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In vitro tick rearing as a replacement for the use of live animals in the maintenance of tick colonies in acarology and parasitology research

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Abstract

The blood-feeding behaviour of ticks highlights their importance as arthropod vectors because majority of pathogens affecting both humans and animals are transmitted during the blood feeding activity. Live animals are conventionally used to maintain tick colonies for laboratory research. There is a need and also interest in replacing this conventional use of live animals for tick rearing with artificial tick feeding/rearing alternatives, as this is considered to be more ethical and cost-effective. This article reviews the feeding biology of ticks, the tick feeding process, how various ticks digest blood meal, how ticks are maintained on live animals in the laboratory, and alternative *in vitro* tick rearing methods which have shown promise for the replacement of the use of live animals for the maintenance of tick colonies for research purposes. *In vitro* capillary feeding is compared to membrane feeding of ticks, and the pros and cons of various membrane feeding types are highlighted. Studies in our laboratory using a silicone membrane-based tick feeding assay optimized for *Ixodes ricinus* is described. These studies yielded good probing levels and tick attachment (≥ 70%), but feeding the ticks to repletion has not yet been achieved. Further research in the area of *in vitro* tick rearing systems is recommended.

Keywords: Ticks, Blood feeding; Tick rearing; Capillary feeding; Membrane feeding; Acarology.

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Introduction

Ticks are very important arthropod parasites second only to mosquitoes as disease vectors because of the wide range of pathogens which they transmit to both humans and animals (Bouchard and Wikel, 2005; de la Fuente *et al*., 2008). Their economic importance, which is directly or indirectly related to their blood feeding activity, is attributed to their ability to induce anaemia, reduction in weight gain and milk yield, stress, depreciation of hide value, hypersensitivity, toxicosis and complications with secondary infections (Jongejan and Uilenberg, 2004; Otranto and Wall, 2008). To undertake research on ticks and tick-borne diseases, it is often desirable to maintain tick colonies in the laboratory. For this, ticks must be blood-fed. Conventionally, cattle, dogs, and laboratory animals including rabbits and guinea pigs are used for this purpose (Jones *et al*., 1988; Ghosh and Azhahianambi, 2007). Several ethical concerns relating to physical harm or otherwise to these animals, escalating to episodes of skin inflammation or subjection of animals to certain restrictions which interfere with their grooming behaviour, for instance the use of Elizabethan collars, have been raised. Apart from ethical concerns, the cost of maintaining these suitable hosts to establish artificial tick colonies in the laboratory for research purposes is usually very high (Waladde *et al*., 1993). Alternative *in vitro* feeding methods for especially hard tick species which feed for prolonged periods hold prospects for the replacement of live animal tick hosts. In addition, aspects of tick physiology or the dynamics of the relationship between haemoparasites and their tick vectors can also be more easily studied due to a higher level of control over certain variables which may interfere with these interactions in living animal systems. There is therefore the need to review literature and the state of working knowledge in the area of *in vitro* rearing of ticks as replacement for the use of live animals for the maintenance of ticks used for acarology and parasitology research.

The Tick Feeding Process

Following questing and attachment to the host, the tick spends a considerable length of time exploring the host in search of a suitable site to begin feeding. The selection of a particular host and a specific site on that host is based on a blend of thermal, hygro, mechanical, olfactory and contact stimuli (Waladde and Rice, 1982; Kuhnert, 1996). During the first few days of their attachment, hard ticks ingest small amounts of blood per unit time. During this period, they equally undergo various physiological changes including maturation of their salivary glands, synthesis of the procuticle, and production of various pheromones (Shipley *et al*., 1993). For successful and prolonged feeding to occur, the tick needs to enhance bleeding, prevent inflammation and wound healing and at the same time suppress the host immune responses (Tajeri *et al*., 2016). For the first two days following attachment, the natural course of wound healing favours the tick. In subsequent days however, the tick would need to secrete extra protein components (vasodilators and immunomodulators) not just to maintain a constant blood supply but also to counter the host immune responses and prevent rejection (Grigorieva and Amosova, 2008). Following the onset of feeding, acini of different kinds in the salivary glands increase in size and synthesize several pharmacologically active substances involved in the feeding process including cytolytic enzymes, anticoagulants and other substances like prostaglandins (Dickinson *et al*., 1976; Higgs *et al.,* 1976). One of the prostaglandins, PGE2, also known as Dinoprostone, leads to dilation of the host's blood vessels, thereby increasing blood flow to the feeding site (Neitz and Vermeulen, 1987).

