

# *In vitro* feeding assays for hard ticks

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**Prevention of tick bites and transmission of tick-borne pathogens requires the use of molecules that target physiological processes crucial to both tick and pathogen survival. These molecules are best tested in standardized *in vitro* assays. Because hard ticks require several days to feed to repletion, the development of *in vitro* feeding assays for these species is challenging. A standard and easily automated feeding assay has been developed for the tick *Ixodes ricinus* that involves feeding on blood through a membrane that mimics the elasticity of skin. The system can be adapted to feed other hard tick species *in vitro*. This assay permits, among others, investigations on the role of tick endosymbionts on tick survival, the identification of potential vaccine candidates and drugs, and the application of genomic tools *in vitro*, including RNA interference experiments.**

## Why feed ticks *in vitro*?

The development of animal health products against ticks requires hundreds of cattle, dogs, rabbits and gerbils for *in vivo* trials with acaricides, placing the annual worldwide use of animals in acaricide research in the tens of thousands. Small mammals used in such trials can suffer from skin inflammation and anaemia, and can be submitted to restrictions by the Elizabethan collar inhibiting grooming behaviour. For controlled studies, dogs have to be kept in small cages and cattle are kept in climatic boxes where their movement is confined. Apart from the ethical aspects of using experimental animals, the costs of maintaining suitable hosts for ticks are high. An *in vitro* alternative to *in vivo* trials should aim to provide key elements of the tick–host interface and allow an adequate assessment of test products.

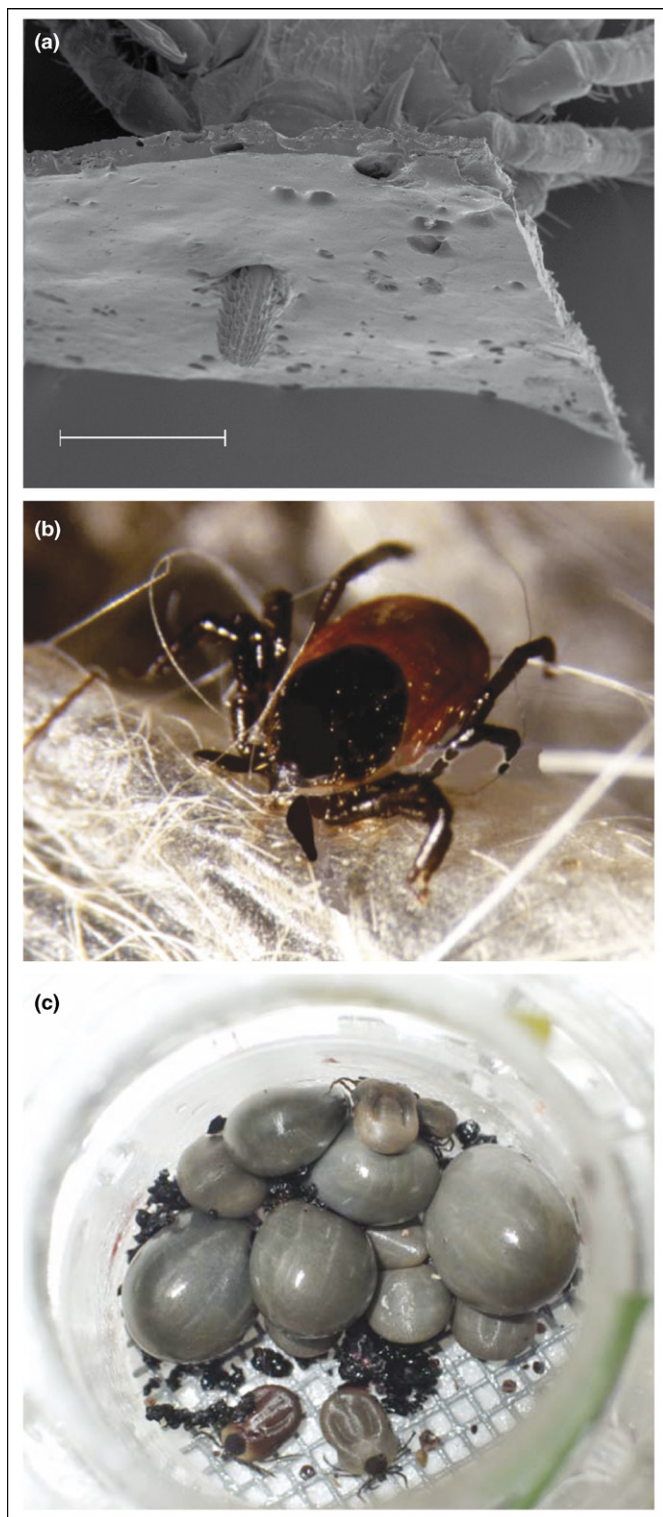
## Parameters affecting ticks feeding *in vitro*

