amount of hair which was required for the experiments. There was a preference for white hair, because it gives a better contrast towards the dark collared ticks.
The hair was cut into pieces (± 1 cm), for a better fit in the units.
In the first 7 experiments, the hair was only inserted into the units during the start-up of the experiment.
From experiment 8 onwards, the hair in the units was refreshed daily, in order to add a fresh smell.

GRID

To provide more grip for the ticks, a grid was used in the first three experiments.
The grid was cut out of a plastic flexible mosquito netting. After cutting it on the right size, it was glued in the units onto the silicone membranes with silicone glue, by using a cotton swab. It was difficult to make the perfect balance between good adhesion of the grid and make sure that the holes weren't covered by glue (which made it hard for the ticks to puncture). This made it a time consuming job.
After the third experiment there was still attachment, even though no grid was used. So the ticks did not necessarily need the grid to attach.

COW EXTRACT → UNKNOWN SOURCE

The first cow-extract that was used originated from an unknown source. When the extract was sprayed on a surface, it clearly smelled like cow. After a few hours the odour was gone. Therefore, it was decided to concentrate the extract.

CONCENTRATED COW-EXTRACT → UNKNOWN SOURCE

For this extract, the cow-extract from an unknown source was used. First, the open bottle was placed before a fan overnight. During the night half of the fluid was vaporized. After this, the cow extract was dripped on the grid (that was already glued on the membrane), until the grid was completely covered in cow-extract. Then, a hair dryer (cold setting) was used to vaporize the moisture.

Concentrated dog extract → unknown source

There was also a bottle of dog extract present at UCTD. The source and how they obtained this extract was unknown as well. Because the odour was minimal, it was decided to concentrate the extract before using it on an experiment.
An open bottle with the dog extract was placed in front of a rotating fan, overnight. During the night, two-thirds of the fluid was vaporized.

Rubbed membranes

Two untreated cows were selected. Dried membrane on the glass plates were cut out around the edges and taped on a plastic surface. Each membrane was rubbed on the shoulder and neck of a cow for 10 minutes. After which the units were glued on the membranes.

Preconditioned ticks

In the fifth experiment, the ticks were preconditioned for 24 hours in a unit with rubbed membranes.
To precondition other ticks, a textile fabric (which was rubbed on several cows, who had not been treated with an anti-parasitic drugs) was put in a glass bell.

Units/Membranes in odour box

To give the units and the membranes an odour that was equal to the odour of a host animal, an odour box was created. The odour box contains a collection of cow hair and other materials (textile rubbed on cows), all from untreated cows.
Units and membranes could be placed in this odour box.
When the units from the TD-system were placed in the odour box, the odour was present in the entire unit (membrane and tick compartment). When the units from the TU-system were placed in the odour box, only the membrane (attached to the blood compartment) would contain the odour.
The membranes could also be placed separately (without units glued on them) in the odour box. The units from the TD-system can be glued on the membranes afterwards, which resulted in only the membranes smelling like the host animals, and not the whole unit.

Bovine sebum extract (Hexane)

There are several ways to make an extract and several methods were tried. The method from Gikonyo et al. was used in this study (Gikonyo et al., 2000). The dichloromethane was replaced by hexane.
Cotton pads were drenched in 240 ml Hexane. These drenched cotton pads were rubbed onto the shoulders and neck of two cows (both not treated with anti-parasitic drugs). After which, all the cotton pads were squeezed, and 50 ml bovine pelage extract was extracted.

Concentrated bovine sebum extract (hexane)

An open bottle with bovine sebum extract was placed in front of a rotating fan, overnight. During the night, all the fluid vaporized. Then, the extract was dissolved in 5 ml hexane.
A second batch of concentrated bovine sebum extract was made. The bottle with the remaining squeezed cotton pads was filled with 200 ml hexane. All the cotton pads were squeezed, and 100 ml bovine pelage was extracted. The open bottle with bovine sebum extract was placed in front of a rotating fan, overnight. During the night, all the fluid vaporized. Then, the extract was dissolved in 10 ml hexane.

Faeces extract

Adult *Dermacentor reticulatus* tick faeces were available at UCTD. The first extract was made with 2 g faeces, which was dissolved in 3 ml hexane. This mix was stored for three days. Then, the mixture was filtered through a lens cleaning paper. The fluid was directly absorbed by the lens cleaning paper, which did not leave any extract. The remaining faeces was mixed with 10 ml hexane and directly filtered through the earlier used lens cleaning paper.
The second faeces extract was produced by dissolving 5 g faeces in 10 ml hexane. This solution was filtered through a clean lens cleaning paper. The extract was put in front of a rotating fan after which it vaporized. Afterwards the remaining extract was dissolved in 10 ml hexane.

WATER BATH

During the experiments, the units had to be stored in a controlled environment. In the first two experiments the units were kept in a water bath. The units floated on the water, while the blood level was below the water level. The temperature of the water was kept at 37°C, to simulate the same body temperature of the host animal. The water bath was covered with a black piece of cloth. Humidity was kept between 70-75% underneath the cloth.

The use of the water bath had several disadvantages. There was a lot moist underneath the cloth, which resulted in moist in and between the units. The six-well plates could only carry four units, because of the weight, otherwise the plates would sink.

INCUBATOR

During the second experiment, it was decided to transfer the units to the incubator because of the disadvantages of the water bath. Also the temperature in the incubator was set to 37°C. To keep the right humidity, a container with (in aquadest) dissolved K_2SO_4 was placed inside the incubator. The humidity was kept between 70-85%. The incubator had the advantage, that the CO_2 could be managed (it was set on ± 5,0%).
A disadvantage of the incubator was that it was used by different scientists. Several times during the day the scientist would open the incubator, which caused a drop in humidity and temperature.

SCHEDULE

Most of the experiments started on Monday. In the morning, the blood was obtained from the Farm Animal Department. The ticks were selected and placed in daylight and room temperature. Thereafter, the units were prepared and the attachment stimuli were applied. By the end of the day, the ticks were placed in their units and the six-well with the units were placed in either the water bath or the incubator. During the week, the units were checked daily.
On Friday morning the ticks were checked and the experiment was stopped. The ticks were counted and checked in detail on the tick racetrack. After the experiment, the units were cleaned.
During the week new attachment stimuli, membranes, complete units and bonbons were produced to replenish the stock.

Some experiments were started on other days of the week.
(see appendix for the protocols: start-up, maintenance and finishing-up)

CHECK-UP MOMENTS

During the week, the units were checked daily for blood colour, attachment rate, mortality of ticks and various other factors in the flow-cabinet. First of all, there was a visual inspection of the units, regarding the blood colour and overall impression of the ticks in the units. After this inspection, the unit was opened, and the ticks could be inspected more closely. Sometimes manipulation of the ticks in the unit was necessary, to make sure if they were attached or not. Because of the excessive movement of the ticks, the bonbon (mostly with a group of ticks on it) was placed in a high glass container, to prevent them from escaping. This made it easier to focus on the unit.

RESULTS

Most of the experiments took five days. The units are indicated with the letter U and a corresponding number. The thickness of the membranes and the differences between the units is noted.
Only in the first experiment, *Dermacentor reticulatus* ticks were used.

EXPERIMENT 1 – TEST WEEK (TD-SYSTEM)

This experiment was conducted to get more familiar with the protocols, methods, proceedings and crafts. The attachment stimuli that were used were the grid, the cow-extract from a unknown source and the cow hair as a basis. The cow-extract was applied twice with a cottonswab. The first time on the grid, the second time after the hair was put in the units. The hair was only applied once, before the ticks were put in there units.
The stirring-technique was the anticoagulation method used for the blood.

Each unit contained 5♀ and 5 ♂ *Dermacentor reticulates* from the colony.
U1: membrane had a thickness of 80 µm
U2: membrane had a thickness of 80 µm
U3: membrane had a thickness of 120 µm
U4: membrane had a thickness of 100 µm

The six-well plates were put in the water bath, after starting the experiment.

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	0/10	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
3	2/10	20	2/10	20	2/10	20	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
4	0/10	0	0/10	0	0/10	0	1/10	10	0/10	0	0/10	0	0/10	0	0/10	0

Unit	Highest attachment (%)	Average attachment (%)
U1	0	5
U2	20	
U3	0	
U4	0	

During the 24-hour check-up, it was noticed that unit 1 was leaking. The unit was removed from the experiment. During the 48-hour check-up it was noticed that the blood had turned black in some wells.
In U3, there was attachment of two male ticks, which were no longer attached during the 96-hour check-up anymore. In U4, there was attachment of one male tick noticed during the 96-hour check-up.

The average attachment is 20%.

EXPERIMENT 2 – CONCENTRATED COW-EXTRACT (TD-SYSTEM)

This experiment was the first experiment with *I. hexagonus.* The attachment stimuli in this experiment were the grid, the concentrated cow-extract form the unknown source and the cow hair.

During the gluing of the grid, it was noticed that the prepared grid was too large (µm to mm) for the units, which made the edges curl upwards, after gluing it with silicone glue. It is possible that this was also the case in the previous experiment. This can be an obstacle for the ticks to attach, because they usually attach around the edges.

The concentrated cow-extract was applied on the grid until the grid was completely covered with fluid. After which the unit was air dried until the moisture was vaporized. A mucous layer was visible on the grid (figure 8).

The stirring-technique was used as the anti-coagulation method for the blood.

Figure 8. Mucous layer on the grid.

Each unit contained 5♀ and 5 ♂ *I. hexagonus* from the colony.

U1: membrane had a thickness of 80 µm

U2: membrane had a thickness of 80 µm

U3: membrane had a thickness of 95 µm

U4: membrane had a thickness of 80 µm

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	1/10	10	1/10	10	1/10	10
2	0/10	0	0/10	0	0/10	0	0/10	0	1/10	10	1/10	10	1/10	10	1/10	10
3	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0
4	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	1/10	10	1/10	10	2/10	20

Unit	Highest attachment (%)	Average attachment (%)
U1	0	0
U2	0	
U3	0	
U4	0	

After the 72-hour check-up the six-well plate was stored in the incubator, because of the high amount of moist in and in-between the units. Figure 9 shows the six-well plate and the high amount of moist in between the units (mixed with some blood from the edge of a well). From this moment on, all of the experiments took place inside the incubator. The dark coloured blood is shown as well.

There was no attachment during this experiment.

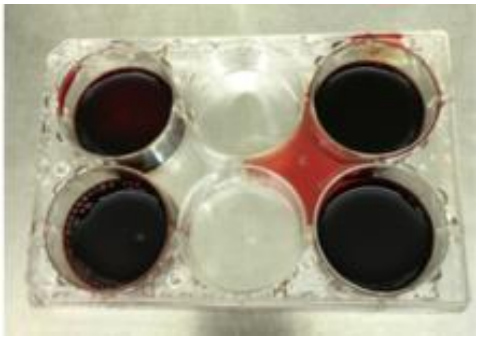


Figure 9. High amount of moist in between the units.

EXPERIMENT 3 – CONCENTRATED DOG EXTRACT (TD-SYSTEM)

The grid, the concentrated dog extract from an unknown source and the cow hair were used as attachment stimuli in this experiment. The grids, used in the units, were cut smaller than the original size, which made them fit better in the units. The extract was dripped on the grids, spread out over the membrane with a cotton swab and dried with a hair dryer. There was no mucous layer visible on the membranes and grids.
The stirring-technique was used as the anti-coagulation method for the blood. The six-well plate was placed in the incubator as well as a container with K_2SO_4, to provide the right humidity.

