Feeding of Hard Ticks *In Vitro*: New Perspectives for Rearing and for the Identification of Systemic Acaricides

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Zusammenfassung: Fütterung von Schildzecken in vitro: Neue Perpektiven für die Zucht und die Identifikation systemischer Akarizide
Versuche zur in vitro Fütterung der Schildzecken Ixodes ricinus, Boophilus microplus, Amblyomma variegatum und Amblyomma hebraeum werden geschildert und ein in vitro Test für systemische Akarizide vorgestellt. Begonnen wurde mit einer ständig von neuem Blut durchströmten Fütterungskammer. Diese war jedoch zu anfällig gegenüber Pilzkontaminationen und zu ineffizient. Einfache Fütterungsgläser mit einem Glasrohreinsatz erwiesen sich als erheblich praktischer. Über das untere Ende des Glasrohres waren matrixgestützte Silikon-Membranen oder klebstoffimprägnierte Baudruche-Membranen gespannt. Das verfütterte Rinderblut wurde wöchentlich unsteril am Schlachthof gesammelt, wo es als Abfall anfiel, und 1–3 × pro Tag gewechselt. Die Fütterungsgläser standen in einem 38 °C-Wasserbad. Das Blut enthielt Mikrobizide. Das Festsetzen der Zecken auf den Membranen wurde durch Kombinationen von Wirtstierhaaren, Kot der jeweiligen Zeckenart, Rinderhaar-Extrakt und synthetischen Aggregations-Fixierungs-Pheromongemischen (Amblyomma) angeregt. Larven der einwirtigen Rinderzecke B. microplus entwickelten sich in vitro über das Nymphen-Stadium zu ungesogenen Adulten. Weibchen erreichten die halbe natürliche Körpermasse und legten Eier. Der Lebenszyklus von A. hebraeum wurde in vitro durch aufeinanderfolgendes Füttern aller Entwicklungsstadien (Larven, Nymphen, Adulte) auf Silikon-Membranen geschlossen. Bei adulten B. microplus, A. variegatum und A. hebraeum nahm die Fortpflanzungsfähigkeit nach der in vitro Fütterung stark ab, vermutlich wegen verminderter Blutqualität. Eine suboptimale in vitro Ernährung hatte größere Konsequenzen für die Fruchtbarkeit als für die Häutung der Zecken, wie das gute Abschneiden der Larven und Nymphen besonders von A. hebraeum zeigt. Die Toxizität des systemischen Akarizids Ivermêctin® für Nymphen dieser Art wurde mit der in vitro Fütterungsmethode bestätigt. Diese Methode bietet neue in vitro Möglichkeiten sowohl für die Zeckenzucht, als auch u. a. für Tests systemischer Akarizide, Fraßhemmer, Repellenzien und Vakzine und erlaubt Experimente zur Übertragung von Krankheitserregern wie auch mit radioaktiv markierten Substanzen.

Summary
Experiments on in vitro feeding of the hard ticks Ixodes ricinus, Boophilus microplus, Amblyomma variegatum and Amblyomma hebraeum are described. An in vitro test for systemic acaricides is presented. Work started with a feeding chamber continuously supplied with new blood. It proved too susceptible to fungal contamination and not efficient enough. Simple feeding units made of honey jars with an inner glass tube were far more practical. Reinforced silicone membranes or baudruche membranes impregnated with glue were stretched across the bottom end of the tube. Weekly open-collected bovine blood from the slaughter house, where it is considered as discard, served as nutrient and was changed 1–3 times a day. The feeding jars were held in a waterbath (38° C). The blood contained microbicides. Attachment of ticks on the membranes was induced with combinations of host hair, conspecific tick faeces, bovine hair extract and synthetic aggregation attachment pheromone mixtures (Amblyomma). Larvae of the one-host cattle tick B. microplus were bred in vitro through the nymphal to the unfed adult stage. Females gained half of the natural body mass and laid eggs. The life-cycle of A. hebraeum was completed in vitro by consecutive feeding of all life-stages (larvae, nymphs, adults) on silicone membranes. In adult B. microplus, A. variegatum and A. hebraeum the reproductive capacity was strongly impaired, possibly in consequence of the low quality of the blood. A suboptimal in vitro nutrition was more fatal for the fertility than for the moult of ticks, as demonstrated by the good results for larvae and nymphs, particularly those of A. hebraeum. The toxicity of the systemic acaricide Ivermectin® for nymphs of A. hebraeum was confirmed using the in vitro feeding method. This method offers new in vitro possibilities for tick rearing as well as for tests with systemic acaricides, antifeedants and repellents. It allows experiments on transmission of pathogens and with radioactive markers.

Keywords: in vitro feeding, artificial feeding, acaricide, Ixodidae, artificial membrane

1 Introduction

Hard ticks (Acari: Ixodidae), a group of parasitic arthropods, are beside the insect order Diptera the most important vectors of diseases of humans and their livestock. Man suffers from, e.g., Lyme borreliosis and spring-summer meningoencephalitis, whereas domestic animals in the tropics and semi-tropics are struck mainly by anaplasmosis, babesiosis, theileriosis and heartwater disease (Uilenberg, 1992). Added to this, the ticks' long blood-meal, which lasts 2–14 days for each of the life-stages (i.e., larvae, nymphs and adults), strongly debilitates the host and may provoke secondary infections and pain. World-wide economic losses of annually billions of US $ are reported. The development of modern, ecologically and economically liable approaches to tick control, such as systemic acaricides, repellents and antifeeding compounds, demands large numbers of all instars of such ixodid ticks. To date, maintenance of laboratory strains of these parasites means for each of the three life-stages an irritating blood-uptake on a range of vertebrate hosts, generally single-use mammals like mice, rabbits, sheep, goats or cattle. Ethical questions surrounding the use of experimental animals lend impetus to the development of alternative methods for the rearing of ticks, and indeed other bloodsucking arthropods, as well as *in vitro* tests for putative acaricidal compounds.

