An *in vitro* feeding assay to test acaricides for control of hard ticks

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Abstract: Animal husbandry could not be practised over large areas of the planet without acaricides. The prevention of tick bite and the transmission of diseases requires the use of pesticides, but this contributes to the development of tick resistance against acaricides. This drives the quest for new molecules that target physiological processes crucial to tick survival. *In vivo* trials involve multiple repetitions because of inherent variations between host animals, requiring large amounts of test products and ticks. An *in vitro* alternative should permit the testing of the ability of a product to restrict attachment and feeding by ticks at precise doses. In this paper an *in vitro* feeding system is described where the European tick *Ixodes ricinus* L. feeds on blood through a cellulose rayon-reinforced silicone membrane. The membrane Shore hardness is modified to imitate the elastic retraction forces of skin that ensure the closing of tick penetration sites on the membrane to prevent bleeding. Tick attachment (75–100%) is achieved by adding chemical and mechanical stimuli to the membrane. Survival curves for different doses of fipronil and ivermectin tested with the method showed highly reproducible acaricide effects within 5–7 days. Significant effects are recorded down to ppb levels in blood. Standardised tests can be made with blood from the same donor animal or culture medium under the membrane.
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1 INTRODUCTION

Ticks affect the health of livestock through the direct effects of blood feeding and by transmitting parasitic diseases. Major pest species include *Rhipicephalus (Boophilus) microplus* Canestrini, *Rhipicephalus appendiculatus* Neumann and *Amblyomma* spp., which transmit babesiosis, theileriosis and heartwater disease respectively. Animal husbandry could not be practised over large areas of the planet without acaricides. This persistent reliance on pesticides has led to the development of resistance in ticks against the major classes of acaricide treatments.[1–3] There is a continual requirement for new types of molecule to target physiological processes crucial to tick survival. The development of animal health products against ticks requires hundreds of cattle, dogs, rabbits and gerbils for *in vivo* trials with acaricides. The use of animals in acaricide research and maintaining suitable hosts for tick rearing add considerably to the costs of developing new acaricides.

Tick control on animals is achieved either through contact with a topically applied product or via ingestion of a systemic acaricide. *In vivo* trials of new products with ticks require multiple repetitions owing to the variation between individual hosts. This approach requires large amounts of products: for a dog of 10 kg, some 100 mg of a product is needed, further contributing to the high costs of trials for developing novel animal health products. An *in vitro* alternative should reflect the *in vivo* situation but allow the dosing of small amounts of a test product under controlled conditions. This assay should permit assessment of the capacity of the product both to affect tick attachment for a blood meal and to restrict feeding once a tick has started to take blood.

In contrast to other arthropods such as tsetse flies, mosquitoes and triatomine bugs, where taking the blood meal can last from just a few seconds to tens of minutes, hard ticks can remain firmly anchored to the feeding site for periods of 2–14 days. Attachment by ticks at predilection sites on hosts is preceded by a behavioural sequence that depends on the presence of an appropriate array of mechanical, olfactory and contact chemostimuli.[4] When ticks start to penetrate the skin with their mouthparts, they enter the uppermost keratin-rich stratum corneum with outward lacerating movements of their cutting mouthparts. Strong retrograde food canal denticles anchor the tick in the skin, allowing the cutting mouthparts to move deeper until the corium containing blood vessels is reached.

Feeding ticks on blood through animal-derived or artificial membranes has long since been used to rear soft ticks.[5] Early attempts to feed hard ticks through animal gut membranes (baudruche) by Kemp *et al.*[6] were followed by success with silicone membranes,[7] culminating in the *in vitro* rearing of all life stages of *Amblyomma hebraeum* Koch.[8,9] In the present study the membrane developed by Kuhnert *et al.*[8] has been modified to feed *Ixodes ricinus* L. adults to repletion *in vitro* on blood. The suitability of this modified system to measure the systemic effects of the acaricides fipronil and ivermectin on feeding and

survival of *I. ricinus* and the effect of applying fipronil and permethrin to the silicone membrane to which *I. ricinus* must attach itself to feed is described.