Typically, ticks usually spend several days on the host during a blood meal. During prolonged periods of their feeding, the three stages of the tick increase in body size and weight through neosomy (Grigorieva and Amosova, 2008). Neosomy in an arthropod is a remarkable enlargement or formation of new external structures or both, resulting from the secretion of new cuticles unrelated to a moult. It is

prominently seen in Acari, including all stages of hard ticks. Adult female ticks engorge fully during the last 24 hours of their feeding, ingesting about 2 to 8 times as much blood as their final weight, with consequent increase in their body weight up to 100 times their initial unfed weight (Rechav *et al*., 1994; Krober and Guerin, 2007). On the other hand, males feed intermittently and do not engorge themselves (Krober and Guerin, 2007). Ticks of *Ixodes* species ingest whole blood containing non-lysed erythrocytes, whereas the *Amblyomma spp* prefer haemolysed blood. In the former, lysis of erythrocytes is achieved by secreting an enzyme (haemolysin) in their saliva (Grigorieva and Amosova, 2008). The accumulation of blood within the body of the tick occurs by secreting water and ions back into the host through specialized salivary gland cells (Kaufman, 1983; Sauer *et al*., 1995). In contrast, argasid or soft ticks complete their feeding within an hour. After completing feeding, their body weight increases about 12 times their initial weight. In the latter case, concentration of the blood meal occurs post-feeding by excretion of the water and salts from the coxal organs (Balashov, 1972).

Hard and soft ticks differ in their feeding habits. In soft ticks, feeding occurs intermittently after which the engorged fertilized females digest their blood meal and lay batches of eggs. Here, virgin female ticks do not digest their blood meal and lay eggs until they are mated. Hard ticks on the other hand, usually mate on the host and take a small quantity of blood before mating. The females engorge fully only after being mated, otherwise the virgin females remain attached to their host for weeks until they are removed by the host either by grooming or scratching (Akov, 1982).

How do ticks digest their blood meal?

The process of digestion of a blood meal in ticks is quite different from what obtains in other haematophagous insects like mosquitoes or tsetse flies (Akov, 1972). In insects, digestion of blood proteins occurs in the gut lumen at an alkaline pH, whereas in ticks, protein digestion is intracellular, in which case intracellular proteases work at a pH of 3 (Tatchell *et al*., 1972; Gooding, 1975). Blood digestion in ticks occurs in the midgut within several branched diverticula called caeca. The blood gets digested in the epithelium of these caeca. The process begins with the concentration of blood where excess water and ions are removed through the salivary glands in ixodid ticks or coxal glands in argasid ticks (Akov, 1982).

In argasid ticks, blood digestion begins after the tick drops off from the host. The process is broadly divided into three phases. During the first phase, the blood meal is concentrated, and the blood cells are haemolyzed. Very little digestion occurs in this phase. The second phase involves very rapid digestion whereby the blood components are taken into the digestive epithelial cells. The third digestive phase is rather slow and helps the tick to survive starvation periods (Akov, 1982).

In ixodid ticks, feeding and digestion occurs together. Araman (1979) reported three different phases of feeding and digestion in hard ticks. These include the preparatory, growth and expansion phases. During the preparatory phase, there is very little feeding and digestion of blood (little protease activity), but the gut epithelium is developed in this phase. The growth phase involves vigorous feeding and digestion (peak protease activity), and the nutrients are diverted towards the expansion of the cuticle in readiness for the expansion phase. The expansion phase involves rapid feeding, but digestion is quite slow (reduced protease activity). After engorgement, the digestion of haemoglobin takes place away from the host and the nutrients are utilized for vitellogenesis and oviposition (Bogin and Hadani, 1973; Akov, 1982).

Maintaining Ticks in Live Animals in the Laboratory

The production of high-quality laboratory-reared ticks is necessary for the advancement of studies on tick biology, tick control and interactions of ticks with pathogenic agents (Sonenshine, 1999).

As a result of the complex life cycles of some species, maintaining ticks in the laboratory usually involves the use of a combination of hosts which include rats, guinea pigs or smaller-sized rodents for immature stages while rabbits, dogs or other medium to large animals are used for the adult stages (Bonnet and Liu, 2012).

