

Utrecht Centre for Tick-borne Diseases

In vitro feeding of *Rhipicephalus sanguineus* ticks



Naam Student:

Jorinda Schellekens

Studentnummer:

3383148

Periode onderzoekstage:

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Locatie:

Faculteit Diergeneeskunde Utrecht

Departement Infectieziekten & Immunologie

Utrecht Centrum voor Teken-gebonden Ziekten

Prof. Dr. F. Jongejan

Begeleider:

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Abstract

The purpose of this study was to optimize the in vitro feeding system for *Rhipicephalus sanguineus* larvae, nymphs and adult ticks. *Dermacentor reticulatus* nymphs and adults and *Dermacentor marginatus* nymphs were also used for the feedings. Attachment of ticks is essential because this makes it possible to study the transmission dynamics of tick-borne pathogens in vitro.

The attachment rate of *R. sanguineus* adults was very low in this study, possible due to the unattractive membranes and/or the quality of the ticks. The mortality rate of the *R. sanguineus* nymphs was high and it is important to decrease this rate by optimizing the environmental conditions and by the use of ticks of a good quality. Also, the use of thinner and attractive membranes is important to let the nymphs attach and feed. *R. sanguineus* larvae were not able to attach, because the membranes are too thick for their mouthparts. *R. sanguineus* ticks are very host specific and they need certain stimuli to attach. Therefore, several feedings have been carried out to find stimuli that attract ticks. It appears that membranes need to have a strong dog odor, so that ticks are stimulated to attach to the membrane.

Adult *D. reticulatus* ticks did attach to the membrane. Thus the in vitro feeding system works for these ticks. The mortality rate of *D. reticulatus* nymphs and *D. marginatus* nymphs was very high, probably due to the environmental conditions during the feedings.

In vitro odor tests were also carried out to investigate if perfumes with animal odor are attractive for ticks. The attraction of perfume with cow odor was tested with adult *D. reticulatus* and *Ixodes ricinus* ticks. *D. reticulatus* ticks were only attracted by freshly made perfumes, whereas *I. ricinus* ticks were not attracted by the perfumes. *R. sanguineus* ticks were used to test perfume with dog odor. Different dog breeds were used to make the odor of the perfume and *R. sanguineus* ticks were especially attracted by the odor of a bouvier. Further research is required to investigate if perfume with animal odor can make membranes for in vitro feeding more attractive.

1. Introduction

Purpose of this study

At the beginning of this study, the main purpose was to investigate the transmission dynamics of *Ehrlichia canis*. During the study the attachment rate of *R. sanguineus* was very low. Therefore, it was not possible to investigate the transmission of *E. canis*. It changed the purpose of this study into how to increase the attachment rate of *R. sanguineus* ticks.

The in vitro feeding system

The development of in vitro systems to feed ticks is very important. Experiments with ticks and tick-borne pathogens can be standardized and carried out under controlled conditions. Laboratory animals are not longer used for these investigations and the experiments are not dependent of a host anymore. The animals do not longer suffer from tick bites or the consequences of a tick-borne disease (1). So, the use of an in vitro feeding system has several advantages compared with in vivo experiments.

The feeding of hard ticks takes a several days to complete and therefore, a system is required to feed ticks for a longer period. In vitro feeding of ticks is possible by the use of feeding units with an artificial membrane. This membrane imitates the skin and ticks penetrate the membrane to reach the blood with the hypostome (1).

Artificial feeding of hard ticks started in 1956 with *Boophilus microplus*. The larvae of *B. microplus* were fed on embryonated hen eggs (1). Later in 1975, slices of cattle skin were used to feed the larvae of *B. microplus*. More than 50% of the engorged larvae molted to nymphs (2). The silicone membrane for hard ticks was successfully introduced in 1993 by Habedank and Hiepe (1). A few years later in 1995, the life cycle of *Amblyomma hebraeum* was completed by the use of silicone membranes in an in vitro feeding system (3). The creation of a silicone membrane that imitates the elasticity of the skin was an improvement in 2004. Closing of the membrane by elastic retraction prevents leaking after detaching of a tick. Also, the development of softer en thinner membranes to feed *Ixodes ricinus* ticks was an improvement. *I. ricinus* was able to penetrate the membrane with its relative short hypostome. (4).

Ixodid ticks do not easily feed on artificial membranes. Therefore, stimuli are needed to induce attachment of these ticks and to let them feed successfully (5). Attachment of ticks depends on thermal, hydro, mechanical, olfactory stimuli and contact chemostimuli (3). Development of membranes with attractive stimuli is required.

Rhipicephalus sanguineus

Rhipicephalus sanguineus, also known as the brown dog tick, is a vector of several pathogens. The ticks are found worldwide and it is thought that *R. sanguineus* originate from Africa (6). The tick is common in warmer areas, but it is also adapted to indoor live. Therefore, it is possible to find *R. sanguineus* in colder climates (7). The main host is the dog, but larvae and nymphs can also be found on rodents and other small mammals. Large *R. sanguineus* populations are only possible in the presence of dogs (6).

The life cycle of *R. sanguineus* consist of four stages: egg, larvae, nymphs and adult. To develop from egg to the adult stage, the tick has to feed on three hosts. After a feeding, the larvae and nymphs moult to the next stage and the female adult ticks lay eggs in the environment (6). The feeding period depends on the stage of development. Larvae and nymphs feed shorter than adult ticks. Also, host species influence the duration of the feeding; ticks need more time to feed on other hosts than on dogs (7),(8). Before a feeding, ticks have to find a host in the environment. Ticks need some stimuli to detect

the presence of a host. These stimuli are CO₂ stimulation, ammonia, airborne vibrations and the host body temperature (6). The odor of a host is also attractive for ticks (9).

R. sanguineus ticks have favorite attachment sites on the dog. When a tick finds its host, the tick crawls to the preferred locations on the animal. These places can be found by the presence of chemical substances. Predilection sites of adult *R. sanguineus* ticks are the head (particularly on ears), interdigital spaces, back, inguinal region and axilla. The ticks have a relative short hypostome and therefore, the ticks attach superficial on the skin (7). The larvae and nymphs have other attachment sites, because they have a lower mobility and a shorter feeding period. Larvae and nymphs can be found on the belly, rump, hind legs, head and neck (10),(11). *R. sanguineus* ticks can also have a preference for a certain dog breed. Some dog breeds (cocker spaniel) are more sensitive for ticks than other breeds (beagles) (6),(7). It is possible that some breeds are resistant against the tick because of substances on their skin that inhibit ticks to attach and feed. This means that *R. sanguineus* ticks are able to distinguish between dog breeds (12).

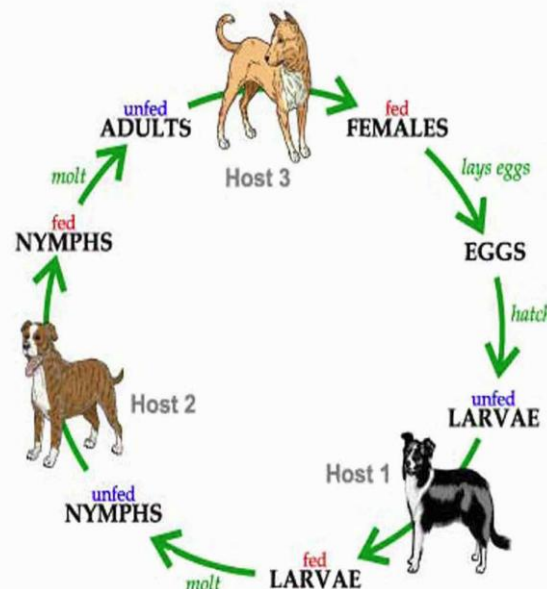


Figure 1: Life-cycle *Rhipicephalus sanguineus* (7)

Dermacentor reticulatus

Dermacentor reticulatus ticks are common in western and central Europe. The tick is not found in Scandinavia and in Mediterranean areas. They live in colder areas and especially on low hills. The last years, the tick is expanding to north-western parts of Europe and *D. reticulatus* has also been found in the Netherlands (13),(14).

D. reticulatus ticks need three hosts to develop from nymph to adult. Adult ticks feed on large mammals, like dogs, cattle, horses, sheep, goats and pigs. Larvae and nymphs only feed on small mammals, especially rodents (13),(14).

Ehrlichia canis

Ehrlichia canis is an important pathogen of dogs and the bacterium can be transmitted by *R. sanguineus* ticks. The bacterium is responsible for canine monocytic ehrlichiosis (CME) and it is found worldwide. In Europe, *E. canis* is mainly found in Mediterranean areas (15). The expansion of *R. sanguineus* ticks to northern parts of Europe cause also an expansion of *E. canis* to countries in North-Europe, such as the Netherlands (16).

R. sanguineus ticks become infected with *E. canis* during feeding of infected blood. The saliva of ticks is infective for dogs and the bacteria invade and develop in the monocytes and macrophages (15). The incubation period of ehrlichiose is 8 to 20 days. The disease consist of three phases: the acute phase, subclinical phase and the chronical phase. The most important symptoms of ehrlichiosis are fever, lethargy, weight loss, anorexia, anemia, lymphadenomegaly, splenomegaly and hemorrhagic diathesis. CME can also be fetal (17),(18).

It is important to gain more information about the transmission of this bacterium. Investigation of the transmission dynamics of *E. canis* is possible by the use of an in vitro feeding system.

2. Materials and methods

In vitro feeding

Ticks

In this study, *R. sanguineus* adults, nymphs and larvae, *D. reticulatus* adults and nymphs and *D. marginatus* nymphs were used for the in vitro feedings. The ticks were stored in an incubator with a temperature of 21°C and a humidity of 70%. The *R. sanguineus* larvae, nymphs and uninfected adults originate from Greece. The 15% *E. canis* infected *R. sanguineus* adults originate from South-Africa. 15 *R. sanguineus* adults came from dogs from Portugal. *D. reticulatus* nymphs and adults originate from the Netherlands and the *D. marginatus* nymphs originate from Portugal.

The following ticks were used for the in vitro feedings in this study:

- 200 *R. sanguineus* larvae, Greece;
- 209 uninfected *R. sanguineus* nymphs, Greece;
- 80 uninfected *R. sanguineus* adults (39♂, 41♀), Greece;
- 121 15% *E. canis* infected *R. sanguineus* adults (56♂, 65♀), South-Africa;
- 15 *R. sanguineus* adults, Portugal;

- 159 uninfected *D. reticulatus* nymphs, the Netherlands;
- 61 uninfected *D. reticulatus* adults (31♂, 30♀), the Netherlands;
- 186 uninfected *D. marginatus* nymphs, Portugal.

In feedings 1 to 5, new *R. sanguineus* adult ticks were used for each feeding. These ticks were not used in previous in vitro feedings. In feedings 6 and 7, *R. sanguineus* adults, 15% infected with *E. canis*, were used. Also, these ticks had never been fed in vitro. Eight ticks from feeding 6 and 7 were re-used in feeding 8. The other ticks in feeding 8 came from the incubator and had also never been used for in vitro feedings. In feeding 9, only ticks from feedings 6, 7 and 8 were used. Feeding 10 contained 10 male and 6 female *R. sanguineus* adults from feeding 9 and 9 new females from the incubator.

R. sanguineus nymphs and larvae, *D. reticulatus* adults and nymphs and *D. marginatus* nymphs were always from the incubator and they were all fed in vitro for the first time.

Blood

At the beginning of a feeding, cow blood was collected at the department of farm animals. Three cows were used to collect the blood for the feedings. Every week, the blood was taken from another cow, so discomfort was minimized.

To prevent contamination of the blood, it is necessary to take the blood sterile as possible. The skin of the cow was disinfected with alcohol and the blood was collected by the use of a needle, catheter and a bottle or an erlenmeyer. In feedings 1 to 6 the blood was collected in a sterile bottle of 0,5 liter. In feedings 8 to 10 the blood was collected in a sterile erlenmeyer of 0,5 liter. To prevent clotting of the blood, a sterile 10 ml pipette was used for stirring of the blood for 10 to 20 minutes. The erlenmeyer was used because of the larger opening. The blood clot is easier to remove from an erlenmeyer than a bottle. Also, not more than 250 ml was collected in the erlenmeyer, so that the blood clot would not become very large. A smaller blot clot is easier to remove.

In feeding 7, the blood was collected in a blood bag. These blood bags are normally used for humans. In these bags anti-coagulants are already added, so that stirring of the blood to prevent blood clots is not necessary. The needle from this bag was too short to stay in the blood vessel of the cow. Therefore, the needle did not stay in the blood vessel and the cow had to be pierced for a several times.

In the laboratory D-(+)-glucose (2g/L) was added to the blood. Then, the blood was distributed over sterile 50 ml falcon tubes and stored at 4°C. Blood in feedings 6 to 10, used for *R. sanguineus* nymphs and *D. marginatus* nymphs was treated with gentamicine (5 µl/10 ml) to prevent bacterial growth.