2 MATERIALS AND METHODS

2.1 Silicone membranes

Ixodes ricinus was fed on bovine blood through a silicone membrane reinforced by Kodak® lens cleaning paper (Eastman Kodak, Rochester, NY, USA), as already described.[8,10] This membrane was modified to facilitate attachment by *I. ricinus* by rendering the silicone softer. For this, a silicone glue was selected with a low Shore A hardness (expressed in degrees) – a measure of the indentation hardness of soft materials. Here, use was made of the silicone glue RTV-1 Elastosil® E4 (Wacker, Burghausen, Germany) with a very low Shore A hardness of about 16°. Admixture of silicone oil (30% DC 200, viscosity ~10 mPa s; Fluka, Switzerland) to the silicone glue further increased softness and reduced the 'frog grip' – the sticky nature of the resulting silicone surface. Pieces of Kodak lens cleaning paper (70 × 120 mm), a non-woven tissue made of regenerated cellulose (rayon), were placed on a layer of kitchen plastic film and impregnated with the silicone mixture which was rendered more fluid for application by adding 150 g kg^{-1} hexane. Excess silicone glue was removed with a 80 mm wide scraper made from a piece of silicone sheet (3 mm thick). Membranes were left to polymerise for about 24 h in advance of gluing them to the feeding tubes (Section 2.2). The thickness of the membranes was measured, and only those of 50–100 μm were used.

2.2 Feeding units and attachment stimuli

The feeding units were made of either acrylic glass tubing (Plexiglas®; Röhm GmbH & Co. KG, Darmstadt, Germany) (26 mm ID, 2 mm wall thickness, 45 mm high) or polystyrene (24 mm ID, 1.25 mm wall thickness, 40 mm high). A ring was fitted around each tube to limit the depth (4 mm) to which the unit sank into the blood in the wells (Fig. 1). The feeding membrane was attached to the bevelled (1°) lower end of the tube using silicone glue (Elastosil® E4; Wacker, Burghausen, Germany) and left to dry (for a minimum of 3 h). Following this, the membranes were cut flush with the wall of the tube and checked for leaks in a petri dish with 70% aqueous ethanol. The criterion was no entry of solvent after 20 min with the ethanol rising to ca. 7 mm around the feeding unit.

The following items were used to improve attachment of the ticks to the membrane. A piece of glass fibre mosquito netting (1.4 mm mesh, 24 mm diameter) was glued to the membrane in the feeding unit with silicone glue (Wacker Elastosil E4) and left to dry (Fig. 2). A plastic cross (2 mm thick tile spacer) was placed on the membrane so as to create additional borders where ticks prefer to attach (Fig. 1). A cow hair extract (0.5 mg lipid extracted from freshly shaven cow hair and dissolved in 75 μL dichloromethane) was
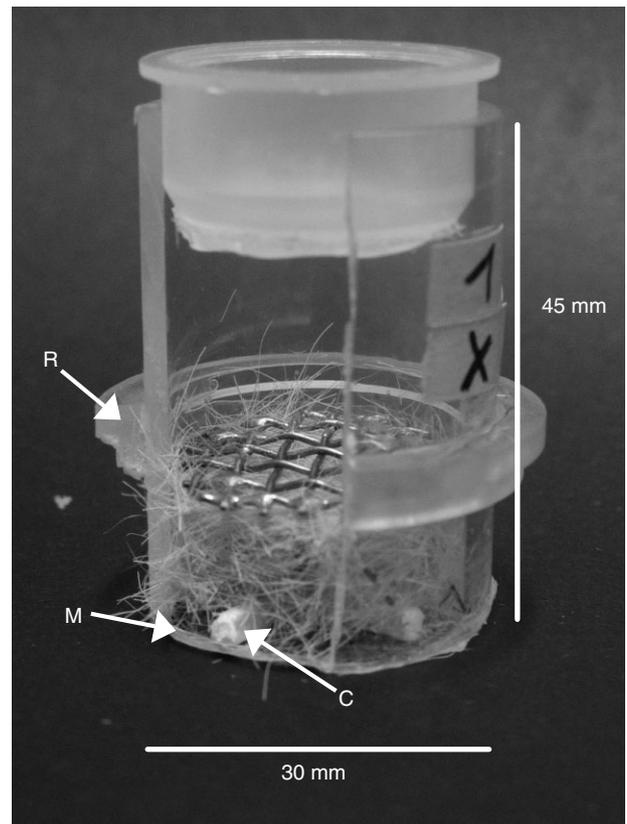

