In vitro feeding of ixodid ticks using a silicone membrane for testing effects of systemic acaricides on tick feeding and mortality

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"In vitro feeding of ixodid ticks using a silicone membrane for testing effects of systemic acaricides on tick feeding and mortality"

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Summary

Ixodid ticks are vectors of disease of humans and animals, worldwide. Use of acaricides is one of the most effective strategies for tick control, and a growing interest in recent years has focused specifically on the discovery of systemic acaricides for ticks. Systemic acaricides are those destined to be dosed in the blood of host animals, and designed to target ticks during the uptake of their bloodmeal. An earlier published study demonstrated the utility of feeding ticks *in vitro* on an artificial membrane for experimentally introducing systemic acaricides into ticks during the bloodmeal and to quantify their effects on tick mortality. The present thesis research had three aims: to describe a simplified method for producing artificial membranes on which to feed ixodid ticks *in vitro*; to compare the feeding behaviours of ixodid ticks fed *in vitro* and *in vivo*; and finally, to characterize and quantify the systemic effects of products from different acaricide classes on tick feeding behavior and mortality *in vitro*.

Silicone membranes for *in vitro* feeding of ixodid ticks are traditionally custom-made and manually produced to a specific thickness, which is dependent upon the hypostome length of the tick species or life stage being fed. In this study, a standardized method has been described for both preparing and manipulating the thickness of silicone membranes for *in vitro* feeding of ixodid ticks. Tick attachment and engorgement using these membranes was highly satisfactory for both *Ixodes ricinus* females and *Amblyomma hebraeum* nymphs. This method should permit production of standardized silicone membranes for olher tick species and life stages in the future.

Little is known about the effects of feeding *I. ricinus in vitro.* This study shows that both *I. ricinus* females and *A. hebraeum* nymphs fed for a longer period of time before drop-off and attained a smaller engorged weight *in vitro* on bovine blood compared with ticks fed *in vitro* on steer. The daily increase in the partial engorged weight of *I. ricinus* females fed *in vitro* and *in vivo* diverged significantly after day 3, but was generally similar over the first three days of feeding. These results may have implications for any generalisations of findings obtained with *in vitro* feeding systems for ixodid ticks.

The LD_{50} values of 12 compounds from five acaricide classes (macrocylic lactones [MLs], phenylpyrazoles, organophosphates, organochlorines, and isoxazolines) have been tested using *I. ricinus* females. The assay proved sensitive enough to detect significant differences in acaricide LD_{50} values both between and within acaricide classes. The MLs were demonstrated to have a significant dose-dependent inhibitory effect on the partial engorged weight attained by feeding ticks, whereas the inhibitory effect on feeding was not significant for the phenylpyrazoles, and the organophosphates significantly increased feeding by the ticks *in vitro*. Consistent effects on tick feeding were demonstrated across all acaricides tested from each class. The feeding assay permits standardized tests of the systemic activity of candidate acaricides for ixodid ticks *in vitro*.

Keywords: *In vitro* feeding of hard ticks, artificial feeding of ticks, tick membrane feeding, tick feeding behaviour, systemic acaricide

Résumé

Les tiques de la famille des Ixodidés sont des vecteurs de maladies pouvant affecter l'homme ou les animaux partout dans le monde. Le contrôle chimique au moyen d'acaricides constitue l'une des méthodes de lutte les plus efficaces à l'encontre des tiques. Ces dernières années, un intérêt croissant pour la recherche dans ce domaine a permis la découverte d'acaricides systémiques contre les tiques. Un acaricide systémique est un produit conçu pour circuler dans le système vasculaire des animaux traités et affectant les tiques durant leur repas de sang. Une récente étude a démontré l'efficacité d'un système artificiel d'alimentation à travers une membrane artificielle permettant la quantification de la mortalité des tiques ayant ingéré différents acaricides systémiques durant un repas de sang *in vitro*. La présente thèse a donc 3 buts: décrire un système simplifié pour la production des membranes permettant le nourrissage des tiques, comparer le comportement alimentaire des tiques ixodidés nourries sur le sang bovins de façon *in vitro* ou *in vivo* et, finalement, caractériser et quantifier les effets de plusieurs produits appartenant à différentes classe d'acaricides sur le comportement alimentaire des tiques nourries sur le sang de bovins *in vitro*.

Les membranes de silicone pour le nourrissage *in vitro* des tiques ixodidés sont généralement faites sur mesure et manuellement de façon à obtenir une épaisseur adéquate en fonction de la longueur de l'hypostome de l'espèce et du stade de vie de la tique utilisée. Dans cette thèse, une méthode standard est décrite pour préparer et ajuster l'épaisseur des membranes de silicone pour le nourrissage des tiques ixodidés *in vitro*. L'attachement et l'engorgement des femelles *Ixodes ricinus* et des nymphes *Amblyomma hebraeum* sur ce type de membrane se sont avérés entièrement satisfaisants. Cette méthode devrait donc faciliter la production de membranes de silicone standardisées pour différentes espèces et stades de vie de tiques dans l'avenir.

Les effets des conditions *in vitro* sur la nutrition d'*I. ricinus* sont encore peu connus. Dans cette thèse, je démontre que les femelles *I. ricinus* et les nymphes *A. hebraeum* se nourrissent durant une période de temps plus longue avant de se détacher et atteignent un poids d'engorgement plus faible sur le sang de bœuf

in vitro que les tiques nourries directement sur des bœufs. La prise de poids journalière durant l'engorgement des femelles *I. ricinus* nourries diffère significativement après trois jours selon que les tiques sont nourries de façon *in vitro* ou *in vivo*, bien qu'elle soit similaire durant les trois premiers jours du repas de sang. Ces résultats pourraient avoir des implications pour la généralisation des résultats obtenus avec des tiques ixodidés nourries *in vitro*.

Les doses létales 50 (DL_{50}) pour 12 composés appartenant à cinq classes d'acaricides (lactones macrocycliques [LMs], phénylpyrazoles, organophosphates, organochlorines, et isoxazolines) ont été calculées pour les femelles *I. ricinus*. Le test s'est avéré être suffisamment sensible pour détecter des différences significatives entre les DL_{50} des acaricides, à la fois entre et au sein des différentes classes des produits. Dans cette thèse, je démontre que les LMs inhibent significativement l'engorgement des tiques en fonction de la dose au cours du repas sanguin. Cet effet inhibiteur n'est toutefois pas significatif pour les phénylpyrazoles, et les organophosphates augmentent même la quantité de sang ingérés par les tiques. Des effets consistants ont été trouvés entre tous les acaricides testés au sein de chaque classe, et ces effets corroborent le mode d'action moléculaire de chaque composé. Ce nourrissage *in vitro* permet de standardiser les tests pour évaluer l'activité d'acaricides potentiels contre les tiques ixodidés.

Mots clés: nourrissage *in vitro* des tiques, systèmes de nourrissage artificiel pour les tiques, nourrissage des tiques sur des membranes, comportement d'alimentation des tiques *in vitro*, acaricides systémiques

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my master's in Amherst, MA, USA, I spent countless hours reading Frank's and Thomas's papers. I remember the surreal moment when I finally found myself one day on the other side of the world rummaging through boxes of Frank's old work in an attic in Germany. The same goes for my first visit to the University of Neuchâtel. It has been a priviledge carrying on the tradition of *in vitro* feeding of ticks, thanks again to Patrick, Frank, Thomas, and Andreas for making all of this possible.

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1. General introduction

1.1. Systematics and biology of ticks

Ticks are obligate blood feeding ectoparasites (Phylum: Chelicerata) of vertebrates, belonging to the class Arachnida (Figure 1). All chelicerate arthropods lack antennae, have a fused body plan, and instead of mandibles for mouthparts (as in the insects), the chelicerates possess chelicerae and pedipalps (or palps), both of which vary considerably from a functional and morphological standpoint throughout the class (see below). Within the Arachnida, ticks, together with mites, comprise the sub-class Acari. All acari, especially ticks, have a fused body plan and lack a clearly defined head. In ticks (Order: Parasitiformes; Sub-order: Ixodida), the chelicerae and palps are housed in a unique sub-division known as the capitulum (Figure 2). The chelicerae in ticks have completely lost the pincer-like structures common to the distal ends of most other arachnid chelicerae (Figure 3; top left). Instead, tick chelicerae bear specialized, lateral-facing, heavily sclerotized cutting denticles (Figure 3; top right). The mobility of the chelicerae in ticks is restricted to the mediolateral plane and they are therefore perfectly suited for lacerating host skin, using cutting and sawing movements during tick attachment (sawing outward from the mid-line). The hypostome in ticks is fused and prominent (Figure 3; bottom) and bears multiple layers of heavy, recurved denticles (Figure 4). During tick attachment, the hypostome is driven into the host at a 45–60 degree angle to the skin, and serves to anchor the tick mouthparts firmly in place in the feeding lesion during tick feeding over several days (Balashov 1972).

There are three families of ticks, the Ixodidae, Argasidae, and Nutalliellidae. More than 800 species of ticks have been described and the geographic distribution of ticks is worldwide. The ixodidae are the most medically and economically important of the three families and this study only deals with ixodid ticks. Ixodid ticks are the so-called “hard ticks” due to a characteristic, hard, anteriodorsal plate, known as the scutum. Following attachment to a host, ixodid ticks synthesize new cuticle around the scutum in order to accommodate the

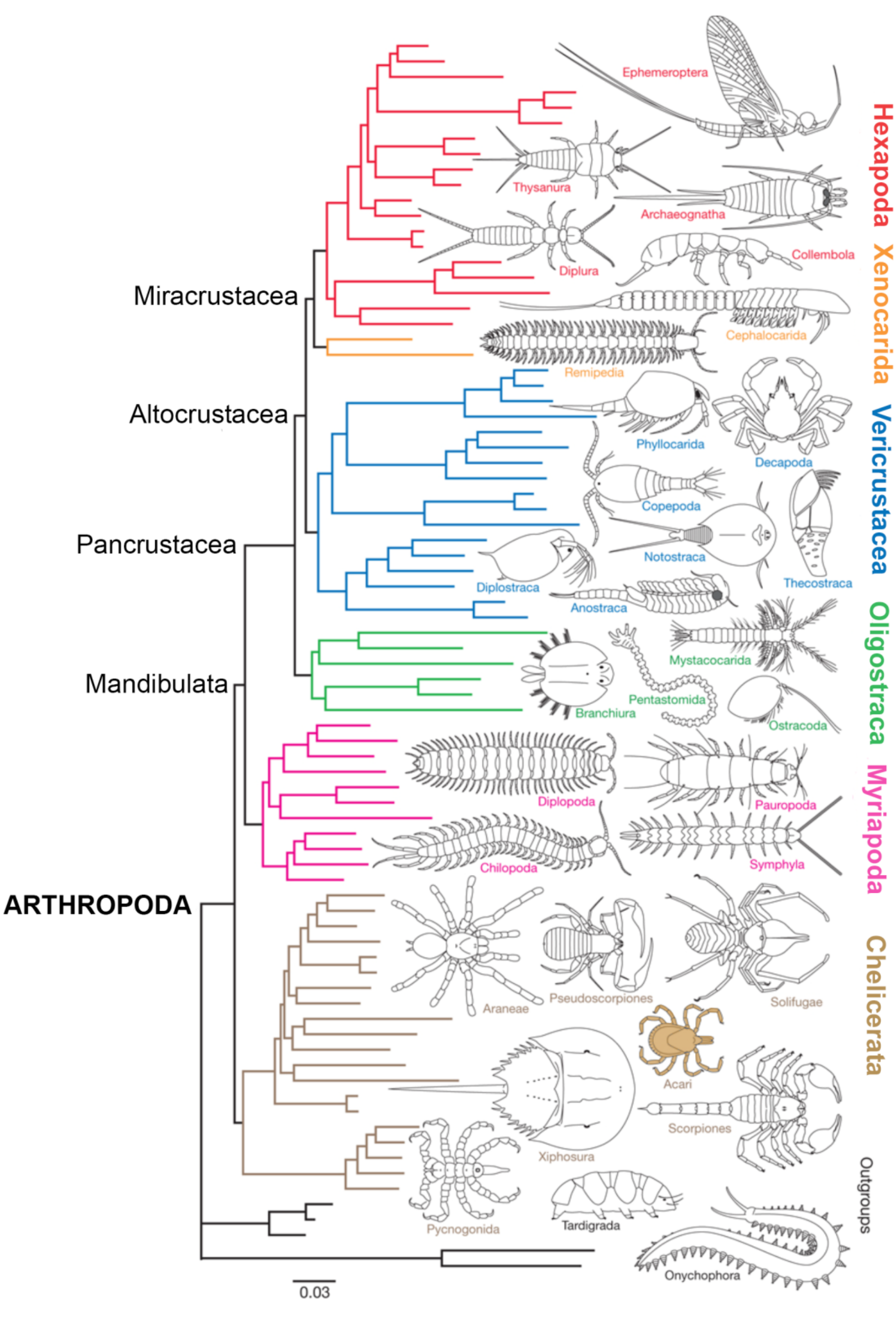

Figure 1. Relationships of the arthropoda. Some two-thirds of the arthropods, including insects (Hexapoda) are mandibulates. The Acari, which include ticks, do not have mandibles and are members of the phylum Chelicerata. A drawing of a tick (colored in brown) shows the general morphology of ixodid ticks (not drawn to scale). Modified from Regier et al. (2010).

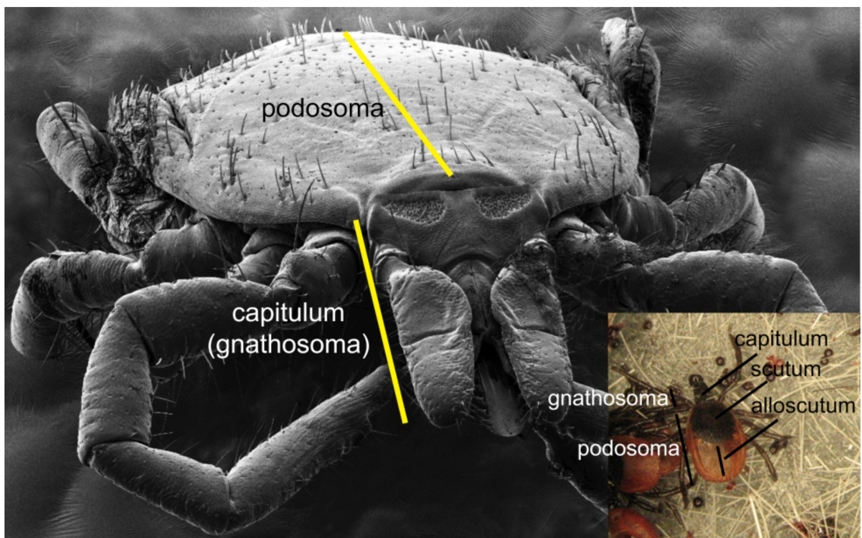

Figure 2. Micrograph (anterior view) showing the tick capitulum with reference to the rest of the tick body (podosoma) in *Ixodes ricinus.* Insert (bottom right) shows *I. ricinus* from a dorsal view. The podosoma includes the scutum and alloscutum. Micrograph provided by Marie Vancova, University of South Bohemia, Czech Republic, and modified.

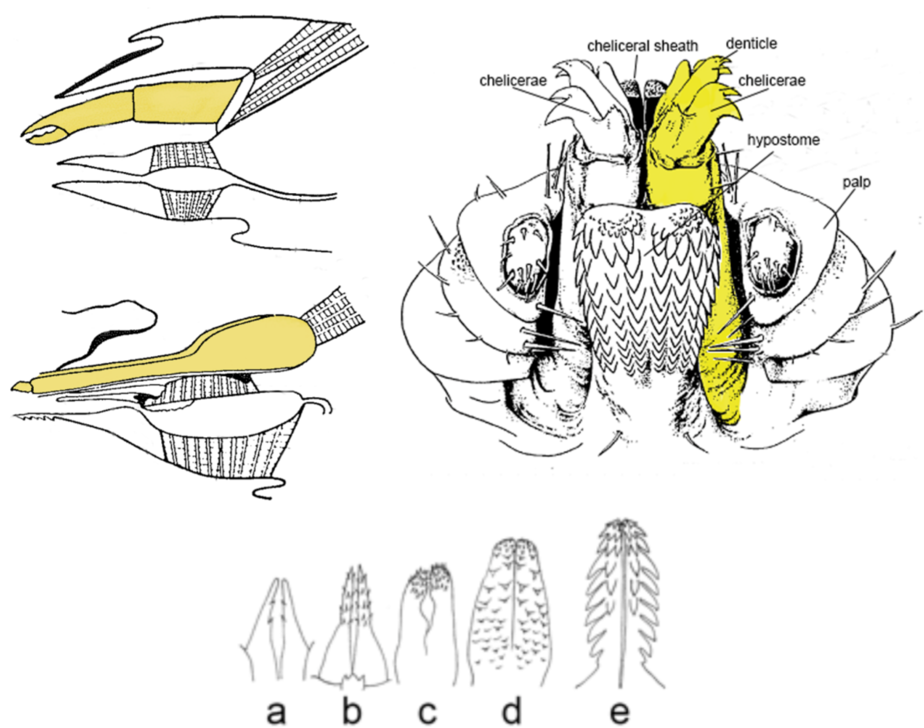

Figure 3. Top left: Sections of the anterior prosoma in a mite (top) and ixodid tick (bottom); homologous chelicerae are colored in yellow; modified from Balashov (1972). Top right: Anterior view of the mouthparts of *Rhipicephalus microplus*; chelicera highlighted in yellow; modified from Kemp et al. (1982). Bottom: Morphological variation in the hypostome of three mites (a-c) and two tick (d-e) species.

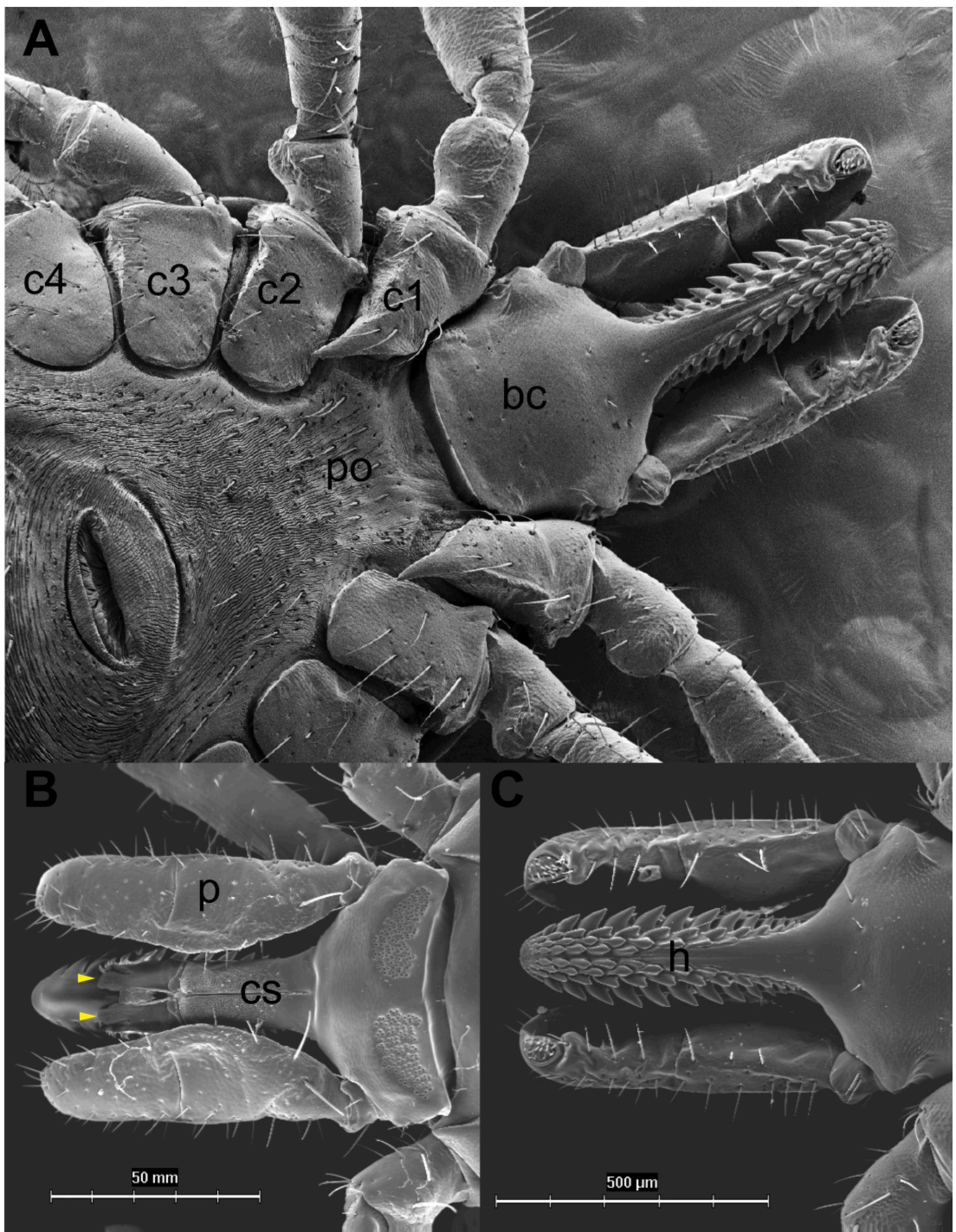

Figure 4. Female *I. ricinus* mouthparts A) ventral view showing position of the basis capitulum with reference to the podosoma and coxae; B) dorsal view; C) ventral view; p: palps; h: hypostome; bc: basis capitulum; po: podosoma; c1 – c4: coxae. Yellow arrows indicate the chelicerae. Photos provided by Marie Vancova, University of South Bohemia, Czech Republic and modified.

tick's enormous bloodmeal. During this initial stage of feeding, termed the preparatory feeding phase, relatively small amounts of blood are consumed and salivary gland maturation, differentiation of digestive cells, and maturation of the ovaries occurs (see Figure 5). The majority of the tick bloodmeal is not consumed

until the final 24 hours of feeding, termed the rapid feeding phase. The tick feeding process and the profound changes in tick physiology that take place during feeding, which takes place over several days and includes a bloodmeal that can reach more than 100-times the initial weight of the tick, are extraordinary and unparalleled in other blood feeding arthropods. During the enormous tick bloodmeal, red blood cells are concentrated in the midgut and excess water and ions are expelled back into the host using the salivary glands. Blood digestion in the tick takes place intracellularly (also unique amongst arthropods) in the midgut and engorged females lay several thousand eggs after feeding. In immature tick stages, because digestion is intracellular, undigested blood remaining in the midgut serves as a food reserve after molting and the tick life span is often measured in years.

The unique feeding process of ticks has important consequences for ticks' associations and interactions with disease-causing pathogens. The tick's prolonged feeding duration on the host provides ample time to acquire disease-causing pathogens in the bloodmeal. Once in the tick, the tick's intracellular digestion process insulates pathogens from digestive enzymes in the midgut. From the perspective of the tick, however, prolonged feeding on the host means increased exposure to the host's immune response. To counter this, ixodid ticks have evolved a pharmacy of bioactive molecules (including histamine binding proteins, complement inhibitors and immunoglobulin-binding proteins; Table 1) that the tick secretes into the feeding lesion, via salivary secretions, in order to evade the host's immune response. These molecules are exploited by tick-borne pathogens both during acquisition into the tick (Narasimhan et al. 2007) and during transmission from the tick into the host (Hovius et al. 2007). Ultimately, the tick blood feeding process is the fundamental interface between tick, disease-causing pathogen, and host, and it is only in better understanding the unique biology and physiology of tick blood feeding that we can better control ticks and prevent the transmission of tick-borne diseases.

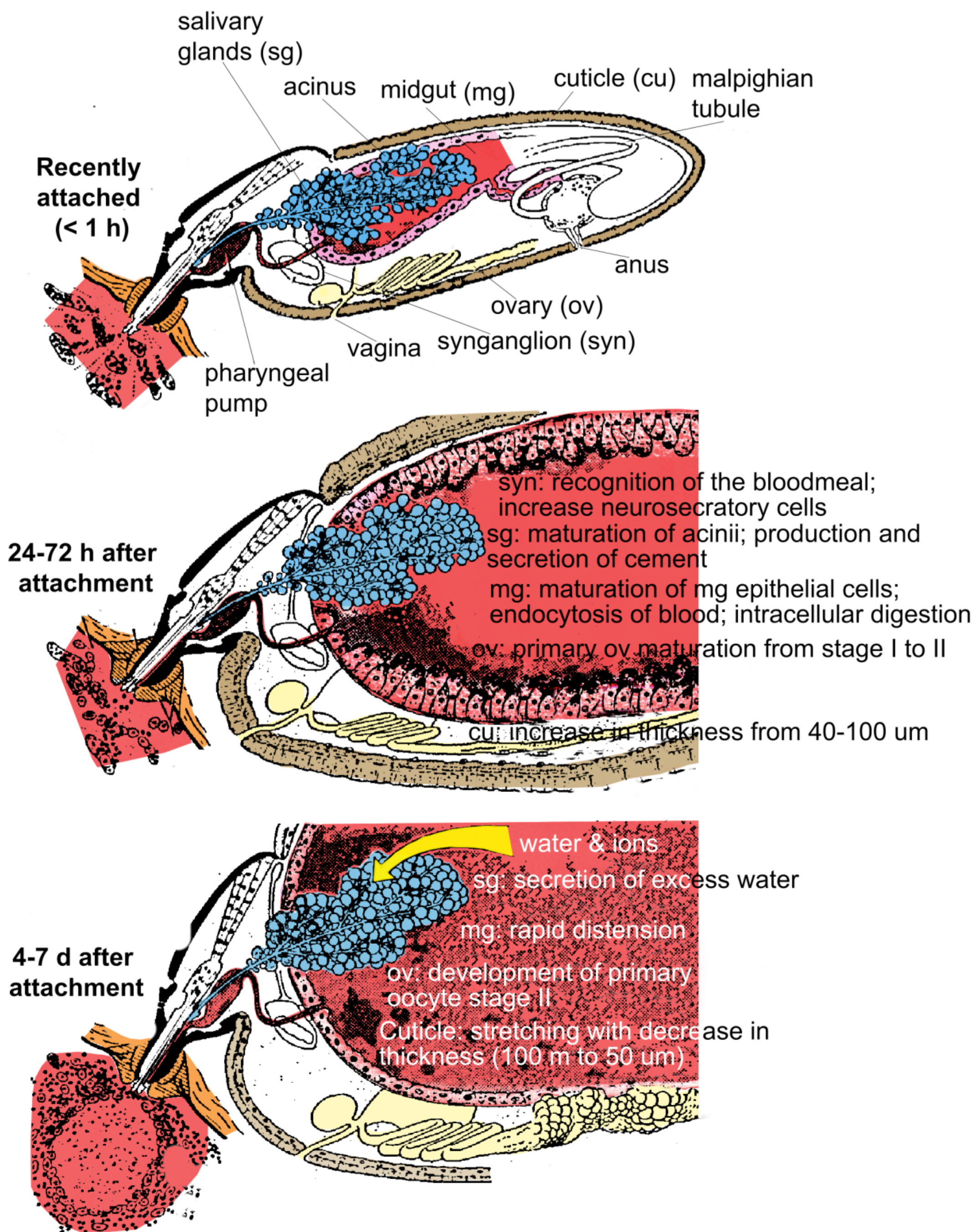

Figure 5. Changes in tick physiology during feeding. Modified from Kemp et al. (1982).

