In vitro feeding of *Rhipicephalus sanguineus* ticks and the attraction of ticks to dog odor

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Abstract

Rhipicephalus sanguineus, *Dermacentor reticulatus* and *Dermacentor marginatus* were fed in an in vitro feeding system for ticks. *D. reticulatus* adults served as a control for this system since feeding this species was successful in previous research. Furthermore, all developmental stages of *R. sanguineus* were allowed to feed in vitro. For the feedings of adult *R. sanguineus* odors of different dog breeds were used, but this tick species did not seem to have a preference for any of these odors. Attachment of adult *R. sanguineus* did not exceed 30%, while mortality reached 90%. *R. sanguineus* nymphs did attach up to 16% in one unit, but they did not engorge. Larvae of this tick species could not feed, because they were not able to penetrate the membrane.
In vitro odor tests were carried out with *R. sanguineus*, *D. reticulatus* and *Ixodes ricinus*. For these tests perfumes of dogs and cow were produced by two different methods. One uses 96% ethanol as a solvent while the other method uses hexane. Results show some preference of *R. sanguineus* for the perfumes, especially the dog perfume made with 96% ethanol.

Introduction

Rhipicephalus sanguineus, *Dermacentor reticulatus* and *Ixodes ricinus* are the most common ticks in Europe (Beugnet et al, 2009). While both *D. reticulatus* and *I. ricinus* are present in the Netherlands (Bodaan et al, 2007), *R. sanguineus* is a Mediterranean tick which thrives best in areas with higher temperatures and high humidity (Estrada-Peña et al, 2004) and is mainly found in countries between 35 °S and 50 °N (Dantas-Torres, 2008; Estrada-Peña et al, 2004).
Rhipicephalus sanguineus is also known as the brown dog tick, referring to its primary host (Dantas-Torres, 2008). However, cases of *R. sanguineus* bites on humans have been reported (Estrada-Pena & Jongejan, 1999) and other mammals and birds can occasionally act as hosts as well (Dantas-Torres, 2008; Taylor et al, 2007). Adult ticks are usually found on the shoulders, neck and in the ears, while nymphs prefer shoulders and ears and larvae the flanks and stomach (Estrada-Peña et al, 2004). *R. sanguineus* is a three-host tick, so every stage feeds on a new host after which the tick will fall off the animal to moult to the next stage (Estrada-Peña et al, 2004; Jongejan & Uilenberg, 2004). It takes 63-91 days to complete the life cycle of *R. sanguineus*, depending on environmental conditions (Dantas-Torres, 2008; Taylor et al, 2007). This species can survive and develop under a broad range of conditions and is endophilic, which means this tick is mainly found indoors (Dantas-Torres, 2010), so *R. sanguineus* may survive in the Netherlands as well. Cases of *R. sanguineus* have been reported in the Netherlands on dogs that have not been abroad, but it was assumed that these dogs got in contact (direct of indirect) with animals that have been to foreign countries (Bodaan et al, 2007).

Some dog breeds are more susceptible to tick infestations than others. For example, *Rhipicephalus sanguineus* prefers Cocker Spaniels over Beagles according to an experiment performed by Louly and others (2010). Ticks seem to be able to differentiate between hosts by picking up specific substances excreted by a host (Carroll, 2002). These substances are called kairomones and are picked up by sensors in the tarsi of the front legs of the tick (Haggart et al, 1980). Due to this property ticks are able to find the right host to feed on. Once attached to the host infected ticks are able to transmit diseases or, the other way round, ticks can pick up diseases from the host. *Rhipicephalus sanguineus* is a vector of *Babesia vogeli*, *B. gibsoni*, *Ehrlichia canis* and *Rickettsia conorii* (Bodaan et al, 2007) and is considered to be a vector of *Bartonella vinsonii* spp. *berkhoffii* (Billeter et al, 2008). Transstadial transmission of *E. canis* is established (Bremer et al, 2005) and this way of transmitting a disease may be important for other pathogens as well.

To gain knowledge about transmission of pathogens, in vitro feeding systems can be used. This in vitro method is relatively easy and is less invasive for test animals compared to an in vivo test (Kröber & Guerin, 2007). Pathogens can be added to the blood to infect ticks, this way pathogen acquisition can be studied. Or infected ticks can infect the blood during a feeding, which gives information about pathogen transmission. Ticks are fed in a controlled environment and blood samples can be taken repeatedly to check pathogen transmission. The advantage of this method is that all of this can be done without discomfort for a test animal.

Dermacentor reticulatus is a vector of multiple pathogens including *Rickettsia slovaca*, *Babesia canis* and *Coxiella burnetii* (Bodaan et al, 2007; Taylor et al, 2007; Zahler & Gothe, 1995). This tick species is a three-host tick, the adults feed on large mammals such as cattle, sheep, but also humans. The larvae and nymphs feed on insectivores and sometimes on birds (Estrada-Peña et al, 2004; Taylor et al, 2007).

For this research *Dermacentor marginatus* has been used as well. This species is a three-host tick and can be found on mammals as sheep, cattle, human (adults) as well as on insectivores and birds (nymphs and larvae) (Estrada-Peña et al, 2004). This tick can transfer *Babesia canis*, *B. divergens*, *Theileria equi* and many other diseases. *D. marginatus* is mainly found in the Mediterranean region (Taylor et al, 2007).

Materials and methods

Ticks

Rhipicephalus sanguineus ticks from the colony of the UCTD as well as *R. sanguineus* ticks collected in South-Africa were used for this research. Fifteen percent of the South-African ticks were infected with *Ehrlichia canis*. The batch only contained adults and was collected during the period of this research. All ticks were stored at 21 °C and 69% relative humidity (RH). All stages of *R. sanguineus* (larvae, nymphs and adults) are used for the in vitro feedings.

Dermacentor reticulatus nymphs and adults have also been used. The adults have been used as a control, since they have successfully attached and fed in previous research. These ticks are all derived from the UCTD colony, except the adults used for the odor tests. The latter were collected in Zeeland (the Netherlands). Finally, the species *Dermacentor marginatus* from the UCTD colony was also used for this research and originated from Portugal. From this species only the nymphs were used. All *Dermacentor* ticks are also stored at 21 °C and 69% RH.

Ixodes ricinus adults from the UCTD collection were used for the in vitro odor tests.

Set-up of in vitro feeding

Ticks are allowed to feed in an in vitro feeding unit. A unit is a tube made of Plexiglas with a silicon membrane on the bottom which resembles the skin of a host (figure 1).
This membrane is made from a lens cleaning paper (6x7 cm, Tiffen®) besmeared with a mixture of Elastosil® E4 silicon rubber (Wacker), silicon oil (SIGMA-ALDRICH®), hexane (SIGMA-ALDRICH®) and white color paste, prepared according to protocol (Appendix I). After drying, the thickness of the membrane is measured (the thickness should be between 70 and 100 µm) and it is taped to the lid of a 6-wells cell culture plate. To make a feeding unit more attractive the odor of the host is transferred to the membrane either by rubbing the animal or placing hairs of the host onto the membrane (see Appendix II). After that the membrane can be glued to a unit with Elastosil® E4 silicon rubber (Wacker). It takes 3 hours to dry, and then the membrane is cut loose from the lid and neatly cut around the unit. After that the unit can be tested for leakage by placing it in demineralized water for 20 minutes. If no leakage occurs, the feeding unit can be used.

Figure 1: In vitro feeding unit

Figure 2: In vitro feeding system. Two units are placed in a 6-wells culture plate filled with blood. The units are closed with a stopper.

A unit can be filled with 10 adults (5 males and 5 females), 25 nymphs or up to 150 larvae. These ticks may escape, so to prevent this, the unit is closed with a 'stopper' wrapped in organza. This stopper needs to be pressed down to the membrane (circa 0.5 cm from the membrane) to force the ticks to stay close to the bottom where they can feed.
For the feeding, a unit is placed in a well of a 6-wells plate filled with 3.1 mL of cow or pig blood. Up to 4 wells of 6-wells plate can be used (figure 2). The blood-filled 6-wells plate is placed in a water bath, 30 minutes before placing the units into a well, to warm up the blood.

During a feeding the ticks have to be placed in an environment comparable to natural feeding conditions (±28 °C and ±90% RH). To meet these needs a water bath is designed. This bath consists of an outer and inner bath. The outer bath is filled with demineralized water (MilliQ) while the inner bath is filled with a potassium sulfate solution (120 gr/L). The level of the K_2SO_4 solution in this aquarium may not exceed the water level of the outer bath, to keep the temperature inside the aquarium the same as the outer bath. The

temperature is set at 37 °C. RH inside the inner bath needs to be circa 90%, and to keep the humidity constant the aquarium is closed with a triangular lid and regularly refilled with K_2SO_4 solution. Feeding units in a 6-wells plate are placed in the inner bath and the bath is covered with a dark cloth.