In feeding 5 pig blood was used. Pig blood was collected by the use of a needle, catheter and a bottle. To prevent clotting a sterile 10 ml pipette was used for stirring of the blood. In the laboratory D-(+)-glucose (2g/L) was added to the blood and the blood was distributed over sterile 50 ml falcon tubes and stored at 4°C.

Membranes

Silicone membranes are a good imitation of the elastic skin. Ticks have to penetrate the membranes, so that they can reach the blood. The first step in the process to make membranes is to cover a glass plate with plastic foil. The foil has to be taped very tight on the glass plate. Then, 8 lens papers (70 x 120 mm) are placed on the plastic foil. To make 16 membranes, a mixture of 15 g silicone-gluce, 4,5 g silicone oil, 2,9 g hexane and 0,15 g Wacker FL color paste is made. The mixture is equally distributed over the lens papers with a scraper of 80 mm wide. Membranes have to dry for 12 hours at room temperature. After drying, the membrane thickness can be measured with a micro calipers.

The hypostome length of *R. sanguineus* male adults is 270 µm and the length of female adults is 370 µm. Therefore, membranes with a thickness between 70 and 100 µm were used for adult ticks. *R. sanguineus* nymphs have a hypostome length of 120 µm and the larvae 50 µm (1). Because the hypostome of nymphs is shorter than the hypostome of adult ticks, membranes with a thickness between 60 and 70 µm were used for nymphs. *R. sanguineus* larvae need a membrane with a thickness of less than 50 µm, but membranes thinner than 50 µm are not elastic anymore. Membranes for *D. reticulatus* adults had also a thickness between 70 and 100 µm. *D. reticulatus* and *D. marginatus* nymphs were fed on membranes with a thickness between 60 and 70 µm.

The membranes for the feedings of *R. sanguineus* ticks are taped on a lid from a cell culture plate. A few hours before the start of a feeding, the membranes are rubbed over a host to obtain the host odor. Membranes for *R. sanguineus* of feedings 1 to 4 and 8 to 10 were rubbed for 3-5 minutes over a dog. In feedings 5 to 7, the membranes for *R. sanguineus* were rubbed for 10 minutes over a dog. The membranes for *D. reticulatus* adults in feedings 1 and 2, *D. reticulatus* nymphs in feeding 1 and 4 and *D. marginatus* nymphs in feedings 5 and 6 were also rubbed over a dog. *D. reticulatus* nymphs in feeding 2 were fed on membranes that were rubbed over the ears of a rabbit. Membranes of *D. marginatus* nymphs in feeding 7 had a mouse odor. These membranes obtained the mouse odor to put them in a box with sawdust with the odor of a mouse.

In feeding 8, the mixture to make the membranes was made with hexane with dog odor. The first step to make hexane with dog odor is to rub 2 cotton pads over a dog. These cotton pads are put in a jar and 10 ml of hexane is added to the jar. Then, the jar is put on the shaking plate and after 24 hours the hexane can be used in the mixture of the membranes.

Feeding units

The feeding units are made of Plexiglas and these have a diameter of 26 mm, a height of 45 mm and a thickness of 2 mm. The unit has also an acryl glass ring, so that the bottom of the unit does not touch the basis of the six-well culture plate. This ensures that 2 mm blood between the membrane and the six-well plate is left (4). Ticks are able to penetrate the membrane and to reach the blood.

The feeding units have to be glued on the membrane with silicone-gluе. The glue is equally divided over the edges of the feeding unit with a paintbrush. Then, the units are placed on the membrane and they have to dry for a minimum of 3 hours. It is possible to place 4 feeding units on one membrane, depending on the quality and thickness of the membrane. After drying, the units can be cut out with a scalpel and a scissor. Before using, the units are tested for leaks. The plastic foil has to be removed and the units can be placed in a six-well plate filled with distilled water. After 20 minutes the units are checked for leaks.

A plastic stopper wrapped with organza fabric is used on the feeding unit to prevent ticks to climb out of the unit. The stopper is pressed down until a space of 0,5 cm height is left. It is also possible to place some hair of the host in the unit to make the membrane more attractive for ticks.

The feeding

At the beginning of a feeding, ticks are identified and counted. The required ticks are placed in a feeding unit. Four feeding units can be placed in a sterile six-well plate. Each well is filled with 3,1 ml blood. The six-well plate with blood is warmed up in a water bath of 37°C. After 15-30 minutes the blood has the right temperature for the feeding. The feeding units with ticks are placed in the wells with the blood. Then, the blood is checked on bubbles with air between the membrane en the six-well plate. When bubbles are present, the unit is placed again till the bubbles are gone. The six-well plate with the units is placed in an aquarium with a cover in the water bath. The aquarium is filled with potassium sulfate (120 g/L), to reach a humidity of 90%. To keep the ticks in the dark for 24 hours a day, the water bath is covered with a blanket.

Every day at 09:00 and 19:00 the blood is changed. The blood on the membranes has to be removed before putting them in the fresh blood. In feedings 1 to 5 the blood on the units was rinsed with PBS (Phosphate Buffered Saline) and in feedings 6 to 10 the units were rinsed with 0,9% Sodium Chloride solution. Every 24 hours the ticks are checked on attachment and mortality. Dead ticks stay in the unit till the end of the feeding.

Samples from the old blood are taken in duplo. These samples are stored at -20°C, so that they can be used later for DNA extraction.

At the end of a feeding, the ticks are carefully removed from the unit. Living ticks are put in a jar and are restored in the incubator. Dead ticks are put in a jar with 70% alcohol. Dead ticks can be used later for DNA extraction. The membranes are removed from the units and the units are cleaned, so that they can be re-used for other feedings.



Figure 2: The water bath



Figure 3: Six-well plate and feeding units

***In vitro* odor test**

Perfume

Perfume with the odor of an animal can be used to attract ticks. To make the perfume the odor of an animal has to be captured in a medium. The first step is to rub an animal for 5 minutes with 2 cotton pads on the preferred locations. Then, the two cotton pads are put in a jar and the jar is closed. The perfume can be made with two different solvents: 96% ethanol or hexane.

The first option is to use 96% ethanol as a solvent. 10 ml ethanol is added to the jar with the cotton pads. The jar is put on a shaker plate and stays there for 48 hours. During the shaking period, the ethanol picks up the odor of the animal. The ethanol is sucked from the jar with a 10 ml pipette. Finally, the ethanol with the odor is put in a perfume bottle with a sprayer.

The second option is to use hexane as a solvent. 12 ml hexane is added to the jar with the two cotton pads. The jar is put on a shaker plate for 48 hours. The hexane picks up the odor of the animal. The hexane with odor is sucked from the jar with a 10 ml pipette and it is put in another jar. Then, the jar is put in the fume hood without the lid, so that the hexane can evaporate. When all the hexane is evaporated, 8 ml ethanol (96%) is added to the residue in the jar. The jar is put on the shaker plate till the residue is solved. When the residue does not solve, the content in the jar can be sucked with a pipette and put in 2 ml eppendorf cups. These eppendorf cups are centrifuged and after centrifuging the liquid can be sucked with a pipette. Finally, the liquid is put in the perfume bottle.

***In vitro* odor test**

The odor test is useful to investigate if the perfume is attractive for ticks. A petri dish and filter paper are needed to test the perfume. The filter paper is divided in three zones: a positive, negative and neutral zone. In the positive zone 25 μ l of the perfume is dropped on the filter paper. In the negative zone 25 μ l ethanol (96%) or hexane is dropped on the filter paper, depending of the solvent of the perfume. Then, 10 ticks are released in the petri dish and the petri dish with ticks is placed in the incubator. The incubator has a temperature of 25°C, a humidity of 80% and a day-night rhythm. After a few hours, the ticks are checked to see in which zone they are. Then, the petri dish is shaken and also CO₂ is blown into the petri dish. A few hours later the ticks are checked again.

3. Results

In vitro feeding

Feeding 1

In this feeding, 20 adults (10♂, 10♀) and 50 nymphs of *R. sanguineus* were used. The adult ticks were evenly distributed over 2 feeding units and the nymphs were distributed over 2 other feeding units.

The adult ticks of *R. sanguineus* did not attach to the membrane and one of the male adults died. Also the *R. sanguineus* nymphs did not attach to the membrane. After 40 hours the mortality of the nymphs raised to 48% and at the end of the feeding the mortality was even 62%. The blood in units 10, 11 and 12 turned dark after 75 hours.

D. reticulatus adults and nymphs were also used in this feeding. *D. reticulatus* ticks are normally easy to feed in an in vitro feeding system and these ticks were used as control. 21 (11♂, 10♀) adult ticks were distributed over 2 units and the 2 other units were filled with each 25 nymphs.

After 15 hours 4 adults in unit 6 were already attached to the membrane. During the feeding all ticks in unit 6 were attached. In unit 5 only 2 adults were attached to the membrane. There was no mortality of adult ticks. At t=15 is seemed that one of the *D. reticulatus* nymphs was attached to the membrane, but other nymphs did not attach. The mortality of the nymphs was very high. At t=40 94% of the nymphs were dead and in unit 8 all nymphs died.

R. sanguineus

Unit	Total	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	Labrador odor	Cow
U10	10 adults (5♂, 5♀)	Labrador odor	Cow
U11	25 nymphs	Labrador odor	Cow
U12	25 nymphs	Labrador odor	Cow

Table 1: F1 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=15	U9	0	0
	U10	0	1
	Mean	0	0,5
	%	0	5
t=40	U9	0	0
	U10	0	1
	Mean	0	0,5
	%	0	5
t=65	U9	0	0
	U10	0	1
	Mean	0	0,5
	%	0	5
t=89	U9	0	0
	U10	0	1
	Mean	0	0,5
	%	0	5

Table 2: F1 *R. sanguineus* nymphs

Time (h)	Unit	Attached	Mortality
t=15	U11	0	2
	U12	0	1
	Mean	0	1,5
	%	0	6
t=40	U11	0	10
	U12	0	14
	Mean	0	12
	%	0	48
t=65	U11	0	10
	U12	0	14
	Mean	0	12
	%	0	48
t=89	U11	0	15
	U12	0	16
	Mean	0	15,5
	%	0	62

D. reticulatus

Unit	Total	Membrane odor	Blood
U5	10 adults (5♂, 5♀)	Labrador odor	Cow
U6	11 adults (6♂, 5♀)	Labrador odor	Cow
U7	25 nymphs	Labrador odor	Cow
U8	25 nymphs	Labrador odor	Cow

Table 3: F1 *D. reticulatus* adults

Time (h)	Unit	Attached	Mortality
t=15	U5	0	0
	U6	4	0
	Mean	2	0
	%	20	0
t=40	U5	0	0
	U6	8	0
	Mean	4	0
	%	40	0
t=65	U5	2	0
	U6	8	0
	Mean	5	0
	%	50	0
t=89	U5	2	0
	U6	11	0
	Mean	6,5	0
	%	65	0
t=98	U5	2	0
	U6	10	0
	Mean	6	0
	%	60	0

Table 4: F1 *D. reticulatus* nymphs

Time (h)	Unit	Attached	Mortality
t=15	U7	1	0
	U8	0	0
	Mean	0,5	0
	%	2	0
t=40	U7	0	23
	U8	0	24
	Mean	0	23,5
	%	0	94
t=65	U7	0	23
	U8	0	25
	Mean	0	24
	%	0	96
t=89	U7	0	23
	U8	0	25
	Mean	0	24
	%	0	96
t=98	U7	0	23
	U8	0	25
	Mean	0	24
	%	0	96

Feeding 2

In feeding 2, 20 (10♂, 10♀) adults and 50 nymphs of *R. sanguineus* were distributed over 4 feeding units. Two units were filled with each 10 adults and the other 2 units with each 25 nymphs.

One male adult was attached to the membrane after 67 hours. Other adult ticks did not attach. After 91 hours 2 ticks and after 102 hours 3 ticks were dead. Some of the *R. sanguineus* nymphs seemed to be attached, but at the end of the feeding none of the nymphs was attached anymore. At the end of the feeding, the mortality rate was very high and only 4 nymphs survived. The blood of units 9 and 10 was already dark after 19,5 hours. The blood of unit 12 turned dark after 67 hours.

D. reticulatus adults and nymphs were also used in this feeding. The adults (10♂, 10♀) were equally distributed over 2 units and the 50 nymphs were equally distributed over 2 other units.

After 19,5 hours, one adult tick was already attached. The highest attachment rate was 35% at t=91. Two adults died during the feeding. The attachment rate of the nymphs was very low; only 2 nymphs seemed to be attached. The mortality rate in this feeding was lower (38%) than in the previous feeding (96%). The blood of units 6 to 8 turned dark after 67 hours.