Table 1. Pharmacologically active molecules in tick saliva and their functions.

Saliva molecules	Activities that may benefit tick-borne pathogens
Histamine-binding proteins (histacalins)	Suppression of inflammatory responses including recruitment of neutrophils and eosinophils
Complement inhibitors	Suppression of inflammatory and immune responses
Immunoglobulin-binding proteins	Protection against antibodies
Cytokine inhibitors	Inhibition of anti-viral activities; T cell suppression; Anti-neutrophil activity
B cell modulators	Reduced antibody and cytokine production
T cell modulators	Polarisation to a Th2 response
Natural killer cell suppressors	Inhibition of anti-viral activities
Phagocyte modulators	Inhibition of microbicidal activity

Source: Labuda and Nuttal (2004)

1.2. The life cycle of ixodid ticks

Ixodid ticks have four developmental stages, namely egg, larvae, nymph, and adult. Ixodid ticks are temporary ectoparasites of vertebrates and the tick life cycle is complex, involving alternating periods off the host (up to several months for egg laying, molting to the next life stage, or host seeking) and shorter periods on the host (less than 10 days in most cases only for feeding and/or mating). All ixodid ticks, except those of the genus *Ixodes*, mate on the host, and all ixodid females require fertilization from a male in order to complete the bloodmeal. It should be noted that Balashov (1972) considers each stage during feeding to be a distinct life stage due to the extraordinary developmental changes that occur in ticks during feeding. After completion of the bloodmeal, engorged females typically drop off the host to gestate before laying a batch of several thousand eggs on the ground. Larvae hatch as the first living life stage and after a brief period of maturation, seek their first vertebrate host. Each life stage feeds to repletion only once on a host, before dropping off to molt into the next life stage. Ixodid ticks therefore only feed three times, typically on three different animals (termed three-host ticks), over their lifetime before laying eggs once and dying, in the case of the adult semelparous female. Periods of gestation, egg incubation periods, and molting times vary depending on many factors, including tick species, life stage, host origin of the bloodmeal, and the environment. Some ticks

complete their life cycle using the same animal (termed one-host ticks, e.g. *Rhipicephalus microplus*) or two animals (two-host ticks).

Ixodes plumbeus, a tick that feeds primarily on sand martins, described in Balashov (1972), provides a useful working example of the tick life cycle and the influence of host and environment on development. Sand martins are migratory birds that return to their nests in warmer months to mate and raise their young. Unfed *I. plumbeus* larvae overwinter in abandoned bird nests, and upon arrival of the birds, larvae attach, feed to repletion, and detach in the same nest. High temperatures (30–32°C) in the bird-inhabited nest facilitate rapid molting of engorged larvae into nymphs, and nymphs emerge just 15–18 days later to feed on the same birds. Nymphs that engorged on the birds then drop off and molt into adults 12–15 days later, and adults complete a final bloodmeal on the juvenile birds before the birds leave the nest. Engorged adult ticks then lay eggs quickly, just 3–8 days after detaching. Egg laying in the same season ensures sufficient numbers of inactive larvae, which tolerate extreme cold and prolonged periods of starvation, are waiting for the birds when they return. The life cycle of *I. plumbeus* from unfed larvae to larvae of the next generation takes 4–4.5 months, matching the seasonal nesting period of the migratory sand martin.

1.3. The global importance of ticks: medical, agricultural, and veterinary perspectives

1.3.1. Medical importance of ticks and tick-borne diseases

Of the more than 1,400 infectious agents known to cause diseases in humans, 60% are zoonotic (i.e. maintained in wild vertebrates and passed to humans) and 75% of newly described or emerging pathogens worldwide are zoonotic (Taylor et al. 2001). Although mosquitoes transmit more human diseases, ixodid ticks parasitize every class of terrestrial vertebrate and are unmatched in the variety of disease-causing pathogens (e.g. bacteria, viruses, protists, helminthes [Otranto et al. 2012]) they transmit during feeding (Oliver 1989; see also Tables 2, 3 and 4 below). Ticks belonging to the *Ixodes ricinus* complex (e.g. *Ixodes scapularis* and

Ixodes ricinus), in particular, serve as bridge vectors for several emerging or re-emerging zoonotic diseases of humans (Xu et al 2003). The expanding of populations of *I. scapularis* and *I. ricinus* in parts of the US and Europe represent one of the most significant public health concerns in the northern hemisphere.

In the United States, 99% of indigenous vector-borne diseases reported annually to the Centers for Diseases Control (CDC) are tick-borne, mainly Lyme borreliosis (95%; Randolph 2001). Recently, the annual estimated incidence of Lyme borreliosis in the United States was increased from 30,000 cases per year to 300,000 cases (Sticker and Johnson 2014). In Europe and Asia, morbidity from tick-borne encephalitis virus (TBEV) has increased 400% over the last 30 years. Although recent aggressive vaccination programmes successfully decreased TBE incidence in some locations (e.g. Austria), 10,000 people are hospitalized annually with TBE and the geographic range of the TBE virus continues to expand into areas where it was previously not recorded from (Süss 2007).

Because tick and other vector-borne diseases are complex in origin, resulting from a confluence of relationships involving the arthropod vector, pathogen, and reservoir host(s), as well as relationships between each of these factors and the environment, management of tick-borne diseases, i.e. the process of disentangling each of these factors for reducing tick bites in humans, is a uniquely complex and challenging process that requires the collaboration of experts from diverse fields (e.g. acarologists, ecologists, epidemiologists, healthcare professionals). The OneHealth initiative for the 21st century was recently introduced to help form transdisciplinary teams of human and animal health experts in order to improve management of tick-borne and other zoonotic diseases (Day 2011).

Table 2. Major tick-borne diseases of humans, geographic distribution, causative agent, and tick vector.

Tick-borne disease	Distribution	Agent	Tick vector
African tick bite fever	Africa, West Indies	*Rickettsia africae*	*Amblyomma hebraeum, A. variegatum*
Human granulocytic anaplasmosis	Europe, North America	*Anaplasma phagocytophilum, Ehrlichia ewingii*	*Haemaphysalis concinna, H. punctata, Ixodes ricinus, I. pacificus, I. scapularis, Rhipicephalus bursa*
Human monocytic ehrlichiosis	North America	*Ehrlichia chaffeensis,*	*Amblyomma americanum*
Lyme borreliosis	Asia, Europe, North America	*Borrelia burgdorferi sensu lato*	*Ixodes hexagonus, I. pacificus, I. persulcatus, I. ricinus, I. scapularis*
Mediterranean spotted fever	Africa, Asia, Europe	*Rickettsia conorii*	*Rhipicephalus sanguineus, R. turanicus*
Rocky Mountain spotted fever	North, Central, and South America	*Rickettsia rickettsii*	*Amblyomma americanum, A. aureolatum, A. cajennense, Dermacentor andersoni, D. variabilis, R. sanguineus*
Tularemia	Asia, Europe, North America	*Francisella tularensis*	Many species of different genera
Babesiosis	Europe, North America	*Babesia divergens, B. microti*	*Ixodes ricinus, I. scapularis*
Colorado tick fever	Western North America	Coltivirus	*Dermacentor andersoni*
Crimean–Congo hemorrhagic fever	Africa, Asia, Europe	Naiovirus	*Amblyomma variegatum, H. punctata, Hyalomma anatolicum, H. marginatum, H. truncatum, R. bursa*
Kyasanur forest disease	Indian subcontinent	Flavivirus	*Haemaphysalis spinigera, H. turturis*
Louping ill	Western Europe	Flavivirus	*Ixodes ricinus*
Omsk hemorrhagic fever	Asia	Flavivirus	*Dermacentor marginatus, D. reticulatus, I. persulcatus*
Powassan encephalitis	Asia, North America	Flavivirus	*Dermacentor andersoni, Haemaphysalis longicornis, I. cookei, I. scapularis*
Tick-borne encephalitis	Asia, Europe	Flavivirus	*Ixodes persulcatus, I. ricinus, H. concinna, H. punctate*

Source: Dantas-Torres et al. 2012

1.3.2. Agricultural and veterinary importance of ticks and tick-borne diseases

Cattle industries provide a vital source of protein for the world and are significant drivers of economic growth worldwide. The beef cattle sector is the largest agricultural sector in the United States with sales from beef cattle exceeding US $40 billion annually (Brandebourg et al. 2013).

Cattle ticks, e.g. *Rhipicephalus microplus* and *Rhipicephalus annulatus*, are one-host ticks that feed almost exclusively on cattle. Compared with other ixodid ticks completing their life cycles over several years on two or three hosts, cattle ticks complete their life cycle (larva to adult) in just 28 days on a single, individual steer. In countries where their populations are not managed, cattle ticks devastate entire herds and profoundly affect economic growth (see below).

Ticks transmit several important diseases of cattle (Table 3), chief among them bovine babesiosis and theileriosis, the causative agents of which are *Babesia spp.* and *Theileria spp*. Although difficult to measure, the economic burden of tick-borne diseases is often estimated in hundreds of millions of US dollars annually. In Brazil, recent estimates of total losses due to tick-borne diseases of cattle were estimated at US $800 million per year (Martinenz et al. 2006). In India, the burden of theileriosis, alone, is estimated to result in annual losses of US $384 million (Jongejan and Uilenberg 2004). In addition to transmitting devastating diseases, tick infestations physically damage cattle hides sold for leather and reduce milk production in dairy cattle by up to 25%; over a 1-year period, minor health problems associated with tick infestations, (e.g. stress and lack of appetite), alone, reduce the weight of a typical 500 kg steer by up to 100 kg (de Leon et al. 2012).

Table 3. Major tick-borne diseases of cattle

Tick-borne disease	Distribution	Agent	Tick vector
Bovine babesiosis	Africa, America, Asia, Australia	*Babesia bigemina*	*Rhipicephalus spp.*
	Africa, America, Asia, Australia	*Babesia bovis*	*Rhipicephalus spp*
	Europe	*Babesia major*	*Haemaphysalis spp.*
	North-west Europe, Spain, United Kingdom, Ireland, Tunisia	*Babesia divergens*	*Ixodes spp.*
Tropical theileriosis	Eurasia, Africa, Central Asia	*Theileria annulata*	*Hyalomma spp.*
East Coast Fever	Africa	*Theileria parva*	*Rhipicephalus appendiculatus*
Anaplasmosis	Worldwide	*Anaplasma marginale, A. centrale*	*Ticks of several different genera*
Heartwater	Sub-Saharan Africa, Caribbean Islands	*Ehrlichia ruminantium*	*Amblyomma spp.*

Source: De la Fuente et al. 2008

Historically, ticks of livestock have been the focus of several of the most intense eradication campaigns ever directed at a bloodfeeding arthropod. Between 1906–1943, the U.S. Cattle Fever Tick Eradication Programme ran from southwestern Texas to North Carolina, covering an area roughly 1.8 million km^2. The campaign cost US $46 million (in the early 20th century) to complete and the resulting eradication of *R. microplus* and bovine babesiosis is estimated to save the U.S. cattle industry US $3 billion annually in losses (Graham and Hourrigan 1977). Today, a permanent quarantine zone is maintained along the lower Rio Grande River in Texas and all cattle from Mexico are systematically dipped in vats containing acaricide, primarily coumaphos (1500–3000 µg/ml), before entering the United States of America.

Switching to the veterinary side, the emergence of ticks and human tick-borne diseases (e.g. Lyme borreliosis) over the last 25 years has undoubtedly increased awareness of ticks and contributed to the perception that pets are also at risk for acquiring arthropod-borne infections. Ticks transmit several important diseases of companion animals, namely canine babesiosis, ehrlichiosis, and

Lyme borreliosis to dogs (Table 4), and cytauxzoonosis to cats (Berrada and Telford 2009). Recently, bartonella species were confirmed as additional pathogens likely transmitted to pets by ticks (Reiss et al. 2011).

Table 4. Major canine tick-borne diseases.

Tick-borne disease	Distribution	Agent	Tick vector
Canine babesiosis	Tropical/semi-tropical worldwide	*Babesia canis canis*	*Rhipicephalus sanguineus, Dermacentor reticulatus, D. marginatus*
	Tropical/semi-tropical worldwide	*B. canis vogeli*	*R. sanguineus*
	Southern Africa	*B. canis rossi*	*Haemaphysalis leachi*
	Africa, Asia, United States of America (USA), southern Europe, Middle East	*B. gibsoni*	*Haemaphysalis bispinosa, R. sanguineus*
Canine monocytic ehrlichiosis	Southern USA, southern Europe, Africa, Middle East, eastern Asia	*Ehrlichia canis,*	*R. sanguineus*
Canine granulocytic ehrlichiosis	USA	*Anaplasma phagocytophilum,*	*Ixodes spp.*
	USA	*E. chaffeensis, E. ewingii*	*Amblyomma americanum*
Lyme borreliosis	North America, Europe, Middle East	*B. burgdorferi sensu stricto*	*Ixodes spp.*
	Europe, Asia	*B. burgdorferi sensu lato*	
Rocky Mountain Spotted fever	USA	*Rickettsia rickettsii*	*Dermacentor andersoni, D. variabilis, R. sanguineus*

Sources: Shaw et al. (2001); Berrada and Telford 2009

Over the last 20 years, in parallel with the emergence of ticks, animal health products for companion animals (CAP) have emerged, globally, as the main drivers of growth in the animal health industry (Horspool 2013). Within CAP, the antiparasitics segment is the fastest growing market in the whole of Animal Health (5–10% annually since 2001) (Beugnet and Franc 2012). While the recent growth of CAP cannot be attributed to ticks, alone, nearly all top-selling products in animal health are antiparasitic drugs, and 75% of antiparasitics used in

companion animals are drugs for treating ectoparasites (Beugnet and Franc 2012).

The emergence of the CAP market, in turn, has had significant effects on research and development in the animal health industry. Where in the past acaricides for tick control were developed for use mainly in cattle and then later adapted for use in pets, the opposite is true today. Demand for CAPs, as well as the attractiveness of CAPs from a drug development standpoint – namely less restrictions on persistence of drug residues, as in dairy and beef products (Graf et al 2004) – has indeed shifted the focus of parasiticides research from the farm animal segment to the companion animal segment. Over the last 20 years, several new insecticides and acaricides with novel modes of action (e.g. imidacloprid, fipronil, spinosad, fluralaner) were introduced to the veterinary market as products for treating ectoparasites of companion animals. Table 5 provides the molecular structure and mode of action of each acaricide used in this study.

Table 5. Molecular structures of acaricides, their mode of action, and chemical class.

Chemical class	Acaricide	Chemical structure	Mode of action
Phenylpyrazoles	Fipronil		Block GABA-Cl and Glu-Cl channels
	Ethiprole		
	Pyriprole		
Macrocyclic lactones	Abamectin		Glu-Cl channel receptor agonists
	Ivermectin		
	Eprinomectin		
	Doramactin		
	Moxidectin		
Organophosphates	Coumaphos		Non-competitive binding to ACH-E
	Chlorpyrifos		
Isoxazolines	Fluralaner		Block GABA-Cl and Glu-Cl channels
Organochlorines	Dieldrin		Block GABA-Cl channels

GABA-Cl, gamma-aminobutyric acid-gated chloride channel; Glu-CL, glutamate-gated chloride channel; ACH-E, acetylcholinesterase

1.4. Defining systemic acaricides for tick control

Whether an acaricide is a systemic or contact acaricide is not always clearly defined and depends on the context of its use, target pest, and formulation. A systemic insecticide in agriculture, for example, imidacloprid, is not always referred to as a systemic insecticide in veterinary medicine. In addition, matters are further confused by some pour-on and spot-on formulations, which are applied topically, but which are transdermally absorbed into the bloodstream. A case in point is selamectin (Beugnet and Franc 2012) and such products can act both systemically (in the blood of the animal during parasite feeding) and on contact when the compound is re-released onto the skin of the animal and where it is directly exposed to the parasite in sebum secretions. In some literature, this absorption into the bloodstream and re-distribution of spot-on acaricides on the skin is referred to as the "systemic action" of the compound. To be clear, systemic action in this case only refers to the systemic absorption of the compound into the animal's bloodstream. It is also important to point out that not all spot-on treatments are transdermally absorbed into the blood, but are nevertheless re-distributed over the skin in sebum secretions (Figure 6). Fipronil, for example, has poor percutaneous penetration and does not enter the bloodstream in treated animals in meaningful concentrations (less than 5%; Brayden 2003). It is nevertheless localized in sebaceous glands after application and is indeed re-distributed over the skin in sebum secretions (Brayden et al. 2010). With regard to the physical mode of action of fipronil, since fipronil is formulated as a spot-on that kills ticks in the sebum and hair of treated animals (and not in blood during parasite feeding), it is referred to as a contact acaricide for tick control. The physical mode of action, i.e. contact versus systemic, is not related to the molecular mode of action (GABA-gated chloride channel blocker) and both are used to fully characterize an acaricide. In this study, systemic acaricides are those acaricides that have been formulated for dosing in blood (e.g. tablets or capsules, injectables, boluses and some spot-ons) and designed to kill ticks during tick bloodfeeding on the animal. Other tick control methods include vaccines, repellents, chitin synthesis inhibitors, and biological control methods (e.g. entomopathogenic fungi and parasitic wasps).

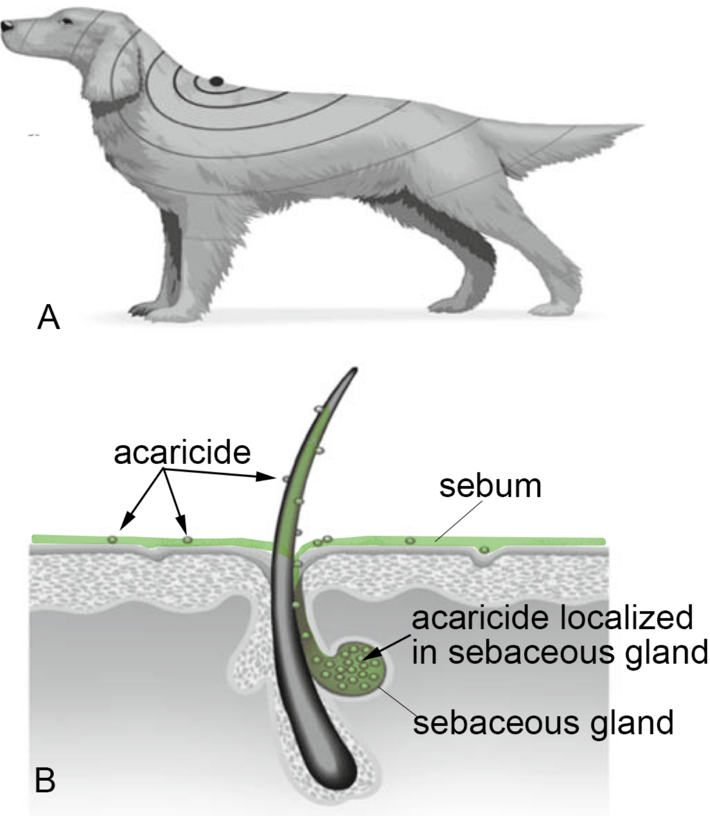

Figure 6. Mechanism of diffusion of fipronil spot-on treatments on dogs. After application of fipronil to the shoulder blades of a dog (A), fipronil localizes in sebaceous glands and is distributed over the dog's body in sebum secretions (B). Modified from Brayden et al. (2010).

1.5. A history of systemic acaricides for tick control

The origin of the use of systemic products for tick control can be traced to the early 1940s when researchers attempted unsuccessfully to feed sulfur to calves in order to control ear tick species infestations (Babcock and Broughton 1943). After World War Two and the introduction of synthetic insecticides, early studies using oral and subcutaneous treatments of chlorinated hydrocarbons (Roulston 1956) reported tick mortality in rodents and cattle (Loomis et al. 1972), and demonstrated for the first time that blood feeding ticks could be killed after feeding on hosts treated systemically with a chemical insecticide. The practice, however, was not recommended due to concerns over mammalian toxicity and the persistence of chemical residues in animal tissues, among other concerns (Loomis et al. 1972). During the 1950s and 1960s, intense research, meanwhile, was being conducted with systemic insecticides for treatment of other

ectoparasites, namely cattle grubs, *Hypoderma spp*. Ironically, these works mainly investigated uses of newly introduced organophosphate insecticides (see below), which are generally regarded as the most toxic insecticides available for mammals, today.

Graham and Drummond (1967) review early success in eradicating cattle grubs in parts of Europe using systemic organophosphate formulations, mainly systemic cromate pour-ons. The success of these programmes (e.g. in Ireland, 1967) came after several failures elsewhere to eradicate cattle grubs using contact formulations, which required laborious and repetitive treatments, and which only affected grubs on the backs of cattle where the compounds were sprayed (Graham and Drummond 1967). The introduction of systemic pour-ons for organophosphates facilitated treatment with persistent efficacy (one treatment per year) and afforded 100% control of grubs over the entire body of the cattle; in addition, pour-on treatments were considered suitable for treating herds of animals and did not require special machinery for application (Graham and Drummond 1967).

The successful cattle grub eradication campaigns of the 1960s had far-reaching effects and generated momentum for increased research on systemic insecticides for ectoparasite control. Shortly after, Drummond and colleagues, among others, focused several projects on discovery of new systemic insecticides and acaricides, including projects focused on systemic acaricides for control of ticks on animals. Drummond and Graham (1964) placed organophosphates in the feed of horses at doses ranging from 1–20 mg/kg/day and obtained 100% control of *Anocentor nitens* using 20 mg/kg dosed for 10 consecutive days. Gladney et al. (1972), using the same model, fed lower concentrations (1.5–7 mg/kg) for longer periods of time (up to 21 days) and concluded that famphur at 5 mg/kg after 7 days of treatment showed promise as a systemic acaricide for ticks. It is important to point out that some organophosphates and dosing regimes in the latter study led to insecticide poisoning and death in horses. In similar experiments using cattle, Drummond et al. (1972) reported limited control of three-host ticks using systemic organophosphates. In summary, early attempts to control ticks with systemic

organophosphates had limited effects on tick mortality, required repetitive dosing regimes over several days, and required relatively high doses of compound (up to 20 mg/kg in some cases) with high mammalian toxicity.

The discovery of the avermectins (mid 1970s), for the first time, introduced many of the ideal characteristics previously envisioned for systemic acaricides for tick control, namely low dose treatments (often measured in µg/kg), low mammalian toxicity, and persistent efficacy (see below). Drummond (1985) reviewed early tests with ivermectin as a systemic acaricide for ticks, noting in cattle, a single subcutaneous injection of ivermectin (200 µg/kg) controlled 3-host species for 3–5 days and 1-host species for 21 days post-treatment. This was unprecedented at the time for systemic acaricides both in terms of dose and residual effectiveness. These works launched decades of research for discovery of improved analogues of ivermectin (e.g. eprinomectin with low persistence in dairy and muscle tissue) and other systemic formulations (e.g. pour-ons, slow-release intra-ruminal boluses, and capsules). As recently as 2009, doramectin and eprinomectin (50–200 µg/kg) were tested in oral capsule form against *A. americanum* on cattle (Lohmeyer et al. 2009).

Within the companion animal products (CAP) sector, selamectin, an avermetin, as previously mentioned, was introduced for systemic control of parasites on pets, however this product is primarily active against fleas and did not receive registration for ticks. Thus, despite advances with avermectins, non-systemic spot-on treatments dominated CAP markets for tick control in companion animals. In the farm animal sector, a similar trend has been realized. Around the same time the avermectins were introduced, the first commercial pour-on for tick control in cattle was introduced in 1985 (Stendel 1985). Despite being non-systemic, the pyrethroid flumethrin in pour-on form demonstrated a remarkable ability to spread to all areas of the cattle body and resisted degradation under environmental conditions (George et al. 2004). In addition, flumethrin pour-ons had higher efficacy and persistence than systemic avermectin pour-ons against ticks. Injectable avermectins proved more efficacious than many pour-ons, but high costs and concerns over transfection through the use of needles limited their use (George et al. 2004).

The upshot of these conclusions is that, despite advances, the avermectins never fully delivered as systemic acaricides for tick control, especially for three-host ticks of companion animals. In 2008, spinosad, a natural macrocylic lactone spinosyn, was introduced as the first drug in tablet form with systemic activity against fleas for up to 30 days in dogs (Snyder et al. 2007). The success of this product significantly shifted market dynamics in the animal health industry and drove intense efforts for discovery of similar products for ticks. During the work contained in this study, afoxalaner and fluralaner, two molecules from the newly discovered isoxazoline class of acaricides, were introduced for treating flea and tick infestations in companion animals; formulated as chewable tablets, these molecules represent the first systemic acaricides in history to provide effective tick control for up to 12 weeks in animals following a single oral treatment (Gassel et al. 2014).

1.6. A brief review of the arthropod nervous system

Nearly all major insecticides and acaricides exert their effects on the parasite nervous system, causing death through disruption of nervous system functions, e.g. disruption of nerve impulse transmission. The arthropod nervous system relevant for understanding the modes of action of acaricides is briefly reviewed below.