Depending on the instar to be fed, a host is selected and anesthetized. The area where the ticks would be retained on the host is shaved using motor clippers. The back is usually used in guinea-pigs, while the ear is used in rabbits especially where ear bags are used. After the back is shaved, 2 – 3 retaining cells are then affixed to the animal's skin using non-irritable glue. Larvae, nymph and adult stages are usually maintained separately, each retaining cell consisting of only one life cycle stage (Jones *et al.,* 1988). After the area is prepared, the ticks are immobilized by placing them on ice for about 5 minutes. The larvae or nymph are then transferred to a petri dish using a fine brush, rolled into a ball and subsequently, pasted on the animal's back. Approximately 200 – 400 larvae or 50 – 100 nymphs are usually placed in each retaining cell. The cells are then covered with a nylon mesh, affixed with glue and trimmed to size. In the case of the adult ticks, equal numbers of males and females are introduced into each retaining cell (to facilitate mating) before the nylon mesh is sealed off using adhesive tape. Fewer numbers of adults (about 10 to 15) are introduced into each retaining cell. As soon as the ticks attach, the host animal is placed in a cage on a rack surrounded by an oil 'moat' to prevent escape of ticks (Jones *et al*., 1988). Retaining cells are then monitored daily until the ticks are engorged. This usually takes between five to seven days in larvae and nymphs and about eight to fourteen days in adult ticks (Sonenshine, 1999). After the ticks engorge, they are removed for storage. The host animal is then humanely sacrificed (Jones *et al*., 1988).

The use of natural hosts for rearing and maintaining ticks in the laboratory provides the best natural relationship and conditions between the parasite and its host to facilitate feeding. However, acquisition, housing, and handling of these animal hosts can be quite complicated, expensive and almost impossible in some circumstances (Kuhnert, 1996). Also, the peculiar feeding behaviour and host specificity of different tick species makes this quite a tedious project (Waladde *et al*., 1991). This is mainly because ticks require blood feeding multiple times throughout their life cycle and most of the rearing procedures are labour and time intensive (Sonenshine, 1999; Bouchard and Wikel, 2005). In addition, ethical concerns associated with the use of live animals for experimental purposes, the advocacy for the 3R (replacement, reduction and refinement) and other administrative requirements further complicate the process hence the search for suitable alternative measures for tick rearing and colonization.

Alternatives to Live Animal Usage for Tick Rearing

Many creative approaches to induce blood-feeding have been attempted for feeding and rearing ticks in the laboratory, mostly based on the original design of the Rutledge feeder (Wade, 1976; Costa-da-Silva *et al*., 2013). One method is the capillary feeding technique which involves placing a micro-capillary tube over the mouth parts of a properly secured semi-engorged tick. The capillary tube is filled with blood which is either manually defibrinated or contains added anticoagulant. The process is quite slow and takes several hours to complete, but even then, the ticks are not replete (Broadwater *et al*., 2002; Billeter *et al*., 2012). Capillary feeding has been employed by many scientists to either artificially feed ticks or to infect the ticks with pathogens (Nuttall and Hindle, 1913; Chabaud, 1950; Burgdorfer, 1957; Purnell and Joyner, 1967; Jones *et al*., 1988; Waladde et al, 1996; Burkot *et al*., 2001). However, it has been suggested that semi-engorged ticks are better candidates for capillary feeding when compared to starving ones (Abel *et al*., 2008; Rangel *et al*., 2008). In addition, it has also been suggested that capillary feeding

does not allow the ticks to reproduce, unless they are also partially fed on the natural hosts (Kuhnert, 1996).