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	78 µm	Labrador odor + hair	Cow
U10	10 adults (5♂, 5♀)	78 µm	Labrador odor + hair	Cow
U11	25 nymphs	51 µm	Labrador odor + hair	Cow
U12	25 nymphs	57 µm	Labrador odor + hair	Cow

Table 5: F2 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=19,5	U9	0	0
	U10	0	0
	Mean %	0 0	0 0
t=43	U9	0	0
	U10	0	0
	Mean %	0 0	0 0
t=67	U9	1	0
	U10	0	0
	Mean %	0,5 5	0 0
t=91	U9	1	1
	U10	0	1
	Mean %	0,5 5	1 10
t=102	U9	1	2
	U10	0	1
	Mean %	0,5 5	1,5 15

Table 6: F2 *R. sanguineus* nymphs

Time (h)	Unit	Attached	Mortality
t=19,5	U11	1	0
	U12	0	0
	Mean %	0,5 2	0 0
t=43	U11	2	1
	U12	0	2
	Mean %	1 4	1,5 6
t=67	U11	2	1
	U12	4	2
	Mean %	3 12	1,5 6
t=91	U11	2	1
	U12	4	2
	Mean %	3 12	1,5 6
t=102	U11	0	22
	U12	0	24
	Mean %	0 0	23 92

D. reticulatus

Unit	Total	Membrane thickness	Membrane odor	Blood
U5	10 adults (5♂, 5♀)	99 µm	Labrador odor + hair	Cow
U6	10 adults (5♂, 5♀)	105 µm	Labrador odor + hair	Cow
U7	25 nymphs	80 µm	Rabbit (ear) odor	Cow
U8	25 nymphs	70 µm	Rabbit (ear) odor	Cow

Table 7: F2 *D. reticulatus* adults

Time (h)	Unit	Attached	Mortality
t=19,5	U5	1	0
	U6	0	0
	Mean %	0,5 5	0 0
t=43	U5	2	0
	U6	2	0
	Mean %	2 20	0 0
t=67	U5	2	0
	U6	3	0
	Mean %	2,5 25	0 0
t=91	U5	5	0
	U6	2	0
	Mean %	3,5 35	0 0
t=102	U5	4	0
	U6	2	2
	Mean %	3 30	1 10

Table 8: F2 *D. reticulatus* nymphs

Time (h)	Unit	Attached	Mortality
t=19,5	U7	0	2
	U8	0	6
	Mean %	0 0	4 12
t=43	U7	0	4
	U8	0	4
	Mean %	0 0	4 16
t=67	U7	0	4
	U8	0	5
	Mean %	0 0	4,5 18
t=91	U7	2	4
	U8	0	5
	Mean %	1 4	4,5 18
t=102	U7	0	9
	U8	0	10
	Mean %	0 0	9,5 38

Feeding 4

In this feeding, 40 adults (19♂, 21♀) of *R. sanguineus* were used. The ticks were distributed over 4 units. Three units contained each 5 males and 5 females and one unit contained 4 males and 6 females.

The adults did not attach to the membrane and there was no mortality. The ticks were not very active and also after CO₂ stimulation there was still no activity. The ticks were attached to the stopper during the whole feeding, thus the ticks had to be alive. At the end of the feeding, the ticks were held to the heating plate and they started to walk. During the feeding the blood became dark. After 40 hours, blood in unit 11 was dark and after 64 hours the blood in units 10, 11 and 12 was also dark.

Also *D. reticulatus* ticks were used in this feeding. 20 (10♂, 10♀) adults and 48 nymphs were distributed over 4 units. The adults were equally distributed over 2 feeding units and 2 other units were filled with nymphs.

The adults did not attach to the membrane and also in this feeding there was no mortality. In several units feces was present. The feces was seen in unit 6 after 40 hours and in unit 5 after 74 hours. Some nymphs were attached to the membrane after 16 hours, but after 40 hours they were detached. During the rest of the feeding no attachment was seen. The mortality of the nymphs was very high, after 40 hours 71% of the ticks were dead and at t=88 all nymphs were dead. The blood of unit 8 became dark after 88 hours.

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	90 µm	Labrador odor + hair	Cow
U10	10 adults (5♂, 5♀)	75 µm	Labrador odor + hair	Cow
U11	10 adults (5♂, 5♀)	70 µm	Labrador odor + hair	Cow
U12	10 adults (4♂, 6♀)	79 µm	Labrador odor + hair	Cow

Table 9: F4 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=16	U9	0	0
	U10	0	0
	U11	0	0
	U12	0	0
	Mean %	0 0	0 0
t=40	U9	0	0
	U10	0	0
	U11	0	0
	U12	0	0
	Mean %	0 0	0 0
t=64	U9	0	0
	U10	0	0
	U11	0	0
	U12	0	0
	Mean %	0 0	0 0
t=88	U9	0	0
	U10	0	0
	U11	0	0
	U12	0	0
	Mean %	0 0	0 0
t=94	U9	0	0
	U10	0	0
	U11	0	0
	U12	0	0
	Mean %	0 0	0 0

D. reticulatus

Unit	Total	Membrane thickness	Membrane odor	Blood
U5	10 adults (5♂, 5♀)	70 µm	Cow hair	Cow
U6	10 adults (5♂, 5♀)	70 µm	Cow hair	Cow
U7	22 nymphs	50 µm	Labrador odor + hair	Cow
U8	26 nymphs	50 µm	Labrador odor + hair	Cow

Table 10: F4 *D. reticulatus* adults

Time (h)	Unit	Attached	Mortality
t=16	U5	0	0
	U6	0	0
	Mean	0	0
	%	0	0
t=40	U5	0	0
	U6	0	0
	Mean	0	0
	%	0	0
t=64	U5	0	0
	U6	0	0
	Mean	0	0
	%	0	0
t=88	U5	0	0
	U6	0	0
	Mean	0	0
	%	0	0
t=94	U5	0	0
	U6	0	0
	Mean	0	0
	%	0	0

Table 11: F4 *D. reticulatus* nymphs

Time (h)	Unit	Attached	Mortality
t=16	U7	3	0
	U8	3	0
	Mean	3	0
	%	13	0
t=40	U7	0	14
	U8	0	20
	Mean	0	17
	%	0	71
t=64	U7	0	21
	U8	0	23
	Mean	0	22
	%	0	92
t=88	U7	0	22
	U8	0	26
	Mean	0	24
	%	0	100
t=94	U7	0	22
	U8	0	26
	Mean	0	24
	%	0	100

Feeding 5

During this feeding, nymphs of *R. sanguineus* were used. The main goal of this feeding was to investigate if *R. sanguineus* nymphs did feed at all. The blood preference of the nymphs was also investigated. 80 nymphs were distributed over 4 feeding units. Nymphs in the first two units were fed on cow blood and the nymphs in the other two units on pig blood.

The results were disappointing because none of the nymphs attached to the membranes. At the end of the feeding, the mortality rate was 11%. After 88 hours the blood of units 9 to 12 was dark.

D. marginatus nymphs were used for the first time in this study. 135 nymphs were distributed over 4 units. The nymphs in the first two units were fed on cow blood and the nymph in the other two units on pig blood.

The attachment rate was very low, only 3 nymphs were attached to the membranes. After 15,5 hours 12% of the nymphs were dead and the mortality rate increased during the rest of the feeding. At the end, 56% of the nymphs were dead. After 40 hours the blood of units 7 and 8 turned dark and after 88 hours the blood of units 5 to 8 was dark.

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	21 nymphs	70 µm	Labrador odor + hair	Cow
U10	16 nymphs	74 µm	Labrador odor + hair	Cow
U11	18 nymphs	75 µm	Labrador odor + hair	Pig
U12	25 nymphs	70 µm	Labrador odor + hair	Pig

Table 12: F5 *R. sanguineus* nymphs

Time (h)	Unit	Attached	Mortality
t=15,5	U9	0	2
	U10	0	0
	U11	0	0
	U12	0	1
	Mean	0	0,75
	%	0	4
t=40	U9	0	2
	U10	0	0
	U11	0	0
	U12	0	1
	Mean	0	0,75
	%	0	4
t=64	U9	0	4
	U10	0	1
	U11	0	1
	U12	0	0
	Mean	0	1,5
	%	0	8
t=88	U9	0	5
	U10	0	0
	U11	0	1
	U12	0	2
	Mean	0	2
	%	0	11

D. marginatus

Unit	Total	Membrane thickness	Membrane odor	Blood
U5	38 nymphs	73 µm	Labrador odor + hair	Cow
U6	40 nymphs	70 µm	Labrador odor + hair	Cow
U7	30 nymphs	72 µm	Labrador odor + hair	Pig
U8	27 nymphs	78 µm	Labrador odor + hair	Pig

Table 13: F5 *D. marginatus* nymphs

Time (h)	Unit	Attached	Mortality
t=15,5	U5	0	5
	U6	0	6
	U7	0	3
	U8	0	2
	Mean	0	4
	%	0	12
t=40	U5	0	6
	U6	1	8
	U7	0	8
	U8	0	9
	Mean	0,25	7,75
	%	1	23
t=64	U5	2	14
	U6	1	14
	U7	0	16
	U8	0	12
	Mean	0,75	14
	%	2	41
t=88	U5	2	22
	U6	0	20
	U7	1	20
	U8	0	14
	Mean	0,75	19
	%	2	56

Feeding 6

In this feeding, 20 adults (10♂, 10♀) and 29 nymphs of *R. sanguineus* were used and distributed over 4 units. The adult *R. sanguineus* ticks came from South-Africa and these ticks were for 15% infected with *E. canis*. Two units were filled with each 10 adult ticks. The other two units were filled with nymphs; one unit contained 13 nymphs and the other unit 16 nymphs.

One of the adult ticks was attached to the membrane after 14,5 hours. The highest attachment rate was 30% at t=38,5. The attached ticks were stuck in a blood clot against the side of the unit. This blood clot was probably mixed with feces. At the end of the feeding, the attached ticks were dead because of the blood clot. The nymphs of *R. sanguineus* did not attach to the membrane. At the end of the feeding, the mortality was 15%. The blood of unit 10 turned dark after 86,5 hours.

The purpose of the feeding with *D. marginatus* nymphs was to investigate how fast and how many ticks would die in the environment of the water bath. The mortality rate was the only important value to investigate and therefore, the unit was not put in blood. After 14,5 hours 27% of the nymphs was already dead and at the end of the feeding 73% was dead.



Figure 4: *R. sanguineus* ticks stuck in the blood clot in the unit

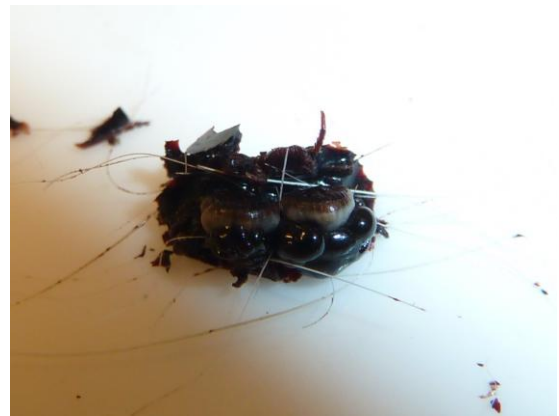


Figure 5: *R. sanguineus* ticks stuck in the blood clot

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	81 µm	Labrador odor + hair	Cow
U10	10 adults (5♂, 5♀)	77 µm	Labrador odor + hair	Cow
U11	13 nymphs	49 µm	Labrador odor + hair	Cow
U12	16 nymphs	50 µm	Labrador odor + hair	Cow

Table 14: F6 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=14,5	U9	1	0
	U10	0	0
	Mean %	0,5 5	0 0
t=38,5	U9	3	0
	U10	3	0
	Mean %	3 30	0 0
t=62,5	U9	3	0
	U10	2	2
	Mean %	2,5 25	1 10
t=86,5	U9	3	0
	U10	2	2
	Mean %	2,5 25	1 10
t=94,5	U9	2	1
	U10	0	2
	Mean %	1 10	1,5 15

Table 15: F6 *R. sanguineus* nymphs

Time (h)	Unit	Attached	Mortality
t=14,5	U11	0	1
	U12	0	0
	Mean %	0 0	0,5 3
t=38,5	U11	0	1
	U12	0	1
	Mean %	0 0	1 7
t=62,5	U11	0	2
	U12	0	3
	Mean %	0 0	2,5 10
t=86,5	U11	0	2
	U12	0	3
	Mean %	0 0	2,5 10
t=94,5	U11	0	3
	U12	0	5
	Mean %	0 0	4 15

D. marginatus

Unit	Total	Membrane thickness	Membrane odor	Blood
U5 test	26 nymphs	49 µm	Labrador odor + hair	-

Table 16: F6 *D. marginatus* nymphs

Time (h)	Unit	Mortality
t=14,5	U5 test	7
	%	27
t=38,5	U5 test	15
	%	58
t=62,5	U5 test	18
	%	69
t=86,5	U5 test	19
	%	73

Feeding 7

In this feeding, 20 adults (10♂, 10♀) and about 200 larvae of *R. sanguineus* were distributed over 4 feeding units. The first two units contained each 10 adult ticks and the other two units each 100 larvae. The adult ticks were for 15% infected with *E. canis*. The attachment rate of adult *R. sanguineus* ticks was not very high. In each unit only one tick was attached to the membrane and at the end of the feeding none of the ticks were attached. In unit 9 one tick and in unit 10 four ticks were dead at the end of the feeding. In units 9 and 10 feces was present. The blood of units 9 and 10 turned dark after 63,5 hours. During the first 40 hours some larvae seemed to be attached to the membrane, but later all larvae were detached. The mortality rate of the larvae was 11%.