Basic functions of the arthropod nervous system include collecting and integrating sensory input signals (i.e. taste, smell, touch, host recognition) and coordinating behavioral output signals (i.e. leg movements, host attachment, feeding and swallowing). Informational signals in the nervous system are transmitted in the form of wave-like, self-propagating electrical impulses, known as action potentials, and the transmission of action potentials – e.g. between sensory organs and the brain, and the brain and muscle tissues – occurs within branching neuronal filaments, known as axons and dendrites. Axons, in turn, network with each other at junctions known as synapses, where action potential transmission might be inhibited or further propagated to connecting neurons or muscle tissue. Voltage-gated ion channels involved in regulation of action potentials and synapses are major sites exploited by parasiticides. Critical

aspects of pre-synaptic and synaptic signal transmission are reviewed in more detail below.

Pre-synaptic action potential transmission within axons is regulated by membrane-bound ion channels and pumps (e.g. voltage-gated sodium channels), which line the length of the axon. At rest, these proteins maintain an unequal distribution of ions with positively charged sodium ions maintained in excess outside of the axon, creating a resting potential of -70 mV inside of the axon. The arrival of a propagating action potential, in the form of a depolarization event (i.e. opening of an adjacent sodium channel) causes rapid localized diffusion of sodium ions into the axon, leading to the inside of the axon becoming positive and the outside negative. This localized reversal in voltage (+40 mV), in turn, triggers opening of the adjacent voltage-gated sodium channel, and it is in this manner – localized depolarization event triggering opening of an adjacent voltage-gated ion channel – that an action potential self-propagates down the length of an axon. Potassium pumps play an important role in maintaining the uni-directional nature of the impulse, however since insecticides do not currently exploit axonal potassium pumps, they are not considered here.

When an action potential reaches the terminal end of an axon, it encounters a synapse, networking the axon with other axons, e.g. motor neurons controlling muscle function. The arrival of the action potential, and subsequent depolarization event in the terminal end of the axon, results in the release of calcium ions, triggering exocytosis of neurotransmitters, which are released into the synapse. The neurotransmitters subsequently traverse the synapse and bind to corresponding ligand-gated ion channels located on any networked post-synaptic neurons. Depending on the neurotransmitter (e.g. gamma-aminobutyric acid (GABA), glutamate (Glu), acetylcholine (Ach)) and the ligand-gated ion channel that is ultimately activated, activation of post-synaptic ion channels might cause further propagation of the impulse, or inhibition. Stimulation of post-synaptic ligand-gated receptors is further controlled by neuromodulators located in the synapse (e.g. esterases), which bind and degrade neurotransmitters, halting their action.

There are therefore three fundamental targets in the tick nervous system that might be exploited by acaricides: 1) axonal voltage-gated ion channels, 2) postsynaptic ligand-gated ion channels, and 3) neuromodulators in the synaptic cleft. The mode of action of each insecticide addressed in this study is reviewed in detail, below.

1.7. Molecular modes of action of acaricides

1.7.1. Organophosphates

Chlorpyrifos and coumaphos, examples of synthetic organophosphate acaricides, inhibit the function of acteylcholinesterase and block degradation of acetylcholine in the synapse (Nauen et al. 2011). During organophosphate poisoning, the esterase residue of the organophosphate molecule binds permanently to the active site of acetylcholinesterase, blocking binding of acetylcholine, and causing a build-up of acetylcholine in the synapse. Accumulation of acetylcholine in the synapse leads to overstimulation of the nervous system and death in the insect.

Organophosphates benefit from an exceptionally short half-life in the environment, ranging from hours to weeks (with most about 2 days) (Nauen et al. 2011). This instability in the environment makes organophosphates attractive as insecticides compared with earlier insecticides, e.g. organochlorines, which were extremely persistent (half-life: 2–15 years). Organophosphates target acetylcholinesterase, a highly conserved esterase in the nervous systems of both invertebrates and vertebrates, leading to cross-reactivity and relatively high toxicity in mammals (rat oral LD_{50}–values between 5–50 mg kg^{-1} for most compounds) (Nauen et al. 2011).

1.7.2. Avermectins and milbemycins

The discovery of the avermectins (macrocyclic lactones derived from natural molecules produced by Actinomycete fungi) in the mid 1970s revolutionized the treatment of endoparasitic infections in humans and animals on a scale similar to that of the discovery of penicillin for treatment of bacterial infections. Both the

avermectins and closely related milbemycins (e.g. moxidectin) potentiate inhibitory post-synaptic glutamate-gated chloride channel receptors (Glu-Cls) leading to paralysis and death, however their mode of action in arthropods, although understood in detail, is not completely understood (see below).

Ivermectin has confusingly been shown to both potentiate (Holden-Dye et al. 1988; Holden-Dye and Walker, 1990) and inhibit (Martin and Pennington, 1988) gamma-aminobutyric acid (GABA)-gated chloride channel receptors in nematodes; however, more recent data strongly suggest the main site of action of the avermectins to be a receptor-binding site on the Glu-Cl receptor (reviewed in Wolstenholme and Rogers 2005). Recombinant Glu-Cls are activated by nanomolar concentrations of ivermectin (McCavera et al. 2009) and ivermectin was shown to bind to a Glu-Cl binding site distinct to that of glutamate (Hibbs and Gouaux 2011). Ivermectin binding to Glu-Cl receptors causes irreversible opening of inhibitory Glu-Cl channels and ultimately paralysis leading to death (reviewed in Wolstenholme and Rogers 2005). Noteworthy, as well, are studies using radiolabeled ivermectin, which was shown to localize in the pharynx of nematodes (where Glu-Cls are known to be localized) and caused paralysis of the pharynx (reviewed in Wolstenholme and Rogers 2005). The avermectins have remarkably low toxicity in mammals due to the fact that Glu-Cls are not present in mammals. Ivermectin also appears to be a remarkably efficient binding substrate for P-glycoprotein pumps. A loss-of-function mutuation in P-glycoprotein pumps in collie dog breeds increases toxicity of the avermectins to this breed (Mealey et al. 2001).

1.7.3. Phenylpyrazoles

Fipronil, pyriprole, and ethiprole, examples of synthetic phenylpyrazole acaricides, block inhibitory GABA-Cl channels, leading to overstimulation of the tick nervous system and death. The fipronil molecule binds deep within the channel of the GABA-Cl, blocking its action (Ozoe 2013). Recent results also suggest fipronil acts on Glu-Cls, where it also binds inside the channel, blocking the Glu-Cl and leading to further overstimulation (Narahashi et al. 2010). Regarding affinity for the two channels in ixodid ticks, Gassel et al. (2014)

showed that the GABA-Cl (IC_{50}: 8.5 nM) is 7-fold more sensitive to fipronil than the Glu-Cl (62 nM) in *R. microplus.*

1.7.4. Isoxazolines

Synthetic isoxazolines, including fluralaner and afoxalaner, represent the newest class of compounds with activity against ectoparasites of companion animals. These molecules block GABA-Cls in a manner similar to fipronil, but at a site distinct from fipronil, and also inhibit Glu-Cls (Gassel et al. 2014). GABA-Cls in *R. microplus* are 52-fold more sensitive (IC_{50}: 1.6 nM) to fluralaner compared with Glu-Cls (82.5 nM). Compared to fiponil, fluralaner is a 5–236-fold more potent inhibitor of GABA-Cls at all five arthropod GABA-Cls tested in the study cited. Their findings ultimately support the model that fluralaner is a molecule with extraordinary inhibitory potency for arthropod GABA-Cls with less, but still significant inhibitory activity on Glu-Cls.

1.8. Feeding behavior in ixodid ticks with reference to *in vitro* feeding using artificial membranes

As ticks attach, they rip and tear host skin with outward cutting and sawing movements with their chelicerae (Balashov 1972). In addition, ticks frequently detach and reattach at different sites on the host (Balashov 1972). After finding a site for attachment, many ixodid ticks then secrete a proteinaceous cement (Bullard et al. 2016) to help anchor the mouthparts in place (Kemp et al. 1982). The tick's pattern of cement use, which is a determinant of hypostome penetration depth (Figure 7; Moorhouse 1969 and see below), has important consequences for *in vitro* feeding using artificial membranes. Females of *R. microplus*, for example, utilize cement extensively and attach only superficially in the first 50 µm of the mostly keratin-rich epidermis (Tatchell and Moorhouse 1968; Figure 8). To reach and feed on blood, they rely on their salivary secretions and the expansion of the feeding lesion (hematoma; Figure 8) that forms below their hypostome (Tatchell and Moorhouse 1968; Berenberg et al. 1972). In female *R. microplus*, the feeding lesion extends up to 1.75 mm, into the capillary-rich dermis, from the tip of the hypostome (Tatchell and Moorhouse 1968). Kemp et al. (1975) note salivary secretions cannot aid in the formation of a feeding lesion in an artificial membrane. In other words, ticks cannot attach superficially to artificial membranes (e.g. comprised of silicone) and then rely on an expanding feeding lesion below their hypostome to reach whole blood; instead ticks must fully penetrate the membrane with their hypostome. It is therefore critical that both hypostome length and pattern of cement use are considered when developing an artificial membrane for an ixodid tick. As an example, adult *R. microplus* have hypostomes 350 µm in length (Moorhouse 1969), however, because of their use of cement and superficial attachment, an artificial membrane for this tick would ideally have a thickness of no more than 50 µm. This extraordinarily thin membrane must be strong enough to withstand the initial ripping and tearing by the tick, and it must be flexible enough to prevent bleeding after ticks detach. Additionally, ixodid ticks feed for several days and they consume more than 100-times their initial weight in blood; the membrane must be resistant to degradation, and it must also be able to support a tick's massive

increase in size and weight over the course of its bloodmeal.

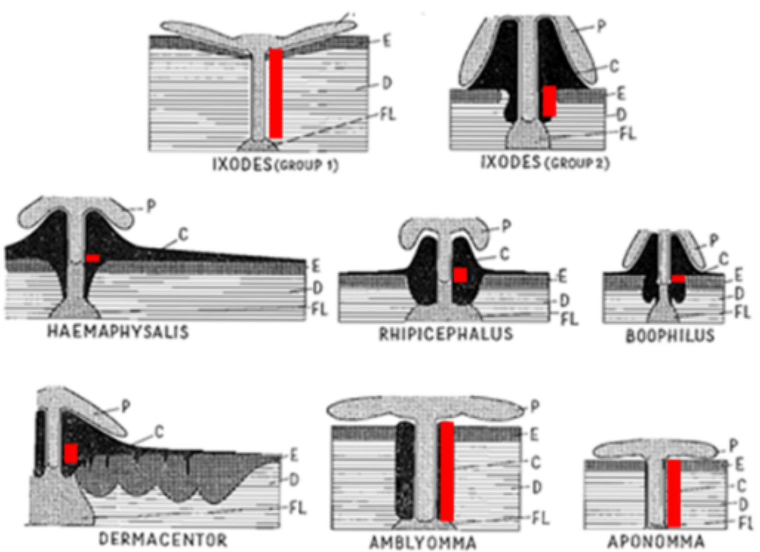

Figure 7. Patterns of cement use in the ixodidae. Red bars highlight the depth of the hypostome of each species in host tissue (modified from Moorhouse 1969).

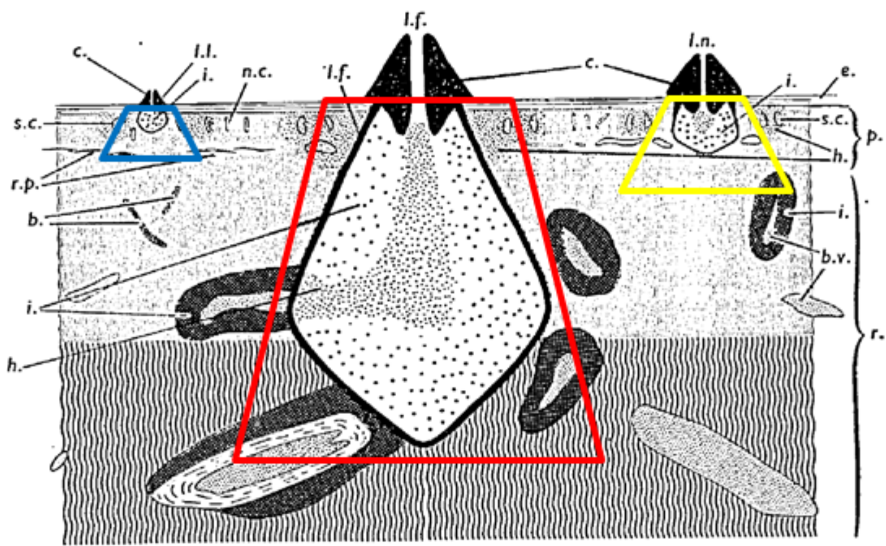

Figure 8. Feeding lesions of larvae (blue), nymphs (yellow) and females (red) of *R. microplus* on cattle (modified from Tatchell and Moorhouse 1968).

1.9. Artificial membranes and attachment requirements for ixodid ticks

Several factors related to the unique attachment requirements of ixodid ticks make it difficult to feed them *in vitro* using artificial membranes. *In vivo*, ixodid tick attachment is described as a multi-step behavioural cascade of events dependent upon the presence of host-associated attachment stimuli (Waladde and Rice 1982).

Burgdorfer (1957) successfully fed several argasid ticks *in vitro* as early as the 1950s using the air sac membrane of an embryonated hen egg, but noted ixodid ticks would not attach to this membrane. Pierce and Pierce (1956) used the same air sac membrane to feed *B. microplus in vitro* and finally obtained attachment after treating the membrane with bovine nasal mucin and other bovine secretions. In years following, it has become increasingly clear that ixodid ticks require considerable efforts on the part of the researcher to induce ixodid tick attachment and feeding to repletion *in vitro* using artificial membranes. Despite attempts by others, it was not until the early 1990s that Waladde et al. (1991) described the first semi-artificial membrane made from bovine intestine impregnated with biodegradable glue for feeding ixodid ticks to repletion. Working with adults of the brown ear tick, *Rhipicephalus appendiculatus*, they treated their membrane with “cattle ear wash”, among other stimuli, and obtained 63% engorgement.

Following Waladde and colleagues, Habedank and Hiepe (1993) introduced the first completely artificial membrane made from silicone for *in vitro* feeding of ixodid ticks. Kuhnert et al. (1995) then described an improved silicone membrane reinforced with cellulose rayon lens paper for *in vitro* feeding of *A. hebraeum.* This membrane permitted tick attachment and engorgement, *in vitro* (see Table 6) and Kuhnert et al. (1995) fed consecutive life stages of *Amblyomma hebraeum, in vitro*, starting with *in vitro*-fed adults (molted from nymphs fed *in vivo* on cattle) and ending with first generation *in vitro*-reared adults, thereby completing the life cycle of an ixodid tick for the first time, *in vitro*. They obtained 95% attachment using *in vivo*-reared nymphs fed *in vitro* and 97% attachment

using first generation *in vitro*-reared nymphs fed *in vitro*. During completion of the life cycle, observations of biological parameters associated with development and reproduction in *in vitro*-fed ticks, specifically engorged weight and molting success (Table 7) and egg production and percent larval hatch (Table 8) were recorded. Although rates of attachment and engorged weights of nymphs fed *in vitro* and *in vivo* were not significantly different, and *in vitro*-reared males had higher attachment *in vitro* than *in vivo*-reared males fed *in vitro*, Kuhnert et al. showed that female ticks fed *in vitro* generally lose their reproductive capacity during *in vitro* rearing. The egg conversion factor (ECF), defined as the number of eggs laid divided by engorged weight, was roughly halved in *in vitro*-fed ticks compared to *in vivo*-fed ticks. Decreased reproductive capacity was especially pronounced in first generation *in vitro*-reared females, which produced 15 mg of eggs per female compared with over 1 g of eggs produced per *in vivo*-fed tick. Feeding duration, as well, was prolonged roughly five days in *in vitro-fed* females and engorged weight of *in vitro*-fed females was less than half that obtained by *in vivo*-fed ticks. Kuhnert et al. (1995) discussed several possible explanations for the reduced fertility of *in vitro*-fed ticks in their system, including possible reduced fertility of males fed *in vitro*, degradation of nutrients in blood during storage for 1-week, reduced levels of oxygen in stored blood, and possible effects of the bacteriostat (gentamicin) and fungistat (nystatin) (used, respectively, to prevent bacterial and fungal contamination in blood during feeding) on gut endosymbionts. They also speculated that it was possible that the membrane thickness they used (500 microns) was possibly too thick and that as females entered the rapid feeding phase the mouthparts were pulled out of the membrane by way of body expansion.

More recently, Kröber and Guerin (2007a,b,c) improved the silicone membrane of Kuhnert et al. (1995); they described a silicone glue with a low Shore A hardness (a measure of the indentation hardness of a material), and treated the glue with silicone oil in order to make the membrane softer and more elastic for *in vitro* feeding of *I. ricinus.* Membranes 50–100 microns thick were used for feeding *I. ricinus* females. Kröber and Guerin (2007a) obtained 77–85% attachment on membranes with *I. ricinus females* and 60% of attached ticks were alive and feeding after 9 days. The engorged weight of detached *I. ricinus* females, fed to

repletion after 16 days, was 160 mg and this weight was comparable to *I. ricinus* fed *in vivo* on rabbits in their laboratory. Fifteen out of 19 engorged ticks laid eggs in their lab and larvae hatched 4–8 weeks later.

More recently, Andrade et al. (2014), building further on the advances of Kuhnert (1997) and Kröber and Guerin (2007a,b), described a simplified silicone membrane for *in vitro* feeding of *Ixodes scapularis* females to repletion. They obtained 49% tick attachment using the membrane (195 microns thick) and 11 out of 19 engorged ticks laid eggs in the laboratory and larvae hatched six weeks later.

Table 6. Attachment rates of *A. hebraeum* life stages fed *in vivo* and *in vitro* during completion of the life cycle *in vitro* (Kuhnert et al. 1995).

Treatment	Males	Females	Nymphs	Larvae
Control fed on steer	95–100	95–100	90–100	No data
In vivo-reared ticks fed *in vitro*	31	46	95	~30
In vitro-reared ticks fed *in vitro*	60	63	97	~30–90

Data are means (95% confidence limits not shown) for 5 – 10 replicates.

Table 7. Drop-off day and molting rates for *A. hebraeum* nymphs fed *in vivo* and *in vitro* during completion of the life cycle *in vitro* (Kuhnert et al. 1995).

Treatment	Drop-off day	Engorged weight (mg)	Molting rate (%)
Nymphs fed on steer	d5	66	98
In vivo-reared nymphs fed *in vitro*	d7	57	96
In vitro-reared ticks fed *in vitro*	d8	63	98

Data are means (95% confidence limits not shown)

Table 8. Reproductive success of *A. hebraeum* females fed *in vivo* and *in vitro* during completion of the life cycle *in vitro* (Kuhnert et al. 1995).

Treatment	Drop-off day	Engorged weight (g)	Pre-oviposition days	Egg conversion factor	% Larval hatch	% Larvae producing females	Fertile egg production (mg/tick)
Females fed on steer	9	2.56	7.8	0.57	87	97	1229
In vivo-reared females fed *in vitro*	8–9	0.98	15.4	0.27	69	46	109
In vitro-reared females fed *in vitro*	14	1.23	19	0.24	39	10	15

Data are means (95% confidence limits not shown) for 4–106 ticks.

1.10. *In vitro* testing of systemic acaricides for ixodid ticks

Despite increased interest in systemic acaricides for tick control in recent years and the introduction of two new systemic acaricides to the veterinary market, Kuhnert et al. (1995) and Kröber and Guerin (2007a) represent the only two studies in the literature on *in vitro* testing of systemic acaricides for control of hard ticks. Kuhnert et al. (1995) demonstrated for the first time that dimethyl sulfoxide (DMSO) could be used as a delivery vehicle for delivering acaricides to ticks in blood. In their experiment evaluating the systemic efficacy of ivermectin (1 and 10 µg/ml), DMSO in the placebo treatment had no effect on mortality or engorged weight of *A. hebraeum* nymphs. Mortality recorded for 1 and 10 µg/ml ivermectin was 54% and 94%, respectively, and Kuhnert et al. (1995) also showed that the development of ivermectin-exposed ticks (expressed as engorged weight) was drastically reduced compared to the placebo.

Kröber and Guerin (2007a) extended the work of Kuhnert et al. (1995), comparing the systemic efficacy of ivermectin with fipronil in their analysis, and covering a dose range of 0.001–10 µg/ml in blood. Mortality plots for fipronil and ivermectin repeated 1-year apart from each other were nearly identical, and using survival curves, they showed significant systemic dose-dependent affects on female *I. ricinus* mortality down to 0.001 µg/ml for fipronil and 0.1 µg/ml for ivermectin. Kröber and Guerin (2007a) also compared the effects of fipronil and ivermectin on *I. ricinus* engorged weight at select concentrations using a subset of the tick population in each treatment. Ultimately, feeding inhibition due to systemic effects of fipronil and ivermectin on bloodfeeding ticks, *in vitro*, was demonstrated using just a fraction of the quantity needed for an *in vivo* test. A total of 5 mg fipronil was used for their test over 9 days at four dose levels, i.e. just 5% of what is required to be effective against ticks on one dog (Kröber and Guerin 2007a).

2. Development of a standardized silicone membrane for *in vitro* feeding of ixodid ticks

2.1 Abstract

Ixodid ticks are one of the most difficult haematophagous arthropods to feed *in vitro*. A simplified silicone membrane was developed for *in vitro* feeding of ixodid ticks, and a standardized method was described to manipulate the thickness of the membrane in order to accommodate the hypostome of *A. hebraeum* nymphs. Silicone glue was dissolved in toluene and the relationship between silicone concentration and membrane thickness was described. The variability in membrane thickness was characterized within and between membranes at five different locations on the membrane. The relationship between solute concentration and membrane thickness was linear, and the range of membrane thicknesses obtained was 61–584 µm. The thickness of membranes, and variability in membrane thickness, increased with increasing solute concentration. Silicone membranes (thickness: 208–540 µm) were tested in *in vitro* feeding trials with *A. hebraeum* nymphs. Tick attachment to membranes increased with decreasing membrane thickness. Using a membrane 208 µm thick (n = 24 feeding units with 20 ticks per unit), mean tick attachment and engorgement (95% confidence interval) was 86% (82–90%) and 79% (74–84%) with *A. hebraeum* nymphs; 95% of the engorged ticks molted into adults. The silicone membrane described, and method for preparing the membrane, simplifies *in vitro* feeding of ixodid ticks.

2.2 Introduction

Artificial feeding systems for ixodid ticks facilitate tick attachment and feeding to repletion, *in vitro*, under controlled conditions, and are among the most useful tools for studying ticks in the laboratory. Uses of *in vitro* feeding systems for ticks include *in vitro* screening of acaricides, controlled introduction of pathogens into ticks, and isolation of bioactive substances secreted by ticks in tick saliva during feeding, among many other uses, for example (Kröber and Guerin 2007b; Stiller and Coan 1995):

- Testing tick susceptibility and resistance to acaricides
- Post-genomic studies on the role of gene products that target tick physiological processes, including RNA interference experiments
- Comparative susceptibility of ticks (vector competence) for disease-causing pathogens
- Comparative infectivity of different species or strains of pathogens for ticks
- Minimum tick feeding time necessary for acquisition of tick-borne pathogens
- Minimum tick feeding time necessary for transmission of tick-borne pathogens
- Minimum pathogen dose required to infect ticks

The major limiting factor for ixodid tick attachment and engorgement, *in vitro*, is an artificial membrane that is able to mimic host skin and support tick feeding over several days (reviewed in Kröber and Guerin 2007b). Specifically, to be suitable for *in vitro* feeding of ixodid ticks, an artificial membrane needs to induce tick attachment, prevent bleeding during tick attachment, feeding, and detachment, and needs to be resistant to degradation in blood over several days. In addition, from a membrane preparation standpoint, it is necessary to be able to manipulate membrane thickness in order to be able to accommodate the different

sized mouthparts of tick species (reviewed in Kröber and Guerin 2007b). Satisfying all of these requirements with a membrane made from artificial materials has proved challenging and simplification of existing methods in the literature for artificial feeding of ticks has been slow. Despite advances with artificial membranes for ixodid ticks over the years, ixodid ticks remain one of the few bloodfeeding arthropods still commonly fed *in vitro* using membranes derived from animal skins (Bonnet and Ye Liu 2012).

Tajeri and Razmi (2011) recently fed *Hyalomma spp*., Fourie et al. (2013) fed *Rhipicephalus sanguineus,* and Campbell et al. (2010) and Bouwknegt et al. (2010) fed *I. ricinus,* using artificial membranes prepared according to methods described in Kuhnert et al. (1995) and Kröber and Guerin (2007a). Kuhnert et al. (1995), as previously discussed, described a method for preparing silicone membranes for *in vitro* feeding of *Amblyomma hebraeum* by impregnating cellulose rayon lens paper with silicone glue. Kröber and Guerin (2007a) improved this membrane by replacing the silicone glue described in Kuhnert et al. (1997) with Elastosil E4 silicone glue (Wacker, Germany), a glue with a low (16) shore A hardness (a measure of indentation hardness), to which they also added silicone oil. These modifications introduced a membrane that was softer and more elastic than the membrane described in Kuhnert et al. (1995), and prevented bleeding or leaking after ticks detached. Kröber and Guerin (2007a) also added hexane to the Elastosil E4 glue-silicone oil mixture, which made impregnating lens paper, as originally described in Kuhnert et al. (1995), an easier process (see below).

Preparation of silicone membranes for *in vitro* feeding of ixodid ticks, as described in Kuhnert et al. (1995) and Kröber and Guerin (2007a,b,c), involves impregnation of lens paper with silicone glue using a custom-made spatula. Briefly, lens paper is laid out over a flat surface and an undefined amount of the silicone preparation is applied to the paper; using the spatula, the glue is then spread into the fibers of the paper. The addition of the hexane to the glue reduces the ‘frog grip’ of the glue and increases its spreadability, making it easier to saturate the fibers of the lens paper using the spatula (Kröber and Guerin

2007a). While improved, this method for preparing silicone membranes (Kuhnert et al. 1997; Kröber and Guerin 2007a), remains a delicate procedure (see below).