An in vitro feeding period takes at least two days, because the ticks need time to attach. But if the ticks need to engorge it will take even longer.
Attachment and mortality are checked in the flow cabinet at 9.30h in the morning every day. Blood needs to be refreshed and blood samples are taken from the old blood at 9.30h and 18.30h. Fresh blood is warmed up as described before as well as the sodium chloride solution (0,9% NaCl) to rinse of old blood from the membrane of the units. For a blood sample 200 µL of every well is taken and pipetted into a 1.5 mL Eppendorf tube. These samples are stored at -20 °C. From every well blood samples are taken *in duplo*.

Blood

Fresh blood was collected on the first day of the feeding. Blood was taken from cows and pigs from the faculty of Veterinary Medicine of the Utrecht University. For the collecting of blood multiple methods have been used.
Glass bottle: a sterile 500 mL glass bottle has been used to collect blood. The bottle can be filled up to 300 mL. Once filled with the desired amount the content needs to be firmly stirred with a sterile 10 mL pipette for 15 minutes to take out the coagulation factors. A blood clot is formed on the pipette and can be easily removed from the bottle. This method was used for feedings 1 to 6.
Blood bag: a 14 Gauge needle is placed onto the tube to collect blood from the animal. While the blood is running the bag needs to be shaken gently to distribute the anticoagulants equally. The blood bag was used for feeding 7 only.
Erlenmeyer flask: a sterile 500 mL flask can be filled up to 200 mL. The blood has to be firmly stirred for 15 minutes with a sterile 10 mL pipette to prevent the blood from clotting. A blood clot is formed on the pipette and can be removed from the flask. Feeding 8 to 10 are done with blood collected in an Erlenmeyer flask.
Once the blood is collected, glucose (2 gr/L) is added to stabilize the erythrocytes. Then, the blood has to be decanted into 50 mL falcon tubes. To prevent bacterial overgrowth, adding gentamicin is an option (5 µL/10 mL) unless the ticks are infected with a pathogen. Now the falcon tubes are stored at 4 °C. Before using the blood for a feeding it should be vortexed for 10 to 15 seconds.

Perfume

Odors from different animal species are captured as attractants for ticks. Perfumes are made according to two different methods. Firstly, odor can be captured in 96% ethanol. The second option is the use of hexane as a solvent. Two cotton pads are rubbed on the host animal and put in two different glass jars. One bottle is filled with 12 mL of 96% ethanol, the other with 10 mL of hexane (SIGMA-ALDRICH®). The bottles are placed on a shaker plate for at least 48 hours at low speed. Odor caught in hexane needs further processing. Hexane needs to evaporate, 5 mL 96% ethanol is added to the residue after which the glass jar is placed on the shaker plate for another 48 hours till the residue is solved. Any residue stuck to the bottom can be scraped off with a stirrer. This process is described in Appendix II.

In vitro odor test

One piece of filter paper is placed on the bottom of a Petri dish. On this paper two sides are marked; one with a (–) mark on which the solvent (96% ethanol) is pored, and the other side with a (+) mark, the side with the animal perfume. The filter paper is now subdivided in a positive, negative and neutral zone.
In the positive zone 25 µL animal perfume is pipetted, and in the negative zone 25 µL of the solvent. Wait 10 minutes for the solvent to evaporate then the ticks (five males and five females) can be placed in the Petri dish. Place the lid on the dish and seal it by taping the lid to the dish on two opposite places. Put it in a stove with light, a temperature of 20 °C and 20% RH.
After a few hours the distribution of the ticks can be observed and documented by taking pictures. The Petri dish can be placed back into the stove straightaway or the ticks can be stimulated to move by blowing into the dish. CO_2 stimulation makes ticks active after which they can choose a side for a second time. Blowing into the dish must be done in the

neutral zone to prevent ticks going to either the (+) or (-) zone, which might influence the outcome of the in vitro odor test.
Eight in vitro odor tests have been performed with five different perfumes and three tick species (see table 1).

Odor test	Perfume (solvent)	Tick species
1	Cow (96% ethanol)	*I. ricinus* + *D. reticulatus*
2	Cow (96% ethanol)	*I. ricinus* + *D. reticulatus*
	Dog Bouvier (96% ethanol)	*R. sanguineus*
3	Cow (96% ethanol)	*I. ricinus* + *D. reticulatus*
	Dog Bouvier (96% ethanol)	*R. sanguineus*
4	Cow (96% ethanol)	*I. ricinus* + *D. reticulatus* (2x)
	Dog Bouvier (96% ethanol)	*R. sanguineus*
5	Cow (hexane)	*I. ricinus* + *D. reticulatus*
	Dog Greece (96% ethanol)	*R. sanguineus*
6	Cow (hexane)	*I. ricinus* + *D. reticulatus*
	Dog Greece (96% ethanol)	*R. sanguineus*
7	Cow (hexane)	*I. ricinus* + *D. reticulatus*
	Dog Bouvier (hexane)	*R. sanguineus*
8	Cow (hexane)	*I. ricinus* + *D. reticulatus*
	Dog Bouvier (hexane)	*R. sanguineus*

Table 1: overview of perfumes used for each in vitro odor test and the ticks species which were used.

Results

During a period of 10 weeks multiple in vitro feedings have been done. Every week at least one feeding was started but most weeks feedings with two different tick species were done, namely *Rhipicephalus sanguineus* and as a control *Dermacentor reticulatus*. Ticks were fed on cow blood, except four units in feeding 5 which were placed on pig blood.
Results shown in the following tables are percentages of the total number of ticks per unit. The numbers are all calculated based on the total number of ticks used for a feeding, so dead ticks are not deducted.
For every feeding a description is given about the ticks and the membranes:

Unit number Tick species (number of ticks) [membrane thickness] – membrane odor

Week 7 - Feeding 1

Dermacentor reticulatus
U5 *D. reticulatus* adults (5♂+5♀) [89 µm] – Labrador odor
U6 *D. reticulatus* adults (6♂+5♀) [89 µm] – Labrador odor
U7 *D. reticulatus* nymphs (25) [+/- 70 µm] – Labrador odor
U8 *D. reticulatus* nymphs (25) [+/- 70 µm] – Labrador odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U5	15	0	0
U6		36	0
U5	40	0	0
U6		73	0
U5	65	20	0
U6		73	0
U5	89	20	0
U6		100	0
U5	98	20	0
U6		91	0

Unit	t=	Attached (in %)	Mortality (in %)
		Nymphs	Nymphs
U7	15	4	0
U8		0	0
U7	40	0	92
U8		0	96
U7	65	0	92
U8		0	100
U7	89	0	92
U8		0	100
U7	98	0	92
U8		0	100

Rhipicephalus sanguineus
U9 *R. sanguineus* adults (5♂+5♀) [?] – Labrador odor
U10 *R. sanguineus* adults (5♂+5♀) [?] – Labrador odor
U11 *R. sanguineus* nymphs (25) [?] – Labrador odor
U12 *R. sanguineus* nymphs (25) [?] – Labrador odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	15	0	0
U10		0	10
U9	40	0	0
U10		0	10
U9	65	0	0
U10		0	10
U9	89	0	0
U10		0	10

Unit	t=	Attached (in %)	Mortality (in %)
		Nymphs	Nymphs
U11	15	0	8
U12		0	4
U11	40	0	40
U12		0	56
U11	65	0	40
U12		0	56
U11	89	0	60
U12		0	64

Membranes are rubbed on a Labrador for 2 minutes.

There is a remarkable difference in attachment percentages of *D. reticulatus* between U5 and U6. After 40 hours the percentage of U6 reached 73% while U5 did not exceed 20%,

despite the corresponding membrane thickness and odor. At the end of the feeding none of the *D. reticulatus* ticks were fully engorged.

D. reticulatus nymphs did not attach in great numbers and at t=40 mortality was high. Attached nymphs did not increase in size.

No attachment is seen with the adult *R. sanguineus* and most of the ticks resided on the stopper. None of the nymphs attached either, but high mortality occurred after 40 hours. The nymphs crawled throughout the unit and did not show a preference for one particular place such as the stopper.

Week 8 - Feeding 2

Dermacentor reticulatus
U5 *D. reticulatus* adults (5♂+5♀) [99 µm] – Labrador odor
U6 *D. reticulatus* adults (5♂+5♀) [105 µm] – Labrador odor
U7 *D. reticulatus* nymphs (25) [80 µm] – rabbit odor (rubbed on the ears)
U8 *D. reticulatus* nymphs (25) [70 µm] – rabbit odor (rubbed on the ears)

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U5	19,5	10	0
U6		0	0
U5	43	20	0
U6		20	0
U5	67	20	0
U6		30	0
U5	91	50	0
U6		20	0
U5	102	40	0
U6		20	20

Unit	t=	Attached (in %)	Mortality (in %)
		Nymphs	Nymphs
U7	19,5	0	8
U8		0	16
U7	43	0	16
U8		0	16
U7	67	0	16
U8		0	20
U7	91	8	16
U8		0	20
U7	102	0	36
U8		0	40

Rhipicephalus sanguineus
U9 *R. sanguineus* adults (5♂+5♀) [78 µm] – Labrador odor
U10 *R. sanguineus* adults (5♂+5♀) [78 µm] – Labrador odor
U11 *R. sanguineus* nymphs (25) [51 µm] – Labrador odor
U12 *R. sanguineus* nymphs (25) [57 µm] – Labrador odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	19,5	0	0
U10		0	10
U9	43	0	0
U10		0	10
U9	67	10	0
U10		0	10
U9	91	10	10
U10		0	10
U9	102	10	20
U10		0	10

Unit	t=	Attached (in %)	Mortality (in %)
		Nymphs	Nymphs
U11	19,5	4	0
U12		0	0
U11	43	8	4
U12		0	8
U11	67	8	4
U12		16	8
U11	91	8	4
U12		16	8
U11	102	0	88
U12		0	96

Membranes with dog odor were rubbed for 5 minutes, the rabbit membranes were rubbed over the ears for 2 minutes.

