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The Life-cycle of the Bont Tick *Amblyomma hebraeum In Vitro*

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Abstract—Kuhnert F., Diehl P. A. and Guerin P. M. 1995. The life-cycle of the bont tick *Amblyomma hebraeum in vitro*. *International Journal for Parasitology* 25: 887–896. The life-cycle of the hard tick *Amblyomma hebraeum* was completed *in vitro* by feeding all life-stages of the tick through silicone membranes on bovine blood from an abattoir. Ticks were placed in a simple feeder consisting of a honey jar containing the blood with a glass tube insert (o.d. 42 mm) across the end of which the membrane was stretched. This feeding unit was held in a water bath (38°C). Larvae and nymphs fed on a membrane (<90 μm thick) made of silicone reinforced with Kodak® lens cleaning paper, and adults on a silicone membrane (0.5 mm thick) reinforced with Terylene® netting. To control microbial growth, gentamicin (5 μg/ml) and nystatin (100 i.u./ml) were added to the weekly open-collected blood, which was manually defibrinated. The blood was changed twice daily for nymphs and three times for adults and larvae. Attachment of ticks was induced with combinations of host hair, tick faeces, a bovine pelage extract and a synthetic aggregation–attachment pheromone mixture. The *in vitro* life-cycle started with unengorged "natural" adults, which had moulted from nymphs fed on steer. The life-cycle closed with unengorged, first *in vitro* generation adults which had moulted from nymphs fed *in vitro*. Although the feeding and development of larvae and nymphs were similar to *in vivo* controls, females fed and developed poorly *in vitro*. The toxicity of the systemic acaricide Ivermectin® for nymphs of *A. hebraeum* was confirmed using the *in vitro* feeding method.

Key words: Ixodidae; *in vitro* feeding; artificial feeding; artificial membrane; acaricide; hematophagous; *Amblyomma hebraeum*; parasite.

INTRODUCTION

Hard ticks (Acari: Ixodidae) are a group of parasitic arthropods well known for the threat they pose to humans and livestock by transmitting diseases, and by their debilitating action on the host during their long blood-meal. There is a strong demand for large numbers of all instars of such ixodid ticks for the development of modern approaches to tick control, such as systemic acaricides, repellents and antifeeding compounds. Maintenance of laboratory strains necessitates the use of a range of vertebrate hosts for each of the three life-stages, i.e. larvae, nymphs and adults, generally on single-use mammals like mice, rabbits and cattle. Ethical questions surrounding the use of experimental animals has lent impetus to the development of alternative rearing methods for ticks, and indeed other hematophagous arthropods.

In vitro feeding methods have been developed for bloodsucking insects such as tsetse flies (Mews, Langley, Pimley & Flood, 1977; Kabayo, Taher & Van der Vloedt, 1985), mosquitoes (Benzon & Apperson, 1987; Kogan, 1990), kissing bugs (Langley & Pimley, 1978; Gardiner & Maddrell, 1972), fleas (Wade & Georgi, 1988) and for soft ticks (Argasidae) (Mango & Galun, 1977; Osborne & Mellor, 1985; Wirtz & Barthold, 1986). In contrast to these arthropods, for which the blood-meal lasts from just a few seconds to maximally 1 h, each hard tick instar remains firmly anchored to the same feeding site for periods of 2–14 days. This consider-

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able 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 & Rice, 1982). These important steps, preceding the actual blood-meal, depend on a specific blend of thermal, hydro, mechanical, olfactory stimuli and contact chemostimuli (Lees, 1948; Waladde & Rice, 1982; Norval, Andrew & Yunker, 1989; Norval, Butler & Yunker, 1989). While injecting numerous physiologically active agents into the lesion, the attached tick evokes strong inflammatory, vasodilatory and immunological responses by the host (Tatchell & Moorhouse, 1968; Walker & Fletcher, 1986; Kemp, Stone & Binnington, 1982).

Only small amounts of blood are imbibed during the first days of feeding, a period during which the tick undergoes a 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 processes prepare the parasite's engorgement, which occurs mainly during the last 24 h of feeding. During this crucial phase, female ticks imbibe up to 4 times as much blood as the final gain in weight (Kaufman & Phillips, 1973). They concentrate this blood, rejecting water and ions (Na^+, Cl^-) back into the host, and multiply their body mass in some cases more than 100-fold with protein-rich nutrient for egg production. All of this renders ixodid ticks rather delicate candidates for *in vitro* feeding, as an adequate diet has to be maintained at the feeding site throughout attachment.