The units with the *D. marginatus* nymphs were put in another water bath. The temperature in this bath was 18-22°C and the humidity was 70-90%. *D. marginatus* nymphs did not attach to the membrane with mouse odor, but the mortality rate of the nymphs was this time lower than the mortality rate in the other feedings. At the end of the feeding, only 6% of the nymphs died. Compared with a mortality rate of 56% in F5 and 73% in F6, the mortality rate in this feeding was very low.

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	79 µm	Labrador odor + hair	Cow
U10	10 adults (5♂, 5♀)	76 µm	Labrador odor + hair	Cow
U11	~ 100 larvae	53 µm	Labrador odor + hair	Cow
U12	~ 100 larvae	54 µm	Labrador odor + hair	Cow

Table 17: F7 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=15,5	U9	1	0
	U10	0	1
	Mean %	0,5 5	0,5 5
t=39,5	U9	1	0
	U10	1	2
	Mean %	1 10	1 10
t=63,5	U9	1	0
	U10	1	3
	Mean %	0,5 5	1,5 15
t=69	U9	0	1
	U10	0	4
	Mean %	0 0	2,5 25

Table 18: F7 *R. sanguineus* larvae

Time (h)	Unit	Attached	Mortality
t=15,5	U11	10	0
	U12	0	0
	Mean %	5 5	0 0
t=39,5	U11	3	10
	U12	0	11
	Mean %	1,5 2	10,5 11
t=63,5	U11	0	10
	U12	0	11
	Mean %	0 0	10,5 11
t=69	U11	0	10
	U12	0	11
	Mean %	0 0	10,5 11

D. marginatus

Unit	Total	Membrane thickness	Membrane odor	Blood
U7	26 nymphs	48 µm	Mouse odor	Cow
U8	25 nymphs	53 µm	Mouse odor	Cow

Table 19: F7 *D. marginatus* nymphs

Time (h)	Unit	Attached	Mortality
t=15,5	U7	0	0
	U8	0	0
	Mean %	0 0	0 0
t=39,5	U7	0	2
	U8	0	1
	Mean %	0 0	1,5 6
t=63,5	U7	0	2
	U8	0	1
	Mean %	0 0	1,5 6
t=69	U7	0	2
	U8	0	1
	Mean %	0 0	1,5 6

Feeding 8

In this feeding, 80 adult (40♂, 40♀) *R. sanguineus* ticks were used and equally distributed over 8 feeding units. The ticks were for 15% infected with *E. canis*. 15 of these ticks were incubated at 37°C for 3 days. These ticks were also used in feedings 6 and 7. At the beginning of this feeding, only 8 incubated ticks were alive and these were distributed over the units. Each unit contained one incubated tick. The other 72 ticks came from the incubator and these ticks were not incubated or used in other in vitro feedings.

The odors of two different dog breeds were used to compare. The membranes of units 9 and 10 were rubbed over the ears of a labrador and the membranes of units 11 and 12 over the ears of a cocker spaniel. For the membranes of units 13 to 16, hexane with a dog odor was used. Membranes of units 13 and 14 were made with hexane with a labrador ear odor and the membranes of units 15 and 16 with hexane with a cocker spaniel ear odor.

Only 2 *R. sanguineus* ticks were attached to the membranes which were rubbed over the dogs. One tick was attached on the membrane with labrador odor and the other tick on the membrane with cocker spaniel odor. 3 ticks died in the unit with labrador odor and 2 ticks in the unit with the cocker spaniel odor.

The ticks on the membranes made of hexane with dog odor did not attach. One tick was dead in a unit with labrador odor and 2 ticks in the units with cocker spaniel odor.

The blood of unit 12 was dark after 39 hours. Blood clots were present on the membrane of unit 10 after 63 hours.

Units 17 and 18 were filled with 15 adult (10♂, 5♀) *R. sanguineus* ticks from Portugal. These ticks had been attached on dogs and the ticks were taken from these dogs. Some ticks were already half engorged.

The membrane of unit 17 was rubbed over a labrador and the membrane of unit 18 over a cocker spaniel. After 15 hours, one of the Portuguese ticks was attached to the membrane with the cocker spaniel odor, but after 39 hours the tick was detached. At the end of the feeding, 3 ticks in unit 17 and 4 ticks in unit 18 were dead.

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	71 µm	Labrador ear odor	Cow
U10	10 adults (5♂, 5♀)	74 µm	Labrador ear odor	Cow
U11	10 adults (5♂, 5♀)	77 µm	Cocker spaniel ear odor	Cow
U12	10 adults (5♂, 5♀)	74 µm	Cocker spaniel ear odor	Cow
U13	10 adults (5♂, 5♀)	75 µm	Labrador ear (hexane) odor	Cow
U14	10 adults (5♂, 5♀)	80 µm	Labrador ear (hexane) odor	Cow
U15	10 adults (5♂, 5♀)	80 µm	Cocker spaniel ear (hexane) odor	Cow
U16	10 adults (5♂, 5♀)	73 µm	Cocker spaniel ear (hexane) odor	Cow
U17	8 adults (5♂, 3♀)	84 µm	Labrador ear odor	Cow
U18	7 adults (5♂, 2♀)	79 µm	Cocker Spaniel ear odor	Cow

Table 20: F8 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=15	U9	0	0
	U10	0	0
	U11	0	1
	U12	0	0
	Mean	0	0,25
	%	0	3
t=39	U9	0	0
	U10	0	0
	U11	0	2
	U12	0	0
	Mean	0	0,5
	%	0	5
t=63	U9	0	3
	U10	1	0
	U11	1	2
	U12	0	0
	Mean	0,5	1,25
	%	5	13
t=70,5	U9	0	3
	U10	0	0
	U11	0	2
	U12	0	0
	Mean	0	1,25
	%	0	13

Table 21: F8 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=15	U13	0	0
	U14	0	1
	U15	0	0
	U16	0	0
	Mean	0	0,25
	%	0	3
t=39	U13	0	0
	U14	0	1
	U15	0	0
	U16	0	2
	Mean	0	0,75
	%	0	8
t=46,5	U13	0	0
	U14	0	1
	U15	0	0
	U16	0	2
	Mean	0	0,75
	%	0	8

Table 22: F8 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=15	U17	0	0
	U18	1	4
	Mean	0,5	2
	%	7	27
t=39	U17	0	3
	U18	0	4
	Mean	0	3,5
	%	0	47
t=46,5	U17	0	3
	U18	0	4
	Mean	0	3,5
	%	0	47

Feeding 9

In this feeding, 50 adults (25♂, 25♀) of *R. sanguineus* were equally distributed over 5 feeding units. These ticks were also used in feeding 8. Two feeding units had a membrane that was rubbed over a labrador. The membranes of the other three units were rubbed over a yorkshire terrier. The yorkshire terrier was however treated with the acaricide certifact™.

The ticks did not attach to the membrane during this feeding. At the end of the feeding, 5 ticks on the membranes with labrador odor were dead. The mortality rate of the ticks on the membranes with yorkshire terrier odor was very high. After 15 hours, 77% of the ticks were dead. The mortality rate was increased to 87% at t=20,5. The feeding of units 11, 12 and 13 was stopped early to spare ticks that were still alive. Blood of unit 10 turned dark after 63 hours.

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	80 µm	Labrador ear odor	Cow
U10	10 adults (5♂, 5♀)	80 µm	Labrador ear odor	Cow
U11	10 adults (5♂, 5♀)	79 µm	Yorkshire terrier ear odor + hair	Cow
U12	10 adults (5♂, 5♀)	74 µm	Yorkshire terrier ear odor + hair	Cow
U13	10 adults (5♂, 5♀)	80 µm	Yorkshire terrier ear odor + hair	Cow

Table 23: F9 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=15	U9	0	1
	U10	0	0
	Mean	0	0,5
	%	0	5
t=39	U9	0	1
	U10	0	2
	Mean	0	1,5
	%	0	15
t=63	U9	0	1
	U10	0	3
	Mean	0	2
	%	0	20
t=65,5	U9	0	2
	U10	0	3
	Mean	0	2,5
	%	0	25

Table 24: F9 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t=15	U11	0	9
	U12	0	7
	U13	0	7
	Mean	0	7,7
	%	0	77
t=20,5	U11	0	9
	U12	0	9
	U13	0	8
	Mean	0	8,7
	%	0	87

Feeding 10

During this feeding, 30 adults (15♂, 15♀) of *R. sanguineus* were used. These ticks were for 15% infected with *E. canis*. All male ticks and 6 female ticks were used in feeding 9. Nine new females came from the incubator. The ticks were equally distributed over 3 feeding units. Each membrane had an odor of another dog breed. The dogs had not been treated with acaricide. The membrane of unit 9 was rubbed over a labrador, the membrane of unit 10 over a heidewachtel and the membrane of unit 11 over a ridgeback. After 49,5 hours the feeding of unit 9 was stopped. The ticks of this unit were placed in unit 12 with a membrane of bouvier odor.

After 16 hours, one tick attached to the membrane with ridgeback odor. After 40 hours this tick was not attached anymore. In the unit with the ridgeback odor, some feces was present after 40,5 hours. In units 9, 10 and 11 one tick was dead in each unit after 40 hours. At the end of the feeding, the mortality rate was 48%. The blood of unit 11 turned dark after 64,5 hours.

R. sanguineus

Unit	Total	Membrane thickness	Membrane odor	Blood
U9	10 adults (5♂, 5♀)	79 µm	Labrador odor + hair	Cow
U10	10 adults (5♂, 5♀)	83 µm	Heidewachtel odor + hair	Cow
U11	10 adults (5♂, 5♀)	73 µm	Ridgeback odor + hair	Cow
U12	9 adults (4♂, 5♀)	80 µm	Bouvier odor + hair	Cow

Table 25: F10 *R. sanguineus* adults

Time (h)	Unit	Attached	Mortality
t ₁ =16	U9	0	1
	U10	0	0
	U11	1	1
	U12	-	-
	Mean	0,3	0,7
	%	3	7
t ₁ =40	U9	0	1
	U10	0	1
	U11	0	1
	U12	-	-
	Mean	0	1
	%	0	10
t ₁ =64 t ₂ =15	U9	-	-
	U10	0	5
	U11	0	1
	U12	0	3
	Mean	0	3
	%	0	31
t ₁ =88 t ₂ =39	U9	-	-
	U10	0	5
	U11	0	2
	U12	0	3
	Mean	0	3,3
	%	0	34
t ₁ =94 t ₂ =45	U9	-	-
	U10	0	5
	U11	0	3
	U12	0	6
	Mean	0	4,7
	%	0	48

***In vitro* odor test**

Odor test 1

During the first odor test, *D. reticulatus* and *I. ricinus* ticks were used to test perfume with cow odor (96% ethanol). At the beginning of the experiment the ticks were equally distributed over the petri dish. After 19,5 hours, 1 *D. reticulatus* tick was in the (+)-zone and 6 ticks were very close to this zone. The *I. ricinus* ticks did not have preference for a specific location.

Table 26: odor test 1

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i>	0	Cow (96% ethanol)	0	7	3
	5		0	8	2
	19,5		1	9	0
<i>I. ricinus</i>	0	Cow (96% ethanol)	1	9	0
	5		1	8	1
	19,5		1	8	1

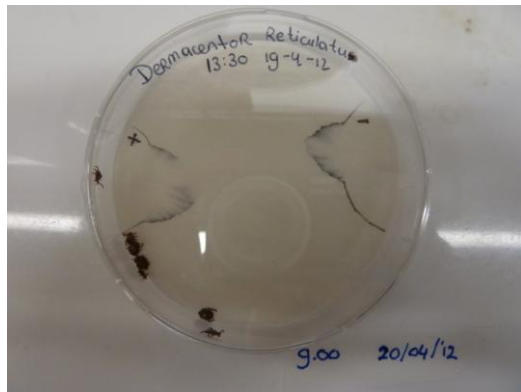


Figure 6: *D. reticulatus* (19,5h)

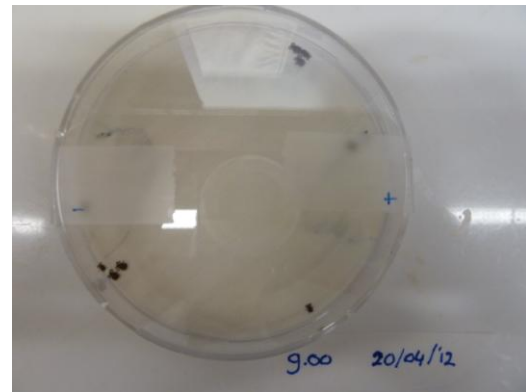


Figure 7: *I. ricinus* (19,5h)

Odor test 2

In this experiment *D. reticulatus*, *I. ricinus* and *R. sanguineus* ticks were used. The perfume in the petri dish of *D. reticulatus* and *I. ricinus* was cow odor (96% ethanol) and the perfume for *R. sanguineus* was bouvier dog odor (96% ethanol). In the beginning of the experiment, *D. reticulatus* and *I. ricinus* were crawling over the filter paper in the petri dish. *R. sanguineus* crawled directly to the perfume side after placing them. After 17 hours, all *D. reticulatus* ticks were on the positive half of the petri dish and after 22,5 hours 7 ticks were in (+)-zone. *I. ricinus* ticks did not have any preference for a specific side of the petri dish during the whole experiment. *R. sanguineus* ticks were directly attracted to the perfume and they were still attracted to the perfume after 17 and 22,5 hours.