In contrast to tsetse flies and mosquitoes, where the bloodmeal takes only a few seconds (tsetse) to minutes (mosquitoes), tick engorgement can take from a few to ten days, depending on the life stage (larva, nymph or adult). Furthermore, attachment by ticks at feeding sites on the host depends on an appropriate array of chemical and physical stimuli [1]. Ticks find a hold in skin, in particular the keratin-rich stratum corneum, using their retrograde rostral denticles (Figure 1a) as an anchor. After attachment (Figure 1b), the female tick establishes a feeding lesion during the first few days and often secretes a cement-like cone to securely attach to the skin (Figure 2). During the

following slow-feeding phase (4–7 days), the female feeds up to 10 times her unfed weight and synthesizes the cuticle that serves to accommodate the third phase of the bloodmeal [2]. Final engorgement occurs during the last 24–36 hours of rapid feeding when the tick imbibes 2–8 times as much blood as it finally gains in weight, and can multiply its unfed body mass approximately 100-fold with protein- and lipid-rich nutrients for the production of thousands of eggs [3,4]. Ideally, an *in vitro* feeding assay should permit both the testing of products that affect a tick's capacity to attach for a bloodmeal, or restrict feeding and transmission of pathogens once the tick has started to take blood. To be reliable, the *in vitro* system must include an appropriate array of attachment stimuli, a non-biodegradable and elastic membrane, and an adequate nutrient supply. Because ticks do not all feed at the same rate (Figure 1c), there is an additional challenge to provide a system that permits some ticks to feed and detach when replete, while allowing others to continue to feed to repletion without any bleeding from neighbouring detachment sites. Such bleeding could cause drowning of the remaining ticks and permit infection of blood exposed at 37 °C. In summary, an *in vitro* feeding assay must reflect the *in vivo* situation to permit tests with physiologically relevant levels of test products under controlled conditions.

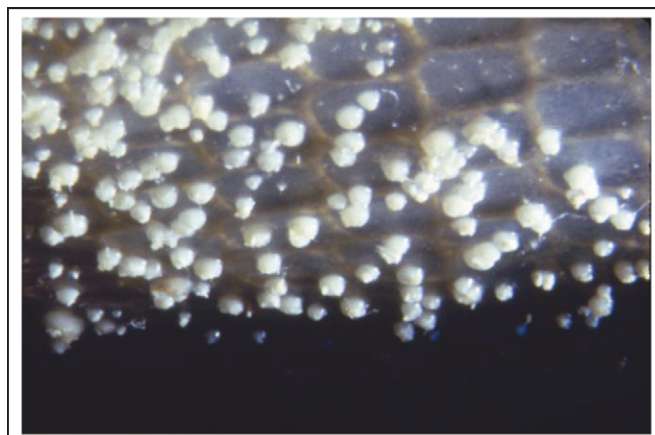
## A brief history of tick-feeding assays