Trials to feed hard ticks through membranes were first carried out at the beginning of this century (Hindle & Merriman, 1912; Totze, 1933), followed more recently by other workers (Kemp, Koudstaal, Roberts & Kerr, 1975; Waladde, Ochieng' & Gichuhi, 1991; Habedank & Hiepe, 1993). Recently, Voigt, Young, Mwaura, Nyaga, Njihia, Mwakima and Morzaria (1993) succeeded in feeding all instars of *Amblyomma variegatum* through animal skin membranes. However, consecutive *in vitro* feeding of moulted *in vitro*-fed instars, a prerequisite for pure artificial rearing, was not accomplished. Here we report on how to complete the life-cycle of *Amblyomma hebraeum in vitro* using artificial membranes in a simple feeding system, and on the successful application of this *in vitro* method in tests with a systemic acaricide.

MATERIALS AND METHODS

Amblyomma hebraeum Koch was chosen as the model ixodid tick because: (1) its widespread use in industry for tests with acaricides, (2) it requires generally 4–14 days for the feeding of each instar, which covers most of blood-meal durations in hard ticks, (3) the dimensions of the mouthparts vary considerably between instars (length of hypostome in larvae 0.08–0.12 mm, nymphs 0.2–0.3 mm, adults 1–1.5 mm) and so mirrors the kind of penetration depth, force and damage an artificial membrane would be subject to by other tick species, and (4) the extreme differences in the weight increase of the replete instars ranging from *ca.* 2 mg in larvae to *ca.* 2.5 g or more in females, is also representative for the requirements of other hard ticks. The unfed 5–7-month-old *A. hebraeum* ticks from a laboratory strain fed on male cattle (Simmentaler breed) at the Ciba–Geigy Agricultural Research Station (CH-1566 St Aubin) were stored at 25°C/85% r.h. (day) and 20°C/95% r.h. (night) in a 12 h : 12 h light/dark cycle.

In vitro *attachment.* As the work detailed here focused on the *in vitro* feeding of ticks, tests with different attachment stimuli were only of interest in so far as they helped to get sufficient numbers of ticks attached on the silicone membranes. To ensure attachment by all development stages, a thin covering of shredded bovine hair (rabbit hair for nymphs) and the synthetic aggregation–attachment pheromone mixture (Apps, Viljoen & Pretorius, 1988) (SPM) of *ortho*-nitrophenol, 2-methyl propanoic acid and benzaldehyde (1 : 1 : 0.1 μg in dichloromethane) dispensed from a rubber septum (after evaporation of the solvent) (Butler & McDonough, 1979) were placed on the membrane. Instead of SPM, unfed males (30 per jar) received about 20 mg faeces (stored at −18°C), produced by congeners in former *in vitro* trials, as attachment stimuli. For larvae the membrane was additionally covered with 50 μl of a bovine pelage extract in dichloromethane (fat content: *ca.* 10 mg/ml; stored at −80°C) and freshly ground tick faeces. Trials with different attachment stimuli and their combinations were also carried out with unfed males (Table 1). The shredded glass wool used here was sprinkled thinly over the membrane. Potential attachment stimulants were manipulated with disposable gloves and all instruments were cleaned with acetone between trials. All attachment tests were carried out with the *in vitro* feeding unit described under "*in vitro* rearing" (below).

Table 1—*In vitro* attachment tests with unfed *Amblyomma hebraeum* males. Data presented as mean % with confidence limits (95%) of 9–10 repetitions with 5 males per feeding unit. Means without a common letter are significantly different ($P<0.05$; Mann–Whitney *U*-test); SPM synthetic pheromone mixture

Stimuli on membrane	% Attachment after 1 h		
SPM	0	(0–7)	a
Glass wool	2	(0–11)	ab
SPM × 10^3 + glass wool	4	(1–14)	ac
Tick faeces	18	(9–31)	cd
Tick faeces + glass wool	20	(10–34)	cd
SPM + glass wool	20	(10–35)	bcd
Bovine hair	24	(13–38)	ad
Bovine hair + SPM	32	(20–47)	d