Membranes 50–100 µm thick were used to feed *I. ricinus* adults (Kröber and Guerin 2007a). As Tiffen® lens paper is roughly 30 µm thick, after impregnating the lens paper with silicone, a final layer of silicone with a thickness of just 20–70 µm must therefore be added to the membrane in order to bring it to a final thickness of 50–100 µm. Addition of this layer of silicone to the membrane, which for reference is roughly 20% of the thickness of a human hair, is a procedure that is difficult to standardize and relies on the researcher's skill with the spatula. All major advances in semi artificial and artificial membranes for ixodid ticks up to 2014, namely glue reinforced with Baudruche membrane (Waladde et al. 1991,1995) and silicone reinforced with cellulose rayon lens paper (Habedanke and Hiepe 1993; Kuhnert et al. 1997; Kröber and Guerin 2007a,b,c), have all used variations of the procedure described above for preparing membranes to the desired final thickness for *in vitro* feeding of ixodid ticks.

In addition to descriptions of new membrane materials, research focused on *in vitro* feeding of ticks over the years has also focused on new and innovative attachment and feeding stimuli, new methods for maintaining controlled temperature, humidity, and lighting in feeding units, different blood sources, anticoagulants, and anti-infectives used to prevent blood contamination, and descriptions of optimized, miniaturized, or simplified feeding units for housing ticks during feeding.

Andrade et al. (2014) recently described a simplified method for preparing silicone membranes reinforced with cellulose rayon lens paper (Kröber and Guerin 2007a) for *in vitro* feeding of *I. scapularis* adults. Attachment rate of *I. scapularis* females to these membranes was 49%, significantly lower than that reported by Kröber and Guerin (2007a) for *I. ricinus* (77–85%). This chapter focuses on characterization of the method of Andrade et al. (2014), improvements made to the method to induce and support high rates of tick attachment and engorgement, and descriptions of an improved and standardized method for *in vitro* feeding of ixodid ticks. To this end, this chapter also focuses

on development of feeding unit design, maintenance of temperature and humidity during feeding, blood sources and treatments, and basic components of the feeding system used for introduction of acaricides into ticks described in the following chapters of this study.

2.3 Materials and methods

2.3.1 Ticks

Unfed *Amblyomma hebraeum* nymphs from a pathogen-free colony bred in-house were used in feeding trials during characterization and development of the *in vitro* feeding assay. Nymphs were starved 4–6 months prior to feeding experiments and stored in environmental chambers at 80% RH, 16:8 light-dark cycle.

2.3.2 Preparation of silicone membranes and feeding units for *in vitro* feeding of ixodid ticks

Silicone membranes for *in vitro* feeding of ixodid ticks were prepared by dissolving RTV Elastosil© E4 silicone glue (Wacker, Burghausen, Germany) in toluene (C_7H_8; solvent grade, Sigma) at concentrations of 168–671 gL^{-1}. The concentration of glue used was manipulated depending on desired membrane thickness, which varied according to the length of the hypostome of the tick species being fed (see below).

Membranes were prepared according to methods described by Andrade et al. (2014), which were significantly modified. For all membranes, 10 g of glue was used and only the volume of toluene (10–55 ml) was changed for manipulating glue concentration and thereby membrane thickness. Ten grams of glue plus silicone oil (30% or 3 g) was weighed out in a 100 ml beaker and briefly mixed using a glass rod stirrer. Depending on the desired thickness, toluene (see table 9) was added to the silicone glue and silicone oil. The mixture was briefly mixed with the toluene using a glass stirrer in order to dissolve the glue at the bottom of the beaker. The mixture was then transferred to an automatic magnetic stirrer (600 rpm) for 20 minutes, using a stirring bar (35X6 mm; PTFE Labware). After being dissolved into solution (Figure 9), a square of lens paper (2.5 cm X 2.5 cm; Tiffen®, New York, USA) was picked up at a corner using thin forceps (Aesculap no. BD215R) and submerged in the glue solution and removed in one motion, fully coating the paper with the glue solution. Excess glue was briefly allowed to

run off into the beaker and the membrane was then hung on a drying line by a corner using a binder clip (Figure 9).

Table 9. Reference table showing the range of membrane thicknesses (61–584 µm) prepared with the method. Data are presented as mean thickness with confidence intervals (95%) of the center of the membrane for nine repetitions per concentration. Membrane codes (T10–T55) are based on the volume of toluene used to prepare the membrane using 10 g of silicone glue.

Membrane code	Volume of toluene (ml)	Concentration of silicone glue (gL^{-1})	Membrane thickness (µm)
T10	10	671	584 (490–677)
T15	15	502	384 (336–434)
T20	20	412	308 (277–340)
T25	25	339	208 (173–244)
T30	30	291	158 (147–170)
T35	35	253	131 (121–141)
T40	40	228	100 (95–104)
T45	45	206	83 (79–88)
T50	50	188	84 (79–89)
T55	55	168	61 (57–66)

Figure 9. Silicone glue before (A) and after (B) dissolving in toluene. Squares of lens paper are dipped into solution B to prepare membranes. Right: The drying line used for air-drying membranes in a fume hood consisting of fishing line and binder clips.

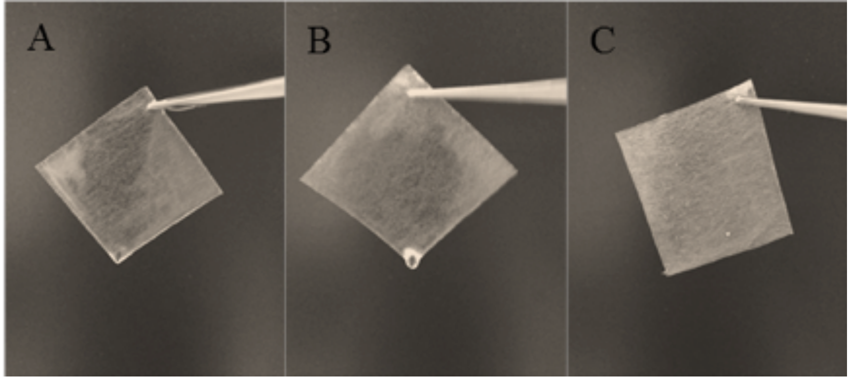

Figure 10. Freshly prepared (A), partially dry (B) and a dry membrane (C). Note the glossy, wet finish of freshly prepared membranes which disappears over time as toluene evaporates, leaving behind the silicone.

To prepare feeding units (see below), membranes were dried (Figure 10) for 24 hours (T10–35) or 5 hours (T40–45), depending on toluene concentration. Using forceps, partially dried membranes were then laid out on kitchen plastic film placed over a sheet of glass. The plastic film was taped at the corners of the glass for a tight seal without air bubbles underneath. Membranes were laid out on the plastic film and care was taken to ensure membranes laid flat on the film without creases. Glass feeding units (custom-made in-house; OD: 22 mm; ID: 18 mm; H: 50 mm; edges rough-cut, i.e. not fire polished) were directly pressed on to the semi-dry membranes and allowed to dry for 48 hours. After 48 hours, a commercial cork borer (diameter: 23 mm; Fisher) was used to cut the feeding units from membranes (Figure 11). A tight-fitting cotton plug (H: 55mm; OD: 21mm; Coltene GmbH, Lengenau, Germany) was used to prevent tick escape and inserted to within 2 cm of the membrane. A custom-made rubber washer (OD: 30 mm; ID: 20 mm; H: 2 mm) was placed around the glass vial (5 mm from the top) in order to control the depth the feeding unit sank into blood (3 mm from bottom) after placement in the 60-well plate.

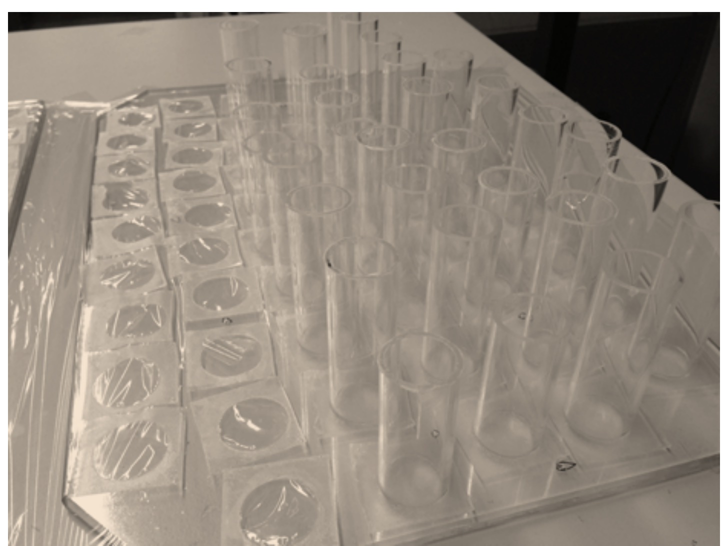

Figure 11. Set-up for preparation of feeding units. Kitchen plastic film is placed over glass and membranes (dried 1–24 hours depending on membrane type) are laid out flat. Feeding units are pressed onto membranes and cut out after 72 hours using a corkborer.

2.3.3 Blood preparation and feeding assay

Whole bovine blood (600 ml) treated with heparin (10 i.u./ml; Sigma, Germany, H3393) was aseptically collected from the jugular vein of Holstein cows and treated with gentamicin (5 ug/ml; Sigma, Germany, G1272) and nystatin (100

i.u./ml; Sigma, Germany, N1638). Feeding trials always began on the day of blood collection using freshly collected blood that was stored at 4°C for up to one week. In preparation for feeding trials, three ml of blood was placed into the wells of a stainless steel 60-well plate (custom-made in-house; Figure 12). *In vitro* feeding took place inside a climate chamber (PVP) at conditions 29°C, 80% RH at ambient CO2. The 60-well plate with blood was placed on an electronically controlled heating foil (Figure 12; Telemeter GmbH, D-86609 Donauwörth), which maintained the temperature of the blood in wells at 37°C throughout the assays. Before placing feeding units into the 60-well plate, blood was pre-warmed until it reached 37°C.

Figure 12. Plexiglas® feeding unit holder with feeding units on top of steel 60-well plate containing blood, and hot plate.

2.3.4 Attachment stimuli

Before placing ticks inside feeding units, cow hair (cut to 5 mm) and a cow hair extract (Kröber and Guerin 2007a; see below) or rabbit hair were added to the membrane for feeding *A. hebraeum* nymphs or *I. ricinus* females, respectively. Cow hair extract was prepared using dichloromethane (Sigma, Germany) according to the following method: 50 g of freshly cut cow hair was placed into a

1 L beaker and dichloromethane (250 ml) was added and exposed to the hair for 20 min under a fume hood. After 20 min, the dichloromethane was poured-off into an air-tight glass container. This process was repeated twice more in order to obtain 600 ml of a dichloromethane-cow hair lipid concentrate stock (stored at -20°C). This concentrate stock was then added to fresh dichloromethane in order to obtain a working stock with a concentration of 0.5 mg lipid/ml (also stored at -20°C). The concentration of the working stock was checked by pipetting 1 ml of extract onto a glass slide placed on a balance; the dichloromethane was allowed to evaporate off, and the weight of the lipids lipids left behind was checked using the balance; this was repeated until 0.5 mg lipid was left behind, indicating a working stock concentration of 0.5 mg lipid/ml. Immediately before feeding trials, 67 ul of the cow hair extract working stock (0.5 mg lipid/ml) was pipetted onto the center of the membrane of feeding units and placed on a metal rack over a hot plate set to 40°C in order to evaporate off the dichloromethane (5 min) and leave behind the lipids. Cow hair (freshly cut and stored at -20°C) was then added to the membrane before placing 10 female *I. ricinus* into the feeding unit. For feeding *Amblyomma hebraeum* nymphs, 20 nymphs were fed per feeding unit and rabbit hair was used as an attachment stimulus instead of cow hair and the cow hair extract.

2.3.5 Characterizing suitability of different solvents for preparing silicone membranes

To establish a suitable solvent for preparing membranes, five different solvents (toluene, hexane, heptane, ethyl acetate, and isopropyl myristate) were tested to dissolve silicone glue. Solvents were characterized based on 1) clarity of the solution when mixed with silicone glue, 2) time needed to dissolve silicone, 3) the range of membrane thicknesses produced with the solvent, 4) membrane drying time needed before glass vials could be attached, and 5) danger. In addition, the possible effects of the solvent on tick attachment were assessed. The time needed to dissolve silicone glue into solution was measured by placing 10 g glue into 25 ml of solvent inside an 80 ml beaker. Using a magnetic stirring bar (3 cm) and magnetic stirrer (600 rpm), the time needed for 100% of the glue to be

dissolved into solution was recorded. Clarity of the solvent-glue solution was assessed by eye and categorized as either clear or cloudy. The range of thicknesses produced by each solvent was determined by dissolving 10 g glue in 25, 30, 35, 40, 45, and 50 ml solvent. Membranes were dried for 72 hours and the membrane thickness was measured at the center with a microcalliper (M7624; Mitutuyo) accurate to 1 µm. It should be noted that the microcalliper is not the most optimal tool for measuring the thickness of soft materials (e.g. silicone membrane), and for this reason a high-quality, digital microcalliper is recommended for the best possible accuracy when using this method. Drying time needed before glass vials could be attached was characterized as x < 12 hours, x < 30 hours, or x > 48 hours. The effect of solvent on tick attachment was determined using 20 *A. hebraeum* nymphs with membranes prepared using 10 g silicone glue dissolved in 25 ml of test solvent. Freshly cut rabbit hair trimmed to 5 mm was applied to each membrane as an attachment stimulus and the number of attached ticks was recorded after 72 hours. Danger was based on the U.S. National Fire Protection Agency (NFPA) section 704. The NFPA 704 classifies hazardousness based on health, flammability, and reactivity on a 0–4 scale with 0 representing no hazard and 4 representing extreme danger.

2.3.6 Intra-membrane thickness variability: Characterizing the relationship between silicone concentration and membrane thickness

To determine the relationship between toluene concentration and membrane thickness and to characterize variability in thickness at different points on the membrane, membranes were prepared using a range of glue concentrations (168–671 gL^{-1} toluene) and thickness was measured at five different points (top, bottom, left side, right side, and center) using a microcalliper. Nomenclature was developed for naming membranes based on the volume (ml; “50–10”) of toluene (“T”) used to prepare the membranes. For example, membranes prepared with 25 ml of toluene are named T25 membranes. All membranes were prepared with 10 g of silicone glue. Table 9 lists the types of membranes used in this study, volume of toluene needed to prepare each membrane, the corresponding final

concentrations of glue (gL^{-1}) in the solution, and the corresponding thicknesses. The "T" nomenclature is used henceforth.

2.3.7 Inter-membrane thickness variability

To measure variability in membrane thickness between membranes in one batch and to test for patterns such as increases or decreases in thickness over the course of production, two complete T30 batches of membranes were prepared (30 membranes per batch). The membranes were dried for 72 hours and measured in the order they were prepared. The thickness of each membrane was measured at the center and compared after 72 hours drying.

2.3.8 Effects of membrane submersion time and size of lens paper on membrane thickness

To determine whether the length of time the lens paper was dipped (submerged) in the glue solution during preparation had an effect on membrane thickness, membranes were prepared by submerging them in a constant glue-toluene solution (T30) for different lengths of time (1, 5, 10 secs). The thickness at the center of each membrane was measured using a microcalliper after 72 hours. To determine whether the size of the square of lens paper used to prepare the membrane affects thickness, squares cut to 1.5, 2.5, and 3.5 cm^2 were dipped in a T30 solution and the thickness at the center was measured and compared after 72 hours.

2.3.9 *In vitro* feeding and optimization of feeding method

Membrane and feeding unit related and tick related parameters were tested for their effects on tick attachment and engorgement using nymphs of *A. hebraeum*. These included 1) membrane thickness, 2) age of the membrane (storage time) before use, 3) age of ticks post ecdysis (2 months versus 6 months) used in feeding experiments and 4) distance the cotton plug used for preventing tick escape from feeding units was placed from the membrane (1, 3, 6 cm). To test for effects of membrane age on attachment and feeding, T25 membranes were

used. Membranes were dried for 24 hours before attaching glass feeding units and then allowed to dry an additional 24 hours (group 1) and 14 days (group 2) before applying ticks. *A. hebraeum* nymphal attachment and engorgement was recorded after 15 days of feeding. To quantify the effect of membrane thickness on tick attachment and engorgement, successful hypostome penetration was quantified by exposing the ticks to a range of membrane thicknesses (200–540 μm). After 72 hours, the number of attached ticks was counted, and using a light microscope, each attached tick's hypostome was assessed as either penetrating or not penetrating the membrane.

2.4 Results

2.4.1 Suitability of different solvents for preparing silicone membranes

All solvents tested dissolved the silicone glue in less than 15 minutes. Table 10 summarizes characteristics of the solvents with regard to their usefulness for preparing membranes. Although ethyl acetate did not dissolve silicone any

Table 10. Summary table showing the suitability of five solvents for preparing silicone membranes for in vitro feeding of ticks. Data for tick attachment presented as mean percent with confidence limits (95%) for 6 feeding units with 20 *A. hebraeum* nymphs per feeding unit.

Solvent	Solution Appearance	Membrane drying time[a] (hours)	Tick attachment (%)	Hazards[b] (health, flammability, reactivity)
Ethyl Acetate	Cloudy	< 12	18 (2–35)	2,4,0
Heptane	Cloudy	< 30	22 (6–37)	1,3,0
Hexane	Cloudy	< 30	10 (0–23)	1,3,0
Isopropyl Myristate	Clear	> 48	no data	0,1,0
Toluene	Clear	< 30	43 (17–70)	2,3,0

[a]Time needed for solvent to evaporate before feeding units can be attached;
[b]United States National Fire Protection (NFPA) 704 guidelines; 0, no danger; 4, extreme danger

faster, the final solution was noticeably less viscous and “thinner” than the others. Also noteworthy was the strong odour given off by ethyl acetate and relatively short time period needed before feeding units could be attached (< 12 h) to membranes. Membranes prepared with isopropyl myristate were still wet more than two days after preparation and feeding units were never attached. The highest tick attachment rates by *A. hebraeum* nymphs was attained using membranes prepared with toluene, followed by heptane and ethyl acetate. The ranges of membrane thickness recorded with these solvents were 80–200, 100–380, and 40–80 µm, respectively (Figure 13). Consistent with the noted differences in viscosity, the slope and 95% confidence intervals of the slope of the ethyl acetate curve were the lowest of the three solvents, suggesting this solvent was the most efficient of the three for dissolving silicone glue.

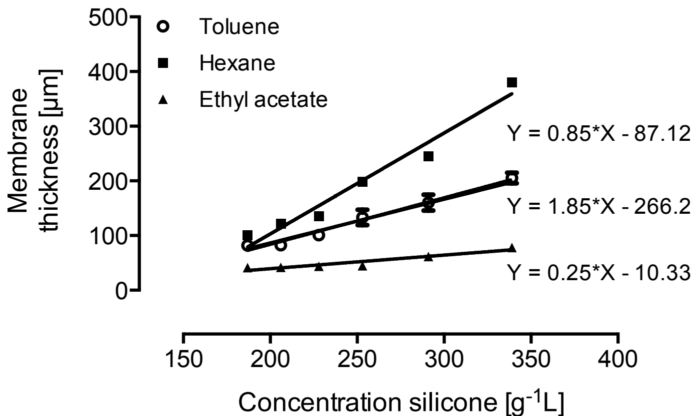

Figure 9. Linear models showing effects of the solvents toluene (P < 0.0001, r^2 = 0.94), heptane (P < 0.0001, r^2 = 0.96) and ethyl acetate (P < 0.0001, r^2 = 0.87) on membrane thickness (mean ± SD; 5 repetitions) as a function of silicone glue concentration ($g^{-1}L$).

2.4.2 Characterizing the relationship between solute concentration and membrane thickness: Intra-membrane thickness variability

The range of membrane thicknesses produced using cellulose-rayon lens paper dipped in silicone glue dissolved in toluene (167.57–670.86 gL^{-1}) was 83–538 µm (Table 9). The minimum thickness attained with T55 membranes was 63 µm, however, these membranes occasionally dried with holes in them. The mean thickness (Standard Deviation [SD]) of Tiffen® cellulose rayon lens paper, alone, (n = 25) was 41.8 (1.6) µm. The method, after drying, therefore coated the paper with a film of silicone 40–630 µm thick depending on toluene concentration. Figure 14 shows the relationships between silicone concentration and thickness at five different regions on the membrane (1: top; 2: right corner; 3: middle; 4: left corner; 5: bottom). Linear models fitted to the data for the different regions did not share a common slope (P < 0.0001). Variability in membrane thickness increased with silicone concentration and membrane thickness (Figure 14). Mean thickness measured at the bottom region (region 5) of the membrane was frequently the thickest region of the membrane (8/10 concentrations) (Figure 15). Of the five points, the thinnest region was frequently the right corner (8/10), the top point was thinnest at two concentrations and points 3 and 4 never registered the thinnest measurement at any concentrations. Together, these data show that the bottom half of the membrane was generally thicker than the top half (Figure 15).

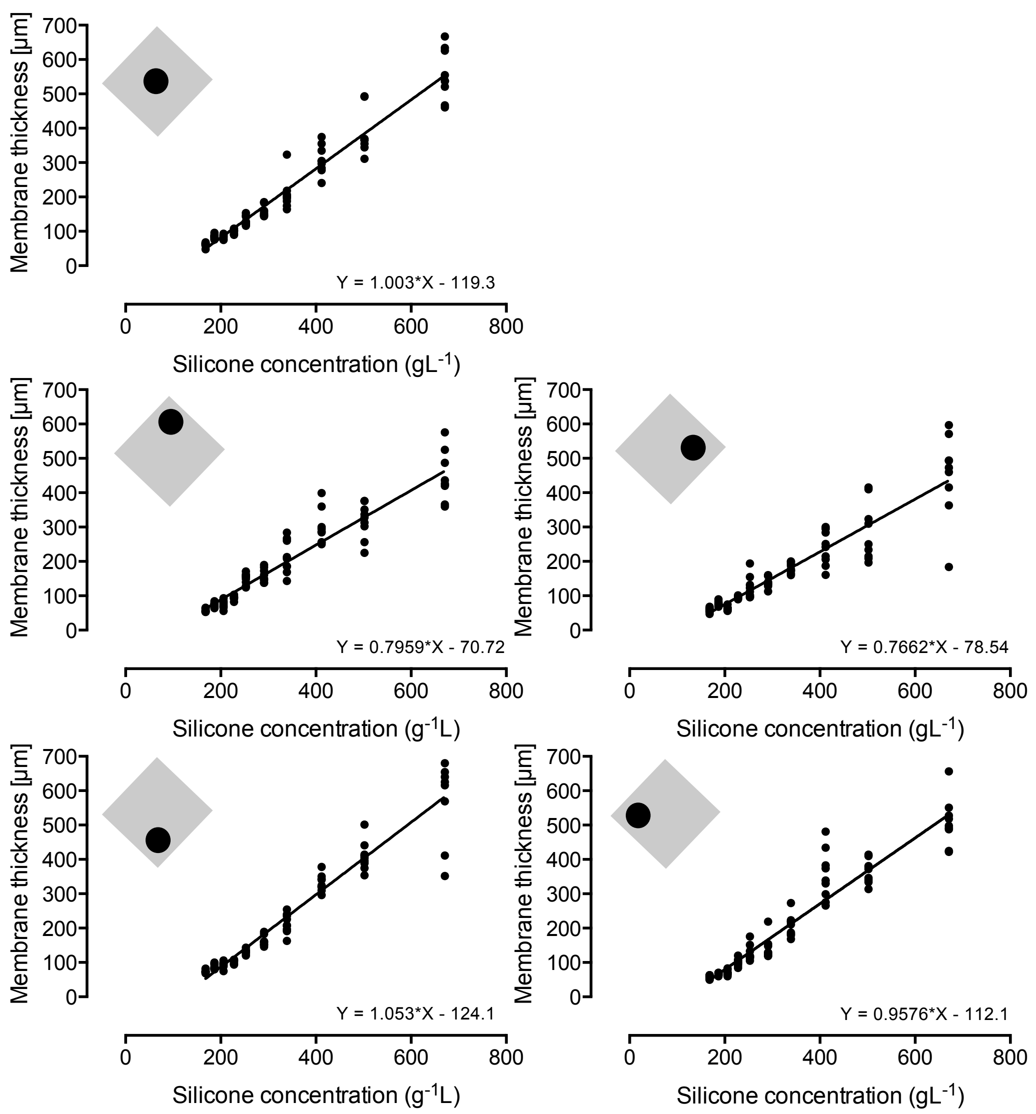

Figure 14. Linear models showing membrane thickness (µm) as a function of silicone glue concentration (gL^{-1}) at five points on the membrane. Data points are individual thickness measurements (n = 9) per concentration. A legend in the top left of each chart shows the region where thickness was measured.