Membrane feeding is another method which has been attempted for feeding/rearing ticks *in vitro* (Kuhnert, 1996). During natural host feeding, ticks penetrate the uppermost keratin rich stratum corneum with outward lacerating movements of their chelicerae. The strong backward directed denticles on the hypostome anchor the capitulum to the host skin helping the cutting mouth parts move deep to the corium level that contains blood vessels (Kuhnert, 1996). After reaching the corium level the ticks start to suck blood. The membrane feeding unit tends to replace the host's skin and provide the tick with a similar surface over the blood. Here, the procedure involves use of feeding chambers with various kinds of membranes made of materials obtained from various sources, which may either be natural or synthetic. Natural membranes such as the air sac of embryonated chicken eggs, bat wing, the skin of bovine animals or biodegradable glue-impregnated Baudruche membrane, have all been used previously with variable success (Pierce and Pierce, 1956; Youdeowei and Mango, 1975; Kemp *et al*., 1975; Waladde *et al*., 1991; Voigt *et al*., 1993). More recently, synthetic membranes made of silicone have been developed and adapted for different tick species by several researchers (Habedank and Hiepe, 1993; Kuhnert *et al*., 1998; Krobër and Guerin, 2007; Fourie *et al*., 2013; Tajeri *et al*., 2016; Krull *et al*., 2017). For instance, Kuhnert *et al*. (1995) developed an alternative feeding system for the bont tick, *Amblyomma hebraeum*, due to its widespread use in testing acaricide efficacy, the varied hypostome length of each instar, the long feeding duration (4 – 14 days) and considerable differences in weight increase of instars. Silicone membranes stretched over the base of a glass tube and defibrinated blood contained in a honey jar were used. Eggs from naturally collected adult ticks were used to produce larvae which were then fed on the membranes to produce 'artificial nymphs' and later 'artificial adults'. The problem with this technique was the reduced fecundity of the 'artificial females' when compared with their natural counterparts and inability to engorge above 38% of the capacity of naturally reared ticks. In 1996, Waladde *et al*. (1996) employed the use of Baudruche membrane (made from cattle intestinal tissue) for feeding *Rhipicephalus appendiculatus*. Two types of feeding systems were tried; the first system had ticks in the upper chamber and blood was stored in the lower chamber and the two chambers were separated by the Baudruche membrane to which the ticks attached, while the second system had blood in the top chamber and the ticks contained in the lower chamber attached to the membrane above. They reported that the second system was more efficient in feeding nymphs than the adults, achieving up to 94% of nymphs moulting to adults, however many of the adults died before oviposition. More recently, Krober and Guerin (2007) developed a membrane feeding system for *Ixodes ricinus*. The apparatus consisted of a cellulose rayon reinforced silicone membrane attached to the base of acrylic glass tubing. Here, the ticks were fed on blood contained in a six well tissue culture plate. The blood meal comprised of defibrinated bovine blood supplemented with glucose, ATP and antibiotics. This blood meal was replaced twice daily. Cow hair extract, mosquito netting and a layer of cow hair were used as chemical and mechanical stimuli within the apparatus. They reported 75 – 95% attachment rate by the ticks using this apparatus.

The addition of some physical and chemical stimuli (semiochemicals) to the feeding system has been reported to improve both tick attachment and engorgement rates on silicone membranes (Waladde and Rice, 1982; Kuhnert, 1996; Krobër and Guerin, 2007). The semiochemicals which originate from the typical host animal and attract the ticks to a potential food source are known as kairomones whereas those secreted by the ticks to elicit various physiologic responses including attracting other ticks, inducing physiological changes for feeding or initiating mating, are known as pheromones

(Waladde *et al*., 1993). These substances are believed to be contained in exudates obtained from either the host or the tick. Both kairomones and pheromones are thought to be important feeding stimuli (Waladde *et al*., 1996).

Temperature sensitivity of blood sucking arthropods such as ticks or lice is also quite pronounced, and the ambient temperature of a potential host has been highlighted as an important consideration in eliciting feeding behaviour in such blood feeders (Carr and Salgado, 2019). Generally, most of the *in vitro* feeding systems developed for ticks are composed of a heating element (for blood warming), a blood reservoir, a feeding chamber, and an artificial membrane surface simulating vertebrate skin. The ticks are usually fed on the blood placed into the feeding chamber. Blood from several animal species may be served, but usually from larger domestic animals like cattle, sheep or horses (Waladde *et al.,* 1979; Stone *et al*., 1983; Kuhnert *et al*., 1998; Krober and Guerin, 2007; Tajeri and Ramzi, 2011). The thickness of the membrane produced depends on the species of the tick to be fed and this is usually determined by the length of the mouth parts of the tick, especially the hypostome-chelicerae complex, which also varies with the instar of the tick (Waladde *et al*., 1996). During membrane feeding of ticks, all the salivary secretions flow into and accumulate in the blood meal on which the ticks are fed. One of the disadvantages of this is that it could result in the rapid deterioration of the blood in the feeding chamber. This happens more rapidly when many ticks are engorging simultaneously, and necessitates the replacement of the blood in the chamber every 8 to 12 hours, making the process laborious and time intensive, and results in disturbance to the feeding ticks. Flow-through systems, with circulating blood that can be changed from a central reservoir, are being investigated by some research groups (Böhme et al*,* 2018) but are not yet commonly used.