This paper deals with the current developments in the *in vitro feeding* of hard ticks and their significance for non-animal based rearings. It outlines *in vitro* feeding experiments with four ixodid tick species (*Amblyomma hebraeum, Amblyomma variegatum, Boophilus microplus, Ixodes ricinus*), and emphasises the successful application of this *in vitro* method testing a systemic acaricide.

1.1 Hard tick biology

In contrast to hematophagous insects such as mosquitoes, tsetse flies, kissing bugs or fleas, and the fast sucking soft ticks (Acari: Argasidae), where the blood-meal lasts from just a few seconds to maximally one hour, each hard tick instar remains firmly anchored to the same feeding site for days on end. This considerable investment on the part of the tick is preceded by a stringent behavioural cascade for acceptance of an appropriate host and for attachment at a suitable site (Waladde and Rice, 1982). These important steps prior to the actual blood-meal, depend on a specific blend of thermal, hygro, mechanical, olfactory and contact chemostimuli (Lees, 1948; Waladde and Rice, 1982; Norval et al., 1989ab; Steullet and Guerin, 1992ab, 1994ab). While injecting numerous physiologically active agents into the lesion, the attached tick evokes strong inflammatory, vasodilatory and immunological responses by the host (Tatchell and Moorhouse, 1968; Kemp, Stone and Binnington, 1982; Walker and Fletcher, 1986).

Only small amounts of blood are imbibed during the first days of feeding, a period during which the tick undergoes a broad variety of physiological changes, i.e., maturation of the salivary glands, considerable synthesis of procuticle to accommodate the blood-meal in females, and the synthesis and emission of pheromones, according to the species, in males or females. These pheromones, volatile and non-volatile, are indispensable for attracting sexual partners and for correct mating behaviour. Most of the hard tick species, except the genus *Ixodes* which can also mate fasting and off-host, have to take up blood before successful mating can occur on the host. Fecundation of the females is a prerequisite for completing their meal and the production of viable offspring (Balashov, 1972; Diehl et al., 1982; Oliver, 1986). Consequently, the quality of the diet is a crucial factor at any time of the feeding period. The tick's engorgement occurs mainly during the last 24 hours of feeding. During this decisive phase female ticks imbibe 2 to 8 times as much blood as they finally gain in weight (Lees, 1946; Balashov, 1964; Seifert et al., 1968; Snow, 1970; Rechav et al., 1994). They concentrate this blood, rejecting water and ions (Na^+,Cl^-) back into the host (Kaufman and Phillips, 1973; Kaufman, 1983; Sauer et al., 1995), and multiply their body mass, in some cases more than 100 fold, with protein- and lipid-rich nutrient for the production of 2000 to 20,000 eggs per female, according to the species, after the drop-off. All of this renders hard ticks rather delicate candidates for *in vitro* feeding, since an adequate diet has to be maintained at the feeding site throughout attachment.

Boophilus microplus (Canestrini, 1887), the cattle tick, is the most important tick in veterinary parasitology. In contrast to the three species described later, *B. microplus* is a one-host tick, that means all three life-stages stay, engorge and moult on the same host animal. The parasitic phase takes only 3 weeks (Roberts, 1968), and the whole life-cycle can be completed in 6 weeks (Nuñez et al., 1985). *B. microplus* is abundant particularly on cattle in areas of Central- and South America, Africa, and Oceania, where it is a vector of Anaplasma (Bacteria: order Rickettsiales) and Babesia (Protozoa: order Piroplasmida). This species serves as one-host tick model in industry for the development of acaricides.

Ixodes ricinus (Linneaus, 1758), the sheep tick, can be found in Central Europe where every life-stage feeds on a different host (three-host tick) - larvae and nymphs mainly on small vertebrates (i.e., birds, mice, rabbits), females on deer, sheep, dogs etc. Humans might serve as hosts, too. This contributes to the tick's role as vector of viral (spring-summer meningoencephalitis) or bacterial (Lyme borreliosis) diseases in man.

Amblyomma variegatum (Fabricius, 1794), the tropical bont tick, lives in most of the tropical and semi-tropical parts of Africa and on the Caribbean Islands. Larvae and nymphs prefer small vertebrates and

ungulates, whereas adults can be found for the most part on ruminants. *A. variegatum* transmits the rickettsial pathogens of heartwater disease (*Cowdria ruminantium*) and Q-fever (*Coxiella burnetti*).

The life-cycle of the bont tick *Amblyomma hebraeum* Koch, 1844, can extend to 6–8 months, even in laboratory colonies (Norval, 1974). The natural occurence of this tick is limited to the south of Africa (Walker and Olwage, 1987). Preferred hosts and vector potential, as well as the diseases transmitted, correspond closely to the former species. *A. hebraeum* is used as a three-host tick model in industrial acaricide research.

1.2 *In vitro* feeding methods

Artificial feeding methods have been developed for bloodsucking insects such as tsetse flies (Mews, Langley, Pimley and Flood, 1977; Kabayo, Taher and Van der Vloedt, 1985), mosquitoes (Benzon and Apperson, 1987; Kogan, 1990), kissing bugs (Langley and Pimley, 1978; Gardiner and Maddrell, 1972) and fleas (Wade and Georgi, 1988; Pullen and Meola, 1995). Feeding through animal-derived or artificial membranes was also successful for soft ticks (Argasidae) (Mango and Galun, 1977; Osborne and Mellor, 1985; Wirtz and Barthold, 1986; Hokama et al., 1987). Reviews have been given by Klunker (1979) and Issmer et al. (1994).