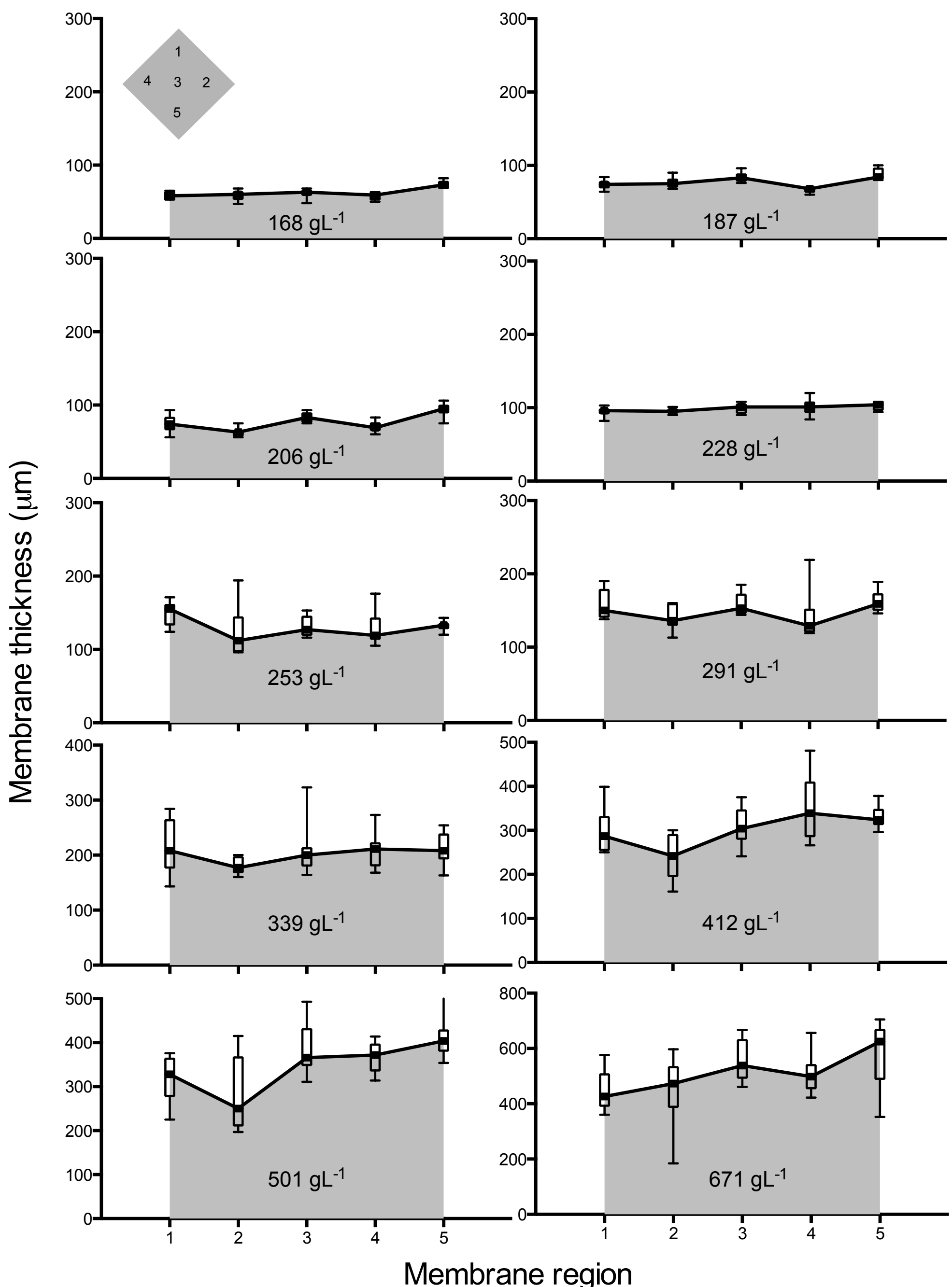

Figure 15. Variability in membrane thickness over five regions on the membrane for membranes prepared with 168-671 gL^{-1} silicone in toluene, n = 9 measurements per region. A legend in the top left shows the regions where thickness was measured. Box plots show the median, interquartile range, and 10–90th percentiles.

2.4.3 Inter-membrane thickness variability

The slope of a linear model fitted to membrane thickness data for individual membranes (T25) in a batch slightly increased over the course of production (Figure 16). In a second batch, the data for individual membrane thicknesses were almost identical (no difference in slope, *P* = 0.59), strongly suggesting membrane thickness increased over the course of producing a batch of T25 membranes.

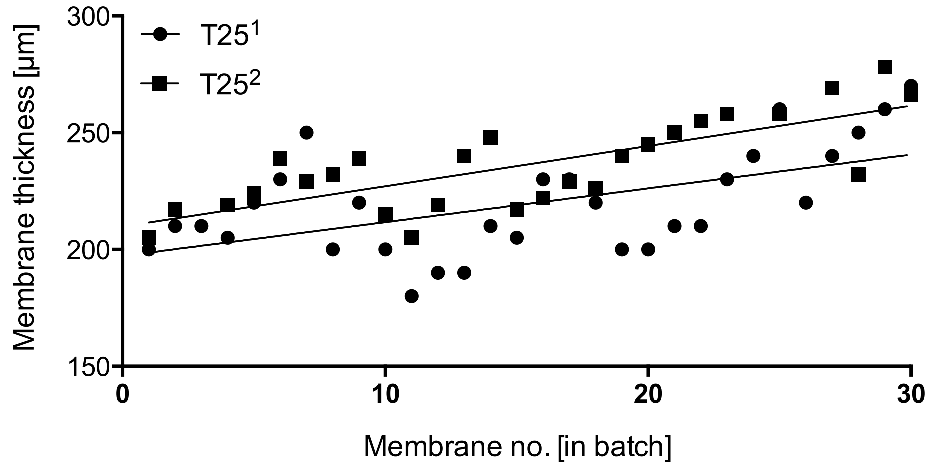

Figure 16. Membrane thickness (center region) of individual membranes over the course of preparing a batch of membranes. Two independent batches are shown (30 membranes per batch) prepared independently. Membranes are plotted in the order in which they were prepared, 1 to 30.

2.4.4 Effects of lens paper submersion time and paper size on membrane thickness

The size of lens paper had no effect on membrane thickness (*P* = 0.45) and paper submersion time was weakly significant (*P* = 0.04; Figure 17). The thicknesses of membranes (median: 151.5) prepared from lens paper submerged for an extreme 10 seconds were slightly thicker than 1-second submerged membranes (median: 146); however 5-second submerged membranes were thinner than 1-second membranes, suggesting that slight variation in submersion time during preparation did not influence membrane thickness.

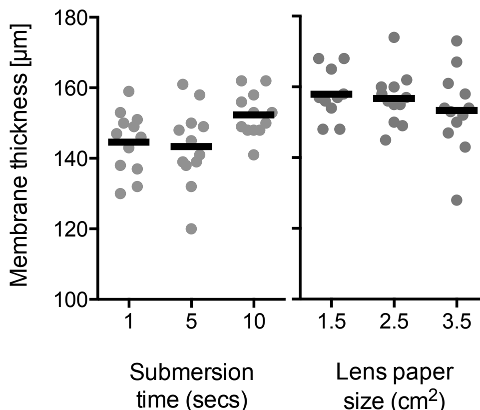

Figure 17. Scatter plots showing the effect of paper submersion time (in toluene mixture during preparation) and lens paper size on membrane thickness. Bars show the mean of 10–12 repetitions per treatment.

2.4.5 *In vitro* feeding and optimization of feeding method

In *in vitro* feeding trials, the effects of membrane thickness on hypostome penetration and attachment and engorgement for *A. hebraeum* nymphs were directly tested. The percentage of attached *A. hebraeum* penetrating the membrane with their hypostome and reaching blood decreased with increasing membrane thickness (Table 11). At 208 and 308 microns, for example, 64% and 65% of ticks were attached and of these attached ticks, 100% and 92% penetrated the membrane with their hypostome. At 366 microns, attachment dropped to 43% (10–75%), however 89% of these ticks still penetrated the membrane. The data suggest that the ticks actively searched for an attachment site, detaching and reattaching until they found a location where they reached blood. Overall, attachment decreased with increasing membrane thickness, and the ticks that did attach were those that

Table 11. Effects of membrane thickness on the ability of *A. hebraeum* nymphs to attach, penetrate, and engorge on silicone membranes. Data presented as median percent with confidence intervals (95%) for 4–5 repetitions with 20 nymphs per feeding unit. Treatments not sharing a letter are significantly different (Kruskall-Wallis test, Dunn's method post-hoc).

Membrane thickness (µm)	Hypostomes reaching blood (%)	Hypostomes not reaching blood (%)	Attached (%)	Engorged (%)
208 (173–244)	100^{abc}	0^{abc}	$64\ (45–83)^{abcd}$	$64\ (45–83)^{abc}$
308 (277–340)	$92\ (91–94)^{bacd}$	$8\ (6–9)^{bacd}$	$65\ (52–78)^{bac}$	$60\ (47–73)^{bac}$
384 (336–434)	$89\ (62–100)^{cab}$	$11\ (0–38)^{cba}$	$43\ (10–75)^{cabd}$	$40\ (6–74)^{cabd}$
584 (490–677)	$7\ (0–25)^{db}$	$93\ (7–100)^{db}$	$20\ (0–41)^{dca}$	$3\ (0–11)^{dc}$

were able to penetrate the membrane. Attachment improved by decreasing the distance of the cotton plug (used to prevent tick escape) from the membrane with the highest tick attachment attained with plugs placed 1 cm from the membrane (Figure 18). In *in vitro* feeding trials using *A. hebraeum*

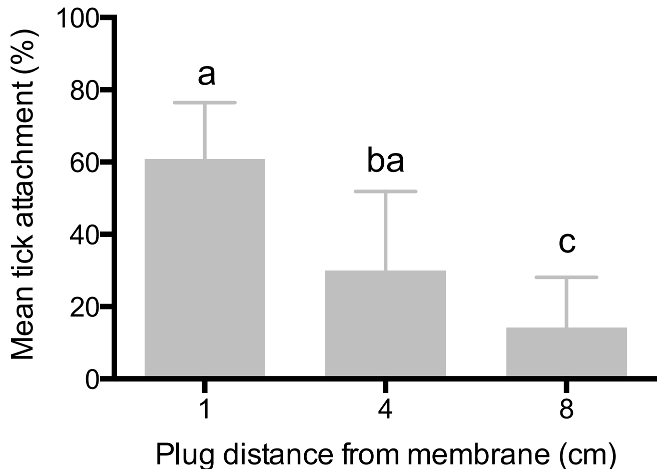

Figure 18. Effect of cotton plug height (distance from the membrane) on tick attachment. Data are mean percentage attachment per feeding unit ± SD for six repetitions containing 20 *A. hebraeum* nymphs per feeding unit. Treatments not sharing a letter are significantly different (P = 0.002; Kruskal-Wallis test followed by Dunn's method post-hoc).

nymphs, T25 membranes, and the cotton plug inserted 1 cm from the bottom of the membrane, a mean of 86% tick attachment was reached and 95% of engorged *A. hebraeum* nymphs molted into adults five weeks later (Table 12).

Table 12. Attachment, engorgement, engorged weight, and molting rate for *A. hebraeum* nymphs fed in vitro. Attachment and engorgement presented as mean percent with confidence intervals (95%); n = 24 feeding units with 20 nymphs per unit.

in vitro feeding of *A. hebraeum*	
Life stage	Nymph
Membrane code	T25
Attached (%)	86 (82–89)
Engorged (%)	79 (74–84)
Mean engorged weight (mg)	49
Molting rate (%)	95

In trials where tick starvation period and membrane storage time varied, these parameters had no effect on tick attachment in *A. hebraeum* nymphs (n = 39 feeding units with 20 nymphs per unit) (Table 13).

Table 13. 2x2 table summarizing the effects of tick starvation period and membrane storage time on nymphal *A. hebraeum* attachment to the membrane. Data are mean number of attached ticks (SD) per feeding unit (20 ticks per unit) for 8–10 feeding units per treatment.

	Membrane dried 48 h	Membrane dried 2 wks	ANOVA
Ticks starved 2 months	16.4 (2.5) (82%)	17.7 (2.4) (89%)	0.4130
Ticks starved 6 months	18 (1.3) (90%)	17.3 (2.3) (87%)	

2.5 Discussion

Over the years, the materials used for artificial membranes for *in vitro* feeding of ixodid ticks have been improved significantly (Kuhnert et al. 1995; Kröber and Guerin 2007a). However, the overall methods used for preparing artificial membranes for *in vitro* feeding of ixodid ticks have generally received less attention in the literature. Specifically, the procedures used to impregnate membrane matrix material with glue in order to produce an artificial membrane for an ixodid tick have generally changed little over the years and manipulating the thickness of artificial membranes to suit the hypostome of different ixodid tick species has long required a considerable amount of skill. Andrade et al. (2014) described a departure from previous methods for membrane preparation whereby a regenerated cellulose matrix (described in Kröber and Guerin 2007a) is saturated and coated with silicone by dipping and removing the cellulose in a silicone glue mixture, a procedure that produces an artificial membrane with a layer of silicone with a defined thickness that is suitable for feeding *Ixodes scapularis* females to repletion *in vitro*. This method has been improved significantly in this study.

After submerging and removing cellulose lens paper matrix in silicone glue dissolved in toluene, the paper is saturated and coated with a layer of silicone between 63–538 µm, depending on silicone concentration. The relationship between membrane thickness and silicone concentration was shown to be linear in this study and therefore standardized manipulations of membrane thickness were obtained by manipulating the concentration of silicone in solution. The length of time the lens paper was submerged in the silicone solution during membrane preparation did not have a meaningful effect on final thickness, but over the course of preparing a batch of membranes, the thickness of individual membranes increased. This increase in thickness was likely due to evaporation of toluene over the course of membrane production, which took roughly 30 min, increasing the concentration of silicone in solution over time. Care should therefore be taken to prepare membranes immediately after dissolving glue in toluene.

The variability in membrane thickness varied for different concentrations and at different points on the membrane; overall, variability in membrane thickness increased with increasing solute concentration and thickness. Region 5, or the bottom point of the membrane, was frequently the thickest region of the membrane. Membranes dried vertically hanging on a drying line, and the bottom of the membrane was likely thicker due to gravity and the silicone slowly falling and accumulating at the bottom over time. This variability in membrane thickness, while important to characterize, did not meaningfully affect tick attachment as 86% (95% CI, 82%–89%) attachment was achieved with *A. hebraeum* nymphs using T25 membranes. The 95% confidence interval of the mean thickness of T25 membranes was 173–244 μm, spanning roughly 70 μm, which is similar to the variability in membrane thickness allowed for in Kröber and Guerin (2007a) (50 μm) for feeding *I. ricinus* females.

While membrane thickness is an important factor affecting tick attachment and engorgement, other factors such as attachment stimuli, temperature, and humidity also influence tick attachment and engorgement *in vitro* (Kröber and Guerin 2007b). With multiple variables affecting ixodid tick attachment *in vitro*, it is important to be able to test artificial membranes with the same thickness and quality in experiments. The method described in this study for preparing artificial membranes provided a simple procedure for reproducibly preparing large numbers of membranes with the same thickness and quality, and facilitated controlled experiments testing the effect of different membrane thicknesses on tick attachment. Figure 19 shows the relationship between silicone concentration and membrane thickness super-imposed over the hypostome lengths of various tick species and life stages. This figure might be used in the future as a reference tool to help estimate the membrane thickness needed to feed an ixodid tick *in vitro* and to determine the concentration of silicone needed to produce that membrane.

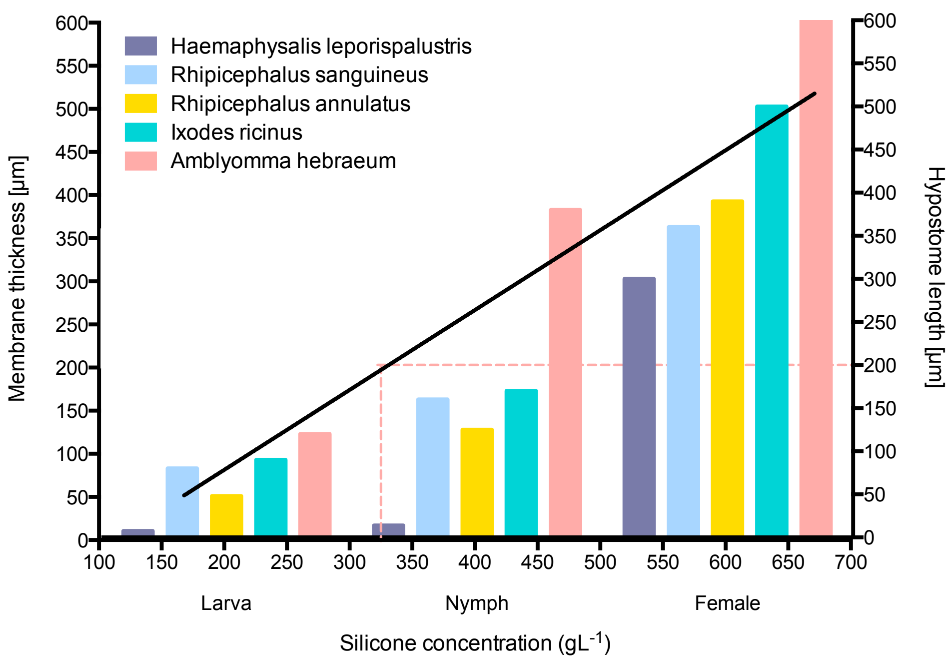

Figure 19. Relationship between membrane thickness and silicone concentration superimposed over the hypostome lengths of different ixodid tick species and life stages. A dotted line illustrates how to estimate a suitable membrane thickness for an ixodid tick (*A. hebraeum* nymph) and how to determine the silicone concentration necessary to produce that membrane. Sources: *A. hebraeum*, Voltzit and Keirans 2003; *I. ricinus*, Kröber and Guerin 2007b; other species, Cooley 1946; Note, that the y-axis cuts off the full length of the female *A. hebraeum* hypostome, which is more than 1 mm in length.

In this study, a membrane with a silicone concentration of 339 gL^{-1} was used to feed *A. hebraeum* nymphs. The length of the hypostome in *A. hebraeum* nymphs is 380 µm (Voltzit and Keirans 2003) and a good starting point for a suitable membrane thickness for a tick species appears to be at least half the thickness of the tick hypostome. However, this should only be used as a starting point. A dotted line in Figure 19 demonstrates how the silicone concentration was determined for preparing membranes for *in vitro* feeding of *A. hebraeum* nymphs; at approximately half the thickness of the *A. hebraeum* nymph hypostome (200 µm), a dotted line was drawn starting from the right y-axis moving left, and the silicone concentration required for this membrane determined by the concentration on the x-axis corresponding to the point at which the dotted line bisected the membrane thickness line.

Membrane thickness obviously plays an important role in determining whether or not a tick is able to penetrate a membrane and reach blood with its hypostome. Despite this importance, few researchers give justification for the thickness of the

membrane used in their study and some neglect to report it. Table 14 summarizes different membranes and membrane thicknesses used to feed various tick species over the years. Here, the 339 gL^{-1} membrane (T25; 208 µm) was experimentally shown to be suitable for *in vitro* feeding of *A. hebraeum* nymphs by comparing attachment and hypostome penetration with 208 µm membranes with other membranes up to 534 µm thick. The percentage of attached ticks penetrating the membrane decreased with increasing membrane thickness, and 100% of ticks attached to T25 membranes penetrated the membrane. In addition to hypostome length, a tick species' pattern of attachment (Moorehouse 1969) should also be considered when estimating a suitable membrane thickness for a tick species. Ticks in the *Rhipicephalus* genus, for example, which attach superficially, will likely require thinner membranes relative to the length of their hypostome in order to feed them to repletion.

While 100% of *A. hebraeum* nymphs attached to T25 membranes penetrated the membranes, the parameter 'percentage of ticks attached penetrating the membrane' was not necessarily a useful indicator, alone, for suitability of membrane thickness, since ticks detached from membranes that were too thick. Kuhnert et al. (1995) used membranes 50–90 µm thick for feeding *A. hebraeum* nymphs, (100–150 µm thinner than the membrane used in this study), and reported mean rates of tick attachment 90–98%. The 86% attachment attained with *A. hebraeum* nymphs in this study therefore might be improved by using slightly thinner membranes.

From a practical standpoint, an advancement described in this study with regard to feeding unit preparation was the finding that feeding units could be directly attached or pressed onto partially dried (polymerizing) membranes. In other words, the feeding units were directly pressed onto the membranes before the membranes fully dried, eliminating the need to glue the membranes to feeding units with extra glue, a process that required a considerable amount of time in previous *in vitro* feeding methods. In addition, gluing membranes to feeding units creates a ring of glue inside the feeding unit that bulges out after pressing the feeding unit onto the membrane.

Table 14. Summary table of various membranes and membrane thicknesses used to feed ticks *in vitro*.

Target species	Common name	Membrane material	Membrane thickness (μm)*	Source
Rhipicephalus microplus	Hard tick	Cattle skin	300–500	Kemp et al. 1975
Ornthidoros ticks	Soft tick	Silicone (Dow Corning General Purpose)	2–5	Butler et al. 1982
Dermacentor Andersoni	Hard tick	Rabbit or mouse skin	NR	Howarth and Hokama 1983
Ixodes holocyclus	Hard tick	Silicone (RTV-M539)	500	Stone et al. 1983
Ornithodorous moubata	Soft tick	Silicone (Expandite silicone sealant - 33)	51	Osborne and Mellor 1985
Ornithodorous moubata	Soft tick	Silicone	70–150	Wirtz and Barthold 1986
Ornithodorous coriaceus	Soft tick	Parafilm	120	Hokama et al. 1987
Rhipicephalus appendiculatus	Hard tick	Baudruche membrane	NR	Waladde et al. 1991
Amblyomma variegatum	Hard tick	Rabbit or bovine skin	500–1500	Voigt et al. 1993
Amblyomma hebraeum	Hard tick	Silicone (no. 251470, Kunststoffe Rehau AG)	10–40 larvae; 50–90 nymph; 500 for adult	Kuhnert et al. 1995
Amblyomma variegatum	Hard tick	Silicone (Gebsicone W; GEB 93012)	NR	Barre et al. 1998
Ornithodorous moubata	Soft tick	Parafilm	NR	Ruheta et al. 2005
Ixodes ricinus	Hard tick	Gerbil or rabbit skin	NR	Bonnet et al. 2007
Ixodes ricinus	Hard tick	Silicone (Elastosil E4 RTV, Wacker)	50–100	Kröber and Guerin 2007a
Ixodes scapularis	Hard tick	Silicone (Elastosil E4 RTV, Wacker)	195	Andrade et al. 2014

NR, not reported.

This ring of glue needed to be carefully removed from each feeding unit in previous methods (otherwise ticks would get stuck in the ring of glue and not reach blood), adding to the labor-intensiveness of the procedure. Finally, in previous methods using membranes attached with extra glue, the membranes were removed using sand paper during clean-up, a process that unavoidably damaged the expensive custom-made glass feeding units. In this study, by directly pressing feeding units onto polymerizing membranes, no ring of glue was created during feeding unit preparation that needed to be removed before feeding experiments to prevent ticks from getting stuck, and membranes were easily removed during clean-up by peeling them off using a gloved hand.

Modifying the size of the glass feeding units significantly streamlined the procedure used to build feeding units. By reducing the diameter of the glass tubes so that they fit inside a commercially available cork borer (20 cm), the tubes were cut easily from membranes in seconds. Previously, excess membrane material was cut from around the edges of the feeding units using scissors.

After ticks were placed in feeding units, by placing the cotton plug used to prevent tick escape closer to the membrane (see below), high rates of mean tick attachment (87–90%) and engorgement (85–88%) were attained; ticks in feeding units with the cotton plug placed at 1 cm from the membrane attached at rates three-times that of ticks in feeding units with the plug placed 8 cm from the membrane. Placing the plug 1 cm from the membrane likely forces the ticks to remain in contact with the membrane and prevents them from walking up (away from the membrane), a common behavior of ixodid ticks.

In this study, nystatin (100 i.u./ml) and gentamicin (5 µg/ml) were added directly to blood to prevent fungal and bacterial contamination, respectively, and sodium heparin (10 i.u./ml) was used as an anticoagulant. Addition of nystatin and gentamicin allowed for blood to be changed every 24 hours, as opposed to every 12 hours in previous methods, without affecting tick attachment or engorgement. Five weeks after drop-off, 95% of engorged *A. hebraeum* nymphs molted into adults, demonstrating blood treatment with nystatin, gentamicin, and heparin did

not affect development in *A. hebraeum* nymphs. The modifications described above (summarized in Table 15) thus significantly standardized and streamlined the in vitro feeding assay for *A. hebraeum* nymphs, while promoting high rates of tick attachment and engorgement.

Table 15. Summary table of *in vitro* feeding assay parameters used in this study and in other assays in the literature.