Each unit contained 5♀ and 5 ♂ *I. hexagonus* from the colony. Unit 1 contained 1 additional female *I. hexagonus*.
U1: membrane had a thickness of 70 µm
U2: membrane had a thickness of 70 µm
U3: membrane had a thickness of 80 µm
U4: membrane had a thickness of 80 µm

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	Number	%	number	%	number	%	number	%
1	2/11	18	3/11	27	0/11	0	2/11	18	0/11	0	0/11	0	2/18	18	4/11	36
2	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	2/10	20	10/10	100	-	-
3	0/10	0	3/10	30	1/10	10	1/10	10	0/10	0	0/10	0	1/10	10	2/10	20
4	0/10	0	0/10	0	0/10	0	0/10	0	0/10	0	1/10	10	4/10	40	6/10	60

Unit	Highest attachment (%)	Average attachment (%)
U1	27	14
U2	0	
U3	30	
U4	0	

During the 24-hour check-up, it was noticed that the plastic foil was not removed before placing the units in the blood. This could be an obstacle for the ticks to attach, however, there was some attachment. The plastic foil was removed during the 24-hour check-up.
During the 24-hour check-up, there was attachment of both a male and a female in U1.
During the 48-hour check-up it was noticed that the male tick had detached in U1, because three female ticks were attached. Also in U3 there was attachment of three female ticks.
Manipulation of the ticks in the unit was necessary, to make sure if they had attached or not. During the 48-hour check-up, two of the three attached ticks in U1 were accidently detached because of manual manipulation. During the 48-hour check-up, a small amount of leakage was found in U2. The blood had dried up and stopped leaking. Two ticks had died in the blood. At the 72 hour check-up, it was observed that U2 had fully leaked, and all the ticks died. Also the mortality rate in U4 on the 96-hour check-up was high, the reason was unknown.

The average attachment was 14%.

EXPERIMENT 4 – RUBBED MEMBRANES (TD-SYSTEM)

The attachment stimuli used in this experiment were rubbed membranes and cow hair. The units were glued on the rubbed membranes and put in a closed box to dry. A container with KCL was also in the box, because the glue dried better in a humid environment.
The membranes were used in experiment 4 on the same day they were rubbed on the cows.
Before the ticks were put in their units, they were put in an incubator at 27°C and lower humidity, to dry them out for some time, and make them (possibly) more eager to attach. From now on, all the ticks, that were used in the experiment were put in the incubator with a high temperature and lower humidity before they were put in the units for the experiments.
The blood used in the experiment was anti-coagulated with heparine instead of the stirring-technique. From then on, blood anti-coagulated with heparine was used in all experiments.
Because of the black coloured blood in the experiment, a blood smear was made and contamination was concluded. From then on, during every experiment, Gentamicin was added to the used blood.

It was decided to increase the size of the groups of ticks in the units to 10 female ticks and 5 male ticks per unit and to add control groups. The control groups are indicated with a 'c'.
Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony.
U1: membrane had a thickness of 85 µm, rubbed membrane
U2: membrane had a thickness of 80 µm, rubbed membrane
U3: membrane had a thickness of 85 µm, rubbed membrane
U4: membrane had a thickness of 85 µm, rubbed membrane
U5c: membrane had a thickness of 95 µm, normal membrane with only cow hair
U6c: membrane had a thickness of 95 µm, normal membrane with only cow hair

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	0/15	0	-	-	-	-	-	-	10/15	-	-	-	-	-	-	-
2	4/15	27	-	-	-	-	-	-	0/15	-	-	-	-	-	-	-
3	0/15	0	-	-	-	-	-	-	8/15	-	-	-	-	-	-	-
4	2/15	13	-	-	-	-	-	-	0/15	-	-	-	-	-	-	-
5c	2/15	13	-	-	-	-	-	-	0/15	-	-	-	-	-	-	-
6c	4/15	27	-	-	-	-	-	-	0/15	-	-	-	-	-	-	-

Rubbed membranes		
Unit	Highest attachment (%)	Average attachment (%)
U1	0	10
U2	27	
U3	0	
U4	13	

Control membranes		
Unit	Highest attachment (%)	Average attachment (%)
U5c	13	20
U6c	27	

During the 24-hour check-up it was noticed that two (U1 and U3) from the four units with rubbed membranes showed leakage. One of the four units with rubbed membranes showed a possible leakage. The risk for more leakage and loss of ticks was the reason to stop the experiment. The ticks that were still alive in U1 and U3 were stored. The other ticks from U2, U4, U5c and U6c were reused in experiment 5.
It stands out that there was a higher attachment rate of 27% in U2 (one male and three female) and in U6c (four female).

The average attachment for the rubbed membranes was lower than the average attachment of the control groups.

EXPERIMENT 5 – RE-USED TICKS FROM EXPERIMENT 4 (TD-SYSTEM)

This experiment contained the two control units (U5c and U6c) including the ticks from those units, used in experiment 4. The ticks from U2 and U3 were placed in units with new basic membranes and cow hair. These ticks were preconditioned for 24 hours on a rubbed membrane during experiment 4.
U1 and U3 were new units, using basic membranes and cow hair, filled up with new ticks.

Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony.
U1: membrane had a thickness of 85 µm, new ticks
U2: membrane had a thickness of 90 µm, ticks from experiment 4, preconditioned
U3: membrane had a thickness of 80 µm, new ticks
U4: membrane had a thickness of 80 µm, ticks from experiment 4, preconditioned
U5c: membrane had a thickness of 95 µm, normal membrane with only cow hair, same units and ticks used in experiment 4
U6c: membrane had a thickness of 95 µm, normal membrane with only cow hair, same units and ticks used in experiment 4

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	1/15	7	0/15	0	0/15	0	-	-	0/15	0	0/15	0	0/15	0	-	-
2	6/15	40	6/15	40	4/15	27	-	-	0/15	0	0/15	0	0/15	0	-	-
3	2/15	13	1/15	7	1/15	7	-	-	0/15	0	0/15	0	0/15	0	-	-
4	6/15	40	5/15	33	3/15	20	-	-	0/15	0	0/15	0	0/15	0	-	-
5c	0/15	0	-	-	-	-	-	-	12/15	80	-	-	-	-	-	-
6c	2/15	13	4/15	27	3/15	20	-	-	0/15	0	0/15	0	0/15	0	-	-

Preconditioned ticks from experiment 4		
Unit	Highest attachment (%)	Average attachment (%)
U2	40	40
U4	40	

New ticks		
Unit	Highest attachment (%)	Average attachment (%)
U1	7	10
U3	13	

Control group		
Unit	Highest attachment (%)	Average attachment (%)
U5c	0	14
U6c	27	

During the 24-hour check-up, one attached tick in U1 and two attached ticks in U3 detached after manipulation. U5c showed leakage and this was removed from the experiment.
What stood out immediately, were the high attachment rates of 40% in U2 and U4 (the units with the preconditioned ticks). The attached ticks were all female, which means that the attachment rates among female ticks for U4 were 60%. The other attachments in the other units were all female ticks.
During the 48-hour check-up, it was noticed that the four attachment ticks in U6c contained one male. This male was observed to be detached at the 72-hour check-up.
During the 72-hour check-up, it was noticed that two possibly attached ticks in U2 were removed from the membrane by pulling the bonbon out of the unit, because these ticks were stuck with their hind body by blood on the bonbon (figure 10). So the attachment rate was possibly higher.

Figure 10.
Tick stuck to the organza.

The average attachment of the preconditioned tick was higher (40%) than that of the control groups (10 and 14%)

EXPERIMENT 6 - UNIT + MEMBRANES 12-24 HOURS IN ODOURBOX (TD-SYSTEM)

This experiment was started in the middle of the week, which made it only last for a total of 48 hours. To make attachment more attractive for the ticks, the units (with the membranes already glued on them) were places in the odourbox for 12 to 24 hours. The membranes were provided with cow hair. The control units contained a normal (odourless) membrane with cow hair.

Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony.
U1: membrane had a thickness of 90 µm, unit + membrane in odourbox
U2: membrane had a thickness of 85 µm, unit + membrane in odourbox
U3: membrane had a thickness of 80 µm, unit + membrane in odourbox
U4: membrane had a thickness of 85 µm, unit + membrane in odourbox
U5c: membrane had a thickness of 80 µm, normal membrane with only cow hair
U6c: membrane had a thickness of 90 µm, normal membrane with only cow hair

	Attachment				Mortality			
	24 h		48 h		24 h		48 h	
Unit	number	%	number	%	number	%	number	%
1	4/15	27	2/15	13	0/15	0	1/15	7
2	3/15	20	-	-	0/15	0	-	-
3	4/15	27	1/15	7	0/15	0	0/15	0
4	0/15	0	-	-	1/15	7	-	-
5c	0/15	0	-	-	9/15	60	-	-
6c	0/15	0	0/15	0	0/15	0	0/15	0

Units + membranes in orourbox		
Unit	Highest attachment (%)	Average attachment (%)
U1	27	19
U2	20	
U3	27	
U4	0	

Control group		
Unit	Highest attachment (%)	Average attachment (%)
U5c	0	0
U6c	0	

During the 24-hour check-up, it was noticed that there was leakage in three units (U2, U4 and U5c). All these units were removed from the experiment. The glued edge was checked and concluded that it was intact. Until this experiment, the membranes were measured with the foil attached, so the membranes were probably thinner than the noted thickness. After this discovery, the membranes were measured without the foil.
The attachment rates in the first 24 hours were higher in U1, U2 and U3. The control units (U5c and U6c) showed no attachment. U4, which was also had been in the odourbox, showed no attachment. During the check-up, one attached tick in U1 was detached through manipulation.

During the 48-hour check-up, it was noticed that two possibly attached ticks in U1 were removed from the membrane by pulling the bonbon out of the unit, because these ticks were stuck with their hind body on the bonbon because of blood/faeces (figure 10). This means that the attachment rate was probably higher.

The average attachment rate of the units and membranes in the odourbox is higher (19%) than the control groups (0%).

EXPERIMENT 7 – BOVINE SEBUM EXTRACT (TD-SYSTEM)

This experiment contained the bovine sebum extract as an attachment stimulus. 50µl bovine sebum extract was added on the centre of the membranes with a pipette. After a couple of minutes, the cow hair was added to the units. The control units only contained cow hair.

Two six-well plates were used during this experiment. One of the units with the bovine sebum extract and one with the control groups.

Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony. Unit 6c contains 1 female *I. hexagonus* more.
Bovine sebum extract group:
U1: membrane had a thickness of 85 µm, bovine sebum extract
U2: membrane had a thickness of 85 µm, bovine sebum extract
U3: membrane had a thickness of 85 µm, bovine sebum extract
U4: membrane had a thickness of 90 µm, bovine sebum extract
U5: membrane had a thickness of 90 µm, bovine sebum extract
U6: membrane had a thickness of 90 µm, bovine sebum extract

Control group:
U1c: membrane had a thickness of 85 µm, normal membrane with only cow hair
U2c: membrane had a thickness of 85 µm, normal membrane with only cow hair
U3c: membrane had a thickness of 85 µm, normal membrane with only cow hair
U4c: membrane had a thickness of 90 µm, normal membrane with only cow hair
U5c: membrane had a thickness of 90 µm, normal membrane with only cow hair
U6c: membrane had a thickness of 90 µm, normal membrane with only cow hair

Bovine sebum extract group																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	5/15	33	3/15	20	6/15	40	7/15	47	0/15	0	0/15	0	0/15	0	0/15	0
2	6/15	40	8/15	53	4/15	27	5/15	33	0/15	0	0/15	0	0/15	0	0/15	0
3	2/15	13	3/15	20	5/15	33	4/15	27	0/15	0	0/15	0	1/15	7	1/15	7
4	7/15	47	3/15	20	3/15	20	5/15	33	0/15	0	0/15	0	0/15	0	0/15	0
5	3/15	20	4/15	27	2/15	13	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0
6	2/15	13	2/15	13	3/15	20	3/15	20	0/15	0	0/15	0	0/15	0	0/15	0

Bovine sebum extract group		
Unit	Highest attachment (%)	Average attachment (%)
U1	47	38
U2	53	
U3	33	
U4	47	
U5	27	
U6	20	

Control group																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1c	3/15	20	3/15	20	3/15	20	3/15	20	0/15	0	0/15	0	0/15	0	0/15	0
2c	2/15	13	3/15	20	4/15	27	6/15	40	0/15	0	0/15	0	0/15	0	0/15	0
3c	5/15	33	1/15	7	3/15	20	3/15	20	0/15	0	0/15	0	0/15	0	1/15	7
4c	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
5c	1/15	7	2/15	13	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0
6c	2/16	13	2/16	13	4/16	25	2/16	13	0/16	0	0/16	0	0/16	0	0/16	0

Control group		
Unit	Highest attachment (%)	Average attachment (%)
U1c	20	22
U2c	40	
U3c	33	
U4c	0	
U5c	13	
U6c	25	

All the ticks that were attached during this experiment were female ticks. To compare the attachment rate of the bovine sebum extract group with the control group, the average attachment rate per 24 hours was calculated. The Bovine sebum extract group had a higher overall attachment rate of 26 to 29%.
During the first check-up, three units (U1, U2 and U4) had attachment rates amongst the female ticks of 50% or higher, respectively 50%, 60% and 70%. During the 48-hour check-up, only U2 had an attachment rate amongst female above 50%, specifically: 80%. During the 72-hour check-up, U1 had an attachment rate of 60% and U3 had an attachment rate of 50%. After 96 hours, the attachment rate amongst females for U1, U2 and U4 was respectively 70%, 50% and 50%.
By opening U2 during the 72-hour check-up, one possibly attached tick was stuck with blood on her hind body on the bonbon.
Also during the 96-hour check-up, one tick in U1 and one tick in U5 were stuck on the organza.

Attachment rates amongst female ticks were only 50% and 60% for respectively U3c and U2c during the check-up at 24 and 96 hours.
During the 24-hour check-up, one attached tick in U1c detached after manipulation.
By opening up U6c during the 72-hour check-up, one possibly attached tick was stuck with blood on her hind body on the bonbon.
Also during the 96-hour check-up, one tick in U1c was stuck on the organza.

The control group showed a lower average attachment rate (22%) than the bovine sebum extract group (38%).

EXPERIMENT 8 – MEMBRANES IN ODOURBOX, 3 TO 9 DAYS (TU- AND TD-SYSTEM)

The membranes from the TD-system, were placed in the odourbox for nine days before the units were glued on them. The membranes connected to the blood compartment were placed in the odourbox for 3 days (with a cap on the blood compartment, to protect it against the cow hair).
Both the TU- and the TD-system contained three control units with a normal membrane (which wasn't placed in the odourbox) and cow hair. The cow hair was replaced daily in all the units.

Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony.
TU-system:
U1: membrane had a thickness of 95 µm, membrane three days in odourbox
U2: membrane had a thickness of 110 µm, membrane three days in odourbox
U3: membrane had a thickness of 90 µm, membrane three days in odourbox
U4c: membrane had a thickness of 100 µm, normal membrane with only cow hair
U5c: membrane had a thickness of 110 µm, normal membrane with only cow hair
U6c: membrane had a thickness of 100 µm, normal membrane with only cow hair

TD-system:
U1: membrane had a thickness of 90 µm, membrane nine days in odourbox
U2: membrane had a thickness of 90 µm, membrane nine days in odourbox
U3: membrane had a thickness of 90 µm, membrane nine days in odourbox
U4c: membrane had a thickness of 90 µm, normal membrane with only cow hair
U5c: membrane had a thickness of 90 µm, normal membrane with only cow hair
U6c: membrane had a thickness of 90 µm, normal membrane with only cow hair

TU-SYSTEM

TU-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
2	0/15	0	2/15	13	1/15	7	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0
3	0/15	0	0/15	0	5/15	33	3/15	20	0/15	0	0/15	0	0/15	0	0/15	0
4c	0/15	0	0/15	0	1/15	7	1/15	7	0/15	0	0/15	0	0/15	0	1/15	7
5c	0/15	0	0/15	0	1/15	7	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0
6c	0/15	0	2/15	13	3/15	20	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0

Membranes three days in odourbox		
Unit	Highest attachment (%)	Average attachment (%)
U1	0	15
U2	13	
U3	33	

Controlgroup		
Unit	Highest attachment (%)	Average attachment (%)
U4c	7	13
U5c	13	
U6c	20	

During the 48-hour check-up, one tick escaped out of the tick compartment and was found under the bonbon. Because some of the bonbons didn't fit tightly in the unit, the ticks were able to crawl in the edges and corners. Some of them managed to crawl along the surface of the bonbon and one escaped out of the unit. The bonbon was provided with an additional layer of organza to better fit in the unit and prevent the ticks from escaping.
In U6c, both attachments (male and female) detached during the picture moment.

During the 72-hour check-up, the attachment rate amongst female was 40% in U3. During this check-up some of the attachments in U3 (one male) and in U4c (one female) were detached because of manipulation.

There is no distinct difference in attachment rate between the units with the membranes from the odourbox and the control units.

The average attachment rate was slightly higher in the group with the membranes from the odourbox (15%), in comparison with the control group (13%).

TD-SYSTEM

TD-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	1/15	7	1/15	7	3/15	20	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0
2	0/15	0	1/15	7	1/15	7	3/15	20	0/15	0	1/15	7	1/15	7	1/15	7
3	4/15	27	2/15	13	5/15	33	3/15	20	0/15	0	0/15	0	0/15	0	0/15	0
4c	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
5c	1/15	7	0/15	0	2/15	13	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0
6c	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0

Membranes nine days in odourbox		
Unit	Highest attachment (%)	Average attachment (%)
U1	20	24
U2	20	
U3	33	

Control group		
Unit	Highest attachment (%)	Average attachment (%)
U4c	7	9
U5c	13	
U6c	7	

Only during the 72-hour check-up, the attachment rate amongst female increased to 50% in U3.
During the 96-hour check-up, one possibly attached tick in U3 was stuck to the bonbon with faeces.
The attachment rates were slightly higher in the units with the membranes from the odourbox in comparison to the control units.

The mean attachment rate was higher in the group with the membranes from the odourbox (24%), than in comparison with the control group (9%).

EXPERIMENT 9 – CONCENTRATED BOVINE SEBUM EXTRACT (TU- AND TD-SYSTEM)

This experiment contained the concentrated bovine sebum extract as an attachment stimulus. 50µl concentrated bovine sebum extract was added on the centre of the membranes with a pipette. After a couple of minutes, the cow hair was added to the units. The control units contained only cow hair.
The cow hair was replaced daily in all of the units.

Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony.
TU-system:
U1: membrane had a thickness of 95 µm, concentrated bovine sebum extract
U2: membrane had a thickness of 95 µm, concentrated bovine sebum extract
U3: membrane had a thickness of 100 µm, concentrated bovine sebum extract
U4c: membrane had a thickness of 95 µm, normal membrane with only cow hair
U5c: membrane had a thickness of 95 µm, normal membrane with only cow hair
U6c: membrane had a thickness of 95 µm, normal membrane with only cow hair

TD-system:
U1: membrane had a thickness of 95 µm, concentrated bovine sebum extract
U2: membrane had a thickness of 95 µm, concentrated bovine sebum extract
U3: membrane had a thickness of 100 µm, concentrated bovine sebum extract
U4: → Unit in use for an experiment with field ticks
U5c: membrane had a thickness of 95 µm, normal membrane with only cow hair
U6c: membrane had a thickness of 95 µm, normal membrane with only cow hair

TU-SYSTEM

TU-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
U1	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	2/15	13
U2	1/15	7	3/15	20	0/15	0	1/15	7	0/15	0	1/15	7	1/15	7	1/15	7
U3	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	2/15	13
U4c	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	1/15	7
U5c	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
U6c	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0

Concentrated bovine sebum extract group		
Unit	Highest attachment (%)	Average attachment (%)
U1	0	7
U2	20	
U3	0	

Control group		
Unit	Highest attachment (%)	Average attachment (%)
U4c	0	2
U5c	0	
U6c	7	

The attachment rate was low during this experiment. The highest attachment rate was seen at the 48-hour check-up in U2 (20%). Only female ticks were attached. The reason of the low attachment is unknown.

The average attachment rate of the concentrated bovine sebum group (7%) is higher than that of the control group (2%).

TD-SYSTEM

TD-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	3/15	20	6/15	40	6/15	40	6/15	40	0/15	0	0/15	0	0/15	0	0/15	0
2	1/15	7	0/15	0	2/15	13	1/15	7	0/15	0	0/15	0	0/15	0	1/15	7
3	3/15	20	3/15	20	6/15	40	6/15	40	0/15	0	0/15	0	0/15	0	0/15	0
x	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5c	0/15	0	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
6c	3/15	20	2/15	13	3/15	20	1/15	7	0/15	0	1/15	7	1/15	7	1/15	7

Concentrated bovine sebum extract group		
Unit	Highest attachment (%)	Average attachment (%)
U1	40	31
U2	13	
U3	40	

Control group		
Unit	Highest attachment (%)	Average attachment (%)
U5c	7	14
U6c	20	

During this experiment there were only female attachments. The control units showed attachment rates from 0% to 30% among the female ticks. In the units with the concentrated bovine sebum extract, the attachment rates among female ticks were 0% to 60%. The units with an attachment rate of 60% among female ticks were U1 at the 48-hour check-up and U3 at the 72-hour check-up.
During the 96-hour check-up, one tick that was possibly attached in U1 and in U2 was stuck onto the bonbon with their hind body in faeces. This means that the attachment rate in these units was probably higher.

The average attachment rate of the concentrated bovine sebum group (31%) was higher than the average of the control group (14%).

EXPERIMENT 10 – MEMBRANES IN ODOURBOX 4 TO 22 DAYS, CONCENTRATED BOVINE SEBUM EXTRACT, FAECES EXTRACT (TU- AND TD-SYSTEM)

This experiment contained several combined attachment stimuli. The initial idea was to put all the membranes in the odourbox and compare the combination of the odourbox membrane and the concentrated bovine sebum extract with odourbox membranes and the faeces extract. Unfortunately, due to certain circumstances, all the TU-system odourbox membranes were adapted with the concentrated bovine sebum extract instead of just the first three units.

The membranes from the TD-system were put in the odourbox for 22 days, before the units were glued on it. The units were used the same day. The membranes (combined with the blood compartment) from the TU-system was put in the odourbox for 22 days. Both of the membranes smelled like cow, before being used in the experiment.

On some membranes 50µl concentrated bovine sebum extract and on some membranes 50µl faeces extract was applied with a pipette. In earlier experiments, the drop with extract was always placed in the middle of the membrane. In past experiments it was seen that most of the ticks attached around the edges of the unit. To see if the ticks preferred to attach in the pure extract drops, the drops were divided in 5 drops of 10µl around the edge of the membrane. After letting them dry for a few minutes, the cow hair was applied in all the units. The cow hair was replaced daily in all the units during the check-ups.

Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony.
TU-system:
U1: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract
U2: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract
U3: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract
U4: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract, faeces extract
U5: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract, faeces extract
U6: membrane had a thickness of 110 µm, odourbox membrane (4 days), concentrated bovine sebum extract, faeces extract

TD-system:
U1: membrane had a thickness of 85 µm, odourbox membrane (22 days), concentrated bovine sebum extract
U2: membrane had a thickness of 90 µm, odourbox membrane (22 days), concentrated bovine sebum extract
U3: membrane had a thickness of 90 µm, odourbox membrane (22 days), concentrated bovine sebum extract
U4: membrane had a thickness of 85 µm, odourbox membrane (22 days), faeces extract
U5: membrane had a thickness of 90 µm, odourbox membrane (22 days), faeces extract
U6: membrane had a thickness of 90 µm, odourbox membrane (22 days), faeces extract

TU-SYSTEM

TU-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
2	1/15	7	2/15	13	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	1/15	7
3	2/15	13	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
4	0/15	0	3/15	20	5/15	33	3/15	20	0/15	0	0/15	0	0/15	0	0/15	0
5	1/15	7	2/15	13	1/15	7	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0
6	0/15	0	0/15	0	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	2/15	13

Odourbox + sebum extract group		
Unit	Highest attachment (%)	Average attachment (%)
U1	7	11
U2	13	
U3	13	

Odourbox + sebum extract + faeces extract group		
Unit	Highest attachment (%)	Average attachment (%)
U4	33	18
U5	13	
U6	7	

During the 48-hour check-up, one female tick was detached by manual manipulation in both U4 and U5. The attachment in the units with the concentrated bovine sebum extract and the faeces extract (U4, U5 and U6) looks higher during the 72- and the 96-hour check-up in comparison with the units with only the concentrated bovine sebum extract. But still, the attachment rates are low. Only the attachment rate among female ticks in U4 during the 72 check-up was 40%.
During the 72-hour check-up it was noticed that the bonbon from U1 had become wet by the cleaning procedure after the 48-hour check-up, so this bonbon was replaced by a new one. In U2, most of the ticks sat in the bonbon (figure 11). Most likely, the organza was punctured during the 48-hour check-up, which gave the ticks an entrance to the bonbon. This bonbon was replaced by a new one as well.
It was difficult to see if the ticks were attached on the extract-drop places.

Figure 11. Ticks in the bonbon.

The attachment rate of the group with the membranes from the odourbox, concentrated bovine sebum extract and the faeces extract (18%) was higher than that of the group with the membranes from the odourbox and the concentrated bovine sebum extract (11%).

TD-SYSTEM

TD-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	5/15	33	5/15	33	6/15	40	4/15	27	0/15	0	0/15	0	0/15	0	0/15	0
2	4/15	27	5/15	33	4/15	27	3/15	20	0/15	0	0/15	0	0/15	0	0/15	0
3	2/15	13	2/15	13	3/15	20	2/15	13	1/15	7	1/15	7	1/15	7	1/15	7
4	4/15	27	1/15	7	1/15	7	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0
5	2/15	13	3/15	20	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
6	5/15	33	4/15	27	2/15	13	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0

Odourbox + sebum extract group		
Unit	Highest attachment (%)	Average attachment (%)
U1	40	31
U2	33	
U3	20	

Odourbox + faeces extract group		
Unit	Highest attachment (%)	Average attachment (%)
U4	27	27
U5	20	
U6	33	

The units with the concentrated bovine sebum extract (U1, U2 and U3) had attachment rates between 13% and 33% during the first two check-ups. The units with the faeces extract (U4, U5 and U6) had attachment rates between 7% and 33% during the first two check-ups. After that, the attachment rates for the faeces extract units (U4, U5 and U6) eventually dropped to 7%, 0% and 13%. U1 (a unit with concentrated bovine sebum extract) contained an attachment rate among female ticks of 50% during the 72-hour check-up.
During the 48-hour check-up, one male tick in U2 had detached by manual manipulation. During the 96-hour check-up, in U1 and U3, one possibly engorged tick was stuck on the bonbon by faeces.
It was difficult to see if the ticks were attached on the extract-drop places.

The attachment rate of the group with the membranes from the odourbox and the concentrated bovine sebum extract (31%) was higher than that of the group with the membranes from the odourbox and the faeces extract group (27%).

EXPERIMENT 11- PRECONDITIONING, MEMBRANES IN ODOURBOX FOR 4 TO 11 DAYS, CONCENTRATED BOVINE SEBUM EXTRACT (2ND BATCH), FAECES EXTRACT (2ND BATCH)(TU- AND TD-SYSTEM)

All the ticks have been in the preconditioning glass bell for 3 days. Also, the membranes of the first three units have been in the odourbox (TU-system 4 days, TD-system 11 days) and the membranes contained 50µl concentrated bovine sebum (2nd batch) extract and 50µl faeces extract.
The control units only contained cow hair.
The cow hair was replaced daily in all the units during the check-ups.

Each unit contained 10♀ and 5 ♂ *I. hexagonus* from the colony.
TU-system:
U1: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch)
U2: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch)
U3: membrane had a thickness of 90 µm, odourbox membrane (4 days), concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch)
U4: membrane had a thickness of 90 µm, normal membrane with only cow hair
U5: membrane had a thickness of 90 µm, normal membrane with only cow hair
U6: membrane had a thickness of 90 µm, normal membrane with only cow hair

TD-system:
U1: membrane had a thickness of 90 µm, odourbox membrane (11 days), concentrated bovine sebum extract, (2nd batch), faeces extract (2nd batch)
U2: membrane had a thickness of 90 µm, odourbox membrane (11 days), concentrated bovine sebum extract, (2nd batch), faeces extract (2nd batch)
U3: membrane had a thickness of 90 µm, odourbox membrane (11 days), concentrated bovine sebum extract, (2nd batch), faeces extract (2nd batch)
U4: membrane had a thickness of 90 µm, normal membrane with only cow hair
U5: membrane had a thickness of 90 µm, normal membrane with only cow hair
U6: membrane had a thickness of 90 µm, normal membrane with only cow hair

TU-SYSTEM

TU-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
U1	1/15	7	5/15	33	2/15	13	4/15	27	0/15	0	0/15	0	0/15	0	0/15	0
U2	3/15	20	5/15	33	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
U3	1/15	7	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
U4c	0/15	0	0/15	0	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	0/15	0
U5c	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
U6c	1/15	7	0/15	0	2/15	13	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0

Odourbox + sebum extract + faeces extract		
Unit	Highest attachment (%)	Average attachment (%)
U1	33	26
U2	33	
U3	13	

Control group		
Unit	Highest attachment (%)	Average attachment (%)
U4c	7	7
U5c	0	
U6c	13	

During the 24-hour check-up, one attachment had detached after manual manipulation in U1, U2 and U6.
During the 48-hour check-up, U1 and U2 stands out with attachment rates among female ticks of 50%.
In U3, two attached ticks had detached after manual manipulation during this check-up.

During the 72-hour check-up was noticed that there was a little dried leakage in U2.
During the 96-hour check-up, U1 had an attachment rates among female ticks of 40%.

The group with the membranes from the odourbox, the concentrated bovine sebum extract and the faeces extract (26%) is higher than the control group (7%).

TD-SYSTEM

TD-system																
	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
U1	2/15	13	4/15	27	6/15	40	6/15	40	0/15	0	0/15	0	0/15	0	0/15	0
U2	6/15	40	7/15	47	7/15	47	9/15	60	0/15	0	0/15	0	0/15	0	1/15	7
U3	6/15	40	5/15	33	4/15	27	5/15	33	0/15	0	0/15	0	0/15	0	2/15	13
U4c	2/15	13	1/15	7	2/15	13	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0
U5c	0/15	0	1/15	7	2/15	13	2/15	13	0/15	0	0/15	0	0/15	0	0/15	0
U6c	3/15	20	1/15	7	2/15	13	3/15	20	0/15	0	0/15	0	0/15	0	0/15	0

Odourbox + sebum extract + faeces extract		
Unit	Highest attachment (%)	Mean attachment (%)
U1	40	47
U2	60	
U3	40	

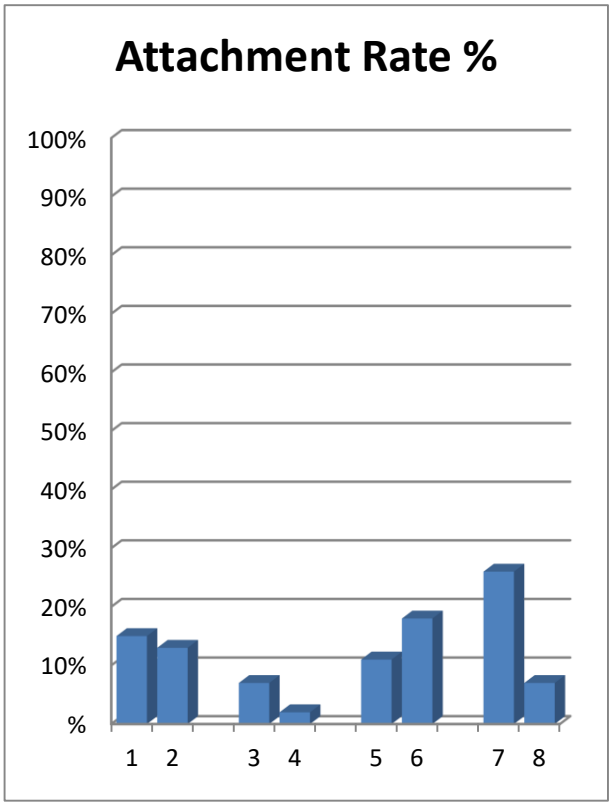
Control group		
Unit	Highest attachment (%)	Mean attachment (%)
U4c	13	15
U5c	13	
U6c	20	

The units with the combination of the odour membranes, the concentrated bovine sebum extract and the faeces extract (U1 to U3), shows high attachment rates. U2 shows even an attachment rate among females of 90%. U3 starts with a female attachment rate of 60% in the first check-up, that drops a little during the second and the third check-up.

The group with the membranes from the odourbox, the concentrated bovine sebum extract and the faeces extract (47%) is higher than the control group (15%).