Feeding ticks on blood through animal-derived or artificial membranes, for example Parafilm<sup>®</sup>, is a long-established means of rearing soft ticks and for investigating soft-tick-transmitted pathogens [5,6]. Hard tick feeding across animal-derived membranes is well documented. In 1956, Pierce and Pierce fed *Boophilus microplus* larvae through the air cell membrane of an embryonated hen egg [7], and in 1975, Kemp *et al.* [8] achieved >50% moult by engorged *B. microplus* larvae fed on tissue culture medium through skin slices of cattle [9]. Following on from Waladde's [10] use of a biodegradable glue-impregnated Baudruche membrane, the introduction of silicone membranes for hard ticks in 1993 by Habedank and Hiepe [11] led to the next advances [12–14]. These membranes permitted high tick attachment rates, engorgement, detachment, moulting of different life stages and oviposition. It was on such a silicone-impregnated membrane that Kuhnert *et al.* completed the life cycle of *Amblyomma hebraeum* *in vitro* in 1995 [13,14]. Subsequently this membrane was modified to accommodate ticks with shorter mouthparts (500 µm in the case of female *Ixodes ricinus* ticks) (Box 1) [12]. A further improvement was the introduction of the elastic characteristic of skin into the membrane structure. It is



**Figure 1.** (a) *Ixodes ricinus* female piercing the silicone-impregnated membrane with its hypostome. Scale bar, 500  $\mu$ m. (b) An attached *I. ricinus* female and (c) nearly engorged *I. ricinus* females in a feeding unit. The un-engorged ticks can readily reattach to complete the blood meal.

this trait that allows skin to resume its natural shape after indentation or injury – a factor that contributes to closing of lesions [12]. Semi-engorged ticks can even withdraw their mouthparts and reattach elsewhere (Figure 1c). This silicone-impregnated membrane provides ticks with a perch over blood for as long as they require to feed to repletion (Figure 1c).



**Figure 2.** Cement cones produced by *Amblyomma hebraeum* that adhered to the underside of the artificial membrane through which they had attached to feed. Subsequent removal of these cement cones from the membrane for analysis demonstrates how products secreted by ticks into the feeding lesion can be selectively recovered *in vitro*.

### An *in vitro* feeding assay for life stages of ixodid tick species

The basic factors ensuring attachment and feeding on blood through a silicone-impregnated membrane have been resolved in this laboratory for two hard tick species, *A. hebraeum* and *I. ricinus* adults [12,13]. Following the success of feeding of *I. ricinus* adults (median attachment rate of 85%, range of 78–100% per feeding unit), larvae and nymphs of this species were fed to repletion on a thinner silicone-impregnated membrane (Box 1), and most of these juveniles moulted to the next life stage. After 9 days, 60% of females were still alive and feeding, 75% of these engorged ticks laid eggs, and larvae hatched within 4–8 weeks. The same attachment stimuli were used for the different life stages of each species, namely the aggregation-attachment pheromone in the case of *A. hebraeum* [15] and a cow-hair extract for *I. ricinus* life stages [12]. A comprehensive review of the stimuli (chemical and physical) used by different workers to enhance attachment by hard ticks to membranes was compiled by Kuhnert in 1996 [16]. Glucose was added to the blood to stabilize erythrocytes and ATP was added as a general tick-feeding stimulus. It is becoming clear from experience that some common principles apply to the nature of the stimuli required to achieve good attachment and engorgement of the hard ticks. Furthermore, only minor modifications are required in the artificial membranes to accommodate feeding by different tick species and life stages *in vitro*. For example, the hypostome lengths for all life stages of *B. microplus* and *Rhipicephalus sanguineus* are similar to those of *I. ricinus*, suggesting that the existing method can be readily adapted for these important tick species (Box 1).

The development of a common *in vitro* assay for hard ticks would permit comparison between species under standardized conditions because the placebo and test products can be assayed in blood from a donor animal or in an artificial nutrient medium. This evidently serves to reduce variance in the data.