Less adults *D. reticulatus* attached compared to week 7, but this week's U5 and U6 are comparable. After 102 hours the first mortality occurs.
For this feeding the membranes for *D. reticulatus* nymphs are rubbed on rabbits. Despite this adjustment the attachment percentage stays low and the same goes for the mortality of *D. reticulatus* adults.

Percentages of attachment of *R. sanguineus* fall behind in comparison with *D. reticulatus*. Again, the adults are situated on the stopper and not on the membrane.
R. sanguineus nymphs attach in higher rates than the nymphs of *D. reticulatus*. At the end of this in vitro feeding mortality among the nymphs is high, especially of *R. sanguineus* nymphs.

Blood of U4 turned black early in this experiment (t=19.5) and the coloration continued till the end of this feeding. After 43 hours blood of U1, U2, U3, U9 and U10 turned dark as well and at the end of the week blood of all units was dark.

Leakage occurred in U6 at t=91, but did not cause notable mortality.

Week 10 - Feeding 4

Dermacentor reticulatus
U5 *D. reticulatus* adults (5♂+5♀) [70 µm] - cow odor
U6 *D. reticulatus* adults (5♂+5♀) [70 µm] – cow odor
U7 *D. reticulatus* nymphs (22) [50 µm] – Labrador odor
U8 *D. reticulatus* nymphs (26) [50 µm] – Labrador odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U5	16	0	0
U6		0	0
U5	40	0	0
U6		0	0
U5	64	0	0
U6		0	0
U5	88	0	0
U6		0	0
U5	94	0	0
U6		0	0

Unit	t=	Attached (in %)	Mortality (in %)
		Nymphs	Nymphs
U7	16	14	0
U8		12	0
U7	40	0	64
U8		0	77
U7	64	0	95
U8		0	88
U7	88	0	100
U8		0	100
U7	94	0	100
U8		0	100

Rhipicephalus sanguineus
U9 *R. sanguineus* adults (5♂+5♀) [90 µm] – Labrador odor
U10 *R. sanguineus* adults (5♂+5♀) [75 µm] – Labrador odor
U11 *R. sanguineus* adults (5♂+5♀) [70 µm] – Labrador odor
U12 *R. sanguineus* adults (4♂+6♀) [79 µm] – Labrador odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	16	0	0
U10		0	0
U11		0	0
U12		0	0
U9	40	0	0
U10		0	0
U11		0	0
U12		0	0
U9	64	0	0
U10		0	0
U11		0	0
U12		0	0
U9	88	0	0
U10		0	0
U11		0	0
U12		0	0
U9	94	0	0
U10		0	0
U11		0	0
U12		0	0

Membranes rubbed on the cows are rubbed until they are slightly brown. The other membranes are rubbed over the entire body of the dog for 3 minutes. *Dermacentor reticulatus* adults are placed on membranes rubbed on cows, while membranes with dog odor are used for nymphs of this species and for the *R. sanguineus* ticks.

Attachment of adult *Rhipicephalus sanguineus* has not been observed during the control moments in the morning. However, in units 5 and 6 feces was present after 74 and 40 hours respectively. Overall, the ticks are not very active and do not respond to CO_2 stimulation.

None of the *R. sanguineus* adults attached or died. On t=40 blood of unit 11 turned black and blood of unit 10 and 12 changed color after 64 hours. After 88 hours the blood of U8 turned dark as well.

The RH of the inner bath was checked with another humidity and temperature meter and the RH turned out to be 83% (a desired RH is circa 90%).

Week 11 - Feeding 5

Dermacentor marginatus
U5 *D. marginatus* nymphs (38) [73 µm] – Labrador odor + hair
U6 *D. marginatus* nymphs (40) [70 µm] – Labrador odor + hair
U7 *D. marginatus* nymphs (30) [72 µm] – Labrador odor + hair ➔ pig blood
U8 *D. marginatus* nymphs (27) [78 µm] – Labrador odor + hair ➔ pig blood

Unit	**t=**	**Attached (in %)**	**Mortality (in %)**
		Nymphs	Nymphs
U5	15,5	0	13
U6		0	15
U7		0	10
U8		0	7
U5	40	0	16
U6		3	20
U7		0	27
U8		0	33
U5	64	5	37
U6		3	35
U7		0	53
U8		0	44
U5	88	5	58
U6		0	50
U7		3	67
U8		0	52

Rhipicephalus sanguineus
U9 *R. sanguineus* nymphs (21) [70 µm] – Labrador odor + hair
U10 *R. sanguineus* nymphs (16) [74 µm] – Labrador odor + hair
U11 *R. sanguineus* nymphs (18) [75 µm] – Labrador odor + hair ➔ pig blood
U12 *R. sanguineus* nymphs (25) [70 µm] – Labrador odor + hair ➔ pig blood

Unit	**t=**	**Attached (in %)**	**Mortality (in %)**
		Nymphs	Nymphs
U9	15,5	0	10
U10		0	0
U11		0	0
U12		0	4
U9	40	0	10
U10		0	0
U11		0	0
U12		0	4
U9	64	0	19
U10		0	0
U11		0	6
U12		0	4
U9	88	0	24
U10		0	0
U11		0	6
U12		0	12

Membranes were rubbed on the body of a Labrador for 10 minutes.

For this feeding recently moulted *D. marginatus* nymphs were used. These nymphs were more active compared to the *D. reticulatus* nymphs used for previous feedings (excluding week 7). Only some of the *D. marginatus* nymphs had attached, but neither these nor *R. sanguineus* nymphs had fed. No difference is seen between feedings with pig blood and cow blood. Furthermore there is high mortality, especially among *D. marginatus* nymphs.

Week 12 - Feeding 6

Dermacentor marginatus
U5 *D. marginatus* nymphs (26) [49 µm] – Labrador odor + hair

Unit	t=	Mortality (in %)
		Nymphs
U5	14,5	27
U5	38,5	58
U5	62,5	69
U5	86,5	73

Rhipicephalus sanguineus
U9 *R. sanguineus* adults (5♂+5♀) [81 µm] – Labrador odor + hair
U10 *R. sanguineus* adults (5♂+5♀) [77 µm] – Labrador odor + hair
U11 *R. sanguineus* nymphs (13) [49 µm] – Labrador odor + hair
U12 *R. sanguineus* nymphs (16) [50 µm] – Labrador odor + hair

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	14,5	10	0
U10		0	0
U9	38,5	30	0
U10		30	0
U9	62,5	30	0
U10		20	20
U9	86,5	30	0
U10		20	20
U9	94,5	20	10
U10		0	20

Unit	t=	Attached (in %)	Mortality (in %)
		Nymphs	Nymphs
U11	14,5	0	8
U12		0	0
U11	38,5	0	8
U12		0	6
U11	62,5	0	15
U12		0	19
U11	86,5	0	15
U12		0	19
U11	94,5	0	23
U12		0	31

Membranes are rubbed on the Labrador for 13 minutes. Rubbing longer would damage the membrane too much. Hairs placed in the units were collected on the same day as the rubbing of the membranes.

Because of high mortality among *D. marginatus* nymphs in previous experiments, the conditions during an in vitro feeding were tested. *Dermacentor* nymphs are exposed to the conditions of the water bath only (temperature of 27 °C and a RH of 88%). The ticks were placed in a regular unit, only this unit is not placed in blood. At the end of this test mortality was still 73%.

The *Rhipicephalus* batch used for these feedings carried *Ehrlichia canis*. Fifteen percent of the adults are infected, so once the ticks attached blood samples were taken every 3 hours, to determine the moment of transmission of the pathogen. Attachment took place after 14.5 hours already and at t=38.5 maximal attachment of this feeding is reached. At the attachment site there is a dark blood smear in which the ticks seem to be stuck (figure 3). Three out of five *R. sanguineus* adults that had attached were dead when ending the feeding.

Figure 3: Two engorged male *R. sanguineus* got stuck in a hard blood clot.