Figure 1. Cut-out view of the *in vitro* feeding unit for *Ixodes ricinus*, made of acrylic glass tubing. Part of a plastic cross (C) placed on the membrane (M) is visible. The cow hair placed on the membrane is held down with a brass grid. The ring (R) around the unit ensures that 2 mm of blood lies under the membrane when placed in a well. A perforated plastic stopper is inserted on top to confine the ticks.

applied to the membrane and the solvent was allowed to evaporate for 15–30 min on a hot plate at 40 °C.

The feeding units were placed in six-well cell culture plates (34.8 mm diameter) (Costar, Schiphol-Rijk, The Netherlands) with 3.1 mL test blood and warmed to 37 °C using a thermostatted water bath (740 mm long ×540 mm wide ×215 mm high) with a tilted acrylic glass cover to keep the air above the feeding units near 100% RH. The six-well plates with the feeding units sat on a metal screen submersed 15 mm below the water surface in the bath. The bath was kept in a windowless chamber with a 16:8 h light:dark cycle.

Ten female and five male *I. ricinus* from an in-house rearing (all unfed, 3–6 months old, kept at 20–23 °C, 85–98% RH and long day conditions) were put into each feeding unit with soft forceps, covered with a 1 cm layer of cow hair cut to about 5 mm length, and the ensemble was held down with a brass grid (25 mm diameter, 3 mm mesh, weighing 1.1 g). Each feeding unit was closed with a perforated stopper (0.5 mm polyester mesh) (Fig. 1).

2.3 Blood

Blood was collected weekly from an abattoir, defibrinated manually, supplemented with 2 g L^{-1} glucose and stored at 4 °C.[8,10] All blood preparation
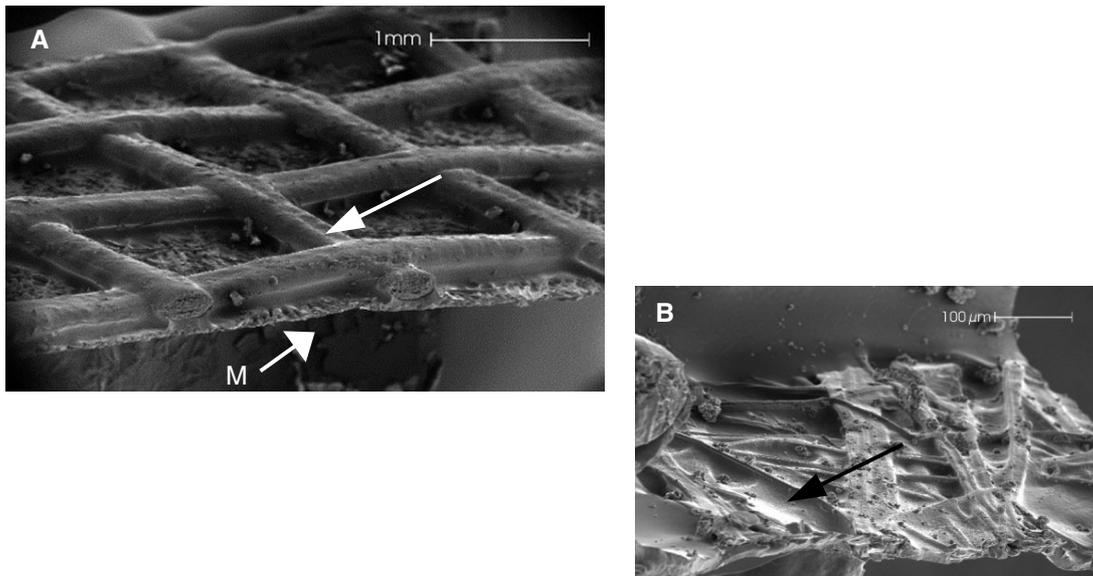
Figure 2. (A) Scanning micrograph of a feeding membrane (M) for *Ixodes ricinus* females with mosquito netting glued to it. A minimum quantity of glue was used to attach the netting to the membrane so as to leave cavities (arrow) which allow ticks to obtain a perch with their mouthparts in the matrix. (B) Spaces between the cellulose fibres of the lens cleaning paper are only partly filled with silicone, providing small regions where the membrane is even thinner (arrow) than the thickness of the paper.