In vitro *rearing*. Batches of larvae and nymphs were fed through a membrane made of Kodak® lens cleaning paper (Eastman Kodak, Rochester, NY), a non-woven tissue made of regenerated cellulose (rayon), which was permeated with a one-component acetate based silicone glue (art. no. 251470, Kunststoffe Rehau AG, D-95111 Rehau) to bring it to a final thickness of 10–40 µm for larvae and 50–90 µm for nymphs (rough side towards the ticks). Adults were placed on a 0.5 mm thick pure silicone membrane reinforced with Terylene® netting, first developed for feeding tsetse flies (Bauer & Wetzel, 1976). The feeding unit consisted of a 200 ml honey jar (93 mm high, o.d. 61 mm) with an inner glass tube (9 cm long, o.d. 42 mm), the upper end of which was set flush in a hole in the lid (Twist-Off®, o.d. 58 mm) of the jar with silicone glue. The membrane (∅ 38 mm) on which the ticks were placed was stretched across the bottom end of the tube and likewise held with silicone glue at 2–3 mm from the floor of the jar. For larvae and nymphs, the membrane was fixed with tape (Tesa 4108, Beiersdorf AG, D-Hamburg) to the outside of the glass tube and disinfected externally with ethanol (70%). All glassware and the glass inserts for adults were sterilised by autoclavation (125°C, 30 min, 1.3 bar) prior to use. To prevent escape of ticks, a cotton wool stopper for immatures and a stainless steel mesh for adults was used to close the top end of the glass tube. Conditions on the climatized workbench where the rearing was done were 23°C/80% r.h. in a 12 h : 12 h light/dark cycle at ambient CO_2 levels. One hour after placing 5–8 pre-fed male ticks and their faeces onto a fresh membrane, the same number of unfed females was added. These males had been allowed to reach sexual maturity by prior feeding for 5–7 days *in vitro* (blood exchange twice daily). Nymphs and larvae were divided into batches of *ca.* 30–50 and 500–700 per feeding jar, respectively. Weekly open-collected bovine blood from the slaughter house served as nutrient. This was manually defibrinated with a stainless steel cooking spoon. At collection, germ-free D (+) glucose was added to the blood (2 g/l) which was then stored at 4°C. The feeding units were held, up to the level of the blood, at 38°C in a water bath. Each contained 10 ml blood for larvae, 15 ml for nymphs or 20 ml for adults, which was replaced 3 times daily (nymphs and three batches of larvae, twice). The blood level for adults reached over the membrane level so as to keep female mouthparts below the erythrocyte-poor serum layer after the blood had settled in the feeding unit. At each blood exchange the fungistat nystatin (100 i.u./ml, Sigma N-1638), the bacteriostat gentamicin (5 µg/ml, Sigma G-1272) and a defrosted portion of a sterile stock solution of the potential feeding stimulants adenosine triphosphate (ATP) plus glutathione (GSH) (Galun & Kindler, 1968) was thoroughly mixed with the fresh pre-warmed blood to obtain a final concentration of 10^{-3} mol l^{-1} for both blood constituents. The underside of the membrane 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). The parameters used to assess the performance of fed females were: body mass (mg), egg conversion factor (ECF = weight of eggs laid/female's drop-off weight), duration of pre-oviposition (d), % larval hatch rate, % larvae-producing females, fertile egg production (mg per female), and "peak" drop-off day (mode). The body mass of the females was obtained by weighing them within 2 days of drop-off, the nymphs within 1 day and the detached larvae in batches of 12–50 at d 10 of an experiment. The number of days between the end of the blood-meal and the onset of oviposition provided the duration of pre-oviposition (at 28°C/ > 90% r.h./constant darkness). Forty-five days after termination of the blood-meal, the weighed egg mass of a female divided by its replete body mass gave the egg conversion factor (ECF). Each egg batch was carefully dispersed in an individual compartment (33 × 38 mm) of a multi-unit tray and, once hatching started, the percentage of eggs containing embryos was assessed by eye on a 5-point scale for each female (i.e. a larval hatch scale); egg batches with < 5% fertile eggs were referred to as "no larval hatch". The percentage of larvae-producing females is calculated with reference to the number of females in a given treatment attached by d 4. An evaluation of the overall reproductive performance of ticks in a given treatment is expressed by the average mass of fertile eggs laid by each female which had attached by d 4 (fertile egg production in mg per female). This was calculated as egg mass × larval hatch × 0.01. To calculate these parameters, females not fulfilling specific criteria were excluded: body mass < 200 mg, female dead at the end of trial or within 1 week, or no eggs laid or all eggs desiccated (not excluded for body mass estimations). If the female was still attached at the end of a trial she was excluded only from "body mass" and "ECF" calculations.