Table 27: odor test 2

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i>	0	Cow (96% ethanol)	4	5	1
	17		4	6	0
	22,5		7	1	2
<i>I. ricinus</i>	0	Cow (96% ethanol)	0	9	1
	17		4	6	0
	22,5		0	7	3
<i>R. sanguineus</i>	0	Dog Bouvier (96% ethanol)	4	6	0
	17		6	3	1
	22,5		7	0	3



Figure 8: *D. reticulatus* (22,5h)

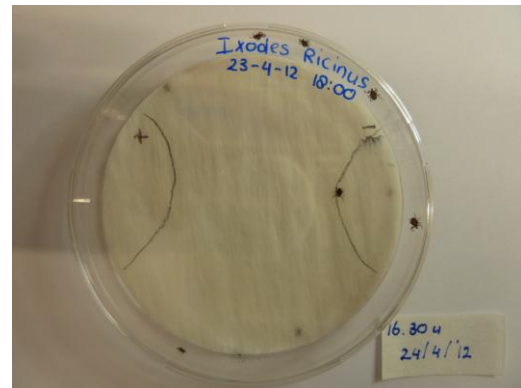


Figure 9: *I. ricinus* (22,5h)

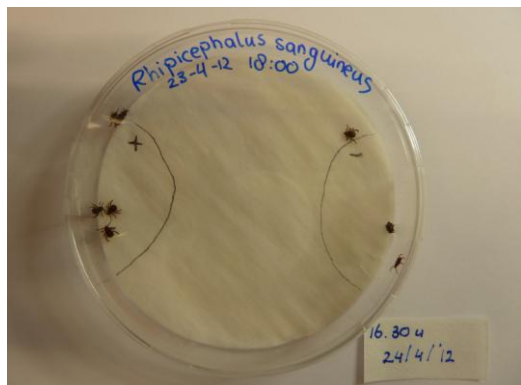


Figure 10: *R. sanguineus* (22,5h)

Odor test 3

In this experiment, *D. reticulatus* and *I. ricinus* ticks were put in a petri dish with cow odor (96% ethanol) and *R. sanguineus* ticks in an petri dish with bouvier dog odor (96% ethanol). During the experiment, the ticks were not attracted to the perfume. Ticks were especially seen in the neutral zone.

Table 28: odor test 3

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i>	0	Cow (96% ethanol)	6	3	1
	18		0	9	1
	22		0	10	0
<i>I. ricinus</i>	0	Cow (96% ethanol)	1	8	1
	18		0	8	2
	22		2	7	1
<i>R. sanguineus</i>	0	Dog Bouvier (96% ethanol)	2	7	1
	18		1	5	4
	22		0	7	3



Figure 11: *D. reticulatus* (22h)



Figure 12: *I. ricinus* (22h)



Figure 13: *R. sanguineus* (22h)

Odor test 4

In this experiment, *D. reticulatus* and *I. ricinus* ticks were used to test the perfume of cow odor (96% ethanol) and *R. sanguineus* ticks to test the perfume of bouvier dog odor (96% ethanol). The ticks of *D. reticulatus*₁ did not have any preference for a certain place. Also, the ticks of *D. reticulatus*₂ did not seemed to be attracted by the perfume, but after 19,5 hours 7 ticks in the neutral zone were in the direction of the (+)-zone. *I. ricinus* ticks were in this experiment more in the direction of the (+)-zone. *R. sanguineus* ticks seemed to be attracted by the perfume. At the end of the experiment, 9 ticks were in the (+)-zone.

Table 29: odor test 4

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i> ₁	0	Cow (96% ethanol)	0	7	3
	19,5		1	8	1
	25,5		1	5	4
<i>D. reticulatus</i> ₂	0	Cow (96% ethanol)	1	9	0
	19,5		1	8	1
	25,5		1	9	0
	44		2	7	1
<i>I. ricinus</i>	0	Cow (96% ethanol)	0	8	2
	19,5		6	4	0
	25,5		5	3	2
	44		7	2	1
<i>R. sanguineus</i>	0	Dog Bouvier (96% ethanol)	2	5	3
	19,5		6	4	0
	25,5		5	5	0
	44		9	0	1

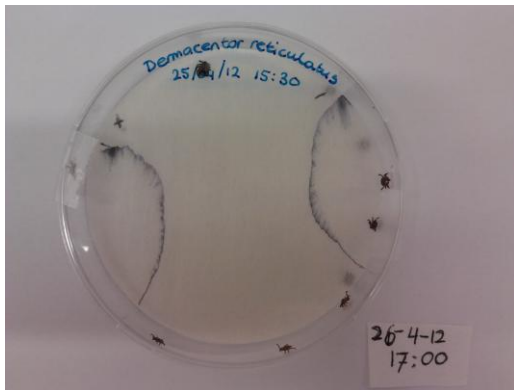


Figure 14: *D. reticulatus*₁ (25,5h)

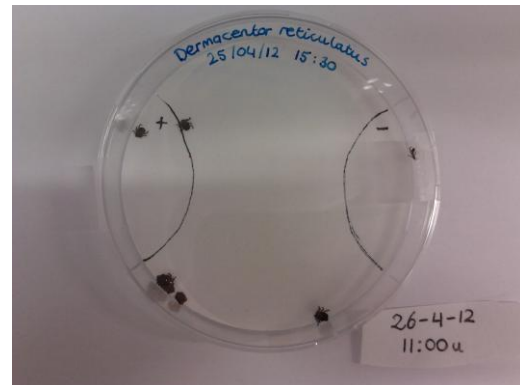


Figure 15: *D. reticulatus*₂ (19,5h)



Figure 16: *I. ricinus* (25,5h)



Figure 17: *R. sanguineus* (44h)

Odor test 5

In this experiment, *D. reticulatus* and *I. ricinus* ticks were put in a petri dish with cow odor (hexane) and *R. sanguineus* ticks in a petri dish with dog odor (96% ethanol) from dogs of Greece. At the end of the experiment, many ticks seemed to have a preference for the positive side of the filter paper. After 95 hours, two *D. reticulatus* ticks were in the (+)-zone and 5 ticks from the neutral zone were in the direction of the (+)-zone. Six *I. ricinus* ticks were in the (+)-zone. Two *R. sanguineus* ticks were found in the (+)-zone and 5 ticks from the neutral zone were close to the (+)-zone.

Table 30: odor test 5

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i>	0	Cow (hexane)	3	5	2
	3		2	6	2
	95		2	8	0
<i>I. ricinus</i>	0	Cow (hexane)	2	8	0
	3		4	4	2
	95		6	4	0
<i>R. sanguineus</i>	0	Dog Greece (96% ethanol)	1	6	3
	3		0	10	0
	95		2	8	0



Figure 18: *D. reticulatus* (95h)



Figure 19: *I. ricinus* (95h)

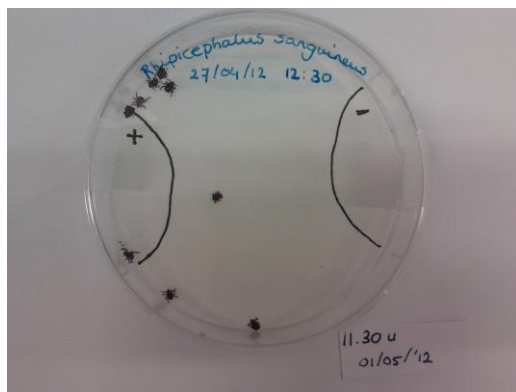


Figure 20: *R. sanguineus* (95h)

Odor test 6

In this experiment, *D. reticulatus* and *I. ricinus* ticks were used to test perfume of cow odor (hexane) and *R. sanguineus* ticks to test Greece dog odor (96% ethanol) *D. reticulatus* ticks did not have a preference for a particular place, but after 24 hours five ticks in the neutral zone were more in the direction of the (+)-zone. *I. ricinus* did not have any preference for a certain place. Also the ticks of *R. sanguineus* were spread over the filter paper during the experiment.

Table 31: odor test 6

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i>	0	Cow (hexane)	3	7	0
	24		1	9	0
<i>I. ricinus</i>	0	Cow (hexane)	1	7	2
	5		1	7	2
	24		3	3	4
<i>R. sanguineus</i>	0	Dog Greece (96% ethanol)	2	4	4
	5		0	3	7
	24		0	7	3

Odor test 7

In this experiment, *D. reticulatus* and *I. ricinus* ticks were put in a petri dish to test the perfume of cow odor (hexane) and *R. sanguineus* to test bouvier dog odor (hexane). *D. reticulatus* did not have a preference for a specific location. After 27 hours, 6 *I. ricinus* ticks were in the (+)-zone. Also the *R. sanguineus* ticks seemed to be attracted by the (+)-zone after 27 hours.

Table 32: odor test 7

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i>	0	Cow (hexane)	1	7	2
	21		0	6	4
	27		3	3	4
<i>I. ricinus</i>	0	Cow (hexane)	3	6	1
	21		2	5	3
	27		6	3	1
<i>R. sanguineus</i>	0	Dog Bouvier (hexane)	2	6	2
	21		2	5	3
	27		6	3	1

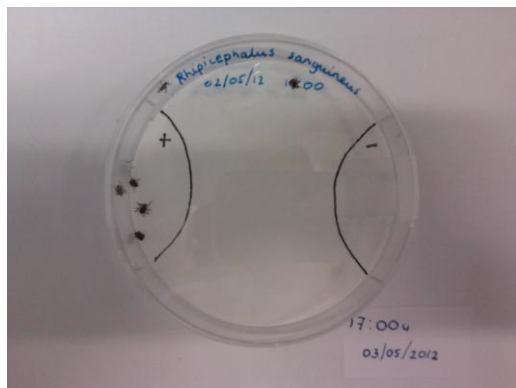


Figure 21: *R. sanguineus* (27h)

Odor test 8

In this experiment, *D. reticulatus* and *I. ricinus* ticks were tested on the perfume with cow odor (hexane) and *R. sanguineus* on perfume with bouvier dog odor (hexane). *D. reticulatus* and *I. ricinus* ticks did not have a place of preference in the petri dish. After 18 hours, *R. sanguineus* ticks were more attracted to the (+)-zone and all ticks in the neutral zone were in the direction of the (+)-zone.

Table 33: odor test 8

Tick species	Time (h)	Perfume	(+)-zone	Neutral	(-)-zone
<i>D. reticulatus</i>	0	Cow (hexane)	0	9	1
	18		2	3	5
<i>I. ricinus</i>	0	Cow (hexane)	1	9	0
	18		4	6	0
<i>R. sanguineus</i>	0	Dog Bouvier (hexane)	1	7	2
	18		5	5	0

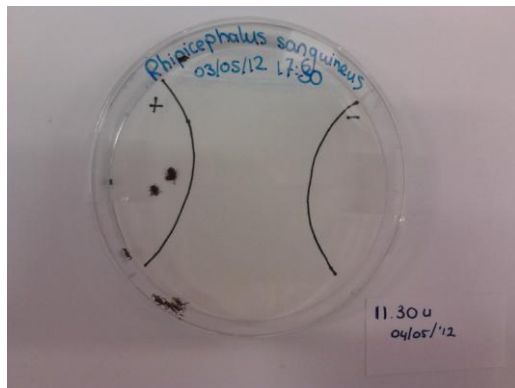


Figure 22: *R. sanguineus* (18h)

4. Discussion

In vitro feeding

Feeding 1

The purpose of this experiment was to feed *R. sanguineus* adults and nymphs in an in vitro feeding system. Although, the adult *R. sanguineus* ticks were active, they did not attach to the membrane at all. A possibility is that the dog odor on the membranes was not strong enough to attract the ticks. The membranes were rubbed over a labrador for 2 minutes. Previous studies used the same dog to obtain dog odor on the membranes, and these membranes were also rubbed for several minutes. In these studies the attachment rate of *R. sanguineus* ticks was higher (19),(20). It is possible the membranes in this feeding were not rubbed good enough to get enough odor to attract the ticks. Also, the nymphs of *R. sanguineus* did not attach. The dog odor on the membranes could also play a role in the feeding of the nymphs. Maybe, the dog odor was not strong enough to attract nymphs. It is also possible that the membranes were too thick to penetrate. By following of the protocol, the membranes were between 70 and 100 μm . It is possible that nymphs need thinner membranes to penetrate and to reach the blood.