was done on a clean bench (Scan USE-2000-120). Commercial gentamycine solution ($5\,\mu g\,mL^{-1}$) (Sigma, Germany) and ATP ($10^{-3}\,mM$ in the blood) (Fluka, Switzerland) were added to the blood just before it was filled into the wells. The well plates were covered and warmed to $37\,^\circ C$ in the water bath prior to adding the feeding units. In all experiments, blood was changed at 12 h intervals in each well and the membrane surface in the blood was rinsed with sterile saline (sodium chloride p.a., Fluka, in demineralised water) before placing the feeding unit in a fresh well. Fungal infections of the membrane occurred only rarely (<10%) and were treated daily with Nystatin solution ($10\,K$ units mL^{-1} DPBS) (Sigma, Germany) for about 10 min during the blood exchange when the daily evaluation of ticks was made.

2.4 Treatments tested
Blood treatments were control (nothing added to the blood), placebo [dimethyl sulfoxide (DMSO) at $2.5\,\mu L\,mL^{-1}$ blood] (Fluka, Buchs, Switzerland) and fipronil (Pestanal®; Riedel de Haën, Seelze, Germany) or ivermectin (Sigma-Aldrich GmbH, Steinheim, Germany) at 0.001, 0.01, 0.1, 1 or $10\,\mu g\,mL^{-1}$ blood dissolved in DMSO at $2.5\,\mu L\,mL^{-1}$ blood. Four feeding units were used for each treatment. After 24 h the ticks were evaluated once a day to count those living and dead attached to the membrane as well as the unattached living and dead ticks; the latter were removed from the feeding units. Feeding experiments terminated after 9 days and the weight of individual female ticks or a representative sample of a group ($n > 10$) was determined.

Treatments applied on to the membrane were dichloromethane alone, permethrin (1 ng and $100\,\mu g\,cm^{-2}$) (Pestanal®; Riedel de Haën, Seelze, Germany) and fipronil (10 ng and $1\,\mu g\,cm^{-2}$) (as the commercially formulated spot-on Frontline®; Merial, Munich, Germany). Three feeding units were used for each treatment. These experiments ended after 30 h and ticks were evaluated as above.

2.5 Statistical analysis
Survival curves were calculated from the numbers of dead ticks recorded per day over the different doses of each treatment using Kaplan–Meier statistics[11] with Peto test of the survdiff algorithm in S-plus (V6.2 build 6713, Insightful). The same software package was used for all other statistics presented.