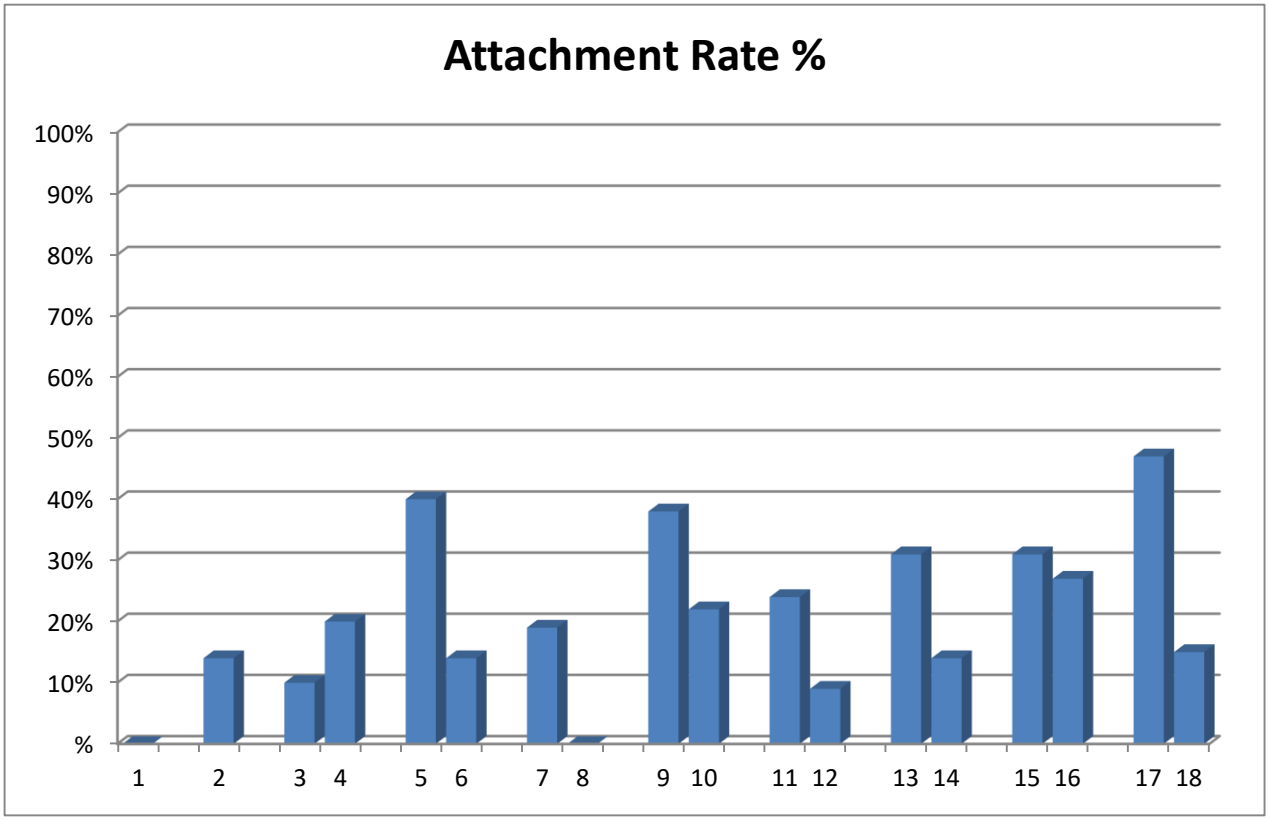
OVERVIEW COMPARISON DIFFERENT ATTACHMENT STIMULI

TU-SYSTEM



1. Experiment 8: Membranes 3 days in odourbox
2. Experiment 8: Control group
3. Experiment 9: Concentrated bovine sebum extract
4. Experiment 9: Control group
5. Experiment 10: Membranes 4 days in odourbox + concentrated bovine sebum extract
6. Experiment 10: Membranes 4 days in odourbox + concentrated bovine sebum extract + faeces extract
7. Experiment 11: Preconditioning + membranes 4 days in odourbox + concentrated bovine sebum extract (2^{nd} batch) + faeces extract (2^{nd} batch)
8. Experiment 11: Control group

TD-SYSTEM



1. Experiment 2: Concentrated cow-extract
2. Experiment 3: Concentrated dog extract
3. Experiment 4: Rubbed membranes
4. Experiment 4: Control membranes
5. Experiment 5: Preconditioned ticks from experiment 4
6. Experiment 5: Control group
7. Experiment 6: Unit + membranes 12-24 hours in odourbox
8. Experiment 6: Control group
9. Experiment 7: Bovine sebum extract
10. Experiment 7: Control group
11. Experiment 8: Membranes 9 days in odourbox
12. Experiment 8: Control group
13. Experiment 9: Concentrated bovine sebum extract
14. Experiment 9: Control group
15. Experiment 10: Membranes 22 days in odourbox + concentrated bovine sebum extract
16. Experiment 10: Membranes 22 days in odourbox + faeces extract
17. Experiment 11: Preconditioning + membranes 11 days in odourbox + concentrated bovine sebum extract (2^{nd} batch) + faeces extract (2^{nd} batch)
18. Experiment 11: Control group

EXPERIMENT 12 – FORCED FEEDING (TU-SYSTEM)

The idea of this experiment was to put the hypostome of a tick through the membrane, and glue the first palpes with superglue, through which the ticks were force fed (figure 12). The tick was held with forceps on the long axis of the hind body. With the tick in this position the membrane was punctured with the hypostome. The tick was freed from the forceps, and the superglue was added on the front legs with the top of a skewer. For this experiment only the TU-system could be used, because the membrane of the TD-system was surrounded with the tick compartment, which makes the space to work in it practically impossible. The main aim of this experiment was to see if the ticks would survive or even engorge during the forced feeding period.

Figure 12. Membrane with force-fed ticks

In the first three units, five ♀ *I. hexagonus* ticks from the colony were glued on the membrane, and each unit was filled up with 5♀ and 5 ♂ *I. hexagonus* ticks from the colony.
During this experiment, it immediately became clear that *I. hexagonus* is a very strong and mobile tick, which made the gluing process almost impossible. To glue down the tick, after puncturing the membrane with the hypostome, the tick had to be let go from the forceps to be able to grab the glue. Immediately when the tick was let loose, it immediately began to struggle and try to liberate itself from the membrane, even before the glue could be applied. The uncooperative ticks made it a time-consuming and intensive job.
Even when the ticks were successfully glued down, they liberated themselves sometimes afterwards.
The other three units were used as control groups.
All the units were provided with cow hair, which was daily replaced during the check-ups.

U1: membrane had a thickness of 100 µm, five ♀ ticks glued on the membrane, one let lose before starting the experiment and is removed from the unit.
U2: membrane had a thickness of 110 µm, five ♀ ticks glued on the membrane, one let lose before starting the experiment and is removed from the unit.
U3: membrane had a thickness of 90 µm, five ♀ ticks glued on the membrane
U4: membrane had a thickness of 100 µm, normal membrane with only cow hair
U5: membrane had a thickness of 100 µm, normal membrane with only cow hair
U6: membrane had a thickness of 90 µm, normal membrane with only cow hair

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	Number	%	number	%
1	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27
2	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27	4/15	27
3	5/15	33	5/15	33	5/15	33	5/15	33	4/15	27	5/15	33	5/15	33	5/15	33
4c	0/15	0	1/15	7	0/15	0	1/15	7	0/15	0	0/15	0	0/15	0	1/15	7
5c	0/15	0	0/15	0	2/15	13	2/15	13	0/15	0	0/15	0	0/15	0	2/15	13
6c	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0

During the 24-hour check-up, all the glued ticks were still attached. Unfortunately, in U1, U2 and U3, four ticks that were glued down, had died. Only one glued tick in U3 was alive and still moving its legs.
In U6c it was seen that one tick was stuck with blood on the edge of the tick compartment, it is possible this tick was attached before.
During the 48-hour check-up, the still living tick in U3 had also died. The attached tick in U4c detached after manual manipulation.
During the 72-hour check-up, both of the attached ticks in U5c detached after opening the unit.
What stood out during this experiment was, that in some units no attachment was seen (U1 and U2), although there were some slightly engorged female tick in the units.

EXPERIMENT FIELD TICKS 1 – CONCENTRATED BOVINE SEBUM EXTRACT (TD-SYSTEM)

The first attachment stimuli in this experiment was only cow hair.
Two partially engorged female *I. hexagonus* ticks send from the field were used.
After the 24 hour check-up it was decided to place the two ticks in a unit with 50µl concentrated bovine serum extract.

U1: membrane had a thickness of 95 µm, normal membrane with only cow hair
U1 after 24-hour check-up: membrane had a thickness of 95 µm, concentrated bovine sebum extract

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0

No attachment or faeces was seen in this experiment.

EXPERIMENT FIELD TICKS 2 - MEMBRANES 22 DAYS IN ODOURBOX, CONCENTRATED BOVINE SEBUM EXTRACT (TD-SYSTEM)

The attachment stimuli used in this experiment were the membranes that lied 22 days in the odourbox, the concentrated bovine serum extract on the membrane and cow hair. The cow hair was replaced daily in all the units during the check-ups.
The unit contained two partially engorged female *I. hexagonus* ticks send from the field.
U1: membrane had a thickness of 90 µm, odourbox membrane (22 days), concentrated bovine sebum extract

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	0/2	0	0/2	0	-	-	-	-	0/2	0	0/2	0	-	-	-	-

No attachment or faeces were seen in this experiment.
After the 48-hour check-up, the two field ticks were used in the force feeding experiment.

EXPERIMENT FORCE FEEDING FIELD TICKS

Because of the lack of attachment among the field ticks, the forced feeding experiment was also tried with field ticks. All of the field ticks were engorged. The engorged body made it impossible to grasp the ticks on the long axis with forceps. When applying force on the forceps, the tick was damaged (figure 13).

Figure 13. Damaged tick.

EXPERIMENT FIELD TICK 3 – 1 HOUR PRECONDITIONING, MEMBRANES IN ODOURBOX FOR 11 DAYS, CONCENTRATED BOVINE SEBUM EXTRACT (2ND BATCH), FAECES EXTRACT (2ND BATCH) (TD-SYSTEM)

The attachment stimuli used in this experiment were one hour of preconditioning, the membranes that had been in the odourbox for 11 days, 50µl concentrated bovine sebum extract (2nd batch) and 50µl faeces extract was added on the membrane. All the units contained cow hair.
The cow hair was daily replaced in all the units during the check-ups.

The units contained several amount of female *I. hexagonus* ticks sent in from the field:
U1: 11 ♀ ticks
U2: 3 ♀ ticks
U3: 3 ♀ ticks

U1: membrane had a thickness of 85 µm, odourbox membrane (11 days), concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch)
U2: membrane had a thickness of 85 µm, odourbox membrane (11 days), concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch)
U3: membrane had a thickness of 80 µm, odourbox membrane (11 days), concentrated bovine sebum extract (2nd batch), faeces extract (2nd batch)

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1	0/11	0	0/11	0	0/11	0	0/11	0	0/11	0	4/11	36	4/11	36	4/11	36
2	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	1/3	33
3	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0	0/3	0

No attachment or faeces was seen in this experiment.
The rate of deaths is relatively high in U1 during the 48-hour check-up.

EXPERIMENT MALES ONLY (TU- AND TD-SYSTEM)

To show that the male *I. hexagonus* ticks also attached, and possibly play a part in the transmission of infections, an experiment was started with male *I. hexagonus* ticks only.
Two systems were used for this experiment. As attachment stimuli the concentrated bovine sebum extract and the faeces extract were used. In the TU-system, 50µl concentrated bovine sebum extract, was added on the membranes because of a shortage of extract. In the TD-system, an alternating concentration of concentrated bovine sebum extract was added on the membranes. In U3 there was no concentrated bovine sebum extract used. All the units contained cow hair. The cow hair was replaced daily in all the units during the check-ups.

In every unit seven ♂ *I. hexagonus* ticks were placed. In U1, U2 and U3 there were tick reused from experiment 9 (TD-system), in U4, U5, and U6 there were ticks reused from the experiment 9 (TU-system)
U1: TD-system, membrane had a thickness of 90 µm, 50 µl concentrated bovine sebum extract
U2: TD-system membrane had a thickness of 110 µm, 20 µl concentrated bovine sebum extract
U3: TD-system membrane had a thickness of 110 µm
U4: TU-system membrane had a thickness of 90 µm, 50 µl concentrated bovine sebum extract
U5: TU-system membrane had a thickness of 110µm, 50 µl concentrated bovine sebum extract
U6: TU-system membrane had a thickness of 110 µm, 50 µl concentrated bovine sebum extract

	Attachment								Mortality							
	24 h		48 h		72 h		96 h		24 h		48 h		72 h		96 h	
Unit	number	%	number	%	number	%	number	%	number	%	number	%	number	%	number	%
1TD	2/7	29	0/7	0	0/7	0	-	-	0/7	0	1/7	14	1/7	14	-	-
2TD	0/7	0	0/7	0	0/7	0	-	-	2/7	29	0/7	0	2/7	29	-	-
3TD	1/7	14	1/7	14	0/7	0	-	-	0/7	0	0/7	0	2/7	29	-	-
4TU	0/7	0	1/7	14	0/7	0	-	-	1/7	14	2/7	29	1/7	14	-	-
5TU	1/7	14	0/7	0	0/7	0	-	-	2/7	29	2/7	29	3/7	43	-	-
6TU	0/7	0	0/7	0	0/7	0	-	-	0/7	0	0/7	0	2/7	29	-	-

In both systems there was attachment of male ticks (figure 14 + 15).
Some of the ticks looked slightly darker and more engorged than other ticks (figure 16 + 17).
After the experiment two ticks were squeezed and a red/brown blood-like liquid came out of the ticks (figure 18).