Tick feeding by the means of capillary tubes put over their mouthparts did not produce viable offspring or else depended on partial feeding on living hosts. This method is useful to infect ticks artificially with pathogens (Chabaud, 1950; Purnell and Joyner, 1967; Joyner and Purnell, 1968; Jones et al., 1988).

Trials to feed hard ticks through membranes were already carried out in the first half of this century (Hindle and Merriman, 1912; Totze, 1933; Lees, 1948). Even the air cell membrane over an embryonated hen egg was offered to the cattle tick *B. microplus* but only larvae fed with a success worth mentioning (Pierce and Pierce, 1956). In a study of Howarth and Hokama (1983), nymphs and adults of *Dermacentor andersoni* only resumed engorgement through modified animal skin *in vitro* after being pre-fed on rabbits for 4–6 days.

Table 1 compiles methods and results of the most important of the more recent work published. Kemp et al. (1975) fed larvae of *B. microplus* successfully through cattle fur on tissue culture medium using a steady flow-through system. However, *in vitro* feeding was not only helpful to study the transmission of pathogens as noted earlier. Developing a vaccine against *B. microplus*, adults of this species fed through animal gut membranes (baudruche) on blood of cattle, which had been stimulated to produce antibodies against ticks' gut epithelium (Kemp et al., 1986) (No table). When experiments ended after 42 hours, both, ticks fed *in vitro* and on these hosts, clearly showed fatal gut damage, as compared with controls.

Forty percent of adult *Dermacentor nuttalli* engorged half of their natural body mass when fed on heparinized, sterile drawn and frozen stored bovine blood through silicone membranes (Table 1) (Habedank and Hiepe, 1993). The diet contained bacteriostats and was changed three times a day. Fifty percent of the eggs laid gave rise to larvae. Feeding *Hyalomma anatolicum excavatum* adults the same way led to similar results (Habedank et al., 1994) (No table).

A short note from Issmer and Grunewald (meeting of the German Association for General and Applied Entomology, D-Jena, March 1993) reported on adult *I. ricinus* and *Hyalomma truncatum* laying eggs after being fed to repletion in a flow-through system with artificial membranes (No table).

Waladde et al. (1991,1993) used modified baudruche membranes with the blood manually changed 3 to 6 times per day to feed adults and nymphs of *Rhipicephalus appendiculatus* to 65 % and 50 %, respectively, of their body mass on the host (Table 1). The nymphs moulted to adults and the females produced offspring normally.

Later, Voigt et al. (1993) succeeded in feeding all instars of *A. variegatum* through rabbit and bovine fur on sterile, heparinized cattle blood containing bacteriostats (Table 1). Larvae and nymphs gained 100 % and 81 % of their *in vivo* body mass, respectively, whereas females did not exceed 38 % of normal weight. Consecutive *in vitro* feeding of moulted *in vitro*-fed instars, a prerequisite for pure artificial rearing, was not accomplished.

Recently the life-cycle of *A. hebraeum* has been completed *in vitro* using a simple feeding system based on honey jars equipped with silicone membranes (Table 1 and see ‚Results') (Kuhnert et al., 1995).

The same year, Waladde et al. combined their modified baudruche membrane with the „upside down" feeding system of Voigt and colleagues. Nymphs of *R. appendiculatus* nourished this way developed almost normally (Table 1).

1.3 Acaricide development

Increasing resistance of hard ticks against chemical attack by organophosphates, carbamates and pyrethroides in the area of cattle farming prompted the industry to look for new compounds (e.g., macrocyclique lactones, growth regulators), new ways of application (systemic), and to develop different strategies in tick control (antifeedants, repellents, vaccines etc.). Therefore, an option to refine or reduce animal-based tests by a simple *in vitro* assay would be desirable. An example is given here on how different tick species and stages accept an artificial feeding system, and how this *in vitro* system can be successfully applied to test a systemic acaricide.

Table 1: Literature review on *in vitro* feeding of hard ticks. In case of more than one mode of feeding per citation, the most successful is presented.