3 RESULTS
3.1 *Ixodes ricinus* feeding *in vitro*
The median attachment rate of female *I. ricinus* in the controls, where only the feeding stimuli were added to the blood, was 77%, and 54% of these females were alive and feeding after 9 days. In the placebo, a median of 85% of females attached to the membrane (minimum 78%, maximum 100% per feeding unit), indicating that DMSO had no effect on *I. ricinus* survival *in vitro* ($P = 0.34$). Mortality data obtained for the placebo-fed ticks in successive experiments were reproducible from feeding units made of either polystyrene or acrylic glass ($P = 0.09$, survival statistics, see Fig. 3). After 9 days, 60% of females were still alive and feeding in control and placebo feeding units. The median weight of engorged detached females by day 16 in the placebo was 160 mg (25–279 mg, $n = 19$), comparable with that for females fed on rabbit ears in the in-house rearing; 15 of these laid eggs and larvae hatched within 4–8 weeks. Here, the control and placebo survival data are pooled and referred to hereafter as controls.
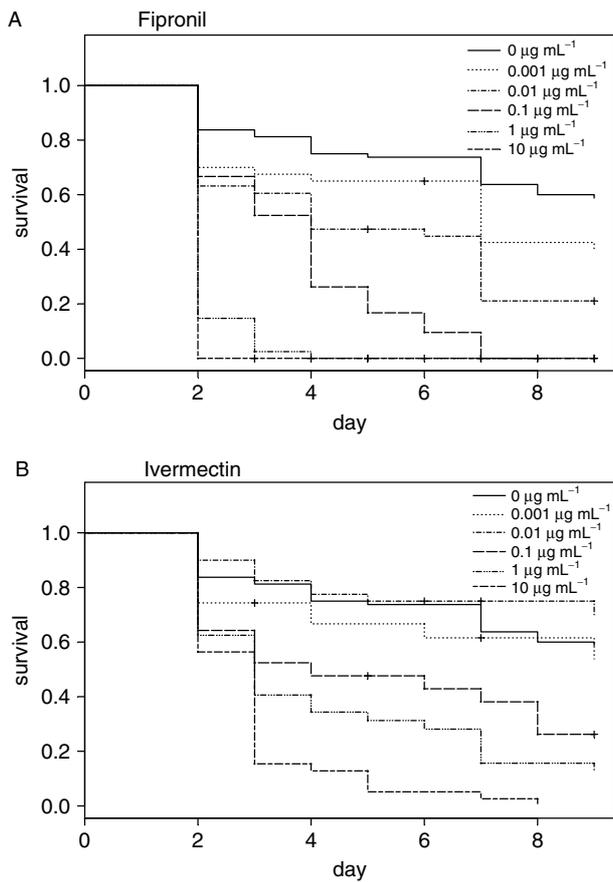
Figure 3. Survival curves for *Ixodes ricinus* females feeding *in vitro* over 9 days through a silicone membrane on bovine blood treated with different doses of (A) fipronil or (B) ivermectin. Compared with the cumulated survival data from controls, fipronil significantly affected mortality at all doses tested ($P \leq 0.0001$ for doses $\geq 0.01\,\mu g\,mL^{-1}$ blood, $P = 0.05$ for $0.001\,\mu g\,mL^{-1}$). Ivermectin showed a clear effect at doses of $0.1\,\mu g\,mL^{-1}$ of blood and higher ($P < 0.0001$ for doses $\geq 0.1\,\mu g\,mL^{-1}$ blood, $P > 0.2$ for 0.01 and $0.001\,\mu g$ ivermectin mL^{-1}).