D. reticulatus adults in unit 6 were all attached to the membrane. This means that the feeding system works for adult ticks. *D. reticulatus* nymphs did not attach to the membrane and the mortality rate was very high. After 40 hours, the mortality increased to 94%. It is possible that the nymphs wanted to feed, but that the membranes were too thick. The nymphs were not able to reach the blood. The temperature and humidity are maybe a reason of the high mortality rate. It may be that *D. reticulatus* nymphs need another environment to feed.

Probably, the darkness of the blood was caused by contamination or because the blood became old. The blood was not free of bacteria, because it was not possible to work sterile during the blood collection. Also, the blood in the six-well plate was kept at a temperature of 37°C, and this is an ideal temperature for bacteria to grow.

Feeding 2

The adult *R. sanguineus* ticks were not very active during this feeding. Only after CO₂ stimulation, the ticks were moving in the unit. The ticks might not have attached to the membrane, because of the inactivity and low quality of the ticks. Active ticks might attach faster to the membrane. Also in this feeding, the membranes were rubbed over the labrador for 2 minutes. It is possible that the membranes were not attractive for the ticks, because the odor was not strong enough.

Some *R. sanguineus* nymphs seemed to be attached to the membrane. Membranes with a thickness of 51 and 57 μm were used in this feeding. These membranes were probably the right thickness for the nymphs to attach. The mortality rate of the nymphs was in this feeding higher than in feeding 1. It is possible that the nymphs cannot stay in an environment with these temperature and/or humidity.

The attachment rate of *D. reticulatus* adults was in this feeding lower than in feeding 1. It is possible that the ticks did not attach or that they detached, because of the bad quality of the blood.

Almost all nymphs did not attach to the membrane. Probably, the membranes were too thick because there were not enough membranes with a thickness between 50 and 60 μm . Therefore, membranes were used with a thickness of 70 and 80 μm .

Feeding 4

In this feeding, the attachment rate of adult *R. sanguineus* ticks was disappointing; none of the ticks attached. The ticks were not very active and even after CO₂ stimulation there was no movement. Anyway, the ticks did not die during the feeding. At the end of the feeding, the ticks were held to the heating plate and they started to walk. Probably, the quality of these ticks was not very good and therefore they did not attach and feed. It is also possible the membranes did not have a strong enough dog odor, because the membranes were rubbed for 2 minutes over the labrador. To obtain a stronger dog odor, it is possible to rub the membranes for a longer period.

D. reticulatus adults did also not attach to the membrane. The odor of the membranes could not be the problem; because *D. reticulatus* ticks are not very host specific and they are normally easily to feed in an in vitro feeding system. It is not clear why they did not feed, but a possibility is that the quality of the ticks was not good anymore.

The attachment rate of the *D. reticulatus* nymphs was very low. The membranes had the right thickness to penetrate, so this could not be the problem. Probably, the low attachment rate was due to the high mortality rate of the nymphs. At the end of the feeding all nymphs were dead. It is possible that the nymphs of *D. reticulatus* cannot live in the environment of the water bath or the quality of the nymphs was not good.

Feeding 5

In this feeding, some changes were made to obtain better attachment results. One of these changes was the time of membrane rubbing. The membranes were rubbed over a labrador for 10 minutes instead of 2-5 minutes. The idea was that the dog odor on the membrane would be stronger. Rubbing longer than 10 minutes is not possible, because the membrane gets loose of the lid where it is taped on. Another change was the warming of the blood. In previous feedings, the blood was warmed for 15 minutes. To be sure the blood had the right temperature (37°C) for the feeding of the ticks, the blood was warmed for 30 minutes. Also, PBS was warmed for 30 minutes so that the ticks were not uncomfortable or that they would detach by cold PBS.

Cow and pig blood were used to investigate if ticks have a preference. The nymphs of *R. sanguineus* were very active, but they did not attach to the membrane. Therefore, nothing is to say about the blood preference of the nymphs.

The nymphs of *D. marginatus* were also very active in the beginning of the feeding. The attachment rate was very low and the mortality rate was high. Also, for the nymphs of *D. marginatus* there is nothing to say about the blood preference. The temperature and the humidity in the water bath are probably the cause of the high mortality rate.

R. sanguineus and *D. marginatus* nymphs were fed on membranes with a thickness of 70-80 µm, because only these membranes were available. The membranes were probably too thick to penetrate. Thinner membranes will be used for next feedings with nymphs.

In the cow blood some blood clots were present. This could be a reason why the ticks did not attach, but this is unlikely because the attachment rate of ticks on pig blood was not better. Also, *I. ricinus* nymphs were fed at the same time with the same blood, and these feedings went very well.

Feeding 6

Adult *R. sanguineus* ticks, 15% infected with *E. canis*, were used for the first time in this study. The attachment rate (30%) in this feeding was higher than in the previous feedings with adult ticks. The higher attachment rate is probably because of the new batch of ticks and these ticks were very active. The membranes were rubbed for 10 minutes over a labrador. Probably, the dog odor was attractive to attach. In the units

feces was present in a blood clot around the attached ticks. At the end of the feeding, the attached ticks were dead because they were stuck in the blood clot. A possibility is to remove these blood clots during the feeding, to prevent that ticks die and that they are able to complete their feeding.

The *R. sanguineus* nymphs did not attach at all and this is maybe because the quality of the nymphs was not good enough anymore. It is also possible that the membranes were not attractive, but these membranes were also rubbed over a labrador for 10 minutes. The mortality rate of the nymphs was low compared with previous feedings with *R. sanguineus* nymphs.

At the beginning of the feeding, the blood was treated with gentamicine (5µl/10ml); to prevent bacterial growth in the blood. Blood with gentamicine was only used for the nymphs. Only the blood of unit 10 turned dark, which means that the gentamicine worked in the blood for the nymphs. Gentamicine is a good solution to prevent bacterial growth. A disadvantage is that it cannot be used in a study about the transmission of bacteria.

A high mortality rate of *D. marginatus* nymphs is probably because of incorrect temperature and/or environment in the water bath. During this experiment, the mortality rate was also high. This means that the environment in the water bath is indeed the cause of the mortality of the nymphs.

Feeding 7

In this feeding, only 10% of the adult *R. sanguineus* ticks were attached to the membrane. This percentage is not very high. The membranes were rubbed over the labrador for 10 minutes. It could be that the labrador is not attractive anymore for the ticks or that the wrong places on the dog were rubbed. Ticks have favorite places to attach on the host, and they have also places on the host which are repellent (11). It could be that the odors of the attractive places are not found or not strong enough on the membranes.

The larvae of *R. sanguineus* seemed to be attached to the membrane at the beginning of the feeding. The hypostome length of the larvae is 50 µm (1), so this means that the larvae could not have reached the blood at membranes of 53 and 54 µm. It is possible that the larvae tried to reach the blood, but that it failed and they detached. It is not possible to make membranes thinner than 50 µm, because the elasticity of the membrane will be lost. At this moment, it is not possible to feed *R. sanguineus* larvae in vitro on these membranes.

The mortality rate of *D. marginatus* nymphs was low (6%) during this feeding. The temperature of 18-22°C and the humidity of 70-90% did not kill the nymphs. This means a lower temperature and humidity decrease the mortality rate of *D. marginatus* nymphs.

The blood in this feeding was collected by the use of a human blood bag. The purpose of blood collection with a blood bag was to obtain the blood sterile as possible, and also to obtain blood without clots. In vitro feedings to study the transmission of tick-borne pathogens cannot use blood with gentamicine, because the antibiotic will also eliminate these tick-borne pathogens. Therefore, sterile blood is needed so that the blood does not become dark during the feeding. Normally, sterile blood is obtained with a blood bag, but the needle of this blood bag was too small to pierce the vein of the cow. The needle felt a several times in the hay and this did not contribute to the sterility of the blood. Bacteria were present in the blood, because blood of units 9 and 10 turned dark. A possibility is to use a needle which is suitable to stay in the blood vessel of the cow.

Feeding 8

R. sanguineus ticks are very host specific and therefore, membranes are needed with only a dog odor to attract ticks and to let ticks attach. The first step to make the membranes attractive was to eliminate the human odor on the membranes. The membranes were made with latex gloves on and taped on sterile lids. Then, two methods to obtain dog odor were carried out. The first method was rubbing of the membranes over the preferred locations of the dog. The second method was to make membranes of hexane with dog odor. In this feeding, two different dog breeds were used to investigate if the ticks had a preference for a certain breed.

Only two *R. sanguineus* adult ticks did attach to the membranes which were rubbed over the dogs. On the membranes made of hexane with dog odor, none of the ticks were attached. Therefore, no conclusions can be drawn about the preference for dog breeds. It is also not sure if the new methods to obtain dog odor on the membrane are useful, because the attachment rate was very low.

The *R. sanguineus* ticks from Portugal had been attached to dogs. The high mortality rate is probably because these ticks were pulled off from the dogs and the ticks were kept in a jar for a few days. Then, the ticks were used for in vitro feeding. The ticks may be starved to dead, because they had to wait and the metabolism was already started.

Feeding 9

The adult *R. sanguineus* ticks in this feeding were also used in feeding 8. The membranes were rubbed over two dog breeds, a labrador and a yorkshire terrier. The ticks on the labrador membrane did not attach and 25% died. It is possible the ticks did not attach because they had already been used for an in vitro feeding. The mortality rate of the ticks on the membranes with yorkshire terrier odor was very high (87%), because the dog was treated with the acaricide certifect™. The acaricide worked very well to kill the ticks. It can be concluded that after rubbing of the yorkshire terrier, the acaricide was present on the membrane. The membranes pick up substances from the hair and skin of a dog during the rubbing procedure.

Feeding 10

During this feeding, the main purpose was to test membranes with different dog breed odors to investigate if adult *R. sanguineus* ticks had a preference for a certain dog breed. Unfortunately, only one tick in this feeding did attach to the membrane and therefore, it is not possible to say something about the preference for a dog breed. The mortality rate was high and at the end of the feeding 48% was dead. It is possible that the quality of the ticks had decreased, because some ticks were already used in other in vitro feedings.

The feeding with the labrador odor membrane was stopped after 49,5 hours because none of the ticks were attached and the labrador odor was already used in other feedings. Therefore, these ticks were placed in another unit with a membrane of bouvier odor.

***In vitro* odor test**

R. sanguineus ticks are very host specific and therefore, it is important to obtain dog odor on the membranes to attract the ticks to feed. In the in vitro feedings which has been done, membranes were always rubbed over a dog. In this study the attachment rate of *R. sanguineus* ticks was very low. It is thought that this low attachment rate is due to the fact that the membranes did not have enough dog odor to attract ticks. Therefore, new methods are needed to make the membranes more attractive.

To increase the attraction of the membranes, perfume was made of dog and cow odor. Perfume made of the host odor has also other advantages. Normally, the membranes are rubbed over a dog and then the membranes can be glued on the feeding units. It is not possible to start directly with the feedings, because the units on the membranes have to dry for 3 hours. After these 3 hours, the units are cut out and tested. It would be easier when the units are already glued and cut out, so that the units are ready when they are needed. The only step that is left is to spray the perfume with the host odor on the membranes.

During the odor tests, *D. reticulatus* and *I. ricinus* ticks were tested on perfume with cow odor and the *R. sanguineus* ticks on perfume with dog odor.

In the first two experiments, *D. reticulatus* ticks were attracted by the cow (95% ethanol) odor perfume. In experiments 3 and 4 the ticks did not have any preference for a certain location. Also, in experiments 5 and 6 the ticks were more attracted to the location with cow (hexane) odor and in experiment 7 and 8 the ticks did not have a favorite location. It seems that *D. reticulatus* ticks are more attracted to the perfume zone when the perfumes are used for the first time. It is possible that the perfumes lose the odor in the bottle and that only fresh made perfumes have an odor that attract *D. reticulatus* ticks.

I. ricinus ticks did not have a certain reaction to the cow (96% ethanol) and cow (hexane) perfume. Almost in all experiments the ticks were found in the neutral zone or equally divided through all zones. Perfume made of cow odor has not an attraction stimulus to *I. ricinus* ticks.

In odor tests 2 and 4, *R. sanguineus* ticks were directly attracted by the perfume of bouvier odor (96% ethanol). Also, the ticks in experiment 7 and 8 with the bouvier odor (hexane) were more attracted to the (+)-zone of the filter paper. It seems that the perfumes with bouvier odor are attractive for ticks. The perfume of dogs from Greece (96% ethanol) did not give a clear indication if the ticks were attracted to it or not. In experiment 5 the ticks in the neutral zone were close to the (+)-zone and in experiment 6 some ticks were in the (-)-zone.