In vitro *acaricide test*. The effect of feeding on the acaricide Ivermectin® (IVM) on *A. hebraeum* nymphs was tested using the *in vitro* feeding system described above. IVM at 1 and 10 p.p.m. was formulated in a mixture of glycerol formal and propylene glycol (1 : 1.5). All treatments and the placebo contained 0.1 vol% solvent, the control none. A treatment consisted of 5 replicates with 32 ± 10 nymphs each, transferred to the experimental conditions of 23°C/80% r.h. 2 h prior to test. Blood treated with IVM was offered to the ticks over 10 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.

RESULTS

In vitro *attachment test*

Practically no males of *Amblyomma hebraeum* attached within 1 h when the synthetic pheromone mixture (SPM) or glass wool was presented alone on

Table 2—Attachment rates of different life-stages of *Amblyomma hebraeum in vitro*. Data presented as ranges or as mean % with confidence limits (95%) and number of replicates. 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*. Data for nymphs and larvae in the third row are for first generation *in vitro*-fed ticks. Results within columns followed by different letters are significantly different ($P<0.05$; Mann–Whitney *U*-test); d day

	Males d1	Males prefed d1	Females d1	Nymphs d4	Larvae d4 (visually estimated)
Control on steer	95–100	95–100	95–100	90–100	No data
In vivo-reared ticks	31a (26–37) (10)	75a (61–86) (9)	46a (32–61) (9)	95a (90–98) (5)	~30
In vitro-reared ticks	60b (53–69) (5)	93b (85–98) (10)	63a (51–74) (10)	97a (95–99) (5)	~30–90

the membrane (Table 1). The addition of tick faeces or SPM to the glass wool or the use of bovine hair improved male attachment. This improvement was significant when the bovine hair was administered together with SPM ($P<0.05$; Mann–Whitney *U*-test). Overdosed SPM, when given in combination with glass wool failed to induce attachment. In pre-feeding experiments in which bovine hair was offered together with 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) (Table 2). 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 1 h 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). Both larvae and nymphs of *A. hebraeum* attached quite readily to the artificial membranes (30–90% and >90%, respectively) on which host hair and SPM had been applied; larvae received the bovine pelage extract and tick faeces in addition. The attachment rate of *in vivo* and *in vitro* reared nymphs did not differ (Table 2).

In vitro *rearing*

The life-cycle of *A. hebraeum* has been completed with the *in vitro* feeding method described. Starting with unengorged "natural" adults, which had moulted from nymphs fed on steer, the life-cycle of *A. hebraeum* was closed by producing unengorged first generation *in vitro* fed adults which moulted from nymphs fed *in vitro* (Fig. 1). "Natural" female ticks fed as adults *in vitro* reached a drop-off body mass 38% ($n = 22$) and an egg conversion factor 47% of that 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 ($n = 4$) (Fig. 2). 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 number of females producing viable offspring from amongst all females which had attached by d 4 (to reduce bias due to different attachment rates) 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. The average mass of fertile eggs

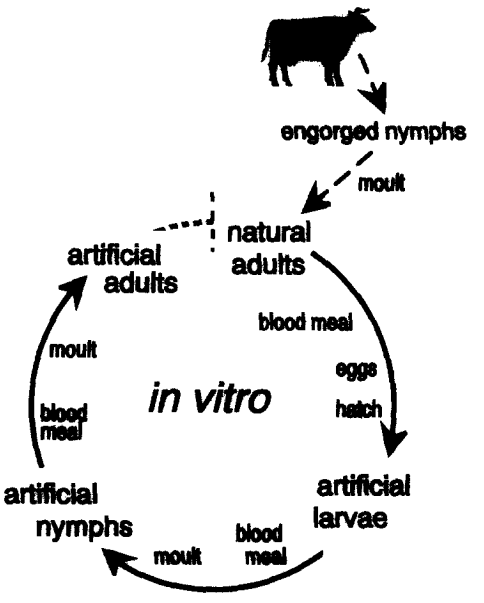

Fig. 1. The life cycle of *Amblyomma hebraeum* completed *in vitro*. The cycle started with unengorged "natural" adults which had moulted from nymphs fed on steer and closed with unengorged first *in vitro* generation adults which moulted from nymphs fed *in vitro*.