3.2 Effects of acaricides tested as systemics *in vitro*

Fipronil at $10\,\mu g\,mL^{-1}$ blood killed all females within 2 days, at $1\,\mu g\,mL^{-1}$ blood no females survived longer than 4 days and at $0.1\,\mu g\,mL^{-1}$ blood all females were killed by day 7 (Fig. 3A). At the lowest doses of 0.01

and $0.001\,\mu g$ fipronil mL^{-1} there still was an effect of the acaricide, with 50 and 30% mortality by day 4 respectively (Fig. 3A). An effect of fipronil on tick survival was still observed at 1 ppb ($0.001\,\mu g\,mL^{-1}$ blood), with 60% mortality after 9 days ($P = 0.051$, survival statistics). The effects of 0.1, 1 and $10\,\mu g$ fipronil mL^{-1} on tick mortality were reproductible between experiments (Fig. 4). Similar effects of fipronil were obtained with *I. ricinus* from different laboratory rearings. Survival analysis indicates significant effects of the different doses of fipronil tested, and the salient data were obtained in the first 5 days of the assay (Fig. 3A). *Ixodes ricinus* females surviving at the lowest doses of fipronil reached 65% of the weight of the placebo. Only females feeding on $0.001\,\mu g$ fipronil mL^{-1} blood laid eggs, but none of these hatched.

The effect of ivermectin was clearly less than that of fipronil: $10\,\mu g$ ivermectin mL^{-1} blood killed all female *I. ricinus* only after 9 days compared with 100% mortality of all females feeding on fipronil-treated blood at this dose by day 2 (Fig. 3B). At $1\,\mu g$ ivermectin mL^{-1} blood some 18% of females survived until day 9, whereas the same dose of fipronil killed all ticks within 4 days. A clear effect of ivermectin was observed down to $0.1\,\mu g\,mL^{-1}$ blood, with 50% mortality by day 4 (Fig. 3B), and the surviving females reached a median weight of 8 mg, i.e. only 20% of that of the placebo on the same day. Mortality at 0.01 and $0.001\,\mu g$ ivermectin mL^{-1} of blood was not different from the control. Eggs were only produced by females fed on blood containing $0.001\,\mu g$ ivermectin mL^{-1}, and larvae hatched. Overall, the percentage of dead ticks attached to the membrane compared with the percentage of dead ticks that had detached varied between 8 and 63%, but showed no correlation with a treatment or dose. Therefore, no special detaching effect of the acaricides dissolved in the blood could be established in these assays.

3.3 Effects of acaricides applied to the membrane

Fipronil applied to the membrane strongly affected tick survival (Fig. 5). The mortality increased from
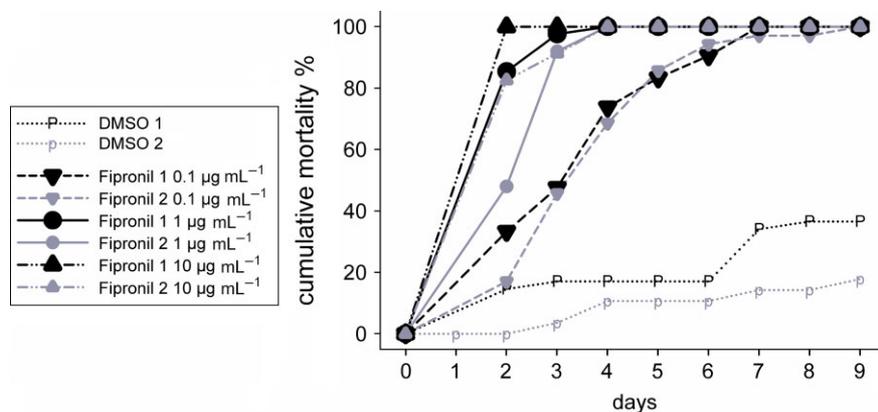

Figure 4. Plots showing the reproducibility of mortality curves for *Ixodes ricinus* females feeding *in vitro* over 9 days through a silicone membrane on bovine blood treated with different doses of fipronil, and repeated a year later. The mortality curves for repetitions with the same dose of fipronil (same line, shade and symbol type) are almost identical.