Perfume with the odor of the host is an ideal method to make membranes attractive. Further investigation about the efficacy is needed to decide which kind of perfume attracts ticks. Different dog breeds can be investigated and also different mediums to dissolve the animal odor. The next step is to spray the perfume on the membranes of the feeding units and to see if the attachment rate of the ticks increases.

5. Conclusion

In this study, *R. sanguineus* larvae, nymphs and adults were fed in an in vitro feeding system. It is difficult to feed these ticks in vitro. It is very important to use ticks of a good quality and also the environmental conditions have to be optimal during the feeding.

It became clear that *R. sanguineus* larvae cannot feed in an in vitro feeding system, because the membranes were too thick for their mouthparts. The mortality rate of the *R. sanguineus* nymphs was high in two feedings and it is important to decrease this rate.

The attachment rate of the adult *R. sanguineus* ticks was very low. Improvements are needed to increase the attachment rate and to make the in vitro feeding system useful to investigate tick-borne pathogen transmissions. Because *R. sanguineus* ticks are very host specific, improvements to make membranes more attractive are essential. Therefore, it is important to obtain attractive stimuli on the membranes. During the in vitro odor tests it became clear that perfume with dog odor attract *R. sanguineus* ticks, especially perfume with the bouvier odor. Further research is needed to investigate what the attraction stimuli are for *R. sanguineus* ticks and how to add these stimuli on or in the membranes.

Also *D. reticulatus* adults and nymphs and *D. marginatus* nymphs were used for the feedings. The in vitro feeding system works for adult *D. reticulatus* ticks. *D. reticulatus* and *D. marginatus* nymphs need other environmental conditions during the feedings to survive.

6. Recommendations

It is recommended to use ticks of a good quality. These ticks will attach faster than ticks in a bad condition. When ticks attach to the membranes, investigation of the transmission dynamics of pathogens can be carried out.

The environmental conditions during a feeding have also to be optimal. Blood collection has to be sterile as possible, so that the blood does not become dark by bacterial growth. The blood needs the right temperature before the unit with ticks is put in the six-well plate. It is also crucial to have the right temperature and humidity in the water bath.

Membranes have to be used with the right thickness and also membranes that are attractive for ticks to attach. Different dog breed odors or the odors of the preferred locations on the dog can be used to make membranes more attractive for *R. sanguineus* ticks. Also, other methods to add the odor on the membranes can be tested. Perfumes are an easy method to give the membranes an odor resembling dogs.

7. References

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8. Appendix

Protocol 'In vitro Feeding of hard ticks'

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Materials & methods

1. Ticks: *preconditioning adult Ixodes ricinus to enhance attachment*

- 3 – 6 month post ecdysis ticks are used: obtained from a laboratory rearing at the University of Neuchâtel. Before placing them in the feeding chamber, they must be preconditioned for at least one week, better 3 weeks, at 20 to 23°C and 85 to 98 % relative humidity, with 10 to 16 h light per day.

! Important: it is critical to avoid low temperatures (i.e. < 14°C) in autumn and wintertime to prevent ticks going into diapause.

2. Blood: *defibrinated blood collection and preparation*

2.1 Blood collection

- Blood is collected weekly from an abattoir, defibrinated manually, by stirring rapidly for twenty minutes with a big spoon to collect the clot which will form attached to the spoon. Blood is poured into 1 litre sterilized bottles and supplemented immediately with 2g/l glucose and stored at 4°C (KUHNER ET AL. 1995, KUHNER 1996).

2.2 Preparation of blood for feeding

- All blood preparation is carried out in a sterile hood (Scan USE-2000-120). Gentamycine and ATP are added to the blood just before the blood is exchanged in the wells. ATP must be applied freshly in order to act as attachment and/or feeding stimulus before being metabolised,

A 5 µl aliquot of a Gentamycine solution (Sigma, Germany, 10 mg/ml in sterile deionised water) is added to 10 ml of blood to achieve a final concentration of 5 µg/ml blood.

A 100 µl aliquot of ATP (Fluka, Switzerland) solution (0.1 molar in NaCl 0.9%), sterile filtered (at 0.2 µm) is added to 10 ml of blood to achieve a final concentration of 10⁻³ molar in the blood.

- The well plates are then covered with the well lid and warmed to 37°C in the water bath prior to adding the feeding units.

! Important: in all experiments, blood must be exchanged twice daily at 12 hour intervals (max interval 14 h) in each well.

- During an experiment, the membrane surface facing the blood is rinsed with sterile saline (9 g NaCl pa, Fluka, in demineralised water) before placing the feeding unit in a fresh well (with ticks still attached).
- Fungal infections under the membrane are treated daily with Nystatin solution (Sigma, Germany, 10,000 units/ml DPBS) for 10 min during the blood exchange when the daily evaluation of ticks is made.

- The amount of blood required for each well is 3.1 ml. For calculated examples for a whole experiment see spread sheet 'blood calc' in file 'IVF Ir method'.

3. Blood treatments: *compound preparation for adding to the blood*

The blood treatments are: control (nothing added), dimethyl sulfoxide as placebo (DMSO, Fluka, Switzerland) at 2.5 µl/ml blood, the reference agent fipronil (Pestanal, Riedel de Haën, Germany) and the test compounds made up at 0.001, 0.01, 0.1, 1, 10 µg in DMSO/ml blood. Four feeding units are used for each treatment concentration.

Worked example: 15 ml of blood is required for 4 feeding units, i.e. 3.1 ml/feeding unit. 37.5 µl of DMSO stock solution (containing 4 mg test product/ml) is added to give 10 µg test compound/ml blood (see spread sheet 'prod conc's' in file 'IVF Ir method').

4. Membrane preparation: *silicone membrane preparation for the tick feeding unit*

Ixodes ricinus is fed on bovine blood through a silicone membrane reinforced by Kodak[®] lens cleaning paper, similar to that already developed in this laboratory (Kuhnert *et al.* 1995, Guerin *et al.* 2000). This membrane is modified to increase the attachment rate of *I. ricinus* by rendering the silicone soft such that it mimics the elasticity of skin. This ensures closure of tick penetration sites on the membrane to prevent bleeding. A silicone glue is selected with a low shore hardness A (expressed in degrees) - a measure of the indentation hardness of soft materials.

4.1 Silicone preparation for the membranes

The silicone glue RTV-1 Elastosil E4, (Wacker-Chemie GmbH, München Germany) with a very low shore hardness A of about 16° is used. Mixing silicone oil (30% DC 200, ~ 10 mPa.s, Fluka, Switzerland) to the silicone glue further increases softness and reduces 'frog grip' - the sticky nature of the resulting silicone surface. 15 % Hexane is added to render the glue more fluid for application to the matrix.

Note: the mixing should be done under dry conditions (as a low relative humidity as possible) to reduce the polymerisation of the silicone to a minimum.

- **Worked example**

Quantities for a smaller amount (i.e. a quarter) given in brackets

60 g (15 g) Wacker silicone E4

0.6 g (0.15 g) Wacker FL colour paste (1 % of the silicone)

18 g (4.5 g) FLUKA DC 200 silicone oil (30 % of the silicone)

11.7 g (2.9 g) Hexane (technical quality, 15 % extra weight)

- Kodak lens cleaning paper (70 x 120 mm), a non-woven tissue made of regenerated cellulose rayon (Eastman Kodak, Rochester, NY) is used as the matrix. The lens paper is placed on a layer of kitchen plastic film (30 cm wide) which has been laid on a glass sheet, making sure that there is a 30 mm working space between each lens paper. The lens

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paper is held down with sticky tape. The silicone mixture is spread evenly over the lens paper using a 80 mm wide scraper made from a sheet of silicone (3mm thick).

- Membranes are left to polymerize for 12 h at room conditions, or to accelerate polymerisation, for 4 to 6 h in 80 to 90 % humidity at 25°C.
- The thickness of every membrane is measured using micro callipers, and only those between 70 to 110 µm are used.

5. Feeding Units: *preparation*

See figures at back of manual

- The feeding units are made of Plexiglas® tubing (26 mm i.d., 2 mm wall thickness, 45 mm high) with a ring made of acrylic glass fixed around each tube to limit the depth (4 mm) to which the unit sinks into the blood in the wells (Fig 1). The feeding membrane is attached to the angled (1 deg) lower end of the tube (Fig 3) using silicone glue (Wacker Elastosil E4) and left to dry (min. 3 h). See Fig. 3 for a construction drawing of an acrylic feeding unit **at the end of the manual.**
- To improve the attachment rate of the ticks to the membrane, a piece of glass fibre mosquito netting (1.4 mm mesh, 25 mm diameter) is cut out by using a 25 mm cutting tool. The netting is glued to the membrane in the feeding unit with silicone glue (WACKER Elastosil E4) and left to dry (Fig. 2).
- Following this, the membranes are cut flush with the outer wall of the feeding unit using scissors and the feeding units are checked for leaks by sitting them in Petri dishes with 70% ethanol for 20 min.
! Important: it is critical that the ethanol does not enter the feeding unit.
- Check for any holes in the membrane under a stereo microscope and repair any small holes using Wacker E4 silicone diluted with 40% toluene with a fine paint brush. Strictly avoid applying thick drops of silicone.
- A plastic tile spacer (2 mm thick tile spacer, size of the 4 arms adjusted to the 26 mm diameter of the feeding unit) is placed on the membrane to create additional borders where ticks prefer to attach (Fig 1).

6. Attachment stimuli: *preparation*

White or light coloured bovid hair is shaven from a non treated animal. The colour of the hair is important in order to see the ticks in the feeding units, but unimportant for the extract (below). Hair is cut into 4 to 7 mm pieces and kept frozen (-20°C) in a jar for adding to the feeding unit and for preparing the cow hair extract.

Preparation of cow hair extract

Hair (50 g) is cut off a young light-coloured cow on one side and collected in a beaker. The hair is extracted in three successive 20 minute steps to increase the yield:

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- add 250 ml of dichlormethane (DCM, Merck, extra pure grade), leave for 20 minutes then remove the solution (about 100 ml) and replace it with a fresh 100 ml of DCM. Leave this for a further 20 minutes, then remove the DCM solution (about 100 ml). Repeat this extraction with 100 ml DCM one more time.
- The three 100 ml extracts are combined. Either the extract is centrifuged at 3000 rpm for 20 minutes and the supernatant is removed or the extract is filtered (Macherey & Nagel glass fiber filter MN GF-2, 0.5- μ m pores, Düren, Germany). This is then concentrated by roto-evaporation to about 100 ml and stored in a freezer at -80°C. The amount of material of low volatility per unit volume (henceforth indicated as the 'low volatile mass', LVM) is estimated by evaporating 1 ml of extract on a glass slide and weighing after 30 min at room temperature. The stock solution is adjusted to 100 mg LVM/ml.
- Prepare a working solution of 7 mg LVM/ml by diluting the stock with DCM. The working solution is kept at -20°C prior to application on to the feeding membrane.

(If the lipid extraction is carried out using methanol or hexane the evaporation time lasts a minimum of 30 minutes).

7. Attachment: application of attachment stimuli and placing ticks in feeding units

- 75 μ l of bovid hair extract (0.5 mg LVM lipids extracted from freshly shaven bovid hair with DCM applied in 75 μ l DCM per feeding unit) is applied to the membrane with a micropipette. The feeding units are placed for 15 to 30 min on a metal grid placed on top of a hot plate at 40°C to evaporate the solvent (DCM).
- The feeding units are placed in six-well cell culture plates (COSTAR, 34.8 mm diameter) with 3.1 ml of the test blood and warmed to 37°C using a thermostat-controlled water bath (740 mm long x 540 mm deep x 215 mm high) with a sloping Perspex hood to keep the air above the feeding units near 100% R.H. A warm plate may also be used but stable temperature in the blood must be assured and high humidity around the feeding units must be maintained.

! Important: the bath must subject to a 16:8 h light: dark cycle, this is critical for attachment.

- The six-well plates with the feeding units sit on a metal support submerged 15 mm below the water surface in the water bath.
- Ten female and five male *I. ricinus* ticks are put into each feeding unit with soft forceps, covered with a 1 cm layer of cow hair, cut to a length of 4 to 7 mm, and the ensemble held down with a brass grid (25 mm diam., 3 mm mesh, 0.55 mm wire). Each feeding unit is closed with a perforated stopper (0.5 mm Sefar plastic mesh, Fig. 1).

! Important: placing the ticks in the feeding units must be carried out towards the end of the 16:8 h light: dark cycle to encourage attachment.

8. Recording data on compounds tested

- Four feeding units are used for each compound at each dose level.
- The ticks are evaluated once a day to count the number of living and dead ticks attached to the membrane as well as the unattached living and dead ticks. All dead ticks are removed from the feeding units. Knock down observations are also made.
- If a large amount of tick faeces accumulates this can be removed by gently tapping the feeding unit upside down, being careful not to dislodge any of the ticks. Sometimes faeces get stuck, especially to mating ticks and need to be removed. This is done by dislodging the faeces with a pair of forceps, being careful not to dislodge the feeding ticks.
- The feeding experiments are complete after nine days, or earlier, depending on the experimental protocol.