	Kemp *et al* (1975)		Waladde *et al* (1991)	Voigt *et al* (1993)	Waladde *et al* (1993)	Habedank & Hiepe (1993)	Kuhnert *et al* (1995)	Waladde *et al* (1995)
species / life-stage	*Boophilus microplus* larvae	dto. females	*Rhipicephalus appendiculatus* adults	*Amblyomma variegatum* all life-stages	*Rhipicephalus appendiculatus* nymphs	*Dermacentor nuttalli* adults	*Amblyomma hebraeum* all life-stages	*Rhipicephalus appendiculatus* nymphs
membrane	bovine fur (0,3–0,5 mm thick)	dto.	baudruche membrane, sealed with glue	rabbit and bovine fur (0,5–1 or 0,8–1,5 mm	baudruche membrane, sealed with glue	silicone membrane (0,16–0,20 mm thick)	silicone membranes (10–40 µm thick for larvae; 50 –90 µm for nymphs; 0,5 mm for adults)	baudruche membrane, sealed with glue
nutrient	sterile tissue culture medium TC 199 + 7 % bovine serum or bovine albumin	dto.	non-sterile steer blood, defibrinated, max. 3 d old	sterile drawn bovine blood (50 i. u. heparin/ml), max. 3 d old	sterile drawn bovine blood (50 i.u. heparin / ml), max.1 d old	sterile drawn bovine blood (2 i.u. heparin/ml), max. 20 weeks stored at –18°C §	non-sterile bovine blood, defibrinated, (glucose 2 g/l, ATP and glutathione 10^{-3} mol · l^{-1}), max. 7 d stored at 4°C	sterile drawn bovine blood (50 i.u. heparin / ml), max.8 h old
change of nutrient	steady flow ca. 8 ml/ h	dto.	3× per day	not reported	6× per day	3× per day	larvae 2–3× per day, nymphs 2×, adults 3× per day	2× per day
microbicide	penicillin G 125 i.u./ml, streptomycin-sulfate 125 i.u./ml, nystatin 100 i.u./ml, the latter also topical	dto.	penicillin 50 i.u./ml, streptomycin 50 i.u./ml	penicillin 100 i.u./ml, streptomycin 100 µg/ml, gentamicin 100 i.u. / ml ‡	no	penicillin 125 i.u./ml, streptomycin 125 i.u./ml	nystatin 100 i.u./ml, gentamicin 5 µg/ml	no
temperature humidity CO_2	incubator 35°C/70 % rh	dto.	water bath 37°C, air 26–27°C/ 70–80 % rh	incubator 35–39°C/90–95 % rh/5 % CO_2	water bath 42°C, air 26–27°C/ 70–80 % rh	incubator 37°C/90 % rh/ 4,5 % CO_2	water bath 38 C, air 23°C/80 % rh/ambient CO_2	incubator 37°C/80 % rh/ 3 % CO_2
attachment stimuli	bovine fur	dto.	cotton wool, iso-tonic saline, *R. a.*-faeces, bovine hair & ear extract	rabbit fur or bovine fur	see Waladde *et al* (1991)	cotton wool bovine hair, bovine hair extract	combinations of host hair, tick faeces, bovine hair extract, synthetic aggregation-attachment pheromone mixture	see Waladde *et al* (1991)
results	body mass after 6–8 d of feeding compared to *in vivo*, 47–83 % engorged fully, 51–71 % moulting success	"partially fed"	body mass 65%, egg conversion factor 82 % of *in vivo;* 63 % engorged fully; egg laying and larval hatch normal	body mass larvae: 100 %, nymphs: 81 %, females: 38 % of *in vivo*, 30 % engorged fully; moult, egg laying, larval hatch normal	body mass nymphs: ca. 50 % of *in vivo*, moult comparable to *in vivo* 50 % larval hatch	body mass females: 50 % of *in vivo*, 38 % engorged fully; eggs layed; 50 % larval hatch	life-cycle completed *in vitro;* body mass larvae: 95 %, nymphs: 87 %, females: 38 % of *in vivo* ; moult normal; 46 % of the females produced offspring; 80 % larval hatch; first generation *in vitro*-reared ticks: larvae & nymphs comparable to natural ticks, fertility of females strongly reduced	body mass of nymphs and moult of nymphs close to *in vivo*

‡ Inner side of animal fur treated with the fungistat amphotericin B. § Blood frozen for more than 4 days supplemented with ATP / glutathion (10^{-3} mol · l^{-1}).

2 Animals, Material and Methods

2.1 The ticks

The *I. ricinus* adults used in this work (10 month old) originated from a laboratory colony reared for years on mice and rabbits at the University of Neuchâtel (Graf, 1976). The other three tick species were laboratory strains fed on steer (Simmentaler breed) at the Ciba Agricultural Research Station (CH-1566 Saint Aubin). Nymphs and adults of *A. hebraeum* were 3–12 months, of *A. variegatum* 3–7 months old. Larvae of *A. hebraeum* were used between 3 to 6 months, those of *B. microplus* at an age of 4 weeks.

2.2 *In vitro* attachment

Attachment of sufficient numbers of ticks onto the membranes was induced with combinations of host hair, conspecific tick faeces, saline, bovine ear wash or bovine hair extract and synthetic aggregation attachment pheromone mixtures for *Amblyomma* (Kuhnert, 1995).

2.3 *In vitro* feeding

Trials were started with *I. ricinus* and a flow-through system, where the nutrient was continuously replaced from a chilled reservoir (Figure 1). Later, for the feeding of the other ticks, a simple system based on modified honey jars and manual blood exchange (1–3 times per day) was used (Figure 2). Details on the manufacturing of the silicone membranes reinforced with Kodak® lens cleaning paper and the sealing procedures of other membranes (baudruche, PTFE-Teflon®), the assemblage of both feeding systems, and the calculation of the results are detailed elsewhere (Kuhnert, 1995; Kuhnert et al., 1995).

Weekly open-collected bovine blood from the slaughter house served as food. This was mechanically defibrinated and stored at 4°C. At collection, germ-free D (+) glucose was added to the blood (2 g / l) (*Amblyomma*) and supplemented weekly (*A. variegatum*) or at each blood exchange (*A. hebraeum*) with defrosted portions of a sterile stock solution of adenosine triphosphate plus reduced glutathione to obtain a final concentration of 10^{-3} mol l^{-1} for both blood constituents. The blood for *I. ricinus* contained only bacteriostats, the other species fed on blood completed additionally with fungistatic agents. The most efficient microbicidal combination was gentamicin (5 µg/ml, Sigma G-1272) and nystatin (100 i.u. / ml, Sigma N-1638), as employed for *A. hebraeum*.