19% in the control to 69% at 10 ng fipronil cm^{-2} ($P \leq 0.001$, Fisher's exact test). Fipronil at 1 µg cm^{-2} on the membrane killed all ticks, and they were found mostly unattached ($P \leq 0.0001$, Fisher's exact test). This dose also killed the ticks more readily compared with adding the same amount to 1 mL of blood fed on by the ticks. Analysis of the tick weights revealed that almost all females on the membrane treated with 1 µg fipronil cm^{-2} were killed without taking any blood. By contrast, permethrin at 1 ng cm^{-2} on the membrane did not affect *I. ricinus* survival within the 30 h of the test ($P \geq 0.7$ compared with the control, Fisher's exact test) nor the attachment rate compared with the control (control 73%, permethrin 72%). At a 10^5 times higher dose (100 µg permethrin cm^{-2} membrane) all ticks were killed and found mostly unattached ($P \leq 0.0001$, Fisher's exact test) (Fig. 5).

4 DISCUSSION

In the *in vitro* feeding system described here, a silicone membrane replaces host skin to provide the tick with a perch over blood (Fig. 6). The membrane used to feed *I. ricinus* is an improved version of one developed to feed *A. hebraeum*.[8,10] The membrane was made softer and thinner (60–150 µm) to facilitate piercing by *I. ricinus* adults with their shorter hypostomes (females 0.5 mm, males 0.28 mm) (Fig. 6) compared with *A. hebraeum* adults (1–1.5 mm). The median attachment rate of 80% for *I. ricinus* females in individual feeding units recorded here is highly satisfactory
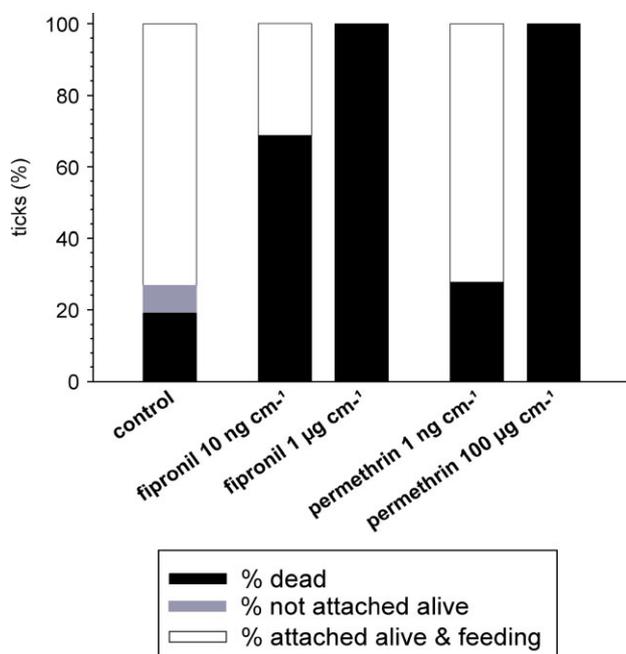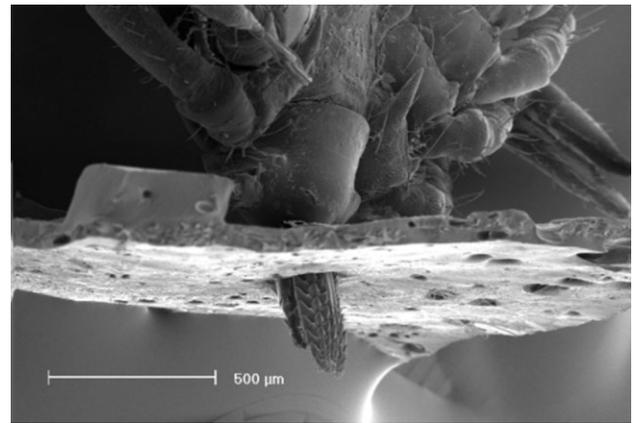

Figure 6. An *Ixodes ricinus* female attached to the silicone membrane. Half of the 500 µm long hypostome has pierced the membrane.