Figure 14. Attached male tick in the TU-system.

Figure 15. Attached male tick in the TD-system.

Figure 166. Engorged male tick.

Figure 15. Not engorged male tick.

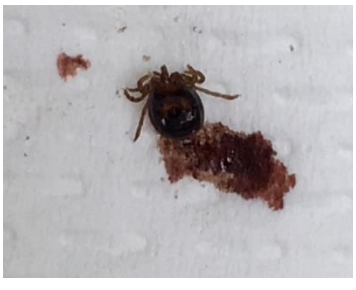


Figure 148. Squeezed male tick with red/brown liquid.

DISCUSSION

During this study, multiple experiments were conducted with all kinds of different approaches and goals. Several of the used methods, results and events during this study can be critically approached and discussed.

The group sizes of all the experiments, were based on the presence of the materials such as the right sized membranes, attachment stimuli and the availability of blood. No statistics were used which makes it hard to draw solid conclusions.

ATTACHMENT STIMULI TESTS

Most of the experiments were done to test which attachment stimuli gave the highest attachment rates. However, several proceedings can disturb the attachment or even prevent it.

For example, as described before, to check the ticks, they have to be manipulated with forceps, to make sure whether they are really attached. This manipulation causes a lot of these ticks to detach, as described in the experiments. It is unknown whether these disturbed ticks were eager or not to attach again. Possibly the attachment rate would have been higher when the ticks were not manipulated.
During the check-up moments, before opening up the unit with the removal of the bonbon, the ticks were always observed by watching through the units. Most of the time, the ticks were very slow and sitting still in the unit. By removing the bonbon, most of the ticks were stimulated to become active. It is unknown whether these stimulations of activity are an advantage or a disadvantage for their eagerness to attach.
During the check-up moments of the TU-system, the blood compartment with the attached ticks on the membrane had to be detached from the ticks' compartment for a check. Several attached ticks detached during this check-up moment, even without manipulation. Potential reasons can be: disturbance by blood, movement or CO_2 stimulants. Norval et al. concludes that ticks become active by high concentrations of carbon dioxide (Norval, Yunker, & Butler, 1987). What speaks against this argument is the fact that the tick in the TD-system, also undergoes movement in the blood and CO_2 stimulants by breathing, but they never detached in the experiments without manual manipulation.
Higher attachment rates might be achievable, when all the check-ups are skipped and the units will only be checked during the finishing-up of the experiment (Andrade et al., 2014).
The blood (used in the units as a food source for the ticks) separated during the night in a layer of white and a layer of red blood cells. This means that the ticks attached in the TU-system, were fed with just red blood cells for some time. The ticks, that were attached in the TD-system were fed with just white blood cells some of the time.
The high amount of leakage during the first six experiments was probably due to the fact that the membranes were measured with the foil on top of it, so the membranes were probably thinner than the noted thickness. After this discovery, the membranes were measured without the foil, and leakage was not a problem anymore.
The experimental groups were relatively small, especially in the experiments with leakage and this means that removal of units was a problem. These small groups made it difficult to draw conclusions because coincidence can play a role.
The odour extracts from the known sources and the materials used in the odourbox all came from cows. The cow is a known host for *I. hexagonus,* but higher attachment rates might be achieved with materials from other host animals, for example the hedgehog.
Several studies use faeces as an attachment stimulus. In two studies of Kuhnert they use species specific faeces (Frank Kuhnert, 1996)(F Kuhnert et al., 1995). In the experiment in this study, the faeces of *D.reticulates* was used in an attachment experiment with *I. hexagonus*. Maybe, attachment rates would be higher if the faeces from *I. hexagonus* were used.
The role of the male *I. hexagonus* is still unclear. They attach only for a short period, which makes it more difficult to find them attached. This brings the average attachment rate down. That is the reason why several times the attachment rate among female ticks was emphasized in this study.
In a study from Yoder et al. they concluded that ticks who were repeatedly pinched in the legs, secreted a fluid from their pores on their dorsolateral surface. The dominant component of this waxy fluid was squalene and it had the characteristics of a defence secretion (Yoder et al., 1993). It is a possibility that the ticks, that were used during this study also secreted this fluid due to too much manipulation of the legs during the check-ups. Maybe, this defence secretion had a negative influence on the attachment rate.

FIELD TICK TESTS

During this study a few experiment with field ticks have been conducted. Unfortunately, none of these ticks showed attachment and no faeces were produced.
A lot was unknown about the field ticks. For example, how long they had been attached on the hedgehog. After they had been removed from the hedgehog, the co-workers from the shelter put those ticks in tubes and send them to the UCTD. The time between the removal from the hedgehog and the transport of the package was also unknown. The transport also took some days, which was usually unknown. When the ticks arrived on the UCTD they had to be sorted out. The ticks came in during the week, but they could only be included in a new experiment on the following Monday.
To summarize, it could take days before a field tick was offered a blood meal. The effect on their eagerness to attach is unknown.
Also the engorgement state of the ticks was unknown. The ticks that were used for the experiment were partially engorged, but some ticks oviposit during the experiment.

The field ticks were removed from hedgehogs. The attachment stimuli (hair, odour and extracts) used in the experiments were obtained from cows (and not from the same animal the ticks were detached from). Maybe this can also be a reason for the ticks to be less eager to attach.
One major disadvantage of the field ticks that were sent in, is that before they were used in the experiments, it was unknown whether they carried any infection. Only after attachment, the infection state can be determined by DNA extraction, PCR and RLB.

MALES ONLY TEST

During the males only test, attachment was seen in both the TU-system and the TD-system. Also engorgement of the ticks was seen. After squeezing the ticks, a red/brown fluid came out of the tick. This all leads to the fact that the tick probably took a blood meal, just like the female tick. To be really sure, a DNA test of the blood should be done.

CONCLUSION

The aim of this study was to achieve high attachment rates of the tick *I. hexagonus* on the artificial membrane by use of several different attachment stimuli, and eventually use in vitro feeding to study its vector role.

The TD-system is a better system to obtain high attachment rates in comparison with the TU-system. The usage of the TU-system needs additional testing.
It is most likely that high attachment rates will only be achieved by using a combination of several attachment stimuli.
The most successful combination of attachment stimuli in the TD-system during this study were the three days preconditioned ticks, membranes that were in the odourbox for 11 days, the concentrated bovine sebum extract (2^{nd} batch), the faeces extract (2^{nd} batch) and cow hair.

Besides the female ticks, the male *I. hexagonus* ticks also show attachment in both the TU- and TD-system. They show engorgement, which makes it seem like they also take blood meals, which means they can probably play a role in the transmission of pathogens. To be sure of this, more research needs to be conducted.

Forced feeding of the tick *I. hexagonus* is not a good method to achieve an in vitro feeding, because of the low survival rate in using the method.

The main research question (if *Ixodes hexagonus*, while being fed in vitro, can transmit *Anaplasma* and *Borrelia* during their feeding process), cannot be answered, because of the lack of attachment of field ticks.

REFERENCES

Andrade, J. J., Xu, G., & Rich, S. M. (2014). A Silicone Membrane for In Vitro Feeding of <I>Ixodes scapularis</I> (Ixodida: Ixodidae). *Journal of Medical Entomology*, *51*(4), 878–879. http://doi.org/10.1603/ME13180

Földvári, G., & Farkas, R. (2005). Ixodid tick species attaching to dogs in Hungary. *Veterinary Parasitology*, *129*(1–2), 125–131. http://doi.org/10.1016/j.vetpar.2004.11.032

Fourie, J. J., Stanneck, D., Luus, H. G., Beugnet, F., Wijnveld, M., & Jongejan, F. (2013). Transmission of Ehrlichia canis by Rhipicephalus sanguineus ticks feeding on dogs and on artificial membranes. *Veterinary Parasitology*, *197*(3–4), 595–603. http://doi.org/10.1016/j.vetpar.2013.07.026

Gikonyo, N. K., Hassanali, A., Njagi, P. G. N., & Saini, R. K. (2000). Behaviour of Glossina morsitans morsitans Westwood (Diptera: Glossinidae) on waterbuck Kobus defassa Ruppel and feeding membranes smeared with waterbuck sebum indicates the presence of allomones. *Acta Tropica*, *77*(3), 295–303. http://doi.org/10.1016/S0001-706X(00)00153-4

Krawczyk, A. I., Van Leeuwen, A. D., Jacobs-Reitsma, W., Wijnands, L. M., Bouw, E., Jahfari, S., … De Bruin, A. (2015). Presence of zoonotic agents in engorged ticks and hedgehog faeces from Erinaceus europaeus in (sub) urban areas. *Parasites and Vectors*, *8*(1), 210. http://doi.org/10.1186/s13071-015-0814-5

Kröber, T., & Guerin, P. M. (2007). In vitro feeding assays for hard ticks. *Trends in Parasitology*, *23*(9), 445–449. http://doi.org/10.1016/j.pt.2007.07.010

Kuhnert, F. (1996). Feeding of Hard Ticks In Vitro: New Perspectives for Rearing and for the Identification of Systemic Acaricides. *ALTEX*, *13*(2), 76–87. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11178446

Kuhnert, F., Diehl, P. A., & Guerin, P. M. (1995). The life-cycle of the bont tick Amblyomma hebraeum in vitro. *International Journal for Parasitology*, *25*(8), 887–96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8550288

Mango, C. K. A., & Galun, R. (1977). Ornithodoros moubata: Breeding in vitro. *Experimental Parasitology*, *42*(2), 282–288. http://doi.org/10.1016/0014-4894(77)90085-6

Nijhof, A. M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L., … Jongejan, F. (2007). Ticks and associated pathogens collected from domestic animals in the Netherlands. *Vector Borne and Zoonotic Diseases (Larchmont, N.Y.)*, *7*(4), 585–595. http://doi.org/10.1089/vbz.2007.0130

Norval, R. A. I., Yunker, C. E., & Butler, J. F. (1987). Field Sampling o f Unfed Adults of Amblyomma hebraeum, *3*, 213–217.

Oliver, J. D., Lynn, G. E., Burkhardt, N. Y., Price, L. D., Nelson, C. M., Kurtti, T. J., … Morzaria, S. P. (2016). Infection of Immature Ixodes scapularis (Acari: Ixodidae) by Membrane Feeding. *Journal of Medical Entomology*, *53*(2), 409–15. http://doi.org/10.1093/jme/tjv241

Osborne, R. W., & Mellor, P. S. (1985). Use of a silicone membrane, 31–38.

Pfäffle, M., Littwin, N., Muders, S. V., & Petney, T. N. (2013). The ecology of tick-borne diseases. *International Journal for Parasitology*, *43*(12–13), 1059–1077. http://doi.org/10.1016/j.ijpara.2013.06.009

Pfäffle, M., Petney, T., Skuballa, J., & Taraschewski, H. (2011). Comparative population dynamics of a generalist (Ixodes ricinus) and specialist tick (I. hexagonus) species from European hedgehogs. *Experimental and Applied Acarology*, *54*(2), 151–164. http://doi.org/10.1007/s10493-011-9432-x

Purnell, R. E. (1973). East Coast fever: The infectivity for cattle of infective particles of Theileria parva harvested in various substrates, *3*, 335–338.