Week 13 - Feeding 7

Dermacentor marginatus
U7 *D. marginatus* nymphs (26) [48 µm] – mouse odor
U8 *D. marginatus* nymphs (25) [53 µm] – mouse odor

Unit	t=	Attached (in %)	Mortality (in %)
		Nymphs	Nymphs
U7	15,5	0	0
U8		0	0
U7	39,5	0	8
U8		0	4
U7	63,5	0	8
U8		0	4
U7	69	0	8
U8		0	4

Rhipicephalus sanguineus
U9 *R. sanguineus* adults (5♂+5♀) [70 µm] – Labrador odor + hair
U10 *R. sanguineus* adults (5♂+5♀) [76 µm] – Labrador odor + hair
U11 *R. sanguineus* larvae (+-100) [53 µm] – Labrador odor + hair
U12 *R. sanguineus* larvae (+-100) [54 µm] – Labrador odor + hair

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	15,5	10	0
U10		0	10
U9	39,5	10	0
U10		10	20
U9	63,5	10	0
U10		0	30
U9	69	0	10
U10		0	40

Unit	t=	Attached (in %)	Mortality (in %)
		Larvae	Larvae
U11	15,5	±10	0
U12		0	0
U11	39,5	±3	±10
U12		0	±11
U11	63,5	0	±10
U12		0	±11
U11	69	0	±10
U12		0	±11

Dermacentor marginatus nymphs are incubated at 20 °C during the weekend and after these two days mortality was low (data not shown), especially compared to the test of week 12. After this test the nymphs are used for an in vitro feeding under the new tested conditions (20 °C and RH of ±75%). Gentamicin was added to the blood for the feeding of these nymphs.
Membranes for the nymphs were placed in a container which was filled with bedding material of mice to make them smell like this host. At the end of this feeding none of the nymphs had attached, but mortality remained low.

For the in vitro feedings of *Rhipicephalus* two different stages are used, larvae and adults. The adults are ticks that survived the previous feeding, supplemented with fresh ticks from the stove. Attachment reaches 10%, but did not last till the end of the week. The attached *R. sanguineus* adult in U10 was turned out to be dead when ending the feeding.

For the feedings of *R. sanguineus* larvae gentamicin was added to the blood.
Checking attachment of the larvae is difficult to do because they are very small (± 0.5mm) and their hypostomes protruding through the membrane cannot be seen. Furthermore, there are so many larvae per unit that distinction between individual larvae cannot be drawn. Nevertheless, some of the larvae seemed to have attached based on some minor knobs seen on the surface of the membrane.
At t=15.5 leakage was discovered in U12. Most larvae crawled onto the stopper and survived so the larvae were rescued and placed into a new unit to proceed the feeding.

The total number of used larvae is an estimate. Because the larvae are small and very active and placing them into another jar was hard to do, counting them was not possible. Therefore, at the end of the feeding mortality of the larvae was not determined.

Week 14 - Feeding 8

Rhipicephalus sanguineus
U9 *R. sanguineus* adults (5♂+5♀) [71 µm] – Labrador odor (ear)
U10 *R. sanguineus* adults (5♂+5♀) [74 µm] – Labrador odor (ear)
U11 *R. sanguineus* adults (5♂+5♀) [77 µm] – Cocker Spaniel odor (ear)
U12 *R. sanguineus* adults (5♂+5♀) [74 µm] – Cocker Spaniel odor (ear)

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	15	0	0
U10		0	0
U11		0	10
U12		0	0
U9	39	0	0
U10		0	0
U11		0	20
U12		0	0
U9	63	0	30
U10		10	0
U11		10	20
U12		0	0
U9	70,5	0	30
U10		0	0
U11		0	20
U12		0	0

Rhipicephalus sanguineus
U13 *R. sanguineus* adults (5♂+5♀) [75 µm] – Labrador odor in hexane
U14 *R. sanguineus* adults (5♂+5♀) [80 µm] – Labrador odor in hexane
U15 *R. sanguineus* adults (5♂+5♀) [80 µm] – Cocker Spaniel odor in hexane
U16 *R. sanguineus* adults (5♂+5♀) [73 µm] – Cocker Spaniel odor in hexane

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U13	15	0	0
U14		0	10
U13	39	0	0
U14		0	10
U13	46,5	0	0
U14		0	10

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U15	15	0	0
U16		0	0
U15	39	0	0
U16		0	20
U15	46,5	0	0
U16		0	20

Rhipicephalus sanguineus
U17 *R. sanguineus* adults (5♂+3♀) [84 µm] – Labrador odor ear
U18 *R. sanguineus* adults (5♂+2♀) [79 µm] – Cocker Spaniel odor ear

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U17	15	0	0
U17	39	0	38
U17	46,5	0	38

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U18	15	14	57
U18	39	0	57
U18	46,5	0	57

Gentamicin was not used for the blood of the experiments with infected *R. sanguineus* adults in units 9 till 16. This week multiple experiments were done, to observe attachment of ticks on membranes with other dog odors. Labrador and Cocker Spaniel odor were used and these odors are caught in two different ways. The first method used is rubbing the membranes over the ears of the dogs. The second method includes trapping the dog odor into the membranes by solving it in the hexane needed for the membrane silicon mixture.
Rhipicephalus sanguineus adults placed on these membranes (U9 to U16) are the ones infected with *E. canis*. Ticks of the previous feedings were used again; the remaining ticks were fresh ticks from the stove. Again, attachment did not exceed 10% per unit and did not last for more than one day. Mortality was 30% at the most.
Two adults had crawled through the organza in U9, but could be placed back into the unit, which was closed with a new stopper. After 39 hours blood had turned black in U12, and kept turning dark till the end. At t=63 clots of blood were found in the well of U10, but have not been seen after refreshing the blood.
Beside the infected *R. sanguineus* batch, adults collected from a dog in Portugal were used in units 17 and 18. All ticks have been attached to a dog, and some of the females were partly engorged before placing them into the feeding units. One male attached in U18, but only at one checking moment. Mortality was high in both U17 and U18.

Week 15 - Feeding 9

Rhipicephalus sanguineus
U9 *R. sanguineus* adults (5♂+5♀) [80 µm] – Labrador odor
U10 *R. sanguineus* adults (5♂+5♀) [80 µm] – Labrador odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	15	0	10
U10		0	0
U9	39	0	10
U10		0	20
U9	63	0	10
U10		0	30
U9	65,5	0	20
U10		0	30

Rhipicephalus sanguineus
U11 *R. sanguineus* adults (5♂+5♀) [79 µm] – Yorkshire Terrier odor
U12 *R. sanguineus* adults (5♂+5♀) [74 µm] – Yorkshire Terrier odor
U13 *R. sanguineus* adults (5♂+5♀) [80 µm] – Yorkshire Terrier odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U11	15	0	90
U12		0	70
U13		0	70
U11	20,5	0	90
U12		0	90
U13		0	80

These *R. sanguineus* adults are the same ticks used for previous feedings. Again, ten adults are placed in a unit provided with the odor of the same Labrador (rubbed for 10 minutes) while the other ten were placed on Yorkshire Terrier odor. Gentamicin was not added to the blood for these feedings.
None of the ticks have attached. *R. sanguineus* placed on the Terrier membranes died in great numbers, with mortality up to 90%. For this reason these feedings were ended after 20.5 hours. When observing these dead ticks they appeared to have a typical posture; their legs were stretched out instead of drawn in.
At t=63 the blood of U10 turned dark.

Week 16 - Feeding 10

Rhipicephalus sanguineus

U9 *R. sanguineus* adults (5♂+5♀) [79 µm] – Labrador odor
U10 *R. sanguineus* adults (5♂+5♀) [83 µm] – Heidewachtel odor
U11 *R. sanguineus* adults (5♂+5♀) [73 µm] – Ridgeback odor
U12 *R. sanguineus* adults (4♂+5♀) [80 µm] – Bouvier odor

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U9	16,5	0	10
U9	40,5	0	10

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U10	16,5	0	0
U10	40,5	0	10
U10	64,5	0	50
U10	88,5	0	50
U10	94,5	0	50

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U11	16,5	10	10
U11	40,5	0	10
U11	64,5	0	10
U11	88,5	0	20
U11	94,5	0	30

Unit	t=	Attached (in %)	Mortality (in %)
		Adults	Adults
U12	0	0	33
U12	24	0	33
U12	30	0	67

This week multiple dog odors have been collected to test the attachment success on one or more of these dog breeds in particular. Labrador odor has been tested before, but serves as a control to compare attachment to the other dog odors. At t=40.5 the Labrador unit was ended, because none of the ticks had attached. Besides, this odor had been tested many times before. Ticks from U9 are transferred to U12, the new unit with Bouvier odor, and a new feeding was started.
From all the units with different odors, only one tick had attached (U11 with Ridgeback odor). At t=40.5 some black spots were observed which could be feces. After 64.5 hours blood of U11 turned dark and stayed dark till the end of the feeding.
Mortality varied from 10% in the Labrador unit up to 67% in the Bouvier unit. None of the dogs were treated with acaricides at the moment of rubbing.

In vitro odor tests

Perfumes of cow and different dog breeds have been developed during this research. Attraction of these odors to ticks is tested by placing 10 ticks into a Petri dish containing one of those perfumes.