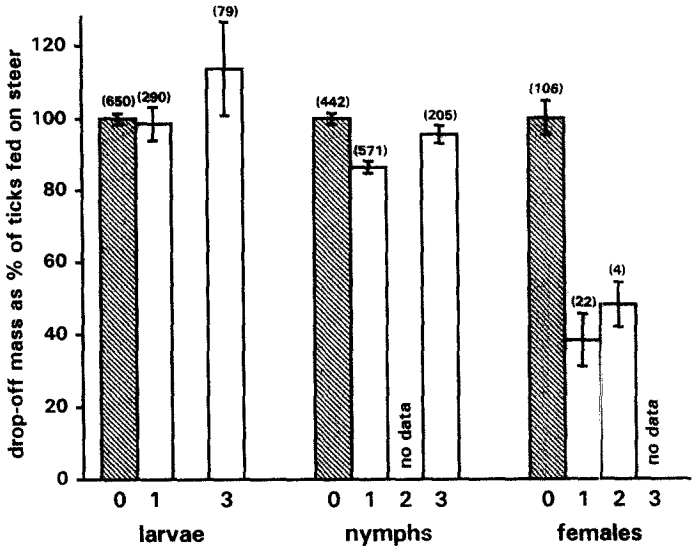

Fig. 2. Body mass attained by all life stages of *Amblyomma hebraeum* ticks *in vitro* as a proportion of the weights achieved on steer. 0 "natural" ticks fed *in vivo* (100%, shaded), pooled data from 3–5 non-parallel infestations; 1 "natural" ticks fed *in vitro*, "nymphs" are pooled data of 5 non-parallel feeding trials; 2 "artificial" ticks fed *in vitro*; 3 first generation *in vitro*-fed ticks. "Nymphs 3" derived from batches of first generation *in vitro*-fed larvae with blood exchanged either 2 or 3 times daily. "Females 1 + 2" were fed on blood from the same animal. Means ± confidence limits (95%) and number of ticks (*n*) are provided. The confidence limits shown for "larvae 3" are based only on 3 batches of 12–50 larvae each.

produced by a female on the host is 1229 mg. This dropped to 110 mg for *in vitro*-fed "natural" females and to 17 mg for *in vitro*-fed "artificial" females.

In contrast with the adult females, nymphs and larvae fed artificially showed, if ever, only minor developmental impairment after drop-off. The body mass was reduced for "natural" nymphs fed *in vitro*, but this improved for first generation *in vitro*-fed individuals (Table 4). The moulting success from nymph to adult was not affected by the way nymphs were reared. This also holds true for the average drop-off body mass of larvae even when blood was only changed twice daily (2.7 mg with a 95% confidence interval of 2.2–3.3 mg; 3 batches with 41–70 larvae each). 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 4).

In vitro *acaricide test*

Body mass gain by nymphs of *A. hebraeum* feeding *in vitro* on Ivermectin® (IVM) added to the blood at 1 and 10 p.p.m. (54 and 93% mortality, respectively) was drastically reduced and none moulted to adulthood (Tables 5 and 6). The placebo showed no difference to the blank *in vitro* control, neither in body mass (64 vs 67 mg), nor in tick mortality (12 and 11%, respectively).

DISCUSSION

The life-cycle of a 3-host tick species has been completed *in vitro* by feeding its different life-stages consecutively on bovine blood through artificial membranes. This blood is freely available at abattoires where it is discarded. Apart from an application of the *in vitro* method for studies on tick metabolism, pathogen transmission, and ethological questions such as drop-off rhythms and mating behaviour, this method could also prove useful for tests on repellents and compounds inhibiting attachment and feeding. Adult hard ticks have been fed with moderate success on skin-derived membranes (Waladde *et al.*, 1991; Voigt *et al.*, 1993; Waladde, Kemp & Rice, 1979; Howarth & Hokama, 1983), but only once on a silicone membrane (Habedank & Hiepe, 1993). Until now the tiny larvae and nymphs have remained problematic, always necessitating the use of animal skin (Voigt *et al.*, 1993).