### Applications of *in vitro* feeding assays for research on ticks

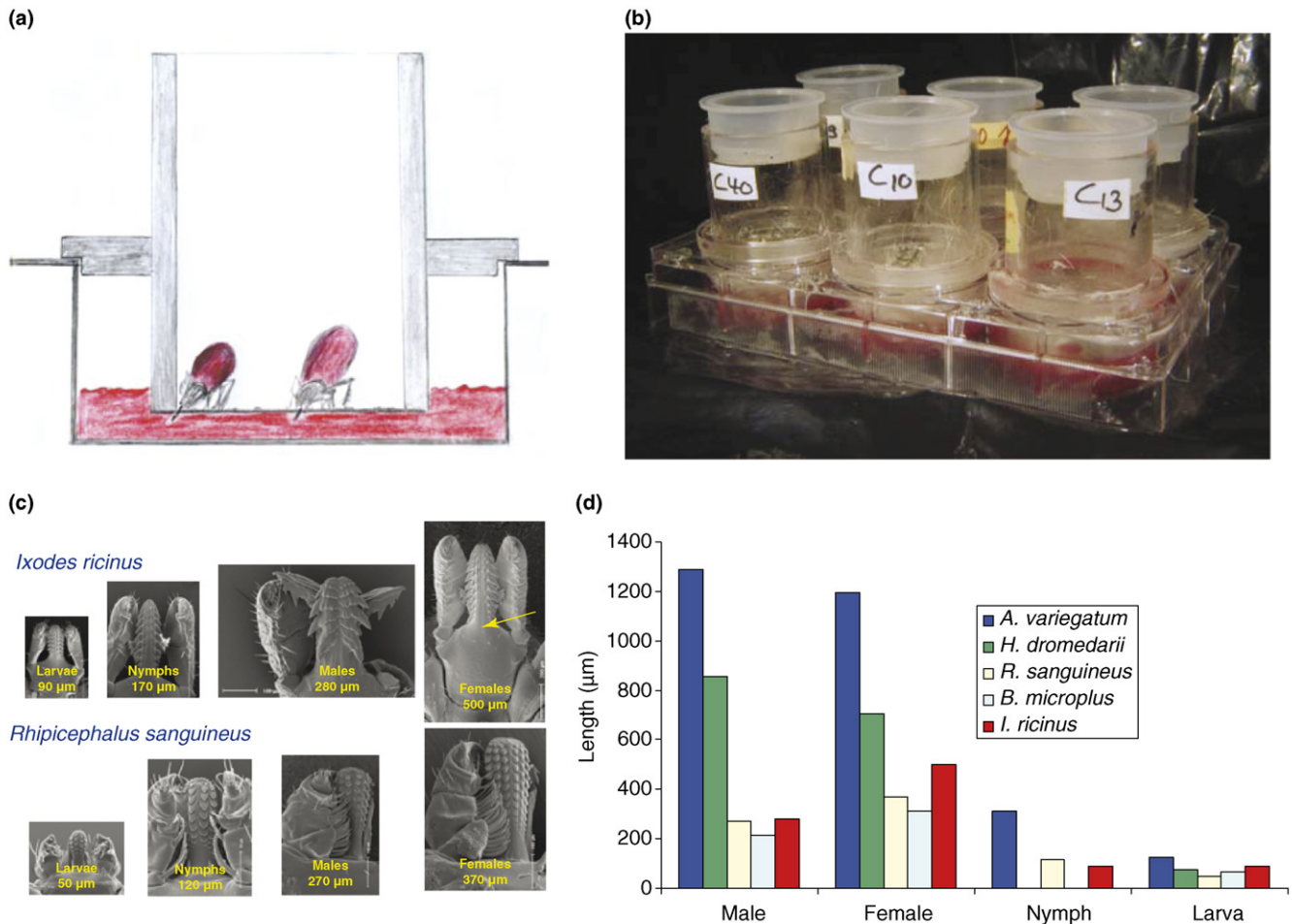
Hard tick *in vitro* feeding assays can be used to carry out refined investigations on a variety of pertinent topics.