Optimizing an Artificial Tick Feeding Assay for *Ixodes ricinus*

In preliminary studies in our laboratory, an artificial tick feeding assay was optimized to conduct *in vitro* tick feeding trials to establish the ideal conditions which would allow attachment and feeding of *Ixodes ricinus* ticks. The assay was prepared according to the method of Krobër and Guerin (2007), with some modifications. Here, Goldbeater's skin was used instead to replace lens cleaning paper as the matrix of the membrane so that thinner membranes of ≤ 40µm could be produced. The silicone mixture used here was also modified to render the resulting membranes softer while retaining their elasticity. The feeding chamber used comprised a transparent tube, artificial silicone membranes and a 6-well tissue culture plate (Figure 1a, b, c and d). Each tube was made of acrylic (28 mm diameter, 2 mm wall thickness and 45 mm high). A ring was fitted around each tube 4 mm from the base to limit the depth to which the chamber sank into the wells of the 6-well tissue culture plate which served as a blood reservoir for the feeding apparatus. A stopper was placed about 2 cm from the base of the tube to prevent ticks from wandering up and down the walls of the acrylic tube and away from the silicone membrane.

Silicone membranes were prepared using RTV-1 Elastosil E4 Silicone rubber (Wacker Chemie, Germany) with a Shore A hardness of 16 degrees. 30 g of the glue was mixed with toluene solvent (BDH Chemicals, England) inside a glass beaker at a ratio of 4:1. 30% Silicone oil (DC 200, viscosity ~10 mPa, Fluka, Switzerland) was also added to the mixture to render the resulting membrane softer. The mixture was then evenly spread over Goldbeaters skin (Preservation Equipment Ltd, UK) using a silicone rubber spreader. The resulting silicone impregnated membrane was then fixed to a cling film using sticky tape and allowed to polymerise and dry overnight. Membranes with thickness of between 40 – 70 µm were used for feeding. Subsequently, the membranes were attached to

the acrylic glass tubes using Elastosil E4 silicone glue and allowed to dry for at least 3 hours. Excess membrane was cut to flush with the tube when dry. The permeability of each membrane attached to the feeding chamber was then tested with normal saline solution. Only membranes that did not permit the entry of saline after 20 minutes were used in experiments. The feeding chambers were then surface sterilized with 70% ethanol before use.