Odor test 1

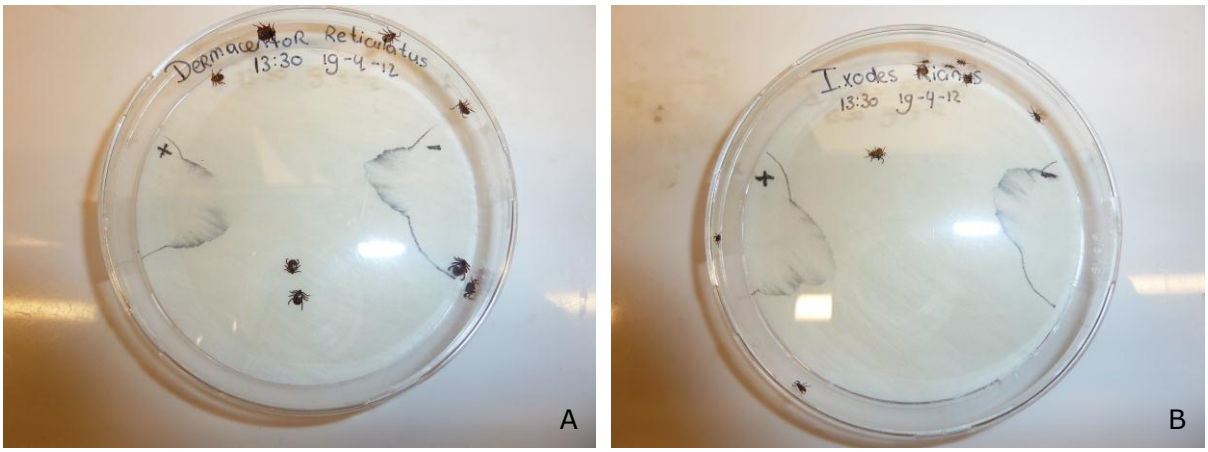

Figure 4: Situation directly after placing the ticks in the Petri dish for odor test 1. The (+) marks the side on which the cow perfume is applied. At the (–) mark 96% ethanol is applied. **A**: *D. reticulatus* **B**: *I. ricinus*.

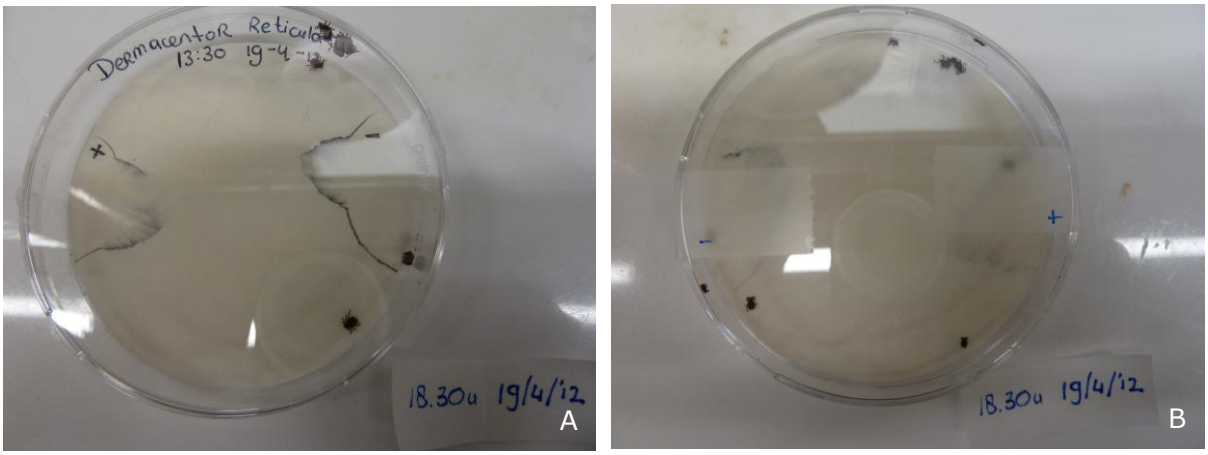

Figure 5: situation after 5 hours. **A**: *D. reticulatus* are gathered at the 96% ethanol side. **B**: *I. ricinus* crawled underneath the paper so the picture is taken from the underside of the Petri dish. The ticks are spread throughout the Petri dish.

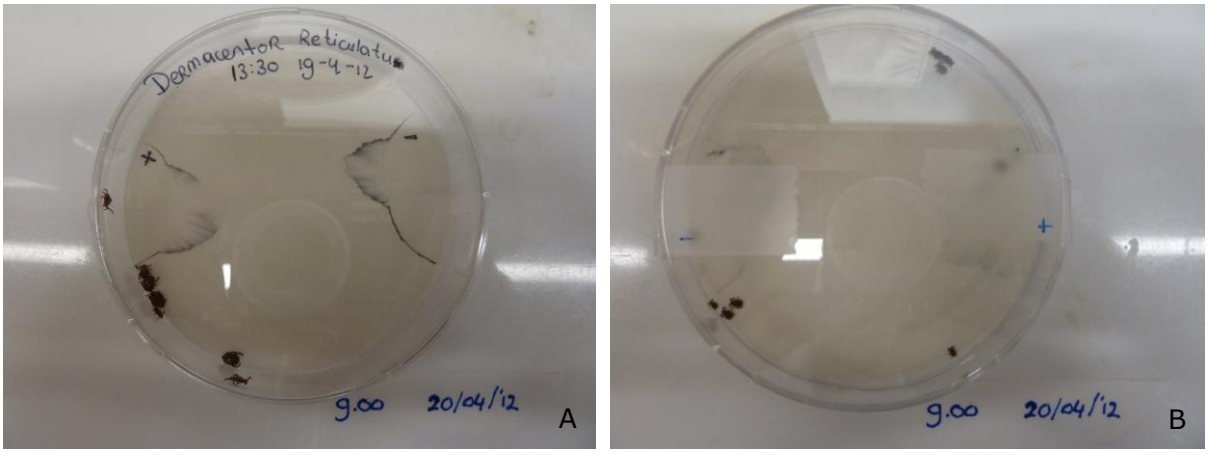

Figure 6: 19.5 hours later. **A**: 7 out of 10 *D. reticulatus* are close to the cow odor. The remaining three are in the neutral zone. **B**: *I. ricinus* ticks are distributed throughout the Petri dish. Two ticks are close to the cow odor, and three ticks are gathered at the (-) side. The other five are situated in the neutral zone.

Odor test 2

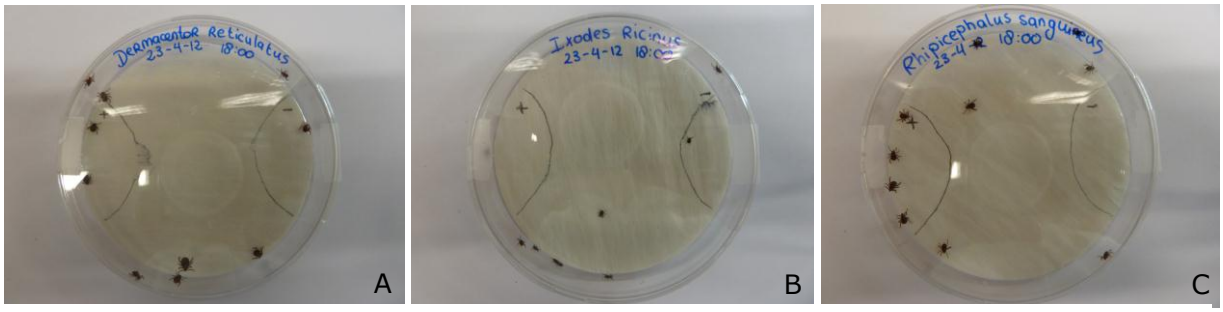

Figure 7: Starting situation of the odor test 2. **A**: *D. reticulatus* tested on cow odor. **B**: *I. ricinus* tested on cow odor. **C**: *R. sanguineus* tested on dog odor. Directly after placing the ticks in the Petri dish, they crawl to the (+) zone, which marks the side of dog odor.

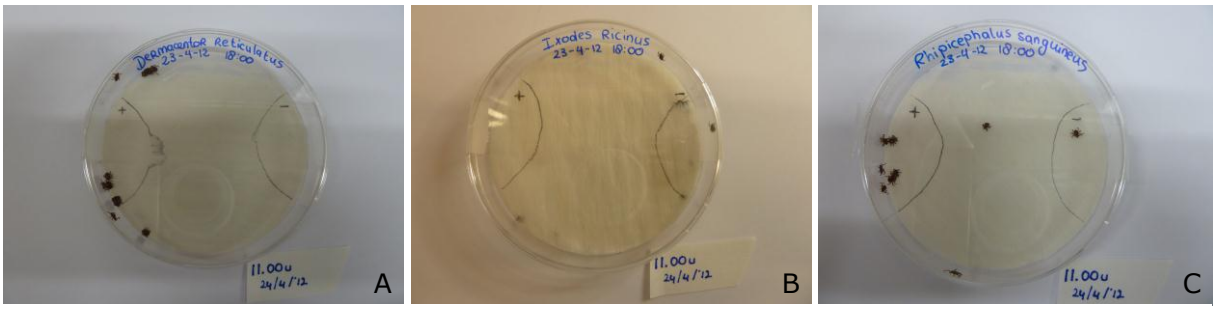

Figure 8: Situation after 17 hours. **A**: *D. reticulatus* ticks all gathered around the (+) zone, but three are clearly situated in the cow odor area. **B**: I. ricinus are distributed throughout the Petri dish. Three ticks settled at the (–) zone at the underside of the paper (not clearly shown on this picture), the rest of the ticks are in the neutral zone. **C**: 6 out of 10 *R. sanguineus* are in the dog odor zone.