The *in vitro* system turned out to be suitable for feeding larvae of *A. hebraeum* to repletion. Their body mass gain matched that of *in vivo*-fed ticks and

Table 3—Biological parameters recorded for *Amblyomma hebraeum* females fed *in vitro*. Data presented as modes ("peak"), ranges or 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 "peak" day	Drop-off days (range)	Drop-off body mass (g)	Pre-oviposition days	Egg conversion factor (ECF)	% Larval hatch	% Larvae-producing females	Fertile egg production (mg per female)
Control on steer	d 9a	d 8–d 14	2.56a	7.8a	0.57a	87a	97	1229a
			(2.44–2.68)	(7.5–8.1)	(0.55–0.58)	(78–96)	(83–100)	(1069–1389)
	(240)		(106)	(29)	(29)	(29)	(1 rep)	(30)
In vivo-reared ticks	d 8/9ab†	d 7–d 14	0.98b	15.4b	0.27b	69b	46a	109b
			(0.80–1.16)	(14.1–16.7)	(0.22–0.32)	(58–80)	(30–63)	(58–160)
	(23)		(22)	(17)	(17)	(17)	(9 rep)	(37)
In vitro-reared ticks	d 14b‡	d 8–d 14‡	1.23b	19.1c	0.24b	39c	10b	15c
			(0.98–1.48)	(17.0–21.3)	(0.17–0.30)	(13–66)	(3–22)	(2–28)
	(8)		(4)	(7)	(4)	(7)	(10 rep)	(50)

†Same number of females dropped off on both days.
‡Most of the females still attached at end of trial (d 14).

Table 4—Biological parameters recorded for *Amblyomma hebraeum* larvae and nymphs fed *in vitro*. Data presented as modes ("peak"), ranges or 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 "peak" day	Drop-off days (range)	Drop-off body mass (mg)	% Moulting rate (to nymph)	Drop-off "peak" day	Drop-off days (range)	Drop-off body mass (mg)	% Moulting rate (to adult)
Control on steer	d5	d4–d6	2.1a	72a	d5	d4–d7	66a	98
			(2.0–2.2)	(70–74)			(65–67)	(96–100)
	(>10,000)		(13 batches)	(39 rep)	(>10,000)		(442)	(2 rep)
In vivo-reared ticks	No data	d6–d10†	2.0a	85b	d7a	d5–d10†	57b	96a
			(1.9–2.1)	(80–89)			(56–58)	(92–98)
			(6 batches)	(6 rep)	(571)		(571)	(12 rep)
First generation *in vitro*-reared ticks	No data	d6–d10†	2.4	84ab	d8b	d5–d10†	63c	98a
			(2.1–2.7)	(79–88)			(61–65)	(95–99)
			(3 batches)‡	(5 rep)	(205)		(205)	(5 rep)

†Some ticks still attached at the end of trial (d 10).
‡Varying batch size of 12–50 larvae.

their moulting success was even better. Changing the blood only twice daily did not affect the results and considerably reduced manpower needs. As already indicated in the literature (Waladde *et al.*, 1979, 1991) and our own preliminary results with larvae of *Boophilus microplus* on animal-derived membranes, the combined use of bovine pelage extract, host hair and tick faeces gave the best attachment results. The pelage extract dispersed on the membranes for larvae might facilitate attachment by first inducing arrestment (Guerin, Steullet, Kröber *et al.*, 1992).

Membrane-feeding of nymphs turned out to be the most practical and reliable step of the *in vitro* rearing of *A. hebraeum*, with attachment, feeding and moulting results consistently close to those achievable on a host. Some 70–80% of nymphs attached after applying only rabbit hair to the membrane (unpublished data). The synthetic pheromone mixture (SPM) was added for nymphs because it is known that this aggregation–attachment pheromone blend, emitted by sexually mature *A. hebraeum* males which have fed for 3–5 days on the host (Apps *et al.*, 1988), attracts not only conspecific males and females but also nymphs to the feeding site (Norval, Peter, Meltzer, Sonenshine & Burridge, 1992). Nymphs did attach, though less so, on our rough home-made membranes even without any olfactory or contact chemostimuli. The skin-like texture of the fibre-reinforced silicone membrane used for nymphs and larvae is probably an important factor for attachment. Lacking this tactile stimulus, neither nymphs of *A. hebraeum* (this study), nor of *A. variegatum* (Voigt *et al.*, 1993) attached to smooth silicone membranes. The matrix-reinforced silicone membrane used here proved ideal for both larvae and nymphs. All life-stages built visible cement cones around their mouthparts, as they do it on the host.