	Voigt et al. (1993)	Waladde et al. (1993)	Kuhnert et al. (1995)	Kröber and Guerin (2007a)	Andrade et al. (2014)	This study (2016)
Tick species and life stage	*A. variegatum* all life stages	*R. appendiculatus* nymphs	*A. hebraeum* all life stages	*I. ricinus* females	*I. scapularis* females	*A. hebraeum* nymphs, *I. ricinus* females
Membrane	Rabbit (0.5–1 mm) and bovine skin (0.8–1.5 mm)	Baudruche membrane impregnated with multi-purpose glue	Cellulose rayon lens paper impregnated with silicone glue (10–40 um for larvae; 50–90 um for nymphs; 0.5 mm pure silicone for adults)	Cellulose rayon lens paper impregnated with silicone glue (50–100 um)	Cellulose rayon lens paper impregnated and coated with silicone glue (195 um)	Cellulose rayon lens paper impregnated and coated with silicone glue (158 um)
Nutrient	Sterile drawn bovine blood (50 i.u. heparin/ml) max 3 d old	Sterile drawn bovine blood (50 i.u. heparin/ml) max 1 d old	Defibrinated non-sterile bovine blood (glucose 2 gL^{-1}; ATP and glutathione 10^{-3} mol), max 7 d old stored 4C	Defibrinated non-sterile bovine blood (glucose 2 gL^{-1}; ATP 10^{-3} mol), max 7 d old stored 4C	Defibrinated non-sterile bovine blood (glucose 2 gL^{1}; ATP 10^{-3} mol), max 7 d old stored 4C	Sterile drawn whole blood heparin (10 i.u./ml), max 7d old, stored 4 C
Frequency of nutrient change	Not reported	6X per day	2–3X per day for larvae; 2X for nymphs; 3X per day for adults	Every 12 h	Every 12 h	Every 24 h
Microbicide	Penicillin (100 i.u/ml), streptomycin (100 ug/ml), gentamicin (100 i.u./ml)	none	Nystatin (100 i.u./ml), gentamicin (5 ug/ml)	Gentamicin (5 ug/ml), nystatin (100 i.u./ml) only as needed	Gentamicin (5 ug/ml), nystatin (100 i.u./ml) only as needed	Nystatin (100 i.u./ml), gentamicin (5 ug/ml)
Temp. and humidity	35–39C, 90–95% RH, 5% CO2	Water bath 42C, air 26–27C, 70–80% RH	Water bath 38C, air 23C, 80%RH	Water bath 37 C, 100% RH, 16:8 L:D cycle	Incubator 37C, 93% RH, darkness	Climate chamber 29°C, 80% RH, ambient CO_2
Attachment stimuli	Skin	Cotton wool, isotonic saline, tick feces, bovine hair, bovine ear extract	Bovine hair, tick feces, bovine hair extract, synthetic aggregation-attachment pheromone mixture	Cow hair extract, tile spacer, cow hair, mosquito netting	Cow hair extract, tile spacer, cow hair, mosquito netting	Cow hair extract, cow hair

3. Comparisons between ixodid ticks fed *in vitro* and *in vivo*

3.1 Abstract

In vitro feeding systems are some of the most useful tools for studying ticks in the laboratory, yet few studies have compared the biology of ixodid ticks fed *in vitro* and *in vivo*. A silicone membrane with a thickness of 158 µm was described for feeding *Ixodes ricinus* females to repletion, *in vitro*. Feeding parameters, including tick attachment, engorgement, increase in weight per day, drop-off time, and engorged weight were compared between *I. ricinus* females fed *in vitro* on bovine blood and *I. ricinus* females fed *in vivo* on steer and on rabbits. Mean (95% confidence interval) tick attachment to the membrane for *I. ricinus* females (N = 2,135) was 89.3% (87.6-91.1%). Of the attached ticks, 94-100% penetrated the membrane with their hypostome; overall, 80-96% of all ticks placed in feeding units were attached and bloodfeeding after 40 hours. The most common drop-off day of *I. ricinus* females fed *in vitro* (n = 217) and *in vivo* on steer (n = 100) was day 13 and day 8, respectively. The median engorged weight attained by *I. ricinus* females fed *in vitro* (n = 215), *in vivo* on a steer (n = 94), and *in vivo* on rabbits (n = 115) was 138.0 mg, 354.5 mg, and 130.5 mg, respectively. *Ixodes ricinus* females fed *in vitro* and *in vivo* on steer experienced similar daily increases in weight over the first three days of feeding, increasing, respecitvely, in weight 239% and 207% overall, compared with their unfed weight. After day 3, the daily weight gain of *in vivo*-fed ticks accelerated, leading up to the rapid feeding phase (day 7), where ticks increased in weight 627% overnight before dropping off on day 8. In contrast, the slow and rapid feeding phases of *in vitro*-fed ticks were prolonged with a maximum daily increase in weight of 127% over 13 days of feeding. These results provide important guidance for the development of *in vitro* feeding assays for ixodid ticks.

3.2 Introduction

In the previous chapter, a method for producing silicone membranes for *in vitro* feeding of ticks was described and successful engorgement of *A. hebraeum* nymphs was achieved. Since, by definition, *in vitro* feeding takes place in the absence of a living host, comparisons must be made that demonstrate tick blood feeding *in vitro* is similar to blood feeding on animals. These comparisons are important in order to have confidence that conclusions reached with ticks fed *in vitro* have relevance for tick feeding on live animals, for example, with regard to systemic acaricides for tick control.

Due to their importance in both human and veterinary medicine, *Ixodes ricinus* was chosen as the target tick species for *in vitro* testing of acaricides in this study. *Ixodes ricinus* females have longer hypostomes than *A. hebraeum* nymphs and penetrate deeply during host attachment and do not secrete cement. Holes left behind after tick detachment cause leakage of blood into feeding units, which can negatively affect tick attachment and engorgement. It is important, therefore, to demonstrate with this new tick species that high rates of tick attachment and engorgement are reproducible using the membrane described in this study.

Kuhnert et al. (1997) used tick attachment rates, survival, feeding duration, engorged weight, and parameters associated with reproduction (e.g. Egg Conversion Factor) to compare the biology of *A. hebraeum* fed *in vitro* and *in vivo*. Kröber and Guerin (2007a) compared the engorged weight of *I. ricinus* females fed to repletion *in vitro* with the engorged weight of *I. ricinus* fed to repletion on rabbits. In this chapter, these parameters, in addition to the size of the bloodmeal over time, are compared *in vitro* and *in vivo* using *A. hebraeum* nymphs and *I. ricinus* females.

3.3 Materials and Methods

3.3.1 Ticks

Ixodes ricinus females *were* purchased commercially in vials with male ticks and used in feeding trials 3–6 months post-ecdysis. Ticks were stored in an environmental chamber at conditions 25°C, 94% RH, 16:8 light-dark cycle. *Amblyomma hebraeum* nymphs originated from an in-house colony (Bayer Animal Health, Monheim, Germany) bred using steer. *A. hebraeum* nymphs used in this study were starved 3–6 months post-ecdysis before feeding trials.

3.3.2 *In vitro* feeding of *A. hebraeum* nymphs and *I. ricinus* females

Details of the *in vitro* feeding assay, membrane thickness for *A. hebraeum* nymphs, feeding units, and feeding conditions are provided in Chapter 1. *In vitro* feeding of *I. ricinus* females took place as described for *A. hebraeum* except for differences in membrane thickness and attachment stimuli applied to the membrane (see below). For *in vitro* feeding of *I. ricinus* females, T31 membranes (158 µm thick) were used and cow hair and a cow hair extract (Kröber and Guerin 2007a) were applied to the membrane as attachment stimuli. Cow hair extract was prepared using dichloromethane as described in Chapter 1 of this study. Immediately before feeding trials, 67 ul of the cow hair extract was added to the membrane of feeding units, and feeding units were then placed on a metal rack over a hot plate set to 40°C in order to evaporate the dichloromethane (5 min) and leave behind lipids. Cow hair (3 mg; freshly cut to 1 cm and stored at -20°C) was then added to the membrane immediately before placing 10 female and 10 male *I. ricinus* into the feeding unit.

3.3.3 *I. ricinus* attachment to the membrane over time

To determine the timepoint where tick attachment to the membrane reaches a maximum inside feeding units, feeding trials (n = 39) were carried out in order to measure tick attachment to the membrane over a series of time points (13, 15, 17, 24, and 40 hours). Ten *I. ricinus* females were placed in each feeding unit

and the number of ticks attached to the membrane, ticks not attached to the membrane and alive, and the number of dead ticks were recorded by eye in each feeding unit over time.

3.3.4 *I. ricinus* survival and engorgement *in vitro*

Ten *I. ricinus* females and 10 males were placed in each feeding unit and allowed 40 hours to attach and begin feeding. After 40 hours, the number of ticks attached to the membrane, the percentage of attached ticks successfully penetrating the membrane and reaching blood, and survival was assessed. Observations on tick attachment and hypostome penetration were made under a light microscope first by recording tick attachment from a top view, looking down inside the feeding unit, and then by confirming hypostome penetration from below the membrane. Attached ticks were considered dead if they did not move after disturbing them with forceps.

3.3.5 Feeding duration, rate of engorgement, and engorged weight of ixodid ticks fed *in vitro* and *in vivo*

To characterize *in vitro* feeding duration and engorged weight, *I. ricinus* females and males and *A. hebraeum* nymphs were fed to repletion *in vitro* and feeding units were checked daily for engorged ticks that had detached from the membrane. The detachment date was recorded and ticks were immediately weighed using a microbalance (Sartorius AG, Germany) accurate to 0.001 mg. Following the experiment recording detachment date and the engorged weight on different days, two additional independent *in vitro* experiments were conducted to fill in gaps for engorged weight on different days. This experiment was repeated *in vivo* with *I. ricinus* placed on steer and rabbits for comparisons of detachment and engorged weight. It was not possible to make observations for *I. ricinus* detachment on rabbits due to the stress this would have caused the animals. Therefore only engorged weight was assessed for *I. ricinus* fed on rabbits. *A. hebraeum* nymphs were fed *in vitro* and *in vivo* on bovine blood and on steer, respectively, for comparing the *in vitro* and *in vivo* detachment days and engorged weight in a second ixodid tick species. In addition to feeding duration

and engorged weight, the size of the bloodmeal per day was compared between *in vitro* and *in vivo*-fed *I. ricinus* females. Two independent *in vitro* and *in vivo* experiments were conducted to collect the partial engorged weights attained by ticks during the early days of feeding; ten female and 10 male *I. ricinus* were placed in feeding units and ticks were chosen at random and manually removed from feeding units every 24 hours. The data from these experiments were then combined with the data obtained from the drop-off day experiments described above where engorged weight was also collected at drop-off.

3.3.6 *I. ricinus* and *A. hebraeum* infestations on cattle

For infestations of cattle with *I. ricinus* and *A. hebraeum*, a ring (outer Ø: 30 cm, inner Ø 15 cm) was shaved into the hair (5 mm) into the short loin region of a Holstein cow using an automatic hair trimmer (center of the ring unshaved). Nylon bags (n = 4, two per side) were then attached to the shaved region of the ring using veterinary adhesive (C10; Henkel GmbH) and allowed to dry for 24 hours. Cattle stood in steel standing stocks at room temperature under a 16:8 light:dark cycle. Forty female and 30 male *I. ricinus* three months post-ecdysis were applied to the unshaven pelage inside the bag and the bag was closed using a rubber band. For feeding *A. hebraeum* nymphs, approximately 1000 nymphs were placed inside each bag.

3.3.7 Infestation of rabbits with *I. ricinus*

Female *I. ricinus* were fed in ear bags on the ears of Giant Chinchilla rabbits. Bag application began by applying general-purpose cloth tape around the base of each rabbit's ear. A layer of C10 adhesive (see above) was then applied to the tape and nylon bags were placed over the ears and attached to the base of each ear by pressing the cloth into to the layer of adhesive applied to the tape previously placed around each ear. A final layer of tape was wrapped around the base of the bag securing the bag to the base of the rabbit's ear. Fifteen females and 10 males were placed on each ear of the rabbit. The bag was then folded down and taped shut at the top. The two ear bags of each rabbit were then taped together; an Elizabethan collar was not necessary. Rabbits stood in cages in a

room with a flooded floor at room temperature, 16:8 light-dark cycle. All ticks were collected from rabbits after nine days and weighed immediately. After weighing, ticks were sorted into glass vials with perforated tops and placed into the incubator to gestate and oviposition was assessed by eye.

3.4 Results

3.4.1 *In vitro* feeding of *I. ricinus* females

Ixodes ricinus attached to and fully penetrated membranes, as shown in Figure 20. Mean tick attachment to the membrane ranged 86–96% per feeding unit in independent feeding experiments (n = 59–87) and 94–100% of attached ticks successfully penetrated the membrane and reached blood (Table 16). Therefore, 80–96% of all ticks placed in feeding units were attached and feeding after 40 hours. Four to 15% of ticks per feeding unit were recorded as dead and unattached to the membrane, and 0–5% were attached to the membrane and dead after 40 hours.

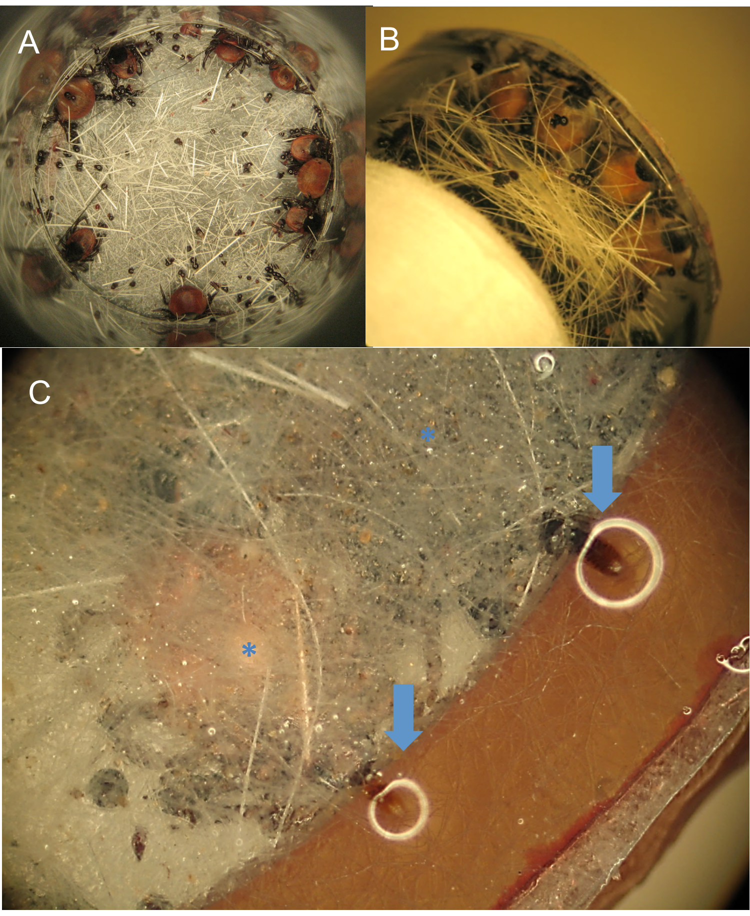

Figure 20. A) Top view; looking down inside feeding unit showing *I. ricinus* females in various stages of attachment to a silicone membrane. B) Side view of feeding unit showing partially engorged *I. ricinus.* C) View of the bottom of the feeding unit, i.e. the side that faces blood showing two hypostomes fully penetrating the membrane (highlighted by blue arrows).

Table 16. Data from independent in vitro feeding trials for *Ixodes ricinus*. Data are mean percentage with confidence intervals (95%) per feeding unit (10 ticks per unit) for tick attachment, hypostome penetration, engorgement, and control death. The number of replicates with the total number of ticks in each trial is reported in the final row of the table. Data in each row sharing a letter are significantly different (ANOVA followed by Tukey's post-hoc test).

		Experiment 1	Experiment 2	Experiment 3	Pooled data
Percentage of tick attachment to membrane		87.7[a] (85.3–94.3)	96.2[bac] (94.3–98.1)	85.5[c] (81.4–89.5)	89.3 (87.6–91.1)
Percentage of attached ticks penetrating membrane with hypostome		99.2[ac] (98.5–99.8)	99.8[bc] (99.5–100)	94.2[cab] (91.6–96.74)	97.8 (96.9–98.7)
Percentage of ticks bloodfeeding in feeding units*		87.0[abc] (84.6–89.5)	95.9[bac] (94.0–97.7)	80.8[cab] (76.2–85.4)	87.6 (85.6–89.5)
Mortality	Attached	0.68 (0.1–1.2)	0.2 (0.2–0.5)	4.68 (2.8–6.6)	1.8 (1.1–2.5)
	Unattached	12.3 (9.9–14.7)	3.9 (2.1–5.7)	14.55 (10.5–18.6)	10.7 (8.9–12.4)
No. of replicates		87 (873)	59 (601)	66 (661)	211 (2135)

3.4.2 *I. ricinus* attachment to the membrane over time

Twenty-four hours after placement in feeding units, a mean of 10% of *I. ricinus* females, per feeding unit, were unattached-alive and 9% were dead (Figure 21). Mean tick attachment to the membrane after 24 hours was 82%. After 40 hours, the percentage of unattached-alive ticks decreased to 4%, dead ticks increased to 12%, and attachment increased to 84%. After 40 hours, 96% of ticks were accounted for as either attached (84%) or dead (12%) and just 4% remained unattached and alive in feeding units.

Unattached-alive ticks post 13 hours mainly attached to the membrane between hours 13–24. The increase in attachment (+6%) between hours 13–24 corresponded with a decrease (-6%) in unattached-alive ticks. After 24 hours, unattached-alive ticks decreased 6%, but attachment increased only just 2.5% with mortality increasing to 3.5%. In other words, in the time period leading up to 24 hours, most unattached-alive ticks attached to the membrane, contributing to overall tick attachment; ticks still unattached-alive after 24 hours did not contribute much to overall tick attachment, and mostly died.

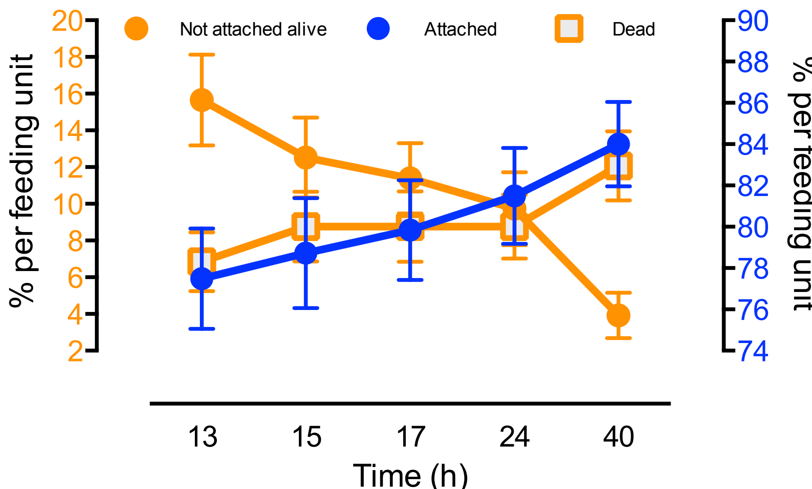

Figure 21. Ticks recorded as alive and unattached (pink), tick attachment to membrane (blue), and ticks recorded as dead in feeding units over time (pink). Data are mean percent (SEM) per feeding unit for 39 feeding units (10 ticks per feeding unit).

3.4.3 *I. ricinus in vitro* feeding duration

Fifty-eight percent (n = 100) and 33% (n = 116) of *I. ricinus* females placed on cattle and rabbits, respectively, fed to repletion and detached from their respective hosts. *I. ricinus* fed *in vitro* took significantly longer to feed to repletion compared with ticks fed *in vivo* on steer ($P < 0.0001$; Figure 22); The most common detachment day, described by the mode, was day eight for ticks fed on steer and day 13 for ticks fed *in vitro* (bovine blood).

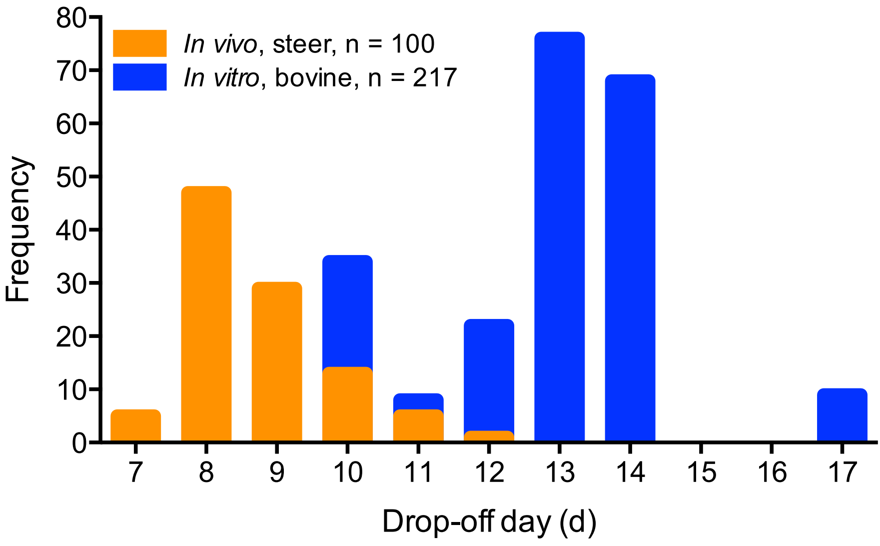

Figure 22. Drop-off day for female Ixodes ricinus fed *in vivo* (cow, n = 100) and *in vitro* (bovine blood, n = 216). Feeding duration was significantly extended *in vitro* (mode: 13 days) relative to *in vivo*-fed ticks (mode: 8 days, $P < 0.0001$; Mann-Whitney)

3.4.4 Engorged weights of ixodid ticks fed *in vitro* and *in vivo*

The source of the bloodmeal significantly affected tick engorged weight (Figure 23). *I. ricinus* females placed on steer (median 354.5 mg) attained an engorged weight more than twice that of ticks fed *in vitro* on bovine blood (median 138 mg) or ticks fed *in vivo* on rabbits (median 130.5 mg). No difference in engorged weight was detected between ticks fed *in vitro* (bovine blood) and ticks fed on rabbits (Figure 23). *Amblyomma hebraeum* nymphs fed on steer (median 71 mg) weighed significantly more than *A. hebraeum* fed *in vitro* on bovine blood (median 47 mg; Figure 24).

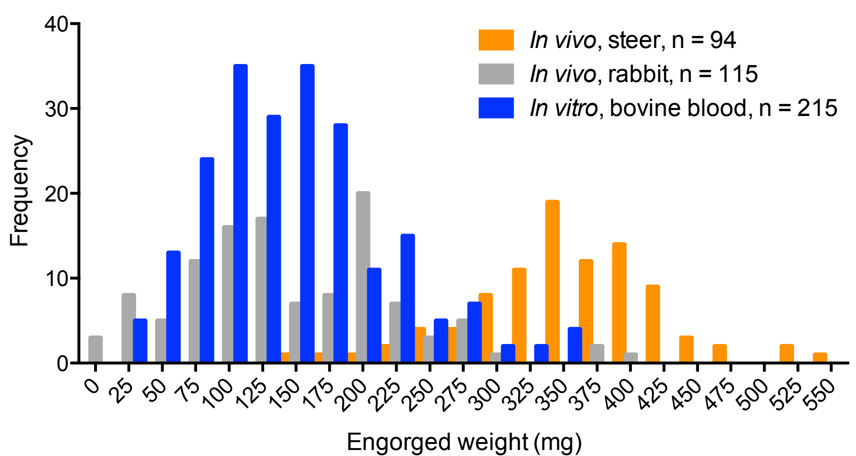

Figure 23. Engorged weight of *I. ricinus* females fed to repletion *in vivo* on steer and rabbits, respectively, and *in vitro* on bovine blood. *I. ricinus* placed on steer reached a median engorged weight (354.5 mg) more than twice that of *I. ricinus* fed *in vitro* on bovine blood (median: 130.5 mg) or *in vivo* on rabbits (median: 138 mg). ($P < 0.0001$; Kruskall-Wallis test followed by Dunn's method post-hoc).

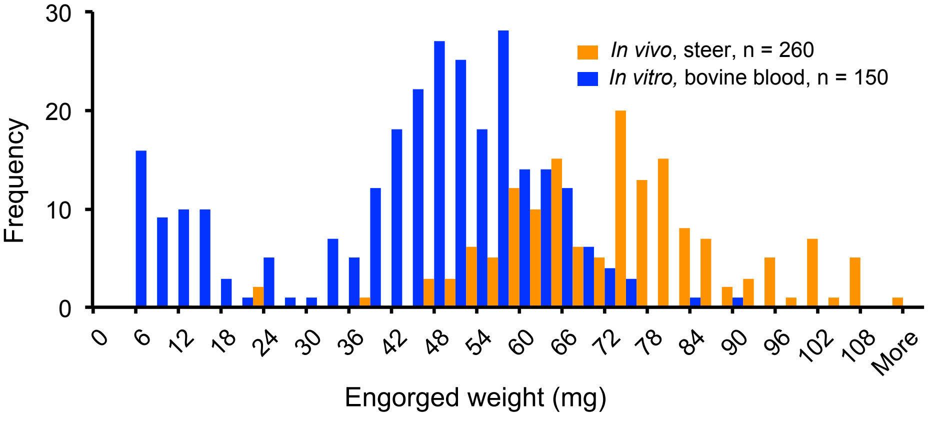

Figure 24. Engorged weight of *A. hebraeum* nymphs fed to repletion *in vivo* and *in vitro*. *A. hebraeum* nymphs fed *in vivo* on steer (median 71 mg) weighed significantly more than ticks fed *in vitro* on bovine blood (median 47 mg; $P < 0.001$; Mann-Whitney U Test).