Remaining odor tests

For odor test 3 new filter paper was used to pipette the perfumes on, and this paper was used in every following test. None of the tick species are clearly gathered around the (+) or (-) zone. Most ticks reside in the neutral zone, and from *D. reticulatus* even all 10 adults are found in the neutral zone.

Odor test 4 shows 9 out of 10 *R. sanguineus* adults at the side of the perfume (table 2), and of *I. ricinus* 7 out of 10 are in the (+) zone. *D. reticulatus* are spread throughout the Petri dish.

Tick species	Time	Perfume	(+)	Neutral	(-)	Observation
R. sanguineus	0	Dog Bouvier (96% ethanol)	2	5	3	
	19,5	Dog Bouvier (96% ethanol)	6	4	0	
	25,5	Dog Bouvier (96% ethanol)	5	5	0	2 neutral ticks close to (+) zone
	43,5	Dog Bouvier (96% ethanol)	9	0	1	Preference for perfume side

Table 2: Results of odor test 4 for *Rhipicephalus sanguineus*. After 43.5 hours 90% of the ticks is found on the (+) zone.

In tests 5, 6 and 7 none of the tick species are obviously present at the perfume side. Mostly they were found in the neutral zone. Once, during odor test 6, seven out of ten *R. sanguineus* were found on the (-) zone after 5 hours.

The last test was done with perfumes made with hexane, but again *I. ricinus* and *D. reticulatus* were not drawn to the (+) zone. All ten *R. sanguineus* adults, on the other hand, were found at the perfume side (figure 9).

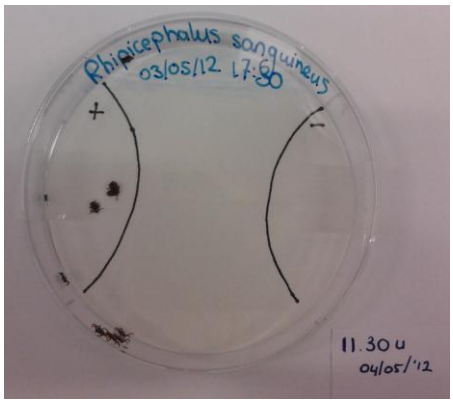

Figure 9: odor test 8 after 18 hours. All *R. sanguineus* reside in or nearby the (+) zone.

Discussion

Feedings

After 10 weeks of in vitro feedings attachment percentages of adult *Rhipicephalus sanguineus* were disappointing compared to results from previous research (Bonga, 2011). The question arises what the cause of this minimal attachment could be. Maybe it is due to bad quality of the *R. sanguineus* ticks. Especially in the first six feedings *R. sanguineus* was inactive and gave minor response to CO_2 stimulation. For feeding 7 a new batch of ticks was used and attachment took place, which supports the assumption that old ticks are less eager to attach. Nevertheless, in the subsequent feedings (7 to 10) these same adult *R. sanguineus* ticks were used and again attachment did not occur. Therefore using the same ticks for consecutive feedings is not recommended.
Another explanation for the lack of attachment results could be that *R. sanguineus* is a very demanding tick species regarding in vitro conditions. Maybe the ticks were not attracted to the membrane because they did not like the odor of it. Since *Rhipicephalus* had attached to membrane rubbed on the same dog before, it is not likely that they are not attracted to Labrador odor. But perhaps this dog had some metabolic problems which had a repellent effect on the ticks during the period of this research. Washing the dog with a different shampoo or even an acaricidic shampoo might have influenced the results. However, on inquiry it turned out that this Labrador is not washed at all, so this assumption can be rejected. Since ticks seem to have preferences for certain dog breeds (Louly et al, 2010) further research should be done to find out more about the right odor to use for in vitro feedings.
Another explanation for low attachment could be minor differences in executing the in vitro feedings. Even though the entire process was explained by a student who had done the same sort of research and protocols were followed, small differences could still occur and influence the results.
For future research it would be best to work with fresh and active ticks. Moreover, it is important to standardize the process of this in vitro feeding system so everybody can perform a feeding in the same way.
When higher attachment percentages are reached, pathogen transmission dynamics can be investigated. For example, transmission and acquisition of the bacterium *Bartonella vinsonii* subsp. *berkhoffii* by *R. sanguineus* can be studied, because this tick species is suspected to have a role in spreading this pathogen (Billeter et al, 2008; Kordick et al, 1999). A small amount of blood is used to feed the tick, which increases the change of picking up transmission of a small amount of pathogens.
When all developmental stages of *R. sanguineus* are able to feed in vitro, studies about transstadial transmission of pathogens can also be done. The advantage of studying this subject with an in vitro feeding system is that the conditions are standardized and therefore comparable with other studies. Furthermore, test animals are not used which precludes parasite-host-pathogen interaction (Kröber & Guerin, 2007).

Blood of multiple units turned dark after approximately 20 hours. This should be prevented because it will not benefit the feedings. During the first two feedings attachment and mortality were checked and blood was refreshed under non sterile conditions. This may be the cause of decoloration of the blood, because dark blood indicates bacterial overgrowth. For this reason some adjustments were done. Firstly, the entire process of checking the units was shifted to the flow cabinet. Secondly, for some feedings gentamicin was added to the blood. However, antibiotics should not be used because bacteria can become resistant to these medicines, which is highly undesired. Therefore, every step has to be performed as sterile as possible. For example, when collecting blood from the cow, the neck of this animal should be shaved to minimize contamination. Working in the flow cabinet, when transferring the units to new blood, is a step in the right direction. Moreover, during the last feedings of *R. sanguineus* gentamicin was not used and this blood was still red at the end of the week.

Working in the flow cabinet has a disadvantage, for it is difficult to check attachment and mortality. If units are opened the ticks crawl to the top and will escape from the unit. Therefore, units are kept closed and the number of attached ticks was determined by counting the hypostomes protruding through the membrane. Mortality was also checked, but because CO_2 stimulation was not possible some ticks could be alive even though they appeared dead. For this reason mortality could only be determined precisely when ending

the in vitro feeding. That is why mortality percentages of, for example, *R. sanguineus* nymphs in week 8 were low during the feeding and increased drastically at the end of the week. This does not necessarily mean these nymphs all died in the last 24 hours, it could also be that dead nymphs were overlooked or it was hard to judge whether they were really dead and therefore were not counted as dead.

In vitro odor tests

The perfumes used for the odor tests did not consistently trigger the same reaction. Maybe the odors were not strong enough. To test this, more concentrated perfumes should be produced to have more 'odor' in the same amount of fluid. It could also be that the ticks were not attracted to the perfume because it is not similar to the natural odor. Perhaps the right odor compounds are lost during the production process or the concentration is too low to attract ticks. This especially applies to the perfumes made with hexane. During this process hexane has to evaporate and for that the jar is opened and placed in the hood for at least two days. Beside losing the hexane, it is plausible that odor compounds are lost as well. If this is the case, another production process has to be considered in which the odor is not lost.

Conclusion

Feedings

Adult *Dermacentor reticulatus* did attach, which was expected since this tick species serves as a control. After four feedings focus was mostly aimed at *Rhipicephalus sanguineus* and partially at *D. reticulatus* and *D. marginatus* nymphs so feedings with *D. reticulatus* adults were put to a halt. Attachment of *R. sanguineus* did not exceed 30% (week 12), which is rather disappointing because higher attachment numbers have been accomplished in previous research (Bonga, 2011; van Dijk, 2011).
R. sanguineus larvae were placed on a membrane with a thickness of 50 µm, but since their hypostome is approximately 50 µm (Kröber, T. 2007) they were unable to penetrate the membrane to feed. Making thinner membranes does not seem to be a solution, because the silicon layer will get to thin to cover the entire lens paper and then leakage will occur. Knobs were seen at the underside of the membrane, so maybe the larvae did try to feed but could not reach the blood. For this reason it is not yet possible to feed the larval stage of *R. sanguineus* in this in vitro feeding system. Nymphs on the other hand, have a hypostome of 120 µm (Kröber, T. 2007), so they are able the get through the membrane. Nevertheless, they were placed on thinner membranes (49 and 50 µm) for feeding 6 (week 12) to test whether they would attach on that thickness, but they did not. Therefore there must be another reason why the nymphs do not attach in greater numbers and engorge. Maybe the odor of the membrane was not strong enough or did not meet their preferred odor. Nevertheless, some attachment did occur, which proves that the nymphal stage of *R. sanguineus* is capable of attaching in an in vitro feeding system.