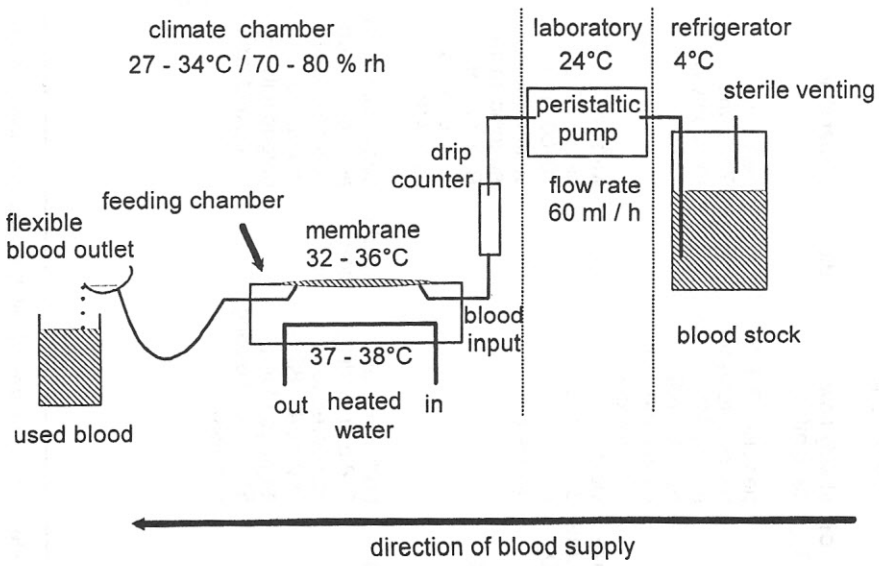

Figure 1: Principle of the flow-through feeding system. Components are not drawn to scale

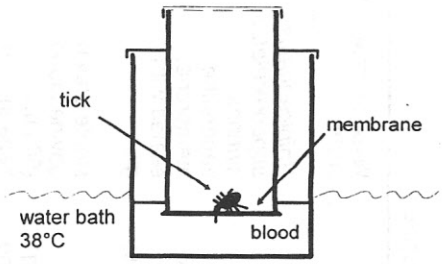

Figure 2: Principle of the feeding jars

The membrane of the flow-through system (*I. ricinus*) was kept at 32–36°C, the surrounding climate chamber at 27°C / 80 % r.h. at ambient CO_2 levels. The feeding jars (*A. hebraeum*) were held (up to the blood level) at 38°C in a water bath, each containing 10 ml blood for larvae, 15 ml for nymphs or 20 ml for adults which was replaced three times daily (nymphs and a few batches of larvae, twice). Here, conditions on the climatized work-bench were 23°C / 80 % r.h.. Experiments with *A. variegatum* and *B. microplus* (*B. m.*: 10 ml blood per feeding jar) were run before the improvements from the trials with *A. hebraeum* could work so that the nutrient was only changed once a day.

The underside of the membrane in feeding jars was washed with sterile distilled water and examined for fungal hyphae daily. Feeding experiments were started in the early evening and ran for 14 days (d) for adults, and 10 d for larvae and nymphs (d 0 = day of infestation).

2.4 *In vitro* acaricide test with *A. hebraeum* nymphs

The effect of feeding on the acaricide Ivermectin® (IVM) was tested using the *in vitro* feeding system described above. IVM at 1 ppm and 10 ppm was formulated in a mixture of glycerol formal and 1,2 propylene glycol (1:1,5). All treatments and the placebo contained 0,1 vol% solvent, the control none. A treatment consisted of five replicates with 32 ± 10 nymphs each, transfer-

red to the experimental conditions of 23°C / 80 % r.h. two hours prior to test. Blood treated with IVM was offered to the ticks over ten days and replaced twice daily. At d 4 non-attached nymphs were removed from the feeding unit. From d 5 the membranes were examined once a day just before a blood exchange and replete detached ticks were weighed and placed for moulting singly in compartments of a closed 96-hole multiwell-plate at constant darkness in an incubator at 28°C / >90 % r.h.. At the end of the trials, all ticks, including those still on the membrane at d 10, were allocated to one of three subgroups: 'alive and developed', 'alive but not developed', and 'dead'. The nymphs were classified as 'developed' if their body mass was at least 7 mg, the lowest limit for successful moult to the next instar.

3 Results

3.1 *In vitro* attachment and feeding

Ixodes ricinus

Adult *I. ricinus* were placed on glue-sealed PTFE-(Teflon®)-membranes covered with rabbit hair, bovine ear wash, saline and conspecific tick faeces. The males started immediately to copulate with the females, without attaching themselves to the membrane. Within a day more than half of all females (17 / 29) were attached. Four out of these gained a body mass worth mentioning (53 / 61 / 168 / 256 mg). None of them dropped off the membrane voluntarily. On rabbits, Graf (1978) reported an average female body mass of 263 mg. A single female laid a few eggs, which shrivelled rapidly, all others died. The flow-through system used for this species was often blocked with fungal mycelium and haunted by numerous leaks.

Boophilus microplus

For this one-host tick species, 7 feeding jars were equipped with a glue-impregnated baudruche membrane on which bovine hair extract, saline and tick faeces had been applied. Fifty to over seventy percent of unfed *B. microplus* larvae attached within one day. Of these, > 80 % engorged and > 80 % of the engorged larvae moulted to nymphs. Re-attachment rate of these so-called artificial nymphs on the same membrane was approximately 70 %. Spreading fungus inhibited nymphal development. From d 16, in only two out of five feeding jars, the gorging nymphs gave rise to adults, which represented roughly 3 % of all larvae set up in them. These artificial adults (32 males, 35 females) were transferred onto a fresh membrane, where 11 and 25 ticks, respectively, were attached at d 3. Since fungus came up again, all adults died before d 5.

In an experiment run at the end of this study, pharate or freshly moulted natural adults were collected from steer at d 11 (males) and d 12 (females) after infestation and transferred on a lens cleaning paper-reinforced silicone membrane (10–40 µm thick) covered with bovine hair and hair extract. Two days later, half of the females (22 / 44) and a similar number of males were attached. At d 11 both trials were stopped by detaching the females manually, except one, who had already dropped off at d 9. The females weighed 110 ± 60 mg (n = 11; *in vivo*: 236 mg). Some of them laid eggs.