3.4.5 Rate of engorgement

After being placed on steer and silicone membranes over bovine blood, respectively, the partial engorged weight attained by *in vivo* and *in vitro-fed I. ricinus* females was not significantly different one day after attachment (Figure 25). After two days, *in vivo-fed* ticks weighed slightly more than *in vitro-fed* ticks (Table 17). No significant difference in engorged weight was detected between *in vivo* and *in vitro-fed* ticks after three days of feeding; after three

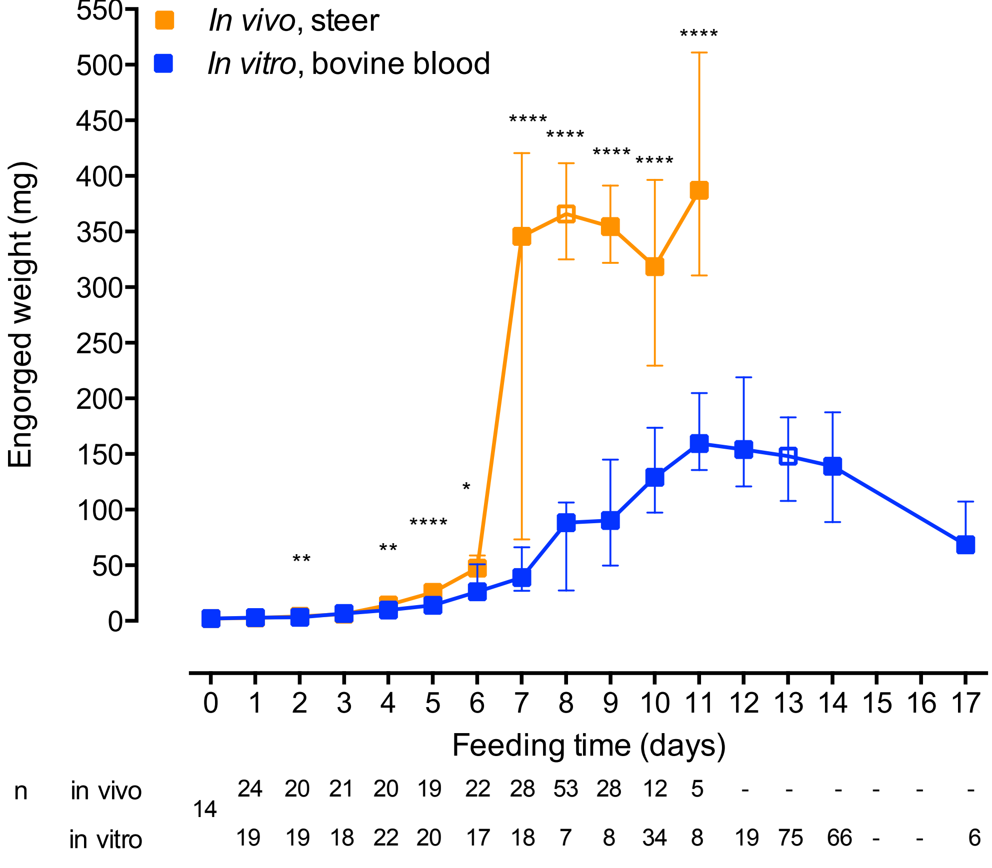

Figure 25. Change in engorged weight over time for *I. ricinus* females fed *in vivo* and *in vitro*. Data points are median weight ± interquartile range. Asterisks denote significant differences between engorged weight for *in vitro* and *in vivo*-fed ticks on each day (Mann-Whitney U-test). The unfilled square in each group denotes the drop-off day.

days, *in vivo* and *in vitro-fed* ticks had experienced similar increases in weight overall compared with their unfed weight (207% and 239%, respectively; Table 18). Between day 3 and 4 and thereafter, *in vivo-fed* ticks experienced consistent greater increases in weight per day compared with *in vitro-fed* ticks (Figure 26);

between days 3 and 4, 4 and 5, and 5 and 6, respectively, *in vivo-fed* ticks increased in weight 133%, 79%, and 85% versus 42%, 45%,

Table 17. Median engorged weight (interquartile range) over time for *I. ricinus* females fed *in vivo* and *in vitro*. Asterisks denote significant differences in engorged weight between treatments (Mann-Whitney U-test)

	Engorged weight (mg)		
Day	*In vivo*	*In vitro*	Significance
Unfed	2.0 (1.7–2.5)		
1	2.8 (1.7–3.5)	2.9 (2.1–4.9)	
2	4.0 (2.4–6.1)	3.2 (2.2–4.3)	**
3	6.2 (4.1–11.3)	6.8 (3.0–12.3)	
4	14.4 (5.2–24.2)	9.6 (4.4–26.5)	**
5	25.7 (18.0–41.2)	14.0 (8.5–37.6)	****
6	47.5 (18.3–68.3)	26.3 (11.7–73.0)	*
7	345.8 (49.6–564.2)	38.9 (9.3–99.7)	****
8	366.0 (181.0–535.0)	88.4 (21.3–258.3)	****
9	354.5 (157.0–477.0)	90.2 (46.3–270.4)	****
10	318.5 (197.0–453.0)	129.0 (37.0–277.0)	****
11	387.0 (295.0–538.0)	159.5 (76.0–230.0)	****
12		154.0 (87.0–283.0)	
13		148.0 (34.0–343.0)	
14		139.0 (33.0–356.0)	
-		-	
17		68.5 (60.0–138.0)	

Table 18. Engorged weight relative unfed weight over time for *I. ricinus* females fed *in vitro* and *in vivo*

	Engorged weight relative unfed weight	
Day	*In vivo*	*In vitro*
1	41%	44%
2	98%	61%
3	207%	239%
4	617%	381%
5	1182%	598%
6	2,270%	1,209%
7	17,147%	1,842%
8	18,154%	4,308%
9	17,581%	4,400%
10	15,785%	6,334%
11	19,202%	7,855%
12		7,581%
13		7,282%
14		6,833%
17		3,316%

and 88%, for in *vitro*-fed ticks (Figure 26). The increase in partial engorged weight per day was always detected as highly significant (*P* < 0.0001) compared to the previous day for *in vivo-fed* ticks, whereas this was not the case for *in vitro-fed* ticks (Figure 26). In other words, *I. ricinus* females fed *in vivo* on steer experienced consistent and substantial daily increases in weight, whereas the partial engorged weight of *I. ricinus* females fed *in vitro* on bovine blood was not always significantly different compared to the previous day, particularly after day 8 (Figure 26). Moreover, *in vivo*-fed ticks experienced a clear two-phase feeding pattern (slow and rapid phase), which included an acceleration in feeding between days 3 and 6 within the slow phase (Figure 26), leading up to the rapid feeding phase; the rapid feeding phase of *in vivo* ticks then generally occurred over one day (day 7) with ticks experiencing a 627% increase in weight compared with the previous day's weight (Figure 26).

The slow feeding phase of *in vitro* ticks did not include a clear acceleration in feeding leading up to the rapid feeding phase, and the slow feeding phase of *in vitro* ticks was prolonged one day compared to *in vivo* ticks (Figure 25). The rapid feeding phase of *in vitro* ticks then occurred over five to six days with a maximum daily increase in weight of just 127% (Figure 26); the maximum engorged weight before drop-off was not reached until day 11 for *in vitro* ticks (Figure 26). On the most common drop-off day for *in vitro*-fed ticks, replete *I. ricinus* females weighed 7,282% more than their unfed weight compared to 18,154% for *in vivo*-fed ticks (Table 18).

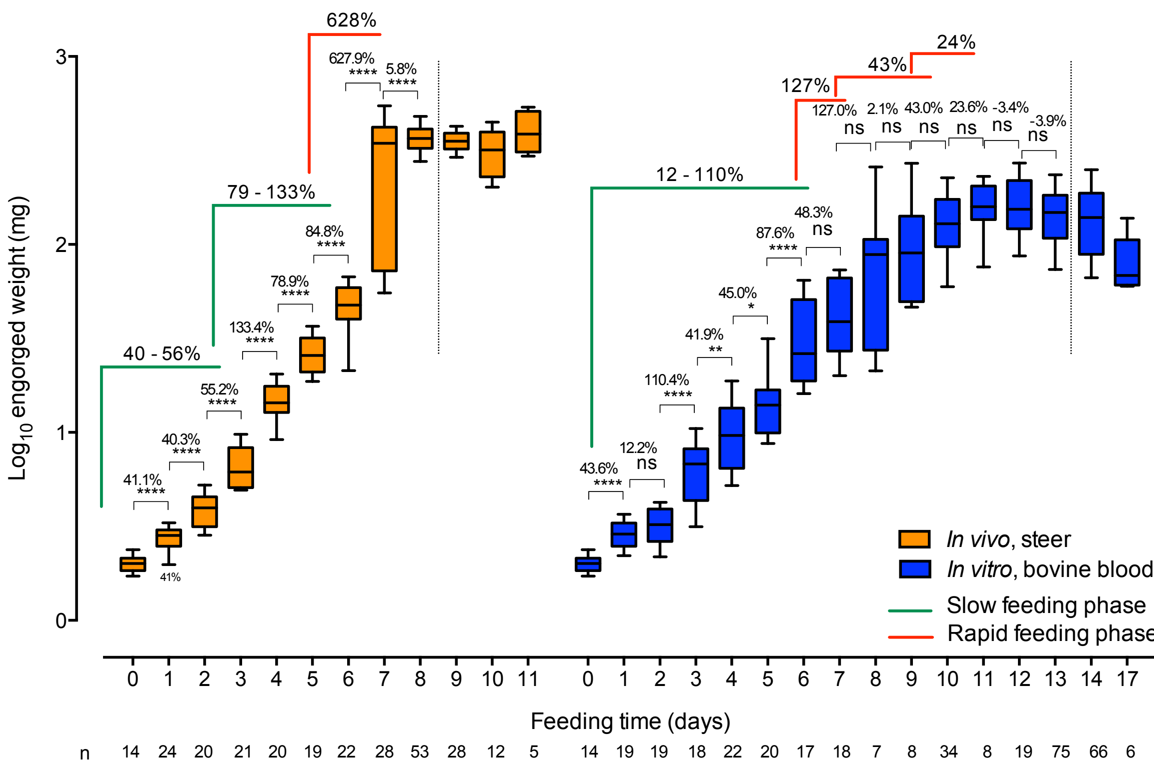

Figure 26. Daily percent change in engorged weight for *I. ricinus* females fed *in vitro* and *in vivo*. Asterisks denote significant differences between the engorged weight on subsequent and preceeding days (Mann-Whitney U-test). Percentages above asterisks report the actual difference in engorged weight between subsequent and preceeding days; dotted lines denote most common drop-off days; green and red lines highlight the slow and rapid feeding phases, respectively. Box plots show the median, interquartile range and 10-90th percentile; ns, not signifcant.

3.5 Discussion

3.5.1 *In vitro* feeding of *I. ricinus*

In this chapter, more than 2,000 *I. ricinus* females were fed in feeding experiments using T31 membranes (thickness: 158 μm). The hypostome of *I. ricinus* females is 500 um (Kröber and Guerin 2007b). Although significant differences in attachment were detected between trials, mean tick attachment to this membrane in feeding units was never lower than 85%. Using the pooled data from three independent feeding experiments, 98% of all ticks that attached to the membrane successfully penetrated T31 membranes with their hypostome and reached blood. Taken together, these results demonstrate that T31 membranes were suitable for *in vitro* feeding of *I. ricinus* females to repletion. In addition, a maximum of just 4.7% of ticks that attached to membranes were recorded as dead after 40 hours, and this number was usually less than 1% in feeding units.

Kröber and Guerin (2007a) discussed a possible need to simplify the attachment stimuli (cow hair, cow hair extract, mosquito netting, plastic cross) used in their study. Here, the cow hair and cow hair extract, alone, were sufficient to attain similar or higher attachment rates than Kröber and Guerin (2007a). The plastic cross in their study was used to create borders where ticks prefer to attach and to prevent clumped attachment by ticks, which caused leaks in their membrane (50–90 μm). In this study, the thicker membranes used prevented leaks without the use of a tile spacer.

3.5.2 *I. ricinus* attachment to the membrane over time

In *in vitro* feeding trials where tick attachment was recorded at hourly intervals, the majority of ticks (more than 75%) attached within 13 hours of being placed in feeding units, the first time point where tick attachment was recorded. A limitation of this study was that attachment was not recorded earlier after placing ticks in feeding units, so to more accurately identify the time needed before ticks attach *in vitro*. However, these experiments were made during precious feeding trials

with acaricides and it was decided that earlier observations of ticks may have disrupted attachment and possibly decreased tick attachment.

After 24 hours, 10% of ticks had not attached and were still walking in feeding units; after 40 hours, 96% of ticks were accounted for as either attached-alive or dead (i.e. only 4% had not attached and were still alive and walking in the feeding units). These data are important to indicate, for example, the optimum timepoint for controlled introduction of test products into blood beneath the membrane. After 40 hours, numbers of alive-attached ticks actively feeding, and dead ticks in feeding units, are easily distinguished before introduction of a test compound into blood. In this manner, the effect of an acaricide on tick mortality is only quantified using ticks exposed to the test product in blood. In addition, it was observed that after 24 hours, unattached-alive ticks mainly died and did not contribute to overall tick attachment in feeding units. This means that when a test product is exposed to ticks at 40 hours, the attached ticks are generally a developmentally homogenous group of ticks, all having mostly attached to membranes within 24 hours after placement in feeding units.

A series of publications testing the systemic efficacy of afoxolaner in dogs against ixodid ticks (e.g. Dumont et al. 2014; Mitchell et al. 2014a,b) included experiments testing the effects of this acaricide on tick mortality using pre-attached ticks (24 hours) on dogs. From a study design standpoint, experiments in this study comparing the systemic efficacy of test products against ticks attached for 40 hours to membranes *in vitro* are closely related to these *in vivo* studies and should allow for direct comparisons of acaricide efficacy results between studies *in vitro* and *in vivo*.

3.5.3 *In vitro* and *in vivo* feeding duration and engorged weight

In this study, the *in vitro* situation significantly affected the feeding duration of *I. ricinus* females, extending the feeding time before drop-off by five days (day 13) compared with the *in vivo* situation (day eight). The source of the bloodmeal also significantly affected the engorged weight attained by ticks, which varied significantly both between the *in vitro* and *in vivo* situation, and between different

hosts *in vivo*. *Ixodes ricinus* females fed *in vivo* on steer attained engorged weights more than twice that of *in vitro-fed* ticks (bovine blood) or rabbit-fed ticks. It is interesting that ticks fed on steer weighed more than ticks fed on bovine blood *in vitro*. It is also interesting that ticks fed on steer weighed more than ticks fed on rabbits. *Ixodes ricinus* females fed *in vitro* on bovine blood did not differ from ticks fed *in vivo* on rabbits. The differences in tick engorged weights due to different *in vivo* hosts and differences in the engorged weight due to the *in vitro* situation are discussed separately, below.

The effect of different hosts on the size of the tick bloodmeal varies depending on the tick species and life stage, and multiple other independent host-related (e.g. host immunity, grooming, attachment site) and environmental factors (e.g. temperature) (Balashov 1972). Balashov (1972) fed six ixodid tick species each on eight different laboratory hosts and showed that the engorged weight did not differ greatly between hosts, albeit these data were for larvae and nymphs only. Given that *I. ricinus* is a well-known generalist tick species, feeding on nearly every class of terrestrial vertebrates (all except amphibians), it is perhaps unexpected that such a large difference in engorged weight was attained by *I. ricinus* females fed on steer (median: 355 mg) versus rabbits (median: 131 mg) in this study. The reason for this result is unclear. Balashov (1972) maintains that the chemical composition of vertebrate blood is generally stable among the different classes of vertebrates that ticks feed on. Galun (1975), however, in her review of phagostimulants of bloodfeeding arthropods, discusses the considerable differences in concentrations of molecules such as adenosine triphosphate (ATP) in different vertebrate bloods. She notes, however, that in spite of these differences, the overall concentration in all classes is nevertheless always many fold higher than the stimulatory concentration for tick feeding (Galun and Rice 1971). Willadsen et al. (1984) showed that *Rhipicephalus microplus* fed different host bloods through capillaries was unable to “concentrate” the blood of any hosts other than bovines. The extent to which generalist ticks like *I. ricinus and A. hebraeum* are able to utilize different host bloods remains unclear.

The reason for the difference in engorged weight between *in vitro* and *in vivo*-fed ticks in this study is also unclear. Kröber and Guerin (2007a) added ATP to the

defibrinated bovine blood used in their study, and although they recorded a slightly higher engorged weight with *I. ricinus* in their study, they still also recorded an engorged weight less than half that of the weight attained by *I. ricinus* fed on steer in this study. An unfortunate missing experiment in this study was a test on the effect of rabbit blood *in vitro* on *I. ricinus* engorged weight. As ticks in this study fed more *in vivo* on steer compared with *in vitro* on bovine blood, and ticks fed *in vivo* on rabbits fed less than ticks fed on steer, it follows that ticks fed *in vitro* on rabbit blood would be expected to feed the least of all if host blood is indeed a main factor affecting engorged weight. As host immunity and other host factors are not present in the *in vitro* situation, *in vitro* feeding systems are well positioned to control for the effect of different host blood on tick engorged weight. This finding would also support the hypothesis that some nutritional, or phagostimulatory factors, are indeed missing from the *in vitro* situation. Unfortunately, I was unable to source a large enough volume of whole rabbit blood to perform tick feeding experiments to repletion using rabbit blood *in vitro*. Future studies should consider this experiment.

Multiple independent factors likely contribute to the difference in engorged weight observed between *in vitro* and *in vivo*-fed ticks, e.g. host immunity, blood nutritional differences, blood pressure, host stimuli, and/or gut endosymbionts in ticks, the latter of which are possibly destroyed by antibiotics in blood, *in vitro* (reviewed in Kuhnert 1996). Unfortunately it was not possible to compare egg laying and other factors related to reproduction (e.g. Egg Conversion Factor; Kuhnert et al. 1995) because *in vitro*-fed *I. ricinus* in this study did not lay eggs. The reasons for this are not known since *A. hebraeum* nymphs were previously fed to repletion in this study under these conditions and more than 90% molted to adults. It is possible that gentamicin and nystatin affected egg laying in the females; however, Kuhnert et al. (1995) also added gentamicin and nystatin to the blood used in their study with *A. hebraeum* (same concentrations as this study, 5 µg/ml and 100 i.u./ml, respectively), and egg laying was not affected. Kuhnert et al. (1995) changed their blood every 12 hours in contrast to every 24 hours in this study. Kröber and Guerin (2007) also changed blood every 12 hours, but did not add nystatin to blood. Blood was obviously not unpalatable to ticks after 24 hours as ticks fed to repletion, but it’s possible that requirements for

egg production are more sensitive to nutrient deficiencies or nutrient degradation than molting requirements. At the time of Kuhnert's review (1996), no endosymbionts of ticks had been identified. Recently, Zhong et al. (2007) treated engorged *Amblyomma variegatum* females with antibiotics and showed that reduced reproductive fitness in ticks treated with antibiotics was associated with a reduction in *Coxiella spp.* bacteria, suggesting that *Coxiella spp.* might indeed provide essential nutrients to ticks in the midgut. It is possible that the combination of gentamicin and nystatin used in this study affected nutrient provisioning by key bacteria in *I. ricinus*, possibly affecting engorged weight and/or egg laying.

3.5.4 Engorged weight and the bloodmeal over time for *I. ricinus* females fed *in vitro* and *in vivo*

As far as I am aware, this is the first study to compare the increase in the size of the ixodid tick bloodmeal over time *in vitro and in vivo*. The results in this study for the pattern and increase in engorged weight over time *in vivo* are in good agreement with previous findings, showing a clear two-phase feeding pattern (Arthur 1965; Balashov 1972; Lees 1952). Lees (1952) showed that over the first five to six days of feeding, coined the slow feeding phase, *I. ricinus* females increase in weight to approximately 50 mg, before increasing to over 200 mg during the last day of feeding (day 7), coined the rapid feeding phase. In this study, *I. ricinus* females fed *in vivo* increased in weight to 48 mg after six days before increasing to 366 mg overnight on day 7. In addition, an acceleration in the growth rate (from a 40–56% increase in weight per day over days 1–3 to a 79–133% increase in weight per day over days 4–6) was observed towards the end of the slow feeding phase, as detailed in Arthur (1965).

Compared to *in vivo*-fed ticks, the daily increase in engorged weight of *in vitro*-fed ticks was similar over the first three days of feeding, but then when *in vivo*-fed ticks began accelerating feeding after day 3, no clear acceleration occurred *in vitro*. Entry into the rapid feeding phase for *in vitro*-fed ticks was then delayed at least one day, and the rapid feeding phase occurred over several days with relatively small daily increases in weight in comparison to one massive increase

in weight overnight for *in vivo*-fed ticks. Note that a statistically significant difference in engorged weight was detected after two days of feeding for *in vitro* and *in vivo*-fed ticks, but the biological significance of this result is not clear; *I. ricinus* females clearly fed similarly *in vitro* and *in vivo* over the first three days of feeding, but more data points are needed between day 1 and 3 to confirm this conclusion. That said, there is no doubt that ticks feeding *in vivo* clearly accelerated feeding after day 3, whereas *in vitro*-fed ticks did not experience a comparable acceleration.

Although we have known for some time that ixodid ticks fed *in vitro* generally weigh less than *in vivo* ticks at repletion, it was not known what was happening leading up to repletion. For example, *in vitro*- and *in vivo*-fed ticks might have fed at similar rates during the slow feeding phase with *in vitro*-fed ticks only consuming less blood during the rapid feeding phase; alternatively, *in vitro*-fed ticks might have fed slower throughout feeding; Kuhnert (1996) speculated that the silicone membrane or the absence of blood pressure *in vitro* might contribute to the smaller engorged weight of *in vitro*-fed ticks (among other factors). There were therefore both biological and physical considerations related to the *in vitro* situation that might have inhibited or slowed feeding *in vitro*. The results in this study therefore have dual purposes: that feeding was similar *in vivo* and *in vitro* over the first three days shows that no physical factors related to the *in vitro* situation inhibit feeding *in vitro* — if this were the case, feeding *in vitro* would have been expected to be slower throughout feeding. Next, that entry into the rapid feeding phase was delayed *in vitro*, and that the rapid feeding phase *in vitro* occurred over several days with relatively small daily increases in weight, as opposed to one massive increase in weight overnight, hints that bloodmeal utilization, or developmental processes occurring during the slow feeding phase, contribute to the smaller engorged weight attained by *in vitro*-fed ticks. Important bloodmeal components may be missing from the *in vitro* bloodmeal, leading to less efficient digestion and utilization and possibly underdevelopment of the midgut or other organs involved in feeding. If this were the case, entry into the rapid feeding phase would then be delayed, as was observed, and less blood would be consumed while feeding on the *in vitro* bloodmeal over time (as observed), because more time is needed for physiological development using *in*

vitro blood before the tick is able to accommodate the bloodmeal during the rapid feeding phase.

A limitation of this study is that ticks were not dissected to compare midgut development *in vitro* and *in vivo* during feeding; if the midguts of *in vitro-fed* ticks were found to be less developed than *in vivo-fed* ticks, particularly after day 3, this finding would strongly support the conclusion that ticks feeding *in vitro* are not efficiently utilizing the *in vitro* bloodmeal. Although ticks are bloodfeeding arthropods, Arthur (1965) notes that ixodid ticks consume significant quantities of “non-blood factors” (e.g. lymph) during the slow feeding phase. The importance of non-blood factors in the bloodmeal for tick development on the host during feeding, which are absent or not as abundant in the *in vitro* situation, may have been overlooked in the past. Without confirmation from midgut dissections, the possibility does still remain that some unidentified factor associated with the *in vitro* situation is physically inhibiting feeding in ticks *in vitro*. As mentioned, if this was the case, slower feeding *in vitro* compared to the *in vivo* situation would be expected *throughout* feeding not only after day 3 (which was not observed). However, it is possible that a membrane-related, or some other factor related to the *in vitro* situation, physically influenced feeding specifically only after day 3. However no observations were made that would suggest this might be the case. The best explanation therefore seems to be that ticks feeding *in vitro* are not able to utilize the *in vitro* bloodmeal as well as the *in vivo* bloodmeal.

The finding that *in vitro* and *in vivo* feeding differs only after day 3 is important to characterize for other reasons: This result has implications for *in vitro* studies interested in, for example, 1) effects of systemic acaricides on tick mortality or 2) tick feeding time needed before pathogen transmission occurs in ticks. The salient data in each of these examples is collected within the first 48 hours of tick feeding; the observation that ticks feeding *in vitro* and *in vivo* feed at similar rates during this time period gives confidence that results obtained using *in vitro* feeding ticks have relevance for the *in vivo* situation

4. Effects of systemic acaricides on feeding behaviour and mortality in *Ixodes ricinus*

4.1 Abstract

As tick populations, and the diseases they transmit, continue to expand into areas where they were not previously recorded, new strategies for tick control are increasingly needed. A simplified semi high-throughput *in vitro* feeding assay is described for *in vitro* testing of systemic acaricides for the control of ixodid ticks. The LD_{50} values and the effects on tick feeding behaviour of 12 acaricides from five compound classes were determined for *I. ricinus* females *in vitro*. The acaricides are the macrocyclic lactones abamectin, doramectin, eprinomectin, ivermectin, and moxidectin, the phenylpyrazoles ethiprole, fipronil, and pyriprole, the organophosphates coumaphos and chlorpyrifos, the organochlorine dieldrin, and the isoxazoline fluralaner. The LD_{50} value (95% confidence interval) calculated for fipronil (0.09 [0.06-0.14] µg/ml) was nearly an order of magnitude lower than the most effective macrocyclic lactone tested, abamectin (0.58 [0.43-0.78] µg/ml). Slopes of linear models fitted to the partial engorged weight attained by ticks as a function of increasing acaricide dose for fipronil, abamectin, and coumaphos, respectively, were -0.07 (P = 0.4555), -0.42 (P < 0.00001), and 0.34 (P = 0.0020), indicating that fipronil did not signifiicantly inhibit tick feeding in this assay, whereas abamectin and coumaphos, respectively, significantly inhibited and significantly increased tick feeding in a dose dependent fashion. Consistent effects were observed across all other acaricides tested in each class. The overall partial engorged weight attained by ticks feeding for 48 hours on blood treated with the phenylpyrazoles, macrocyclic lactones, and organophosphates (10 µg/ml), respectively, was 30-40% lower than the placebo, 80-90% lower than the placebo, and 80-90% higher than the placebo. In video recordings of ticks feeding *in vitro* on treated blood, abamectin was associated with rapid paralysis of ticks 5-10 minutes after exposure, and the size of the tick alloscutum decreased over time during exposure to this acaricide. Fipronil and coumaphos, respectively, were associated with the onset of rapid uncontrolled leg movements in ticks 29 and 148 minutes after exposure, and the size of the tick alloscutum increased in size over time during exposure to both acaricides. The effects of systemic acaricides on tick feeding behaviour in this study are consistent with the molecular mode of action each acaricide. The assay permits comprehensive comparisons of the effects of systemic acaricides on ixodid tick feeding behaviour and mortality *in vitro*.

4.2 Introduction

Advances in functional genomics in recent years have led to increased interest in target-based approaches for discovery of new antiparasitic drugs in the animal health industry. Target-based, or mechanism-based approaches to drug discovery, differ from traditional random screening methods in that target-based approaches are undertaken with a validated drug target, i.e. known parasite ion channel, and seek to find new drugs active at that target site (Woods et al. 2010). High-throughput screening methods using cloned recombinant parasite targets expressed in cells in 384-well plates are now standard in animal health and are used to screen 1000+ compounds per day against a molecular target (Woods et al. 2010).

Although mechanism-based screening approaches are undoubtedly the future of drug discovery in animal health, investment in a single molecular target involves great risk. Failures of target-based approaches to deliver validated leads after more than a decade of focused screening are noted in the literature (Geary et al. 2009). In recombinant gene expression assays, screening conditions at a target site are greatly simplified and activity in these assays does not always translate into activity in a parasite (Wolf and Gunkel 2009). Compounding on this is the unique requirement in the animal health industry for drugs to have a wide spectrum of activity against parasites, e.g. fleas and ticks, which are not closely related. Moreover, animal health antiparasitics are formulated in both contact and systemic formulations that affect availability and activity of compounds at target sites in different ways and in different ways for different parasites. For these reasons, mechanism-based approaches to drug discovery in animal health depend critically on specific *in vitro* whole organism bioassays to confirm the activity of compounds at molecular targets in target parasite species. Whole organism bioassays should therefore provide quantitative endpoints that are consistent with, and predictive of, a compound's molecular mode of action, and should also permit comparisons between compounds in order to facilitate the ranking of compound activity before compounds are progressed to *in vivo* models.