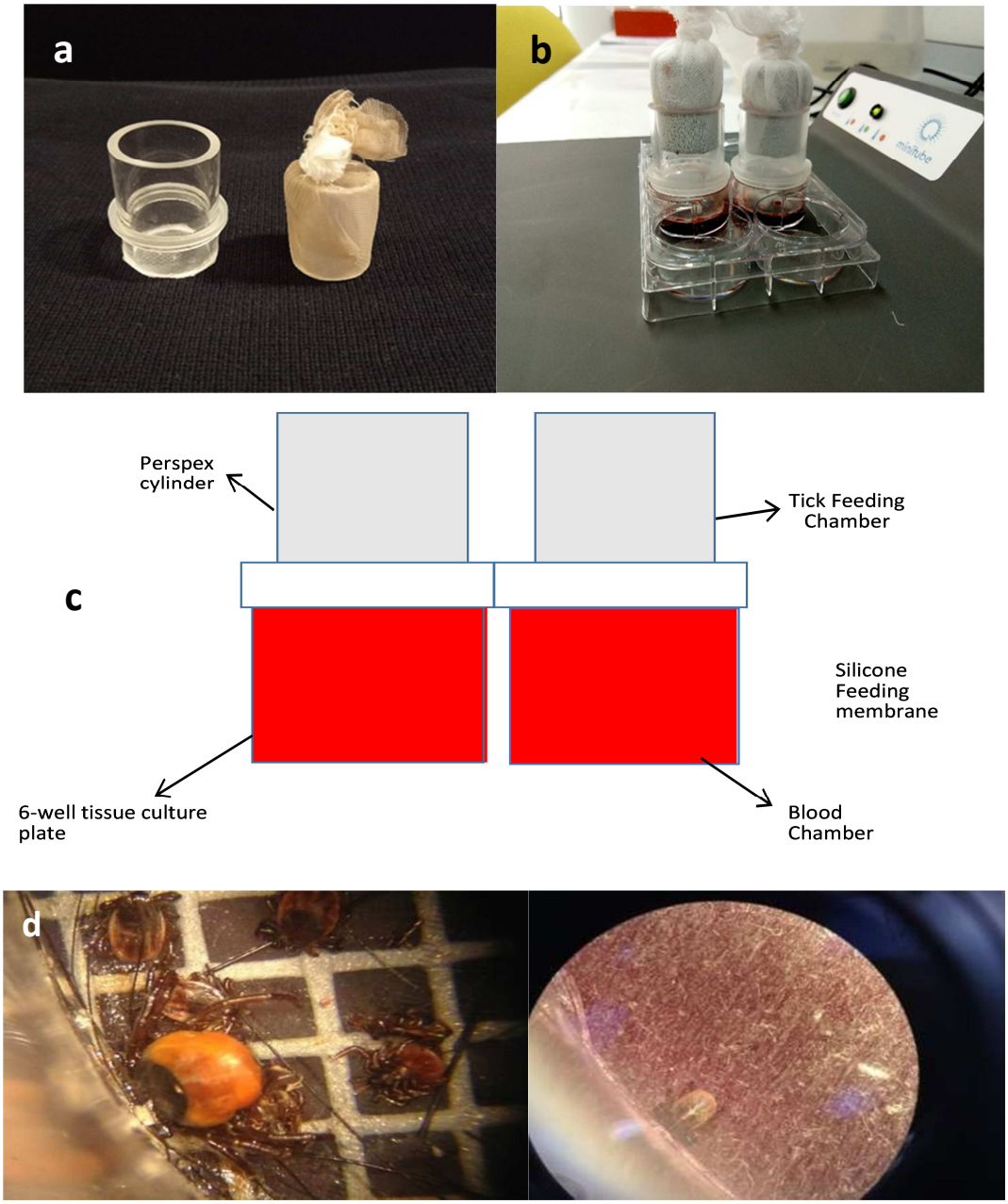

Figure 1. Components of the tick feeding assay optimized for *Ixodes ricinus*: **a**. Acrylic tube and perforated stopper secured with cloth netting. **b.** Assembled feeding chambers in 6-well tissue culture plate containing blood. **c.** Schematic diagram of the tick feeding assay. **d.** *Ixodes ricinus* ticks engorging on the membrane.

Bovine hair extract and granules of tick faeces (assumed to contain attachment and aggregation pheromones) were used as attachment stimuli in the feeding chambers. These were added to the membrane at the bottom of the feeding chambers just before the ticks were introduced. Bovine hair extract was prepared by chopping fresh clipped cattle hair into a beaker and immersing in dichloromethane for 3 days at room temperature to extract the lipophilic compounds. The resulting tan-coloured fluid was collected and stored in a sealed glass container until required. This was then applied to the membrane and allowed to dry for 2 hours before ticks were added into the feeding chambers.

Using this setup, the results indicated that good levels of probing and attachment (≥ 70%) was achieved and ticks were able to attach and commence feeding, however feeding to repletion was more difficult to achieve due to the prolonged feeding required by *I. ricinus*, and the frequency of blood changes (every 12 hours) required which possibly interrupted feeding in already attached ticks.

Concluding Remarks

Rearing and maintaining ticks in the laboratory without the use of animal hosts is an arduous task because achieving artificial feeding of hard ticks is especially difficult to standardize due to the variable feeding duration of most species (Kuhnert, 1996; Krobër and Guerin, 2007). Unlike tsetse flies or mosquitoes, ticks are not easily fed through synthetic membranes and usually will require a complex combination of living host attractants and tick pheromones to initiate the feeding process. Many research groups have attempted to establish *in vitro* feeding systems for rearing ticks in the laboratory with only partial success (Kemp *et al.,* 1986; Bonnet and Liu, 2012). Developing an effective *in vitro* tick feeding system would offer several advantages for various purposes which include quantifying dose effects of newly developed tick control products, testing the efficacy of various acaricides or repellents, or studying tick-haemoparasite interactions. Further research in this direction is highly recommended.

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Conflict of Interest Satement

The authors declare no conflict of interests.

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