Amblyomma variegatum

Nymphs of this three-host tick attached at 39 % (134 / 345) on a baudruche membrane impregnated with glue, hardened under UV-C light and treated with bovine hair extract, saline and a low dosed synthetic pheromone mixture (*ortho*-nitrophenol / methylsalicylate). All of them dropped off within 10 days (body mass: 44 ± 13 mg; *in vivo* 70 ± 11 mg, n = 200) and moulted to adults.

For natural adult *A. variegatum* thick silicone membranes (0.5 mm) reinforced with Terylene® netting were used. Beforehand they had served for mass rearing of tsetse flies at the IAEA (Entomology Unit, Seibersdorf, A-Vienna) and were a kind donation from Drs. E. Luger and U. Feldmann. Unfed males attached at 48 % (d 1; 29 / 60) when the combination of attachment stimuli was given as for the nymphs, but supplemented by nonanoic acid. Fifteen unfed females were added to nine males, who had previously fed *in vitro* for 5–6 days to enable sexual maturation. After 4 days, two thirds of the females had attached. Within 14 days of the experiment, 5 females dropped off with an average body mass of 1,8 ± 0,5 g. The egg conversion factor (ECF) was very low (3 females, 0,20 ± 0,04) and the larval hatch rate came to 0 %, 42 % and 85 %. One fifth of the females attached at d 4 produced viable offspring.

Amblyomma hebraeum

The life-cycle of *A. hebraeum* has been completed with the *in vitro* feeding method based on feeding jars and silicone membranes as described above. Starting with unengorged 'natural' adults, which had moulted from nymphs fed on steer, the life-cycle of this three-host tick was

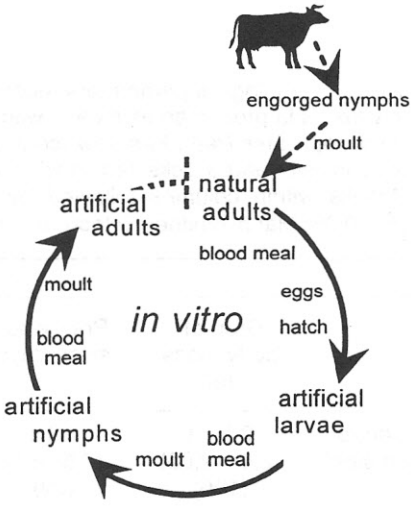

Figure 3: The life-cycle of *Amblyomma hebraeum* completed *in vitro*. The cycle started with unengorged 'natural' adults which had moulted from nymphs fed on steer and closed with unengorged first *in vitro* generation adults which moulted from nymphs fed *in vitro*

closed by producing unengorged first generation *in vitro*-fed adults which moulted from nymphs fed *in vitro* (Figure 3). After gathering experience with the three tick species presented previously, work concentrated later on *A. hebraeum* which serves also as a tick model in acaricide research and is normally reared on steer. Since all results with *A. hebraeum* were treated comprehensively elsewhere (Kuhnert et al., 1995; Kuhnert ,1995), a brief extract is given here.

Larvae and nymphs. *In vitro*, both larvae and nymphs attached quite readily to the thin silicone membranes with embedded lens cleaning paper (30–90 % and > 90 %, respectively) on which host hair and a synthetic pheromone mixture (*ortho*-nitrophenol, 2-methyl propanoic acid and benzaldehyde) had been applied; larvae received the bovine hair extract and tick faeces additionally. The attachment rate of *in vivo* and *in vitro*-reared nymphs did not differ. The average drop-off body mass of larvae, even when blood was only changed twice daily (2,7 mg, 3 batches with 41–70 larvae each), was not worse than on steer. Larval moulting rate to nymphs was significantly better for 'natural' larvae fed *in vitro* compared with those fed on steer, and not different from first generation *in vitro*-fed larvae (Table 2). The body mass was slightly reduced for 'natural' nymphs fed *in vitro* but this improved for first generation *in vitro*-fed individuals (Table 2). The good moulting success from nymph to adult was not affected by the way nymphs were reared.

Adults. In pre-feeding experiments where bovine hair was offered together with conspecific tick faeces, the 31 % attachment by 'natural' males on the silicone membrane at d 1 was significantly less than the 60 % of their *in vitro* counterparts ($P < 0,05$; Mann-Whitney *U*-test) (No table). However, after being pre-fed, *in vitro* males of any provenance re-attached more readily on another feeding unit. 'Natural' females, added to a membrane after introducing sexually mature males one hour earlier, attained attachment rates of 46 % on d 1 (d 4: 69 %). For 'artificial' females attachment rates were 63 % on d 1 (71 % on d 4). 'Natural' female ticks fed as adults *in vitro* reached a drop-off body mass of 38 % ($n = 22$) and an ECF of 47 % of their counterparts fed on steer. 'Artificial' females which moulted from 'natural' *in vitro*-fed or first generation *in vitro*-fed nymphs and fed under the same conditions achieved approximately equal levels ($n = 4$) (Table 3). The duration of pre-oviposition was more than doubled *in vitro*, and larval hatch was strongly diminished from eggs of *in vitro*-fed females (Table 3) The

Table 2: Biological parameters recorded for *Amblyomma hebraeum* larvae and nymphs fed *in vitro*. Data presented as means with 95 % confidence limits and number of ticks (*n*), batches (50 larvae each) or replicates (rep). First row (control): *in vivo*-reared ticks fed on steer; second row: *in vivo*-reared ticks fed *in vitro*; third row: first generation *in vitro*-reared ticks fed *in vitro*. Results within columns followed by different letters are significantly different ($P < 0,05$; Mann-Whitney *U*-test); d day