9. Statistical analysis

Survival curves are calculated from the numbers of dead ticks recorded per day over the different doses of each treatment using the Kaplan-Meier Statistics (KLEINBAUM 1995) with Peto test of the survdiff algorithm in S-plus (V6.2 build 6713).

10. Relevant references

Detailed accounts of this hard tick feeding assay have already been published (KRÖBER AND GUERIN 2007a and 2007b). See section 12 (Literature) of this manual

11. List of materials and suppliers

Chemicals, catalogue numbers and suppliers

NaCl (Fluka 71380, pa, > 99.5 % (AT)), for saline at 9 g/l (<http://www.sigmaaldrich.com>)

Glucose (D(+)-Glucose Monohydrate, Fluka 49159, >99 % (HPLC))

ATP (Fluka 02060, > 95.0 % (HPLC)), 10⁻³ mmolar in the blood

Gentamycine solution (Sigma G1272, sterile filtered 10 mg/ml), 5 µg/ml blood or
Gentamycine sulfate (Sigma G3632)

DMSO (dimethyl sulfoxide, Fluka 41650, > 99.0 % (GC)), 2.5 µl/ml blood as solvent for test products

Fipronil (Riedel de Haen, Pestanal 46451, > 97.5 % (HPLC)), reference acaricide

Nystatin solution (SIGMA N-1348, 100 units/ml) for treatment of fungi, 10 min daily when necessary (<http://www.sigmaaldrich.com>)

Hexane (technical grade)

Toluene (Merck, supraSolv, No 1.08389.1000)

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Dichlormethane (DCM, Merck, SupraSolv, No 1.06054.1000)

Silicone oil DC200 ~10 mPa.s 250 ml FLUKA No. 85411

Feeding units, catalogue numbers and suppliers

Tubes Plexiglas® XT clear 29070 (<http://www.roehm.de/en/plexiglas.html>)

External diameter 30 mm, internal 26 mm, wall 2 mm (for corpus)

External diameter 40 mm, internal 30 mm, wall 5 mm (for ring)

ACRIFIX® 106, glue for ring around the feeding unit, from Plexiglas or Rhoem

Stopper (PE-Caps), 26 mm with 15 mm hole, PET netting glued with hot glue (BOSCH)

Polyester Netting Sefar, Switzerland, PET 1000 18-180W PW (www.sefar.com)

Glass fiber mosquito netting; grey, HSB Phifer Inc. Tuscaloosa, AL, USA (www.phifer.com), Art-No 257251, on membrane

Tile spacer, 2 mm cross, white plastic, Germany

Filters

Glass fiber filter MN GF-2, 0.5-µm pore (Macherey & Nagel, Germany) or other.

Membranes, catalogue numbers and suppliers

Kitchen roll of PE cling film (Tangan No11, house brand from Migros Switzerland)

Silicone oil DC 200, ~10 mPa.s, Fluka 85411, Switzerland,

Wacker ELASTOSIL® E4 RTV-1 Silicone Rubber

(<http://www.wacker.com/internet/noc/Products/ProductsAZ>)

Wacker ELASTOSIL® COLOR PASTE FL white RAL

Wacker ELASTOSIL® E41 RTV-1 Silicone Rubber (contains Toluene, used for repairing small holes in the membrane or the sealing between the acrylic glass and membrane.

Ticks

Ixodes ricinus from lab rearing at Neuchâtel (www.unine.ch) 10 females + 5 males per feeding unit.

Illustrations

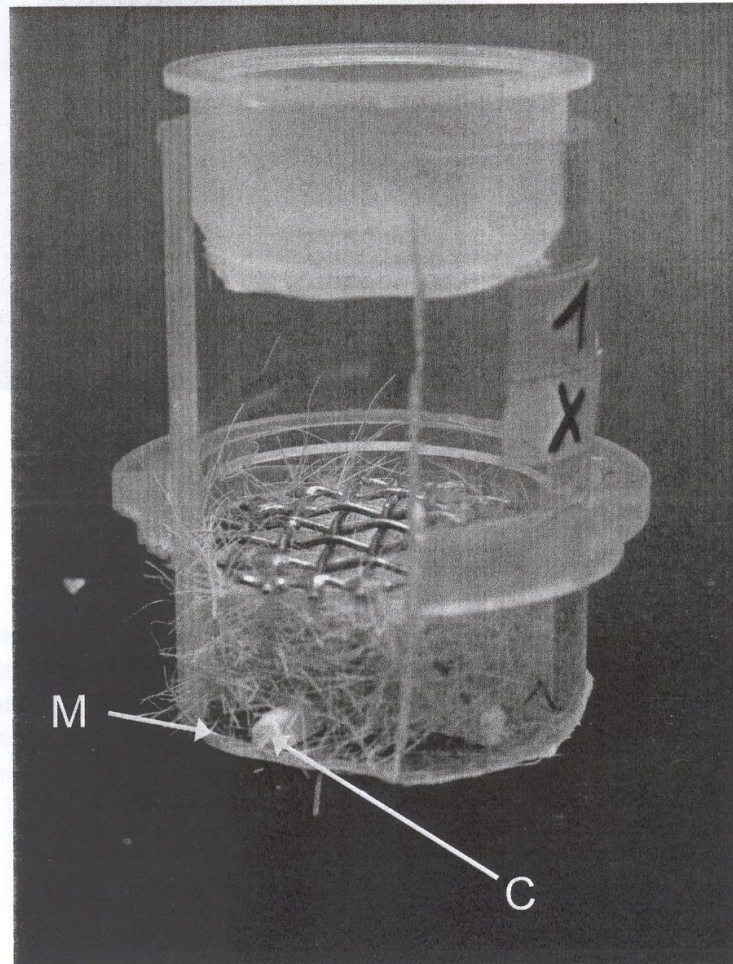


Figure 1

Cut out view of the *in vitro* feeding unit for *Ixodes ricinus* made from an acrylic glass tube (45 mm high x 30 mm o.d., 2 mm thick wall). Part of the plastic cross (C) placed on the membrane (M) is visible, and the layer of cow hair placed on the membrane is held lightly down with a brass grid. The ring around the unit assures that a layer of 2 mm of blood lies under the membrane when placed in the well. A perforated plastic stopper is inserted on top.

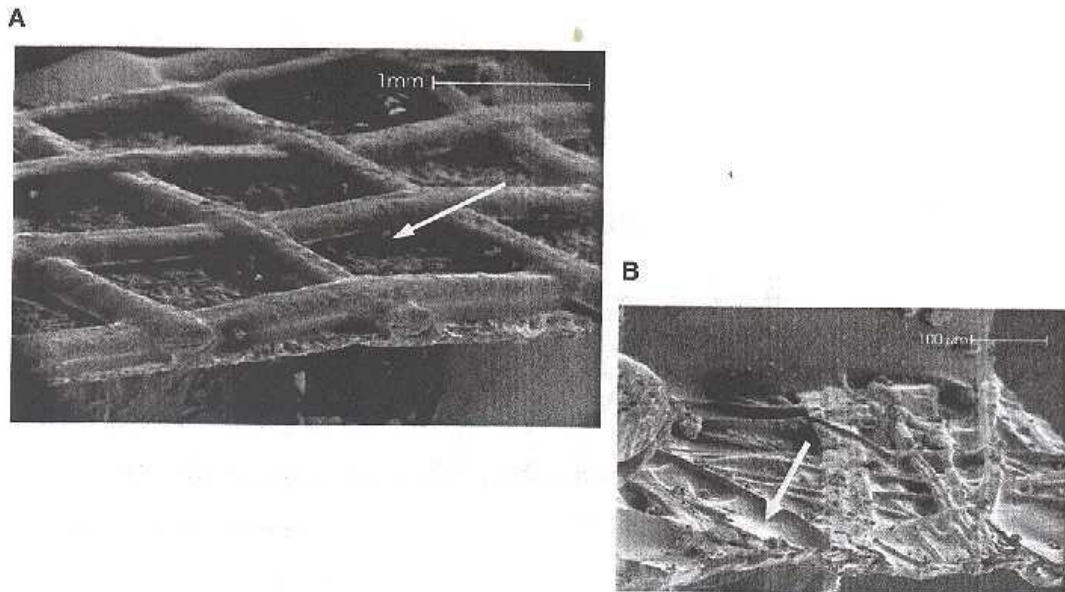


Figure 2

(A) Scanning micrograph of a feeding membrane with mosquito netting glued on to it. Only a minimum quantity of glue was used to attach the netting to the membrane so as to leave cavities (arrow) which allow the ticks to obtain a perch with their mouthparts in the membrane. (B) The spaces between the cellulose fibres of the lens cleaning paper are only partly filled with silicone providing small regions where the membrane is even thinner (arrow) than the thickness of the paper.

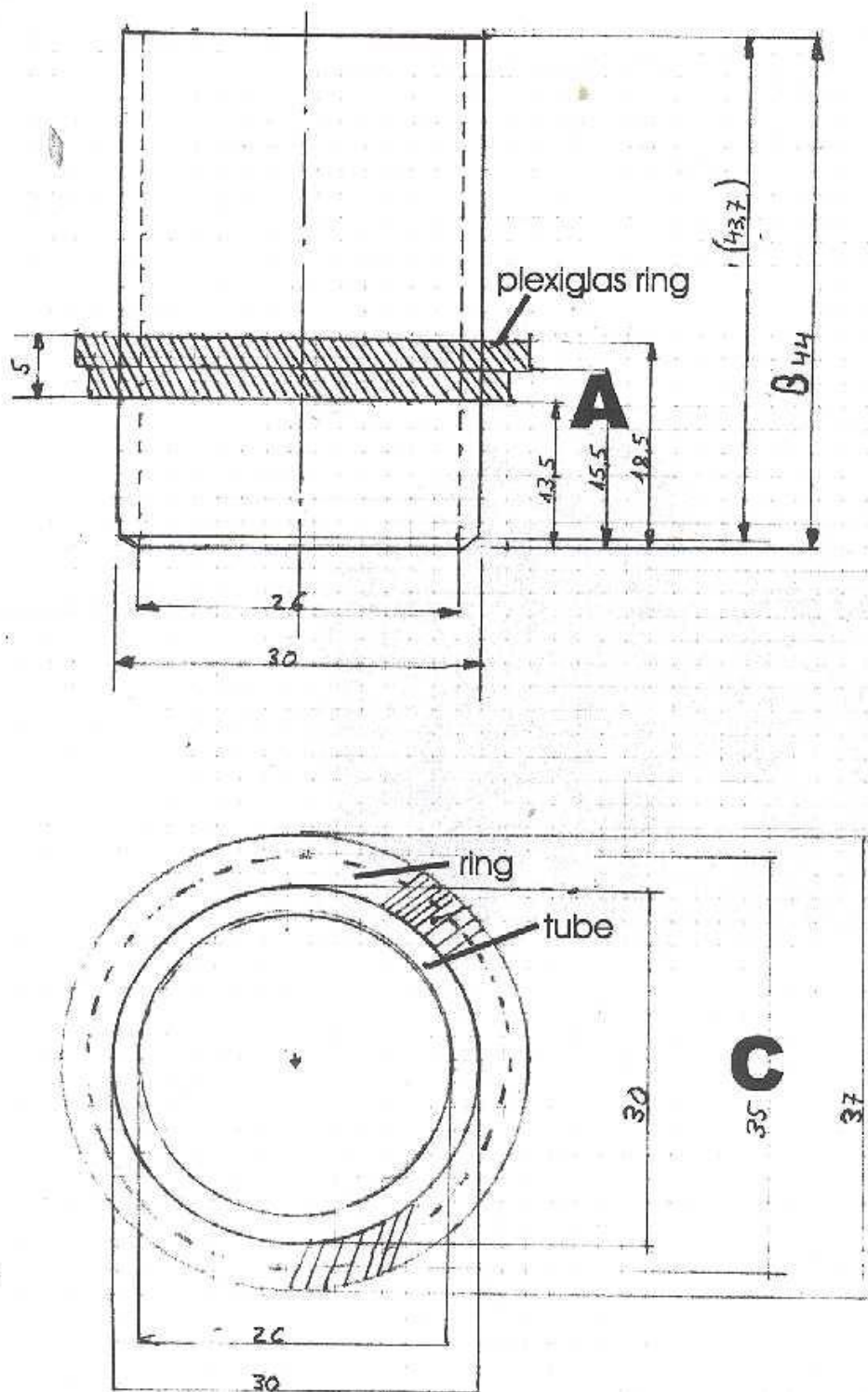


Figure 3

Construction drawing of the feeding unit made from acrylic glass tubing, scale 2 : 1 in mm; measure (A) should have ≤ 0.1 mm tolerance to ensure an equalized layer of blood under the membrane. Measure (C) should be checked for easy fitting without too much play in the well.