Bovine hair and faeces of congeners were chosen to induce attachment of the unfed males *in vitro*, since the use of glass wool as a mechanical attachment stimulus is not convenient for staff, and cotton wool failed for adults (unpublished data). The bovine hair probably provides mechanical stimuli (shelter and thigmotactic) and contact chemostimuli (skin lipids) for the ticks (Waladde *et al.*, 1979, 1991). Tick faeces from former feeding trials is most probably impregnated with the aggregation–attachment pheromones (Diehl, Guerin, Vlimant & Steullet, 1991) and it tends to facilitate attachment of *A. hebraeum* males to the same extent as SPM (Table 1). Tick faeces have already been used as an attachment-improving stimulus in trials with *Rhipicephalus appendiculatus* (Waladde *et al.*, 1991).

"Artificial" males (moulted from *in vitro*-fed nymphs) attached twice as well to silicone membranes as "natural" males (60 vs 31%, respectively). About one-quarter of the nymphs applied got lost during *in vitro* feeding, either through failure to attach, death or inability to moult to adulthood. Therefore, ticks surviving to adulthood *in vitro* are strongly selected. This might render "artificial" adult males less sensitive to suboptimal stimuli of the *in vitro* system and lead to a better acceptance of the artificial membrane.

When an attached sexually mature male is approached by a female he tries to clasp her and this normally leads to the attachment of the female, venter to venter with the male, for copulation after the female has fed for some days. This typical

Table 5—Biological parameters recorded for *Amblyomma hebraeum* nymphs fed *in vitro* on Ivermectin-treated blood. Data presented as means with 95% confidence limits and number of ticks (*n*). Results within columns followed by different letters are significantly different ($P<0.05$; Mann-Whitney *U*-test); d day

	Drop-off "peak" day	Drop-off days (range)	Drop-off body mass (mg)
Control *in vitro*	d 7/8a† (125)	d 5–d10‡	67a (64–70) (120)
Placebo	d7b (122)	d 5–d10‡	64a (61–67) (122)
Ivermectin 1 p.p.m.	*	*	<7§
Ivermectin 10 p.p.m.	*	*	<7§

†Same number of nymphs dropped off on both days.
‡Some nymphs still attached at the end of trial (d 10).
*Not applicable as nymphs were found detached (dead or alive) already at d 4.
§Mostly dead nymphs, either detached or still attached at d 10.

Table 6—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–d4 in a treatment with 5 replicates. Results within columns followed by different letters are significantly different ($P<0.05$; Mann–Whitney *U*-test)

	Dead	Alive but undeveloped	Alive and developed
Control *in vitro*	11a (2–29)	1a (0–13)	88a (66–96)
Placebo	12a (2–27)	2a (0–12)	86a (68–96)
Ivermectin 1 p.p.m.	54b (35–75)	47b (25–65)	9b (0–13)
Ivermectin 10 p.p.m.	93b (77–99)	6c (0–18)	9b (0–12)

clasping behaviour was also observed *in vitro*, though less spontaneous and successful, since most males attach close to the border of the membrane where it meets the glass tube, some of them in an unfavourable position for clasping, i.e. venter towards the glass. Some 50% of "natural" and "artificial" females attached within 1 day in the presence of the *in vitro* sexually mature males and their excrement. Nymphs also attached in the membrane/glass tube corner, mostly beside each other. Such behaviour was not observed for larvae.

Considered as a whole, the current limitation for a high yield of artificially fed ticks is the reduced fertility of the *in vitro*-fed females as expressed by the lower drop-off body mass and ECF, their prolonged pre-oviposition period, the impaired larval hatch from their eggs, and especially, the poor number of females which, once attached, are capable of giving rise to viable offspring. The *in vitro* method shows that the blood-meal size and reproductive performance of *in vitro*-fed females is halved. The drop in body mass, ECF and duration of pre-oviposition *in vitro* seems to be related to the absence of a living host *per se*, and not to the provenance ("natural" or "artificial") of these females. In contrast, the number of non-larvae-producing females increases dramatically for females fed *in vitro*. Though the reproductive performance of females gives the most detailed data on the effect of an *in vitro* rearing method, the role of the male ticks should not be neglected. The alternation of generations involves gametogenesis, genetic recombination and embryonic growth, which are well-adapted to natural conditions and which could be profoundly affected by a deficiency or an excess of critical biochemical components in the blood-meal. Suboptimal *in vitro* nutrition is more likely to have consequences for the production of viable offspring than on the moulting process.