	Larvae		Nymphs	
	Drop-off body mass [mg]	% Moulting rate (to nymph)	Drop-off body mass [mg]	% Moulting rate (to adult)
Control on steer	2,1 a (2,0–2,2) (13 batches)	72 a (70–74) (39 rep)	66 a (65–67) (442)	98 (96–100) (2 rep)
In vivo-reared ticks	2,0 a (1,9–2,1) (6 batches)	85 b (80–89) (6 rep)	57 b (56–58) (571)	96 a (92–98) (12 rep)
First generation *in vitro*-reared ticks	2,4 (2,1–2,7) (3 batches) ‡	84 ab (79–88) (5 rep)	63 c (61–65) (205)	98 a (95–99) (5 rep)

‡ Varying batch size of 12–50 larvae

Table 3: Biological parameters recorded for *Amblyomma hebraeum* females fed *in vitro*. Data presented as means with 95 % confidence limits and number of ticks (*n*) or replicates (rep). First row (control): *in vivo*-reared ticks fed on steer; second row: *in vivo*-reared ticks fed *in vitro*; third row: *in vitro*-reared ticks fed *in vitro*. Results within columns followed by different letters are significantly different ($P < 0,05$; Mann-Whitney *U*-test); d day

	Females				
	Drop-off body mass [g]	Pre-oviposition days	Egg conversion factor (ECF)	% Larval hatch	% Larvae-producing females
Control on steer	2,56 a (2,44–2,68) (106)	7,8 a (7,5–8,1) (29)	0,57 a (0,55–0,58) (29)	87 a (78–96) (29)	97 (83–100) (1 rep)
In vivo-reared ticks	0,98 b (0,80–1,16) (22)	15,4 b (14,1–16,7) (17)	0,27 b (0,22–0,32) (17)	69 b (58–80) (17)	46 a (30–63) (9 rep)
In vitro-reared ticks	1,23 b (0,98–1,48) (4)	19,1 c (17,0–21,3) (7)	0,24 b (0,17–0,30) (4)	39 c (13–66) (7)	10 b (3–22) (10 rep)

number of females producing viable offspring from amongst all females which had attached by d 4 diminished from a level of 97 % on steer to 46 % for *in vitro*-fed 'natural' ticks but this level fell to 10 % for females of *in vitro* provenance.

3.2 *In vitro* acaricide test with *A. hebraeum* nymphs

Body mass gain by nymphs of *A. hebraeum* feeding *in vitro* on IVM added to the blood at 1 ppm and 10 ppm (54 % and 93 % mortality, respectively) was drastically reduced and none moulted to adulthood (Table 4). The placebo showed no difference to the blank *in vitro* control, neither in body mass (64 mg versus 67 mg), nor in tick mortality (12 % and 11 %, respectively).

Table 4: Effect of feeding on Ivermectin®-treated blood on the *in vitro* development of *Amblyomma hebraeum* nymphs. The effect achieved is expressed as mean % with confidence limits (95 %) for all nymphs attached at least once within day (d) 1–d 4 in a treatment with 5 replicates. Results within columns followed by different letters are significantly different ($P < 0,05$; Mann-Whitney *U*-test). Nymphs were classified as 'developed' if their body mass was at least 7 mg, the lowest limit for successful moult to the next instar.

	Dead	Alive but undeveloped	Alive and developed
Control *in vitro*	11 a (2–29)	1 a (0–13)	88 a (66–96)
Placebo	12 a (2–27)	2 a (0–12)	86 a (68–96)
Ivermectin 1 ppm	54 b (35–75)	47 b (25–65)	0 b (0–13)
Ivermectin 10 ppm	93 b (77–99)	6 c (0–18)	0 b (0–12)

4 Discussion

4.1 Membranes and feeding systems

Removed fur or skin of laboratory animals or animals for slaughter have been widely used in artificial feeding of hard ticks (Hindle and Merriman, 1912; Totze, 1933; Kemp et al., 1975; Doube and Kemp, 1979; Howarth and Hokama, 1983; Voigt et al., 1993). Baudruche, a membrane commercially prepared from animal gut, is usually applied in the manufacture of gold leaf. For the long feeding period of hard ticks, baudruche could only be used after being treated with glue (Waladde et al., 1979, 1991, 1993, 1995; Kuhnert, 1995), which made its standardisation difficult. Though it is not a complete artificial membrane, it seems to be at present the only possibility to feed the tiny *B. microplus* larvae *in vitro*. PTFE-(Teflon®)-membranes proved to be too expensive and only useful after glue coating similar to the baudruche and were therefore abandoned. A thick (0.5 mm) Terylene®-reinforced silicone membrane worked well for adults of both *Amblyomma* species with their long and powerful mouthparts. The breakthrough for the smaller ticks and stages was achieved with thin silicone membranes based on Kodak® lens cleaning paper. They were readily accepted by larvae and nymphs of *A. hebraeum* and adults of *B. microplus*. Since this type of membrane proved reliable and easy to standardise, it was applied for the *in vitro* acaricide test.

In this study, the presumed advantages of the flow-through system were neutralized mainly by the growth of fungus mycelium, which blocked the outlet and caused numerous failures. An effective fungistat like nystatin could prevent this in future trials. The low efficiency of a system continuously run through by new blood, led to the development of the feeding jars. This way, the amount of blood and substances needed was reduced by a factor of 20 to 50. Fungus could be controlled with nystatin (100 I.E. / ml).

4.2 *In vitro* attachment

The work detailed here focused on the *in vitro* feeding of ticks. Investigations on attachment were only of interest in so far as they helped to get sufficient numbers of ticks attached on the membranes. The attachment of *A. hebraeum* nymphs was consistently close to what is achievable on hosts. The skin-like texture of the fibre-reinforced silicone membrane used for nymphs and larvae of this species is probably an important factor for attachment. *In vitro* attachment rates of pre-fed males and unfed females in presence of these males were sufficient. Attachment of unfed males of *A. hebraeum* could be improved, for example, by testing a thinner silicone membrane or a higher CO_2-concentration in air. The other species used attached fairly well, which shows, that most of the stimuli relevant for attachment had been present. All species and life-stages (except *I. ricinus*) built visible cement cones around their mouthparts, as they do on the host.