### Box 1. The feeding unit

A feeding unit (Figure 1a) consists of a silicone membrane reinforced with cellulose rayon stretched across one end of acrylic glass tube (44 mm high and 26 mm in diameter). Feeding units are set up in six-well plates with 3 ml of blood applied per well (Figure 1b). The temperature of the blood is kept at body temperature during feeding. The use of six-well plates renders the system amenable to automation and this has the added advantage of running the assays under sterile conditions. A combination of chemical and mechanical stimuli is applied to the membrane [12]. The physical properties of the membrane are very important: the particular combination of cellulose rayon fibre and silicone results in a membrane with a low Shore hardness that mimics the elasticity of skin to ensure closure of tick penetration sites on the membrane to prevent bleeding [12].

Ten female and five male *I. ricinus* can be put into each feeding unit. Detached ticks (by themselves or with forceps) can readily reattach to

complete the blood meal. For example, ticks removed for weighing to measure the effect of a treatment on engorgement rate can be returned to continue feeding as their removal from the membrane causes no damage to the mouthparts. The thickness of the membrane must be adapted to the hypostome length of the species and life stage in question. The hypostome, or food canal, is composed of the chelicerae and rostrum, and is flanked by the palps that remain splayed against the skin when the tick is feeding (see Figure 1b in the main text). The hypostome length is defined as the distance from the tip of the rostrum to the depression containing two intracuticular mechanoreceptors at the junction with the basis capitulum (indicated by the arrow on the adult female *I. ricinus*, Figure 1c). There is a greater need to modify the membrane thickness to accommodate the different hypostome lengths of adult stages of some of the economically important tick species than for the hypostomes of the nymphs and larvae (Figure 1d).



**Figure 1.** (a) An outline drawing of the feeding assay, that is, the feeding unit with ticks over a reservoir of blood. (b) A multi-well plate holding blood under six feeding units. (c) Electron micrographs showing hypostome lengths (in µm) of the three life stages of *Ixodes ricinus* and *Rhipicephalus sanguineus*. (d) Measurements of hypostome lengths of life stages of the hard tick species *Amblyomma variegatum*, *Hyalomma dromedarii*, *R. sanguineus*, *Boophilus microplus* and *I. ricinus* were made from scanning micrographs (median values;  $n = 1-14$ ). Interspecific variability in larval hypostome lengths is lowest. No data are presented for nymphs of *H. dromedarii* and *B. microplus*. The *B. microplus* larval value is derived from W.K. Jorgensen, PhD thesis, University of Queensland, Australia, 1984.

Details of the complex pharmacology of tick saliva [17,18] can be examined as the secretion is readily collected in the feeding medium beneath the membrane (Figure 2). This pharmacopoeia is used by these ectoparasites as an anti-haemostatic, to combat the host's inflammatory responses and to manipulate the host's immune response. Elucidation of the structures of biologically active substances secreted in tick saliva can lead to the development of

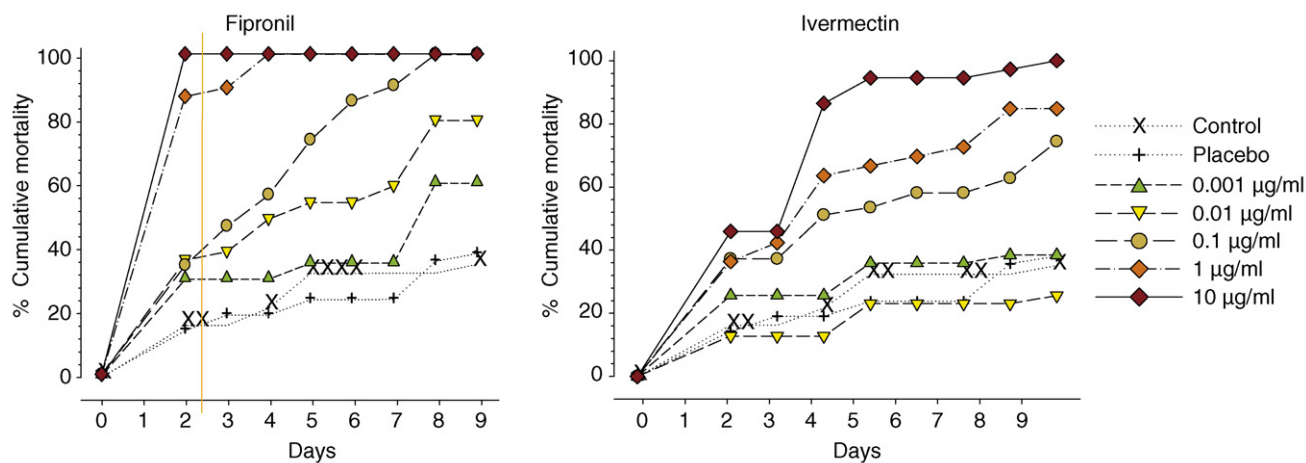
new drugs [19] (Box 2). Use of a defined nutrient medium would facilitate the isolation of such biologically active substances. Feeding assays could also be used for studies on the dynamics of pathogen transmission – from the nutrient medium to the tick, from the tick to the medium, and between infected and uninfected ticks feeding in the same feeding unit – without having to take into account parasite–host–pathogen interactions. The contribution of