Conditions

The *R. sanguineus* larvae did survive for 69 hours under the normal in vitro feeding conditions and were still active at the end, which is positive. Now the membrane has to be modified to make in vitro feeding of these larvae possible.
D. marginatus nymphs, however, were not able to survive under the normal in vitro feeding conditions concluding from the feeding of week 12, so for week 13 temperature and RH were adjusted to 20 °C and 75%, respectively. This adjustment seems to have positive effects on the survival of the *D. marginatus* nymphs, because mortality decreased to less than 10%. Even though mortality decreased, attachment still did not occur, so the environmental conditions are not the only factors which have to be adjusted to successfully feed *D. marginatus* nymphs.

Dog breeds

Because few *R. sanguineus* adults attach to membranes rubbed on a Labrador, other dog breeds were tested to see whether this lacking attachment was due to a wrong odor. Tested dog breeds were; Cocker Spaniel, Bouvier, Ridgeback, Heidewachtel and Yorkshire Terrier, but none of these odors had better results. Feeding 9 was done with the odor of a Terrier and already showed a mortality of 90% after 15 hours. This high percentage is due to the fact that this dog was treated with an acaricide (Certifect™). When ending units 11, 12 and 13 ticks appeared to have suffered from neural symptoms, which explain the typical posture. In feeding 10 (U11) Ridgeback odor seems to have triggered one adult *R. sanguineus* to attach, but this tick was detached when looking for the second time (after 40.5 hours). All the other used dog breeds were not attractive enough to get *R. sanguineus* attached during this research.

In vitro odor tests

Perfumes made of Bouvier did smell like dog, unlike the perfumes of the Greek dog. *Rhipicephalus sanguineus* responded positive to Bouvier odor (96% ethanol) during odor tests 2 and 4, but not in odor test 3. Response to Bouvier odor solved in hexane did occur, but only convincingly in odor test 8, because 5 out of 10 were in the (+) zone and the other 5 were very close to it (figure 9). Experiments with the odor of the Greek dog did not trigger the ticks at all.
The cow perfumes have a strong cow odor, both made with 96% ethanol and hexane. *Dermacentor reticulatus* seemed to prefer the cow odor (96% ethanol) in the second test, but these results could not be repeated with the same perfume. *Ixodes ricinus* did not seem to have a clear preference for the perfumes. But during the fourth odor test 7 out of 10 ticks were present in the positive zone which was surprising since they were randomly

spread throughout the Petri dish in the previous three tests. However, they did not respond to the cow perfume made with hexane.
Over all, most positive responses to the odors were triggered by perfumes made with 96% ethanol rather than with hexane, which suggests that these perfumes contain more or better odor compounds for the attraction of ticks. To gather reliable results about the attraction of ticks to these perfumes, more odor tests should be performed with the same odors.

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Appendix

Appendix I - Protocol in vitro voeden van teken

Het waterbad voor het in vitro voeden dient 37˚C te zijn. Het buitenbad is gevuld met gedestilleerd water en het niveau bevindt zich tot de bovenste streep. Het binnenbad dient gevuld te zijn met KSO_4 (120 g/L) tot iets onder het niveau van het buitenbad. Iedere ochtend wordt de luchtvochtheid in het aquarium gemeten; deze dient rond de 90% te zijn.

Voorbereiden voeding
Membranen maken

1. Pak een gladde glasplaat, desinfecteer met alcohol en span op met keukenfolie. Trek het keukenfolie strak met stukjes plakband.
2. Plak 8 lenspapiertjes met gelijke afstand van elkaar vast op het keukenfolie.
3. Siliconen mengsel maken volgens protocol. Benodigdheden voor 4 platen: 30 g siliconen lijm, 9 gram siliconen olie, 5.8 g hexaan en 0.5 g witte kleurpasta.
4. Plak een stukje karton met de gladde kant boven op de tafel en plak deze vast.
5. Pak de strijker met de gladde kanten en bestrijk alle lens papiertjes met het siliconen mengsel. Probeer de druk zo gelijk mogelijk te houden om het membraan overal even dik te maken. Gewenste dikte: 87-117 µm (inclusief keukenfolie van 17 µm).
6. Laat de membranen 24 uur drogen.
7. Meet de membranen met de micro callipers. Hiervoor snijd je de membranen los van de glazen plaat en meet je op 6 plaatsen de dikte. Kruis eventueel plaatsen af waar het membraan te dik of te dun is en het overige deel van het membraan wel te gebruiken is. Plak vervolgens het membraan op de deksel van een 6-wells plaat.
8. Wanneer je het membraan gaat gebruiken, dient het de geur van de gewenste gastheer te bevatten. Mocht dit een hond zijn, neem de deksels dan ingepakt in een tissue ter bescherming mee naar de proefhonden afdeling van de faculteit Utrecht en wrijf de membranen minimaal 15 minuten over de labrador Kelly. Zorg dat alle plekken van het membraan de hond raken en neem ook plekken mee als de oren en naast de anus.

Units maken en controleren

1. Selecteer gave plexiglas buisjes en desinfecteer deze met alcohol. Controleer op barsten. Wanneer het hergebruikte units betreft, moeten alle lijmresten verwijderd zijn. Zet de buisjes op het membraan om uit te meten hoeveel units er op één membraan passen.
2. Plak een stukje karton op tafel vast en leg daar een kleine hoeveelheid lijm op.
3. Strijk deze lijm uit met de strijker met niet-gladde kanten tot een dun laagje overblijft.
4. Pak een buisje en zet deze met een draaiende beweging in de lijm en haal hem er ook weer met een draaiende beweging uit om te voorkomen dat er veel lijm aan de binnenkant van de unit komt te zitten. Check of er overal op de ring lijm aanwezig is.
5. Plaats het buisje op het membraan en druk stevig aan (let op: de unit niet meer bewegen).
6. Wanneer er veel lijm aan de binnenkant van de unit zit kan dit weggehaald worden met een kwastje.
7. Laat de units 3 uur drogen.
8. Knip de units los van de deksel, waarbij er zo min mogelijk randen uitsteken, en haal daarna heel voorzichtig met een pincet het stukje keukenfolie van het membraan af.
9. Plaats de units minstens 15 minuten in een zes-wells platen gevuld met (gedestilleerd) water om te checken op lekkage (druppels aan binnenkant units). Controleer de rand van het membraan goed.
10. Spuit de onderkant va het membraan goed af met ethanol en laat dit verdampen. Eventueel kunnen de units enkele minuten ondersteboven op het hitteblok (40˚C) gezet worden.
11. Plaats de units in een steriele 6-wells plaat.
12. De units worden afgesloten met een ‘bonbonnetje’. Om deze te maken dient de rand van een dopje afgeknipt te worden, om er vervolgens een voile stukje stof om te bevestigen. Knip een vierkantje van 6 bij 6 cm en bindt deze eromheen met een boterhamzaksluiter.

13. Plaats de bonbon op de unit.

Voeding voor de teken

1. Voor het starten van de voeding dient er vers bloed gehaald te worden. Neem een glazen pot mee voor het benodigde volume en twee 10 ml pipetten (één reserve). De grootte van de pot is afhankelijk van de hoeveelheid bloed die je nodig hebt voor het aantal voedingen dat je gaat inzetten.
2. Ga naar departement Landbouwhuisdieren, kleed je om en vraag een dierverzorger je te helpen.
3. Pak een naald, slangetje en watje met alcohol uit de kar in de diffco ruimte.
4. Pak koe 9751, 4104 of 0142 (rouleer) en bind haar kop uit naar de zijkant.
5. Koppel de naald aan het slangetje en neem zo steriel mogelijk bloed af uit de v. jugularis. Vang het bloed op in de glazen pot.
6. Roer 20 minuten met de 10 ml pipet in het bloed om alle stollingsfactoren eruit te halen. Er vormt zich een flinke klont aan je pipet. Haal de pipet voorzichtig uit de pot en gooi deze in de gele bak.
7. Doe de deksel op de pot.
8. Werk het welzijnsdagboek in het kantoor van Thijmen bij (datum, koe nr, handeling).
9. Neem de pot mee naar het UCTD, doe er 2 g/L glucose bij en zwenk de pot.
10. Verdeel het bloed in tubes, waarbij je de randen van de pot en tubes voor het schenken door de blauwe vlam haalt.
11. Plaats de tubes met de datum erop en de diersoort in de koelkast.