4.3 *In vitro* feeding

Ixodes ricinus

Results for *I. ricinus* were insufficient due to fungal contamination and practical problems with the flow-through feeding system. A more reliable and nearly sterile blood delivery system (Issmer and Grunewald, 1993) or the principle of feeding jars (Bouvier, unpublished) allows female *I. ricinus* to perform close to *in vivo*.

Boophilus microplus

B. microplus, developed in the simpler feeding jars from natural larval to artificial adult stage, though slower and less numerous than on steer (Roberts, 1968). The *in vitro* body

mass of fed natural females was only half as in nature. The insufficient blood quality, as discussed below, probably accounts for this.

Amblyomma variegatum
Even changing blood only once per day, the nymphs gained 63 % of their *in vivo* body mass and moulted successfully to adults. A few natural females feeding through silicone membranes reached nearly 90 % of their normal weight, but fertility was strongly reduced. As shown by Voigt et al. (1993), *in vitro* feeding of this species can bring larvae to 100 %, nymphs to 81 % and females to 38 % of their *in vivo* body mass, with moulting and fertility comparable to natural conditions.

Amblyomma hebraeum
The nutritive quality of the blood offered in feeding jars below a thin silicone membrane met the requirements of larvae and nymphs for quasi normal engorgement and moulting. However, the *in vitro* development of adults, that is the sexual maturation of the males after 5–7 days of pre-feeding, the drop-off body mass and ECF of the females, the larval hatch from their eggs and in particular, the number of females who, once attached, are capable of giving rise to viable offspring were impaired and need to be improved. The reduced feeding performance of the adults is thought to be due to a deficiency of feeding stimuli or essential nutrients, or an excess of critical biochemical compounds, such as toxic metabolic products, in the blood-meal.

The adverse effects of *in vitro* feeding on the adults might be caused by several and yet unknown factors, some of which are discussed below. In this study, the one-week storage of refrigerated whole blood could have led in the presence of free hemoglobin to the peroxidation of blood-phospholipids (Knight et al., 1993), some of which are likely to be essential for tick nutrition. Another reason might be the 50 % decrease in 2,3-diphosphoglycerate, a potential feeding stimulus for the kissing bug *Rhodnius prolixus* (Smith and Friend, 1982; Mumcuoglu and Galun, 1987; Friend and Stoffolano, 1990), within three weeks, as described for human blood (Widmann, 1985).

However, it is unlikely that females ran short of red blood cells for the main blood-meal on d 8 and d 9. Assuming a 3 g body mass gain in the last 8 hours of feeding, an *Amblyomma* female retaining only 20% of the imbibed defibrinated blood as a concentrate while rejecting most of the rest as water and ions (Na^+,Cl^-) back into the feeding unit, would require a total of 15 ml of blood per feeding unit. But each unit contained 20 ml, changed three times daily. The feeding performance of either one or five ticks coming to repletion on the same day on separate membranes was not different, so the amount of blood available per female during engorgement does not seem to be a limiting factor. This holds even more true for females of smaller species, i.e., *I. ricinus* or *B. microplus.*

The bacteriostatic and fungistatic agents employed here proved indispensable for successful tick feeding. In their absence, bacterial and fungal (mainly *Aspergillus spec.*) growth was prolific and caused rapid degradation of the blood and of access by tick mouthparts to the nutrient. Are there symbiotic micro-organisms in ticks which might supply them with substances such as essential vitamins, and which could have been adversely affected by these substances? There is no proven evidence for intestinal symbionts in ticks. The bacterial genus *Wolbachia* (Rickettsiales), abundant in all tissue of most tick species, is considered rather as a commensal than an indispensable symbiont (reviewed by Balashov, 1972). It is possible that the fungistats used might have affected yeast-like extracellular organisms (*Adlerocystis spec.*), which are known from the accessory genital glands of males of all hard tick species (Feldman-Muhsam, 1970, 1974, 1986). In the argasid tick *Ornithodoros parkeri* secretions of these glands promoted female oogenesis (Oliver, 1986). The possible involvement of these organisms in tick reproduction, not yet understood, could be one reason why only the adults fared so badly on blood which contained doses, albeit low, of bacterio- and fungistats.

Heparinized blood is more complete than the defibrinated blood used here, and the feeding performance of larvae, nymphs and females of *A. variegatum* has been much improved for ticks feeding on blood with this anticoagulant (Voigt et al., 1993). Moreover, they recorded higher engorgement weights by elevating the level of CO_2 (4,5–5 %) in the surrounding air. This option, together with heparinized whole blood, should be tested in future feeding experiments with any tick species.

4.4 *In vitro* acaricide test with *A. hebraeum* nymphs

The good results feeding nymphs of *A. hebraeum in vitro* and the significance of this species as a three-host model tick for acaricide research led to the application of the *in vitro* feeder to assay acaricides, which proved very useful. Ivermectin®, first developed for the control of nematodes, has known acaricidal activity (Kaufman et al., 1986; reviewed by Campbell, 1989). This was confirmed here. Already from 1 ppm no nymphs took up sufficiently enough blood to develop to the next instar and half of the ticks were dead by d 10. This *in vitro* assay allows an experiment to be run with control, placebo, standard-acaricide and different compounds using blood from the same donor animal. This is a crucial advantage over *in vivo* tests with systemic agents because of more controlled conditions and less variability with a standardised nutrient as compared to effects arising from individual test animals. The *in vitro* test presented is now routinely used in the Swiss chemical industry to screen systemic compounds for their acaricidal activity.

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