## Box 2. Applications of an *in vitro* feeding assay for hard ticks

Feeding units (see Box 1) can be used to compare the effects of systemic acaricides. Here, the effects of fipronil and ivermectin on survival of female *Ixodes ricinus* are demonstrated (Figure 1). The sensitivity of the system is very high as a significant effect of fipronil on tick survival is still observed at 1 p.p.b. ( $0.001 \mu\text{g ml}^{-1}$  of blood) with 60% mortality after 9 days ( $P=0.051$ , Kaplan–Meier survival statistics with Peto test, S-Plus [25]). Such dose-dependent assays require only a total of  $\sim 5 \text{ mg}$  of a test product with the toxicity of fipronil, that is, just 5% of what is required to be effective against ticks on one dog. The system is highly reproducible and conclusive data can be obtained within the first 5 days [12]. The sensitivity of the method is such that the choice of material for construction of the feeding units becomes crucial in assays with low doses of test products where adsorption to plastics can occur.

### Applications of a hard tick *in vitro* feeding assay include:

- testing novel acaricidal compounds *in vitro* as a high-throughput screening tool;
- testing tick susceptibility and resistance to acaricides;
- isolation and structure elucidation of biologically active substances secreted in tick saliva into the feeding medium;
- isolation of tick-borne pathogens (injected with their saliva into the medium);
- studies on vector competence and pathogen-infection barriers (incompatible with host blood or tick species);
- post-genomic studies on the role of single and multiple gene products that target tick physiological processes, including RNA interference experiments;
- testing vaccines and antibodies that target tick-protective antigens.



**Figure 1.** Mortality curves depicting effects of fipronil and ivermectin on female *Ixodes ricinus* feeding *in vitro*. Fipronil at  $10 \mu\text{g/ml}$  killed all ticks by day 2 (vertical orange line), whereas a similar dose of ivermectin took 9 days to achieve the same effect. Data derived from Ref. [12].

tick-endosymbiotic *Rickettsia* [20] and the more recently identified symbionts [21–24] to tick survival and fecundity can be investigated as the symbionts can be selectively knocked out using antibiotics added to the blood in the feeding assay. Knowledge obtained in relation to these topics from studies on ticks can also be of relevance to insect vectors of disease.

Moreover, the simplicity and inexpensive nature of the feeding method means that it can be readily transferred to laboratories in tropical areas where ticks have the greatest impact on animal health. Such methods would allow practitioners in these regions to optimize the use of ethnobotanicals against ticks: plant extracts could be applied to the membrane to assay for products that inhibit tick attachment, and to optimize the dosing and storage of such natural products.

### Future perspectives

It will be necessary to develop appropriate membranes and attachment stimuli in order to adapt existing feeding assays for the *in vitro* feeding of the major livestock-infesting tick species. Clearly, the replacement of blood by an artificial nutrient medium would not only facilitate further applications of *in vitro* feeding assays but could also serve to standardize another parameter of the assay. Finally, miniaturisation of feeding units and their deployment in multi-well plates would make it possible to establish

high-throughput systems to test products against this group of ectoparasites and the pathogens they transmit.

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