Skuballa, J., Oehme, R., Hartelt, K., Petney, T., Bücher, T., Kimmig, P., & Taraschewski, H. (2007). European hedgehogs as hosts for Borrelia spp., Germany [8]. *Emerging Infectious Diseases*, *13*(6), 952–953.

http://doi.org/10.3201/eid1306.070224

Taylor, M. A., Coop, R. L., & Wall, R. L. (2016). Facultative ectoparasites and arthropod vectors, 921–973.

Toutoungi, L. N., & Gern, L. (1993). Ability of transovarially and subsequent transstadially infected Ixodes hexagonus ticks to maintain and transmit Borrelia burgdorferi in the laboratory. *Experimental and Applied Acarology*, *17*(8), 581–586. http://doi.org/10.1007/BF00053487

Waladde, S. M., Ochieng, S. A., & Gichuhi, P. M. (1991). Rhipicephalus appendiculatus , to repletion, *11*, 297–306.

Yoder, J. A., Pollack, R. J., Spielman, A., Sonenshine, D. E., & Johnstons, D. E. (1993). Secretion of squalene by ticks. *Journal of Insect Physiology*, *39*(4), 291–296. http://doi.org/10.1016/0022-1910(93)90059-Z

APPENDIX

Protocols used in the experiments:

- Blood preparation
- Unit preparation
- Start up and finishing up
- Maintenance

Graphical guides:

- Graphical guide – Blood preparation
- Graphical guide – Starting up
- Graphical guide – Finishing up
- Graphical guide – Maintenance
- Graphical guide – Unit preparation (membranes + TD-system)
- Graphical guide – Unit preparation (TD-system + Forced feeding)

IN VITRO FEEDING - BLOOD PREPARATION (TICKS DOWN SYSTEM)

Cow number	
Blood volume	
Glucose weight	

	Meal preparation	**Done**
1	Take a 250 ml sterile glass jar from room 325 (PCR room), a pen and a cup to collect cow hair. Plastic cups can be found in the acaridarium (room 324). Add 1ml of Heparin/250ml blood in the glass jar.	
2	Take five 50 ml tubes from the cabinet in room 322 (Lab 1, DNA extraction and storage) and place them in the flow cabinet, clean the surface with alcohol and turn on the UV-light.	
3	Go to the farm animal department (LHD) and dress appropriately.	
4	Go the office of Thijmen den Ouden (room 0.434) to pick up the welfare notation sheet.	
5	Ask an animal care taker for help. They will collect blood from the cow.	
6	The caretaker will use a tube to direct the blood into the glass jar. Collect 250 ml of blood. Also collect white hair from a cow (only collect hair from a cow that has NOT been treated with acaricides) and store the hairs in the plastic cup.	
7	Let the animal care taker fill in and sign the welfare notation sheet and bring it back to the office of Thijmen den Ouden (room 0.434).	
8	Take the sterile glass jar to the UCTD. Add glucose to a concentration of 2g/L (for 250 ml of blood, use 0.5 gr of glucose). Add the glucose in the flow cabinet to maintain sterility. Swerve the jar after glucose has been added. Glucose can be found in storage (room 325), the amount that needs to be added to the blood has to be weighed in room 325 as well.	
9	Transfer the blood from the sterile glass jar into the 50 ml tubes that were already placed in the flow cabinet, use a pipette to transfer the blood. Use a new pipette every time a new tube is filled to maintain hygiene levels.	
10	Label the 50 ml tubes with cow number, date and purpose. Store at 4 °C (blood can be stored for one week).	
11	Clean the workspace with 70% ethanol and clean the glass jar in room 325 until all traces of blood are removed.	

IN VITRO FEEDING (TICKS DOWN SYSTEM) - UNIT PREPARATION

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. Tight the foil with tape on the backside and on the front side of the plate(s). Make sure there are no wrinkles in the foil on the front side.	
3	Remove the air underneath the foil by wiping the surface with lens paper.	
4	Tape the top of 8 lens papers on the foil of equidistant per glass plate. Make sure that de tape is the same size or longer than the lens paper to prevent crinkles in the membrane during following steps.	
5	Prepare the silicone mix without the hexane in the chemicals storage room	
6	Pipette the hexane in the lab and combine it with the rest of the silicone mix. Mix well.	
7	Apply the silicon mix onto a piece of carton.	
8	Cover the lens papers with an equal layer silicone mix, with an clean hand applicator at a 45° angle while applying light pressure.	
9	Dry the membrane for 24 hours in a closed environment with 97% humidity.	
10	Dispose all silicone waste in a seal bag into the biological waste bin.	
11	Clean workspace and used tools with 70% ethanol.	

SILICON MIX	**Weight/volume (For 8 lens papers)**	**Weighted (g)**
WHITE COLOR PASTE	0.15g	
SILICON GLUE	15.00g	
SILICON OIL	4.50g	
HEXANE	4.5ml	

	Day 2	Done
1	Clean workspace with 70% ethanol.	
2	Put 70% ethanol on a cotton wipe and squeeze it well, so it is moist, not wet. Clean the membrane by wiping once, with the moist cotton wipe.	
3	Apply some silicone glue onto a plastic petri dish and smooth it to a thin layer with the backside of a plastic spoon.	
4	Place a tubule with a rotation 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 removed with a spoon.	
5	Place a 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 or cotton swab.	
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 to loosen the tubules from the membrane or to loosen the foil from the membrane. Take in account where the tubules came from when measuring the thickness of the membrane.	
9	Cute a square around the remaining parts of the membranes on the glass plate. Remove the foil from the remaining parts of the membranes on the glass plate.	
10	Measure the membrane with the micro calipers at different spots around each tubule. Before measuring, calibrate the micro calipers by turning the handle gently, so the gab closes. After that push the blue origin button twice. Now is the micro calipers calibrated, and measurement can be started. The membrane should be between 70 and 100 µm thick. Write down the thickness on the unit.	
11	Check the attachment of the membrane to the tubule.	
Bonbon preparation		
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.	

IN VITRO FEEDING – STARTUP & FINISHING UP (TICKS DOWN SYSTEM)

Tick species		**Stage**	
Number of units		**Number of ticks per unit**	
Smell/hair/perfume		**Cow blood number**	

Wear gloves

		Done
1	Turn on the flow cabinet and clean the flow cabinet with 70% ethanol. Wear gloves when working in the flow cabinet.	
2	Take a 6-wells plate and a 50 ml tube and place it in the flow cabinet. (Use more 6-wells plates if indicated, use ONE 50 ml tube per 6-wells plate that is used).	
3	Turn on the UV-light for 15 minutes.	
4	Turn on the tick race track in the acaridarium (room 324). Place ticks from the incubators in the acaridarium in the tick race track to expose them to light, keep the ticks inside their tubes (selection will come later)	
5	When the 15 minutes have passed, take a tube of blood and disinfect it with 70% ethanol before placing it in the flow cabinet.	
6	Pipette 20 ml of blood into the empty 50 ml tube. (If more than one 6-wells plate is used, a corresponding number of 50 ml tubes need to be filled with 20 ml of blood).	
7	Add 20 µL of ATP and 2 µL Gentamicin to 20 ml of blood. (When more blood is used, make sure to use the correct amounts of ATP and Gentamicin). Swerve the blood after adding each substance.	
8	Pipette 3.0 ml in each well of the 6-wells plate. Cover the other wells with the lid when pipetting.	
9	Cover the plate with the lid and incubate it in the incubator at 37 °C for 15 minutes. In the meantime, select ticks that will be used. Select the ticks inside the tick race track and place them in tick colony tubes according with the number of units that will be used.	
10	Clean the flow cabinet with 70% ethanol and turn on the UV-light.	
11	Go to the acaridarium with previously prepared units. Place selected ticks into the units. Work inside the tick race track.	
12	Place the bonbon onto the unit until 0.5 cm above the membrane, make sure no ticks are stuck between the bonbon and the side of the unit.	
13	Turn off the race track and take the units back to the flow cabinet.	
14	Take the 6-wells plate(s) from the incubator and wipe the bottom with 70% ethanol before placing the wells plate in the flow cabinet.	
15	Remove plastic foil from the bottom of the membranes carefully. Place the units with ticks in the wells with blood. Place the units sideways to avoid creating air bubbles between the membrane and the blood.	
16	Place the 6-wells plate(s) with the units in the incubator at 37 °C.	

17	Clean the flow cabinet with 70% ethanol, turn on the UV-light for 15 minutes and then turn off the flow cabinet.	

	Finishing up	**Done**
1	Turn on the flow cabinet and clean it with 70% ethanol.	
2	Disinfect a 50 ml tube with 70% ethanol and place it in the flow cabinet. Also place a petri dish and 4 sterile cryo tubes in the flow cabinet	
3	Turn on the UV-light for 15 minutes.	
4	Turn on the tick track in the acaridarium (room 324).	
5	Take the 6-wells plate(s) and a bottle of PBS from the incubator and clean the outside of both with 70% ethanol before placing the items in the flow cabinet.	
6	Check the units and write down your findings. Rinse the units with PBS above a petri dish before placing them on a clean lid of a 6-wells plate.	
7	Take samples. Pipette the blood up and down to homogenize, then take a 1 ml sample and save them in **accordingly labeled sterile cryo tubes**. The label on the cryo tube **must** match the unit where the sample came from!	
8	Remove left-over blood from the wells plate with a large pipette and dispose of it in the 50 ml tube. Also dispose of left-over PBS in the petri dish in the 50 ml tube.	
9	Dispose of waste in a plastic bag.	
10	Clean the flow cabinet with 70% ethanol and turn on the UV-light for 15 minutes.	
11	Take the lid of the 6-wells plate with the used units to the acaridarium to remove the ticks. Work inside the tick race track.	
12	Remove the bonbon and make pictures for documentation. Check if all ticks are present and write down findings.	
13	Inform what has to be done with the ticks.	
14	Either put the ticks in a 1,5 ml tube, fill it with 70% ethanol and store at room temperature after labeling the tubes accordingly OR store them alive. To store the ticks alive, use separate tick collection tubes (1 tube per unit) and place the tubes in the incubator with the right colony.	
15	Turn off the tick race track and clean the workspace in the acaridarium.	
16	Turn off the flow cabinet and fill in the log.	

IN VITRO FEEDING - MAINTENANCE

Wear gloves

	Maintenance and sampling
1	Clean the flow cabinet with 70% ethanol.
2	Place a 50ml tube, 6-wells plate(s) and cryo 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 and the units 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	Place the 6-wells plate(s) with the units in the water bath and close the water bath.
15	For sampling: pipette the old blood up and down and take a sample of 1ml each into sterile cryotubes. **Label the tubes accordingly**.
16	Remove the leftover blood from the used 6-wells plate(s) by pipetting. Dispose it in the 50ml tube in a bio waste container.

17	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).
18	Store the blood samples at 4°C for usage within the next few days or at -20°C for long term storage.

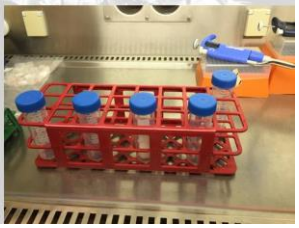
20	Empty the glass petri dish and clean with 70% ethanol.
21	Clean the flow cabinet with 70% ethanol.
22	Turn on the UV-light for 15 minutes.
23	Turn off the flow cabinet.