Teken uitzoeken en sorteren

1. Zet de tekenrenbaan in het tekenlaboratorium aan, zodat deze op de juiste temperatuur is voordat begonnen wordt met het sorteren van de teken.
2. Als er gebruik gemaakt wordt van adulten, dan dienen er in elke unit (indien mogelijk) 5 mannelijke en 5 vrouwelijke teken geplaatst te worden. Plaats deze op de renbaan en plaats ze vervolgens samen in één (klein) potje met gaatjes in de deksel, afgesloten door gaas, om ze zo alvast per unit klaar te zetten.
3. Plaats de potjes terug in de stoof of indien incubatie gewenst is alvast in het waterbad bij 37°C.

Inzetten voeding

Verdelen en opwarmen van het bloed

1. Zet de flowkast aan (15 minuten van tevoren), met daarin alvast de benodigde materialen.
2. Haal een tube met bloed uit de koelkast, vortex deze gedurende enkele seconden zodat het bloed gemengd wordt en plaats deze in de flowkast.
3. Verdeel met behulp van een 10 ml pipet het bloed over de vier buitenste welletjes (let op: dek tijdens het vullen van een well de overige wells af). Indien nodig wordt een vijfde well gevuld als controle well.
4. Elke well wordt voorzien van 3.1 ml bloed (transmissie voeding); de controle well wordt gevuld met 2.5 ml bloed.
5. *Bij acquisitie voedingen wordt er aan het bloed, voordat het bloed over de welletjes wordt verdeeld, Ehrlichia Canis toegevoegd.*
6. *E. Canis dient te worden opgehaald vanuit de celkweek. Neem de benodigde hoeveelheid uit de kweek en doe dit in een tube. Neem de tube mee terug naar de eerste flowkast en vul hier de tube aan met bloed (niet direct uit de koelkast!).*
7. *Pipetteer het bloed een aantal keer op en neer om de kweek goed te mengen.*
8. *De hoeveelheid E. Canis kweek per welletje is 0.2 ml; dit wordt aangevuld met 2.9 ml bloed.*
9. Warm het bloed vervolgens in de 6-wells plaat in het buitenste bad van het waterbad tenminste 15-30 minuten op.

Units verder klaarmaken

1. Zet de flowkast aan.
2. Afhankelijk van de soort teek wordt er vers of ingevroren koeien- of honden haar gebruikt.
3. Knip de haren heel fijn met een schaar en leg voorzichtig een beetje op de bodem van de unit.
4. Neem deze units mee naar het tekenlab.

5. Stop de gesorteerde teken uit de potjes in de stoof in de desbetreffende units.
6. Plaats de bonbon op de unit en schuif deze voorzichtig met twee vingers naar beneden tot plusminus een halve centimeter boven het membraan.
7. Let op dat er geen teken bekneld zitten: indien dit het geval is trek je de bonbon weer iets naar boven zodat de teek loskomt, om de bonbon vervolgens weer naar beneden te duwen.
8. Zet de units in de flowkast.
9. Haal het opgewarmde bloed uit het waterbad en plaats dit ook in de flowkast.
10. Plaats de met teken gevulde units voorzichtig in het opgewarmde bloed.
11. Controleer op luchtbellen tussen het bloed en het membraan: mochten deze er zitten, plaats de unit dan voorzichtig opnieuw in het bloed (schuin plaatsen).
12. Zet de 6-wells plaat in het binnenste bad en plaats de deksel er op.
13. Dek het waterbad af met de doek zodat de teken in het donker zitten.

Verversen, monstername en monitoring van de voeding

Het nemen van de monsters gebeurt twee maal per dag tegelijk met het verversen van de voeding. Eenmaal per dag wordt ook gekeken of de teken aangehecht zijn. Het verversen van het bloed dient ’s ochtends en ’s avonds plaats te vinden, het liefst met interval van 12 uur. Indien dit niet mogelijk is kan het om 09:00u ‘s ochtends en om 17:00u ’s avonds plaatsvinden.

1. Zet de flowkast aan.
2. Vul een 6-wells plaat met nieuw bloed en warm deze op (zie ‘inzetten voeding’, ‘verdelen en opwarmen van het bloed’).
3. Verwarm PBS/NaCl in het waterbad.
4. Haal na 15-30 minuten de platen met de teken uit het waterbad en zet deze in de flowkast, evenals het de platen met het nieuwe bloed.
5. Haal de unit voorzichtig uit het bloed en spoel deze af met warme PBS/NaCl (laat de deksels van de platen er zo lang mogelijk opzitten).
6. Controleer op aanhechting, mortaliteit en sexe van de teken. Hierbij kan het bonbonnetje eventueel iets omhoog getrokken worden. Doe dit zo snel mogelijk en noteer de bevindingen.
7. Plaats de units in het nieuwe bloed en zet deze 6-wells plaat daarna terug in het aquarium (laat de oude plaat met deksel in de flowkast staan).
8. Voor het nemen van de monsters wordt het oude bloed eerst enkele malen op en neer gepipetteerd (600-700 µl) om een homogene samenstelling te verkrijgen. Voor iedere well wordt een nieuwe pipetpunt gebruikt.
9. Neem met een nieuwe pipetpunt van elke well twee monsters van 200 µl en doe deze in twee verschillende epjes (zorg dat deze allemaal correct gelabeld zijn).
10. Plaats de epjes in de vriezer bij -20°C.
11. Zuig het resterende bloed in de wells met een 10 ml pipet op en leeg deze in een tube. Spuit hierna wat ethanol in de wells en pipetteer ook dit restant in de tube.
12. Gooi alles wat met bloed in aanraking is geweest in de gele bak en maak de flowkast schoon met ethanol.

Appendix II - Protocol for the collection of host odors

Option 1: rubbing the membrane
To transfer the odor of the host into a feeding unit, the membrane should be rubbed on the host. Because most ticks have a preference for a specific location on the host, it is recommended to rub membrane over this particular place.

1. Wrap the membrane, taped on a lid of a 6-wells plate, in a paper tissue and put it in a seal bag to take it to the animal.
2. Carefully rub the membrane over the preferred location. (The surface of the membrane is sticky, so first pat it on the animal so hair and fat get on the membrane to facilitate the rubbing.)
3. Rub the animal for 3 minutes and frequently check if the membrane is still intact.
4. Wrap the plate in a paper tissue and put it back into the seal bag.

Note: the animal used for the rubbing of the membrane should not be treated with acaricides, because then the tick will not attach and may even die!

Option 2: placing hairs in the feeding unit
Hairs of an animal contain odor as well, so hairs can be placed on the membrane of a feeding unit.

1. Collect hairs of the host by shaving of collecting loose hairs.
2. Wrap the hairs in a paper tissue and put them in a seal bag.
3. Take a small amount of hair (10 to 15 hairs), and cut them into small pieces of approximately 0,5 cm.
4. Carefully place the cut hairs on the membrane of a feeding unit with a tweezer. Make sure they stick to the membrane, because the odor should be at the bottom of the unit only.

Option 3: animal perfume
The odor of the host can also be captured in a medium to spray onto the membrane of a unit. To do so, an extract should be made. Odor can be collected by rubbing a cotton pad over the host. If the animal does not have a strong odor, the cotton pad can be moistened, so the odor will be stronger ("wet dog" odor).

1. Put some cotton pads, a glass jar and vinyl hand gloves in a seal bag and take them to the animal.
2. Take out a cotton pad (with hand gloves on) and chose one of the following options.
    a. Firmly rub a cotton pad over the preferred location of the host during 5 minutes.
    b. Moisten a cotton pad en firmly rub it over the preferred location of the host for 5 minutes.
3. Put the cotton pad in a glass jar and close it.
4. Write down the animal species and date on the glass jar.
5. Place the glass jar in the seal bag.
6. Repeat step 2 to 5 with a new cotton pad.
7. Add 12 ml of ethanol (96%) to one of the jars, and note "ethanol" on the jar.
8. Add 10 ml of hexane to the other jar and note the "hexane" on the jar.
9. Place both jars on a shaker plate for 48 hours.

After 48 hours the extracts can be processed to make "perfume" out of it.
- Hexane (work in the hood!)
1. Press the cotton pad to the bottom of the jar with a 10 ml pipette to squeeze out the hexane.
2. Pipet the hexane at the same time.
3. Pore the hexane into another glass jar.
4. Leave the jar open in the hood so the hexane can evaporate. This will take about 2 days.
5. Add 5 ml ethanol (96%) to the residue.
6. Place the jar on a shaker plate so the residue can resolve. If necessary the residue can be scraped of the bottom with a stirrer. This step will take at least 2 days.
7. Filtrate the perfume to get rid of the pieces of residue by placing a piece of gauze on a new glass jar. Pipette the liquid into the new jar.

8. If there are still small particles left, pipette the perfume into 2 ml Eppendorf tubes.
9. Centrifuge for 2 minutes.
10. Pipette the solution into a spray bottle for perfume.
11. Label the bottle and write down the odor and solvent used to make the extract (in this case hexane).

- Ethanol 96%

1. Press the cotton pad to the bottom of the jar with a 10 ml pipette to squeeze out the ethanol.
2. Pipette the ethanol at the same time.
3. Pipette the solution into a spray bottle for perfume.
4. Label the bottle and write down the odor and solvent used to make the extract (in this case 96% ethanol).