compared with the 32–74% attachment rate for *A. hebraeum* females on another silicone membrane.[8] Even with eight- to ten-month-old *I. ricinus* females the attachment rate still approached 75%. The softer membrane permits the tick to withdraw its mouthparts to reattach elsewhere as the previous penetration site closes by elastic retraction forces in the membrane, preventing blood leaking into the unit. Females regularly left their feeding location to reattach elsewhere, and males were also regularly observed attached on the membrane. This underlines the suitability of this membrane for penetration by tick mouthparts and even for reattachment by ticks in a semi-engorged state. Males were also frequently observed copulating with females attached to the membrane. In spite of some mortality recorded in controls, most females engorged and produced eggs from which larvae hatched. The mechanical stimuli provided in the feeding unit, especially the plastic tile spacer, serve to increase borders where the ticks prefer to attach. This also obviates clumped attachment by ticks which can lead to a risk of leak through the membrane. The contribution of each of the attachment stimuli employed here still needs to be clarified and possibly simplified.

4.1 Advantages of the *in vitro* feeding assay

In vivo trials of disease vector control agents require repetitions owing to the inherent variation between individual animals. Furthermore, large amounts of products and ticks are needed for such tests. By contrast, valid *in vitro* assays should adequately quantify dose effects of tick control products.[12] Feeding inhibition was achieved here through direct toxic effects of fipronil and ivermectin on the feeding ticks with just a fraction of the quantity needed for an *in vivo* test: a total of 5 mg fipronil was used for a test over 9 days at four dose levels, i.e. just 5% of what is required to be effective against ticks on one dog. In addition to mortality, a knockdown effect of fipronil at 1 and 10 µg mL^{-1} blood was noted where ticks were found with trembling legs 1–2 days before the same

Figure 5. Effects of fipronil and permethrin applied to a silicone membrane through which *Ixodes ricinus* females could feed on bovine blood *in vitro* for 30 h. The different doses of the acaricides strongly affected the proportions of dead ticks (attached and not attached, black bars) and alive ticks (attached, white). Fipronil at 1 µg cm^{-2} and permethrin at 100 µg cm^{-2} killed all ticks. Fipronil at 10 ng cm^{-2} still had an effect, whereas permethrin at 1 ng cm^{-2} showed no effect compared with the control.

individuals were recorded as dead on the membrane. Fipronil is a GABA-gated chloride channel blocker that disrupts ion flow, causing CNS hyperexcitation.

The clear dose effects of the two acaricides permitted a valid comparison of their efficacy. Survival curves calculated over the different doses of fipronil and ivermectin in different feeding experiments showed that the acaricidal effects were highly reproducible and that the salient data were obtained within the first 5 days (except for the effect of $0.001\,\mu g$ fipronil mL^{-1} blood which became significant only after 9 days). In addition, just 40 female ticks were required per dose of product tested. This *in vitro* assay also permits tests under standardised conditions with blood from the same animal. The choice of material used for the feeding units should be considered, as adsorption of low doses of a test molecule on the walls may influence the effective dose to which the ticks are exposed.

Application of an acaricide to the surface of the membrane in this feeding unit also permits assessment of the capacity of the product to affect tick attachment for the blood meal. Fipronil and permethrin are toxic to ticks via contact and are available as commercial formulations for application to animals as spot-ons in the fur. Fipronil at $1\,\mu g\,cm^{-2}$ killed all ticks within 30 h, even more readily than by adding the same amount to 1 mL of blood fed on by the ticks. Cloth impregnated with $4-250\,\mu g$ permethrin cm^{-2} kills *I. ricinus*[13] in accordance with the strong mortality observed here with the pyrethroid applied at $100\,\mu g\,cm^{-2}$ to the membrane.

This paper has described a standardised *in vitro* feeding method for a hard tick that can be used as an assay system for acaricides. This assay yields highly reproducible effects of test products down to ppb levels in blood.

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