Blood preparation

IN VITRO FEEDING

Preparing for blood collection

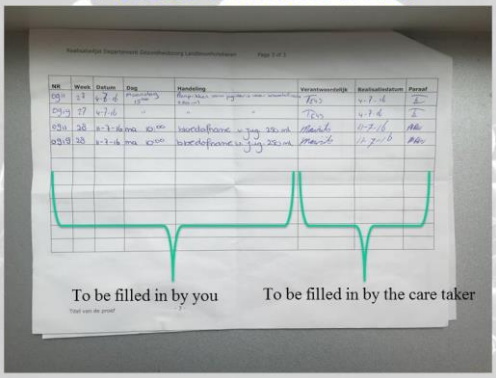
- Supplies:
  - 250 ml sterile glass bottle/jar
  - Plastic cup
  - Pen
- Preparation:
  - Five 50 ml tubes need to be placed in the flow cabinet



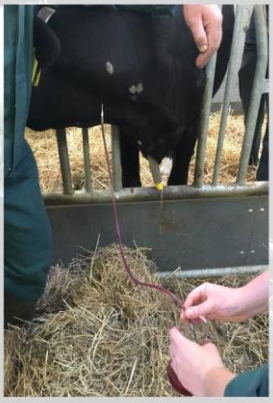
Blood collection

- Go to the farm animal department
  - Go to room 0.434 and pick up the welfare notation sheet
  - Ask an animal caretaker for help, they will collect blood from a cow
  - Collect hair from a cow that has NOT been treated with acaricides!

Welfare notation sheet



Blood collection



Blood preparation

- After the welfare notation sheet has been filled in by the animal caretaker, return to the UCTD for further blood preparation

Blood preparation

- Add glucose (2gr/L)
- Glucose needs to be added to the blood in the flow cabinet
- Wear gloves when working in the flow cabinet
- Maintain sterility

(Glucose was added to 260 ml of blood in this case)

Blood transfer

Blood transfer

- After glucose has been added, use a pipette to transfer the blood into 50 ml tubes
- Work in the flow cabinet and wear gloves

Blood storage

- Label 50 ml tubes with cow number, date and purpose (for example: in vitro feeding)
- Store at 4 °C (for one week)

GRAPHICAL GUIDE – STARTING UP

Starting up

IN VITRO FEEDING

Preparation

- Supplies:
  - 6-wells plate(s)
  - Tube with blood from 4 °C storage
  - One 50 ml tube per 6-wells plate
  - ATP
  - Gentamicin

Preparation

- Turn on the flow cabinet and the tick race track

Preparation

- Place ticks from the incubators in the tick race track to expose them to light

Blood preparation

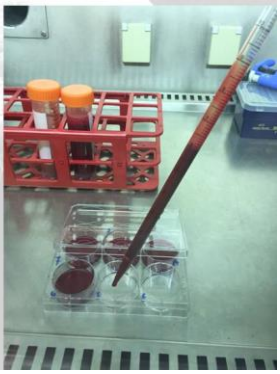
- Take a tube of blood from 4° C storage, disinfect it and place it in the flow cabinet

Blood preparation

- Pipette 20 ml of blood into an empty tube per 6-wells plate
- Add ATP (1 µl/ml
- Add Gentamicin (0,1 µl/ml)

Blood preparation

- Pipette 3,0 ml of blood in each well
- Cover other wells when pipetting
- Incubate 6-wells plate at 37 °C



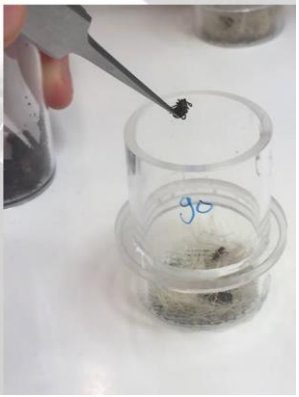
Tick selection

- Select the ticks inside the tick race track and place them in tick colony tubes according with the number of units that will be used

*Place the ticks back in the incubator to expose them to dry air after selection

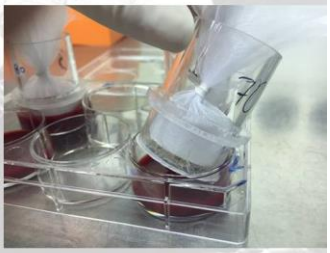
Preparing units

- Take previously prepared units to the acaridarium if ticks have been sufficiently exposed to dry air
- Place ticks inside units and seal the unit with a bonbon
- Take the units to the flow cabinet



Completing in vitro feeding system

- Take the 6-wells plate with blood from the incubator
- Place units in according wells of the 6-wells plate
- REMOVE PLASTIC FOIL FROM THE BOTTOM OF THE MEMBRANE



*Place the units sideways to avoid formation of air bubbles

Clean up

- Turn off the tick race track
- Clean the tick race track with 70% ethanol
- Clean the flow cabinet with 70% ethanol and turn on the UV-light for 15 minutes
- Turn off the flow cabinet

GRAPHICAL GUIDE – FINISHING UP

Finishing up

IN VITRO FEEDING

Preparation

- Supplies:
  - 50 ml tube
  - Petri dish
  - Sterile cryo tubes
  - PBS
  - Lid of a 6-wells plate

Finishing up

- Place a 50 ml tube, a petri dish and sterile cryo tubes in the flow cabinet
- Turn on the tick race track
- Check units
- Place the units on the lid of a 6-wells plate
- Take samples
- Dispose of waste

Place units on lid

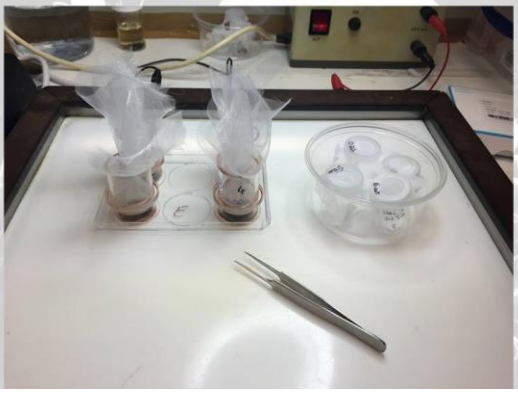
Take samples

Dispose of waste

Acaridarium

- Take the lid with units to the acaridarium
- Remove bonbon
- Make pictures
- Write down findings (check if all ticks are present)

Acaridarium



Storage of ticks

- Alive

Use separate tick collection tubes (1 per unit, label them accordingly) and place them in the incubator (select the right colony)

- Dead

Place ticks in a 1,5 ml tube and fill with 70% ethanol and store at room temperature

Clean up

- Turn off tick race track
- Clean tick race track with 70% ethanol
- Clean flow cabinet with 70% ethanol and turn on UV-light
- Turn off flow cabinet

Maintenance

IN VITRO FEEDING

Supplies

- 6-wells plate(s)
- Cryo tubes
- Petri dishes
- 50 ml tube

Prepare the blood

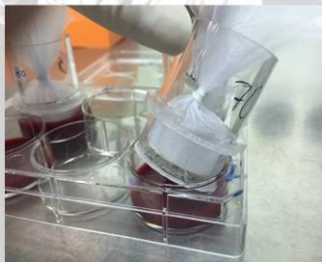
- Pipette the total amount of blood that is required for the experiment (20 ml blood for a 6-wells plate).
- Pipette 1 µL ATP/ml blood
- Pipette 0,1 µL Gentamicin/ml blood
- Swerve the blood between every step
- Pipette 3,0 ml blood per well in the 6-well plate(s).
- Put the 6-well plate(s) in the incubator

Rinse the membranes

- After checking the units, rinse the membranes with PBS.

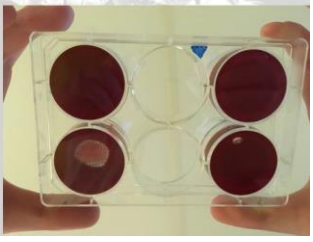
Place the units in the new 6-wells plate(s)

- Place the units side-ways onto the fresh blood.



Avoid air bubbles between the membrane and the blood

- If there are bubbles try to remove them by manipulating the units.



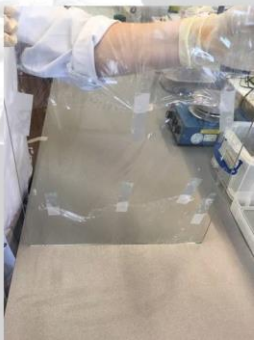
GRAPHICAL GUIDE – UNIT PREPARATION (MEMBRANES + TD-SYSTEM)

Unit preparation

IN VITRO FEEDING

Tight the foil with tape

- Tight the foil with tape on the backside of the plate.



Tight the foil with tape on the frontside of the plate.

- Make sure there are no crinkles in the foil on the frontside.

Remove the air

- Remove the air underneath the foil by wiping the surface with lens paper.

Tape the top of 8 lens papers on the foil

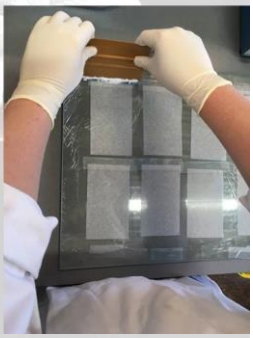
- Make sure that the tape is the same size or longer than the lens paper to prevent crinkles in the membrane during following steps.

Apply the silicon mix onto a piece of carton

- Apply the silicon mix on the applicator like the picture below.

How to applicate

- Place the applicator on the upper side of the lens paper and tilt the applicator towards yourself.



How to applicate

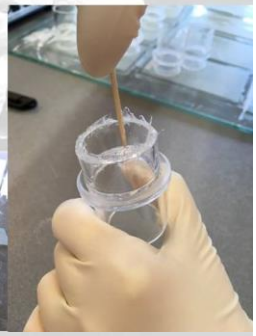
- Cover the lens papers with an equal layer silicone mix, with an clean hand applicator at a 45° angle while applying light pressure.

Day 2

- Place a tubule with a rotation movement in the glue and remove it from the glue with a rotating movement. Check if the ring is completely covered in glue

Remove excess glue

- Place a tubule on the membrane and slightly rotate while applying pressure.

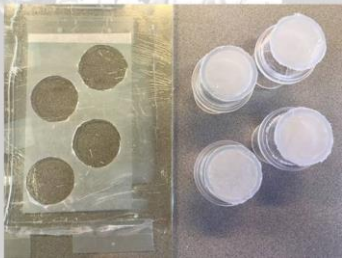


Remove excess glue

- Excess glue on the inside of the unit can be removed with a small brush or cotton swab.

Remove the units from membrane

- Take in account where the tubules came from when measuring the thickness of the membrane.



Measure the thickness of the membrane

- Remove the membrane from the foil.
- Calibrate the Mitutoyo
- Measure the membrane on several places

Check the quality of the membrane

- On the right side good attachment, on the left side bad attachment (see lighter surface and glue residues on the membrane).

Bonbon preparation

- 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.

GRAPHICAL GUIDE – UNIT PREPARATION (TD-SYSTEM + FORCED FEEDING)

Unit Preparation

Ticks Up System

In Vitro Feeding

Unit Preparation

- For preparation of membranes, attachment of units to membranes and preparation of lids, see pp 'Unit preparation Ticks Down System'

Storage of units

- Untreated units can be stored in a closed box or vacuum-sealed bag
- Units can be treated with odour by placing them in a cow odour box
  - In box until use
  - Certain period of time in the box, then in a vacuum-sealed bag

Cow Odour Box

Assembling units

Assembling units

Assembling units

Forced feeding