The adverse effects of *in vitro* feeding on the adults might be due to several and yet unknown factors, some of which are discussed below. The 1-week storage of refrigerated whole blood with glucose supplement in this study is short compared to the accepted storage time of human transfusion blood of 5 weeks (Widmann, 1985). However, peroxidation of lipids already occurs after 1 week (Knight, Searles & Blaylock, 1993) (some of which might be essential for tick nutrition) and a 50% decrease in 2,3-diphosphoglycerate, a potential feeding stimulus (Smith & Friend, 1982; Mumcuoglu & Galun, 1987; Friend & Stoffolano, 1990) happens within 3 weeks (Widmann, 1985). It is unlikely that females ran short of red blood cells for the main blood-meal on d 8 and 9. Assuming a 3 g body mass gain in the last 8 h of feeding, a female retaining only 20% of the imbibed defibrinated blood as 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 3 times daily. The feeding performance of either 1 or 5 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.

In vivo, the feeding lesion around the tick's mouthparts is infiltrated by a high number of polymorphonuclear neutrophil leukocytes as part of the inflammatory response of the host (Walker & Fletcher, 1986; Tatchell & Moorhouse, 1968). These cells are of course missing in the *in vitro* situation. However, when the number of such cells was reduced artificially in dogs parasitized by adult *Rhipicephalus sanguineus* using nitrogen mustard, the female ticks engorged normally and produced viable larvae (Tatchell & Moorhouse, 1970). This allows us to argue that the absence of such cells in the *in vitro* feeder does not limit female development.

In vivo, strong irrigation by oxygenated blood is recorded at the feeding site as the female approaches repletion (Gregson, 1973). This contrasts with the reduced O_2 level of our stored blood, a factor which may limit the females' feeding rate. Heparinized blood is more complete than the defibrinated blood used here, and the feeding performance of females is much improved for ticks feeding on blood with this anticoagulant (Voigt *et al.*, 1993), even though the best relative engorgement weights reported by these authors do not exceed the performances of our ticks fed on defibrinated blood. It has been found that elevated levels of CO_2 (4.5–5%) were correlated with higher engorgement weights, but even under ambient CO_2 levels (0.03%), our experiments led to comparable feeding performances by females.

The bacteriostat and fungistat employed here proved indispensable for successful tick feeding. In their absence, bacterial and fungal (mainly *Aspergillus* sp.) growth was prolific and caused rapid degradation of the blood and of access by tick mouthparts to the nutrient. Are there micro-organisms in ticks which could have been adversely affected by these substances? There is no proven evidence for intestinal symbionts in ticks which might supply them with substances such as essential vitamins. The bacterial genus *Wolbachia* (Rickettsiales), abundant in all tissue of most tick species, is considered more as a commensal than an indispensable symbiont (Balashov, 1972). It is possible that the fungistat used might have affected yeast-like extracellular organisms (*Adlerocystis* sp.), which are

known from the accessory genital glands of males of all hard tick species (Feldman-Muhsam, 1970, 1974). Their possible involvement 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 a bacteriostat and fungistat. Nystatin is an inhibitor of ergosterol synthesis and as such has an affinity for cholesterol, an essential tick nutrient. However, the amount of unbound cholesterol remaining in the blood was estimated as sufficient for normal tick development. This opinion is backed up by the fact that the feeding performance of females in other studies (Voigt *et al.*, 1993), where no antimycotic agent was used in the nutrient, was not superior to ours.

There could be a simple mechanical reason for the reduced *in vitro* body mass of females: feeding ticks attach to the membrane near the glass tube. During the dramatic increase in body volume in the final phase of the blood-meal they might involuntarily pull the mouthparts through simple expansion of the body back into the 0.5 mm thick membrane before reaching the normal engorged weight. Feeding trials with adults on thinner membranes with similar mechanical properties could resolve this question.

Application of the *in vitro* feeder for assay of acaricides proved successful. Ivermectin®, first developed for the control of nematodes, has known acaricidal activity (Kaufman, Ungarian & Noga, 1986; Campbell, 1989). This was confirmed here. The advantage of the *in vitro* assay over *in vivo* tests with systemic agents is the presence of more controlled conditions, and less variability with a standardized nutrient. Effects arising from variability between individual test animals can complicate results in *in vivo* assays.

An improvement in the reproductive capacity of adults needs to be achieved so as to extend the rearing trials to other ixodid species. In order to reduce the use of vertebrates in tick research, the *in vitro* feeding system should be introduced into the relevant laboratories, possibly in parallel with traditional tick-rearing at the outset. Refinement or automatization of certain steps such as blood-changing would help with the transition to *in vitro* rearing.

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