The unique feeding requirements of ticks have traditionally made it very difficult to quantify the effects of systemic acaricides on tick feeding behavior. Tick attachment to the host, and blood feeding, is a complex and slow process with the tick bloodmeal occurring over several days. Researchers in the past attempted to circumvent the complex feeding requirements of ticks by using injection assays to measure systemic efficacy of acaricides (Amaral 1993), whereby acaricides are dissolved in solvent and injected directly into the body of an engorged female tick through the tick's cuticle. In injection assays, however, there is no guarantee the compound is introduced into the midgut of the tick; moreover, the compound is not delivered to the tick in blood. In addition, the quantitative endpoint in injection assays is effect on oviposition, which may not be related to the intended effect of the compound on the tick in the *in vivo* situation, namely inhibition of tick feeding and tick mortality during feeding. For these reasons a relative paucity of information is available in the literature on the effects of systemic acaricides on tick feeding behavior. The farm animal sector has tested systemic acaricides against ticks of cattle for decades, however most of these studies are limited to *in vivo* studies on cattle. Since it is inherently difficult to observe the effects of systemic acaricides on ticks *in vivo* on cattle, little information on systemic acaricide phenotypes could be gained from these studies outside of effects on tick mortality.

Kröber and Guerin (2007a) described a silicone membrane-based *in vitro* feeding assay for ixodid ticks that permits tick attachment and feeding to repletion *in vitro*. This assay facilitates controlled introduction of acaricides into ticks during blood feeding and therefore permits direct observations of tick mortality during feeding on blood that is dosed with precise concentrations of systemic acaricides. Here, the throughput of this assay was increased from six to 60 wells and the test period needed for data collection reduced to four days. Twelve acaricides from five compound classes were tested and the LD_{50} levels of acaricides in blood exposed to *Ixodes ricinus* females were quantified. In addition, the effects of systemic acaricides on partial engorged weight attained during blood feeding was quantified and the assay was combined with a time-lapse video camera for qualitative and quantitative descriptions of real-time effects of systemic acaricides on tick feeding behavior.

4.3 Materials and methods

4.3.1 Ticks

Ixodes ricinus females aged 3–6 months post-ecdysis were purchased commercially and stored in an environmental chamber at conditions 25°C, 94% RH, 16:8 light-dark cycle.

4.3.2 Test products and doses tested

Eleven acaricides were tested (0.001–100 µg/ml): The phenylpyrazoles fipronil, ethiprole, and pyriprole (> 97%, Pestanal®, Fluka); the macrocyclic lactones ivermectin, abamectin, eprinomectin, doramactin and moxidectin (Sigma-Aldrich GmbH, Germany); the organophosphates coumaphos and chlorpyrifos (> 97%, Pestanal®, Fluka); the organochlorine dieldrin (> 97%, Pestanal®, Fluka); and the isoxasoline fluralaner (> 94%, synthesized in-house).

4.3.3 Controlled introduction of acaricide into ticks during *in vitro* feeding

Details for membrane preparation, feeding unit construction, and *in vitro* feeding conditions are provided in Chapter 1. For testing systemic efficacy of acaricides, 10 *I. ricinus* females were placed inside feeding units and allowed 40 hours to attach and begin feeding on control blood in a 60-well steel plate. Blood was pre-warmed to 37 °C before exposure to ticks and treated with heparin (10 i.u./ml; Sigma, Germany, H3393), gentamicin (5 µg/ml; Sigma, Germany, G1272) and nystatin, as described previously. After 40 hours, ticks that had died in feeding units were removed and feeding units containing attached and alive ticks were re-arranged into treatment groups (3 feeding units per dose of test product) so that each treatment contained equivalent numbers of attached and feeding ticks. Feeding units were then transferred to wells containing blood treated with different test products. Stock solutions of acaricide test products were prepared using dimethyl sulfoxide (DMSO) (Sigma, Germany, D8418) as a solvent and final concentrations of test products in treated blood (0.001–100 µg/ml) were

obtained using 0.005% DMSO (v/v) in blood. Placebo treatments received DMSO only in blood.

4.3.4 Quantifying tick mortality and meal size in response to acaricide

Ticks were exposed to treated blood for 48 h and blood was changed and re-dosed with test product at 24 h. After 48 h, ticks were removed from membranes using forceps and transferred to a hot plate for assessment of mortality. No more than one feeding unit was handled per assessment. Ticks were placed ventral side up on a hot plate set to 55° C and considered dead if no leg movements were observed after 30 secs on the plate. The partially engorged weight obtained by individual ticks after feeding on treated blood was recorded immediately after mortality assessments using a microbalance (Sartorius, Germany) accurate to 0.001 mg. Total feces produced in each feeding unit was assessed by transfer to a 2.5 ml tube and weighed using the same balance. Figure 27 provides a visual summary of the experimental set-up, described above, for testing systemic efficacy of acaricides against ticks.

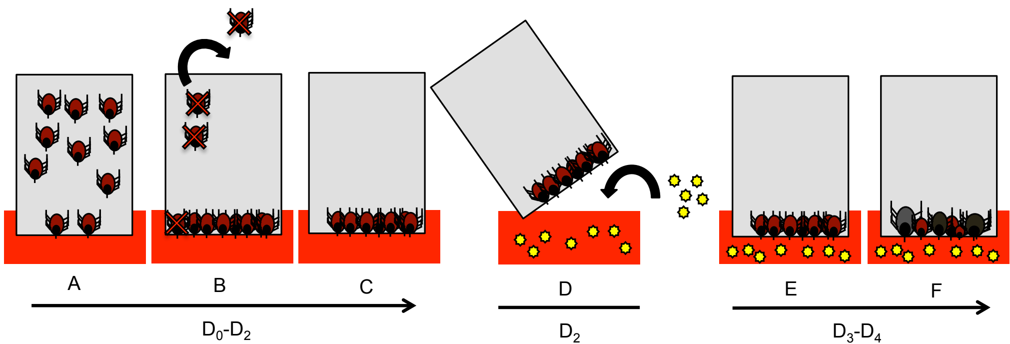

Figure 27. Experimental set-up for quantifying systemic efficacy of acaricides. Ticks are placed in feeding units (A) and given 40 hours to attach and begin feeding. After 40 hours, dead ticks are removed from feeding units (B), leaving only attached-alive ticks in feeding units (C). Attached-alive ticks are then transferred to wells containing blood treated with an acaricide (D) and exposed to acaricide treated blood for 48 hours (E–F). After 48 hours (F), ticks are removed from feeding units for assessing mortality and recording engorged weights.

4.3.5 Experimental set-up for testing acaricides

For direct observations on the effects of acaricides in blood on tick feeding behavior, a colour video camera (WAT-250D2; Watec Inc., NY, USA) with a c-mount was coupled to a 28 mm endoscope lens adapter and rigid endoscope

(diameter: 10 mm, working length: 30 cm; 0° viewing direction, 70° field of view with adjustable focus range of 1.0 mm to infinity; 32mm industry standard eyepiece; ACM-Wolf-Stortz light guide connection; custom made by RVA Synergies, Uxbridge, UK) and the endoscope was threaded through a 12 mm hole, fitted with a 10 mm airtight gasket, in the ceiling of an environmental chamber at conditions 29°C, 80% RH, ambient CO2, 16:8 light:dark cycle (Figure 28).

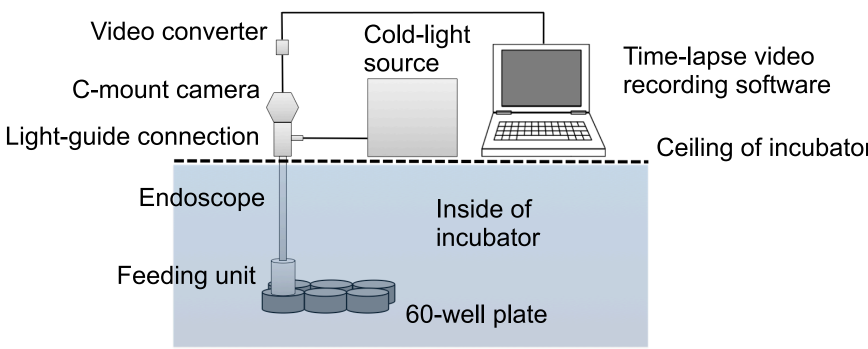

Figure 28. Experimental set-up for time-lapse video recordings of I. ricinus feeding behavior in vitro.

The height of shelving inside the incubator was adjusted so that the endoscope rested above the feeding unit 20 cm from the membrane. A cold light source (Zeiss AG, Jena, Germany) was connected to the light-guide connector on the endoscope and light intensity was set to more than 100 lux. Time-lapse video recordings of ticks feeding *in vitro* were captured using VideoVelocity time-lapse video recording software (CandyLabs, Vancouver, BC, Canada) using a frame rate 29 FPS and one image capture every second. Image quality was set to 70%. Files were created every eight hours for 48 hours and stored on an external hard drive. Ticks were allowed 40 hours to attach and begin feeding on untreated blood as in acaricide experiments and video filming of feeding was captured for the period 40–72 h after attachment. Videos of feeding behaviour on blood containing a placebo or acaricide (10 ug/ml) treatment began immediately following tick exposure to treated blood, i.e., at 40 hours. Final video editing, which included removing the blood change from videos, and video splicing, was carried out using Adobe Premiere Pro CS6 Version 6.0.2 and Adobe After Effects CS6 (Version 11.0.2.11) for Mac OS X 10.8.4. Feeding behavior of *I. ricinus* females feeding *in vitro* on placebo and treated blood was analyzed descriptively

with emphasis on leg movements, muscle contractions associated with feeding and defecation, and changes in the alloscutum associated with cuticle formation and body expansion.

4.3.6 Quantifying time to onset of acaricide poisoning in ticks using time-lapse video recording

The time to onset of acaricide poisoning in ticks by different acaricides was compared using the onset of uncontrolled leg movements as the indicator of poisoning. The time (min) was recorded when all 8 legs of *I. ricinus* females began moving uncontrollably. For data collection, each 32-hour video was condensed into a five-minute version (playback speed increased by a factor of 576). The video was scanned through in VLC media player (Version 2.1.0 for Windows) for recording the general time point when symptoms began. The original files were then played back at normal speed for recording exact time points for onset of symptoms.

4.3.7 Effect of acaricides in blood on tick body size during feeding using time-lapse video recording

The effect of different acaricides, over time, on female *I. ricinus* body size during feeding was quantified from immediately after exposure to acaricide at hour 40. Body size was measured every two hours for 22 hours between the period 40–62 hours attachment and determined by measuring total area (mm^2) of the tick alloscutum. Area of the alloscutum was measured in ImageJ (version 1.48g for Mac) using the elliptical Region of Interest (ROI) selection tool and image stacks consisting of ‘image slices’ recorded every two hours. The contour of the ROI was fitted around the tick alloscutum (Figure 29) at hour zero and adjusted at each time point in a semi-automated fashion by scrolling through the image slices in the stack and adjusting the contour for changes in the alloscutum over time. It was not possible to quantify changes in body size for placebo treatments due to buildup of feces in feeding units which obscured visibility of ticks and made it impossible to measure body size area.

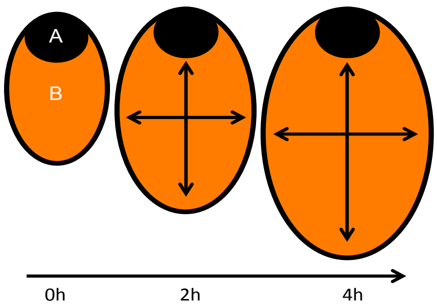

Figure 29. Illustration of the scutum (A) and alloscutum (B) on the dorsal side of the ixodid tick body. Contours were fitted around the alloscutum at each time point and the area of this shape (region depicted in orange above) was measured over time during the bloodmeal.

4.3.8 Statistical analyses

LD_{50} values for acaricides were calculated using dose-response curves fitted using a 4-parameter logistic model with variable slope in GraphPad Prism® version 5 (GraphPad Software, Inc., San Diego, CA). The upper and lower values for tick mortality were constrained to 0 and 100, respectively, and mortality was plotted against the log-transformed concentration of each acaricide. Placebo treatments were plotted with 0.00001 µg/ml assigned as the x-value. Differences in LD_{50} values between acaricides were calculated in Graphpad using the extra sum-of-squares F-test. To compare the effects of acaricides in blood on *I. ricinus* partial engorged weight in response to acaricide or placebo treatments, weights at different concentrations were compared within and between treatments and the slopes of linear models fitted to the data for each product were compared. The weights of partially engorged ticks were analyzed using a Linear Model (LM) for the macrocyclic lactones and a Generalized Linear Model (GLM) for all other acaricides, in order to compensate for differences in variance, using R (V. 3.0.2).

4.4 Results

4.4.1 Mortality of *I. ricinus* females after 48 h of feeding *in vitro* on blood containing acaricide

Dose response curves were initially calculated using fipronil with a dose range of 0.01–100 µg/ml in blood. This dose range encompassed 0–100% mortality for *I. ricinus* females *in vitro* after 48 hours. In four independent experiments with fipronil, the LD_{50} values calculated from three out of four dose response curves were not significantly different from each other (*P* = 0.8524; Figure 30). The LD_{50}-values in independent tests with fipronil never overlapped with ivermectin (Figure 30). As up to 100% mortality was recorded at 10 and 100 µg/ml for fipronil, the dose 100 µg/ml was excluded and a dose range 0.01–10 µg/ml was used in all subsequent experiments. Figure 31A depicts the pooled data from the differerent tests with fipronil and ivermectin. Fipronil was the most active molecule among all

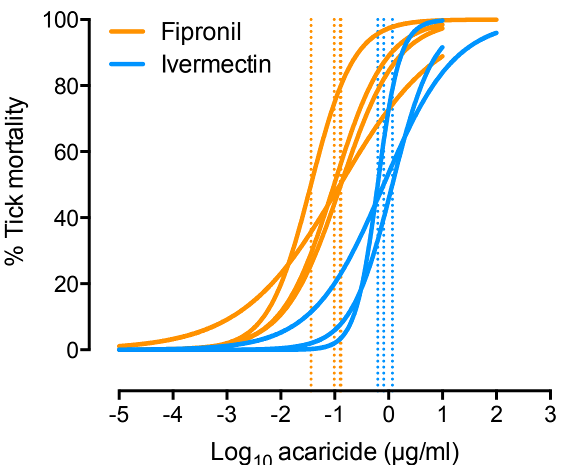

Figure 30. Independent dose response curves showing female *I. ricinus* mortality in response to fipronil (n = 4 experiments with 3 replicates per dose, 10 ticks per replicate) and ivermectin (n = 3). Mortality in the placebo is plotted at 0.00001 µg/ml. Dashed lines show the LD_{50} calculated for each experiment.

acaricides tested in this study and the LD_{50} for fipronil (0.08 µg/ml) was ten times lower than for ivermectin (0.89 µg/ml) ($P < 0.0001$, extra-sum of squares F-test, Figure 31A). Figure 31B shows the dose response curves for all other acaricides tested from the macrocyclic lactones, phenylpyrazoles, and organophosphates, as well as fluralaner, an isoxazoline molecule. Dose response curves for

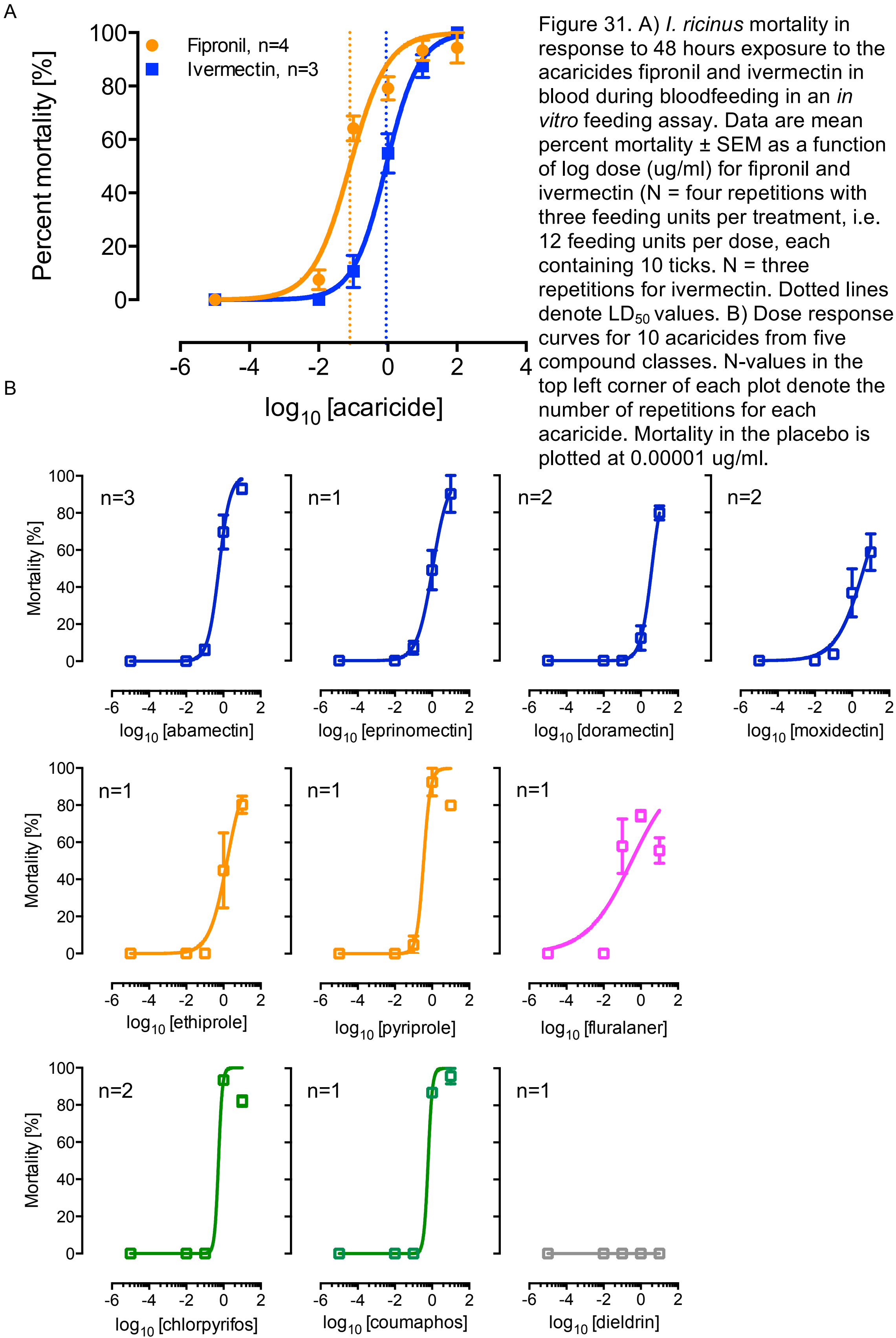

Figure 31. A) *I. ricinus* mortality in response to 48 hours exposure to the acaricides fipronil and ivermectin in blood during bloodfeeding in an *in vitro* feeding assay. Data are mean percent mortality ± SEM as a function of log dose (ug/ml) for fipronil and ivermectin (N = four repetitions with three feeding units per treatment, i.e. 12 feeding units per dose, each containing 10 ticks. N = three repetitions for ivermectin. Dotted lines denote LD_{50} values. B) Dose response curves for 10 acaricides from five compound classes. N-values in the top left corner of each plot denote the number of repetitions for each acaricide. Mortality in the placebo is plotted at 0.00001 ug/ml.

Ivermectin, abamectin, and eprinomectin were very similar, with abamectin having the lowest LD_{50} of the three macrocyclic lactones (Figure 31B). The LD_{50} for abamectin was significantly lower than eprinomectin but was not different from ivermectin. Doramectin and moxidectin were less active in this assay and tick mortality in response to these macrocyclic lactones did not approach 100% mortality at the highest doses tested against *I. ricinus* females (Figure 31B). Ethiprole, a phenylpyrazole, was less active than fipronil ($P < 0.0001$; extra sum of squares F-test), but did not differ from ivermectin ($P = 0.11$; extra sum of squares F-test). Pyriprole, another phenylpyrazole, and the organophosphates coumaphos, and chlorpyrifos were highly effective against *I. ricinus* females at 1–10 µg/ml in blood (80–100% mortality), but these compounds had no effect on tick mortality (0%) at the lowest doses tested in this assay. *I. ricinus* mortality in response to fluralaner, an isoxasoline, was comparable to fipronil at 0.1 and 1 µg/ml, but less than 60% mortality was reached at 10 µg/ml. Table 19 provides LD_{50} values and summary statistics for all compounds tested.

Table 19. Summary statistics for dose response curves

Product	LD_{50} (95% CI) (µg/ml)	R^2	*P* value[a]
Abamectin	0.58 (0.43–0.78)	0.91	-
Ivermectin	0.89 (0.63–1.27)	0.89	0.0597
Eprinomectin	1.07 (0.68–1.68)	0.93	0.0388
Doramectin	12.10 (very wide)	0.86	NC
Moxidectin	13.84 (4.99–38.35)	0.27	NC
Fipronil	0.09 (0.06–0.14)	0.85	< 0.0001
Ethiprole	1.55 (0.75–3.19)	0.85	NC
Pyriprole	0.35 (0.17–0.73)	0.94	NC
Coumaphos	0.61 (0.05–7.87)	0.99	NC
Chlorpyrifos	0.53 (very wide)	0.98	NC

[a]Significantly different from abamectin; *P* values were calculated using the extra sum of squares F-test using Graphpad Prism; NC, not calculated - *P* values could not be calculated for acaricides where mortality did not approach 100% at 10 µg/ml , or where mortality was low at intermediate doses or where variability in mortality was high.

4.4.2 Effects of acaricides introduced into *I. ricinus* females in blood on engorged weight

Figure 32 presents plots of the partial engorged weight attained by *I. ricinus* females feeding on blood dosed with acaricides as a function of increasing

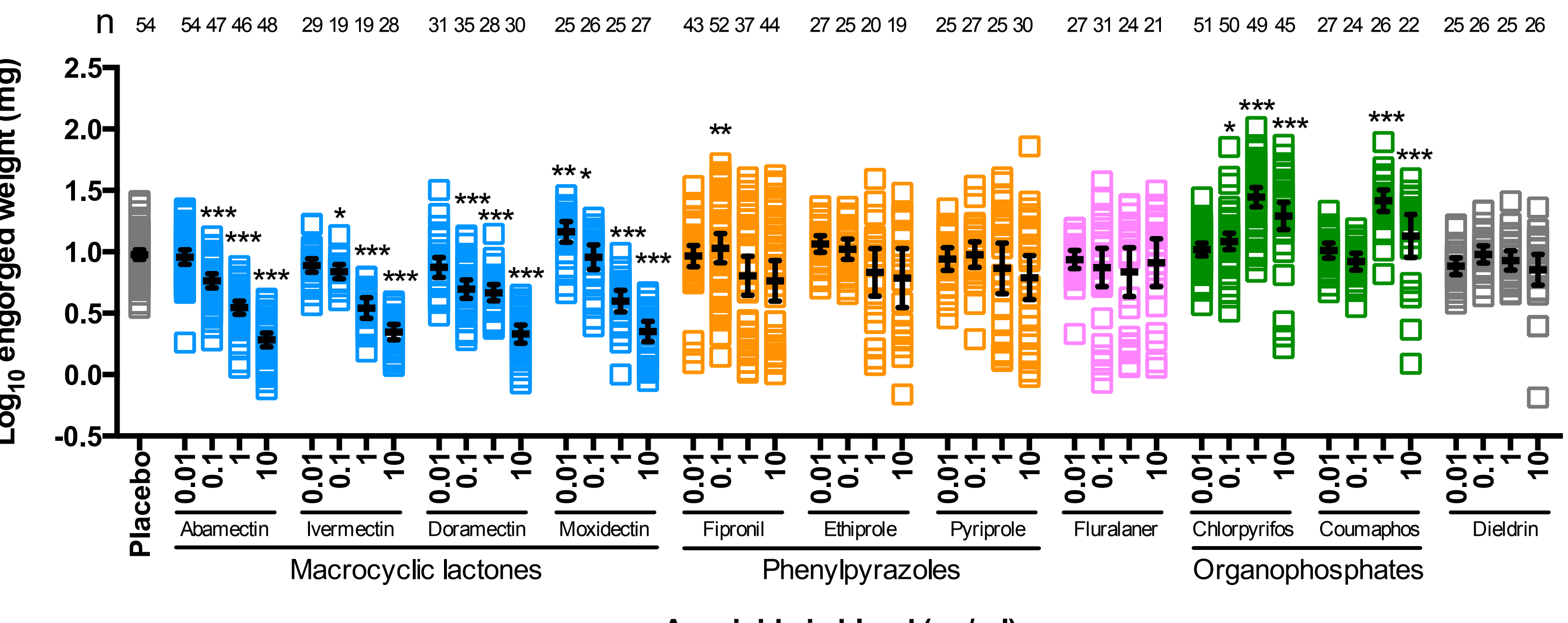

Figure 32. Scatter plots showing the engorged weight attained by *I. ricinus* females feeding *in vitro* on blood containing different acaricides. Boxes represent individual tick weights. N-values above each dose are from two independent experiments for abamectin, fipronil, and chlorpyrifos, and from one experiment for all other acaricides. Black lines and error bars show the mean engorged weight ± 95% confidence interval. Asterisks denote a significant difference versus the placebo (LM for macrocyclic lactones, GLM for all other compounds).

acaricide dose for abamectin, ivermectin, doramectin and moxidectin, representing all macrocyclic lactones (ML) tested in this assay, slopes of linear models fitted to each data set were significant and negative (Table 20); all MLs significantly inhibited tick feeding in a dose-dependent manner.

Table 20. Summary statistics for linear models fitted to engorged weight data as a function of increasing acaricide dose.

Acaricide	Slope	Slope significant?	*P*-value*
Abamectin	-0.42	Yes	< 0.00001
Ivermectin	-0.39	Yes	0.0005
Doramectin	-0.24	Yes	0.0210
Moxidectin	-0.52	Yes	< 0.00001
Fipronil	-0.07	No	0.4555
Ethiprole	-0.10	No	0.3465
Pyriprole	-0.01	No	0.9029
Fluralaner	-0.03	No	0.8298
Chlorpyrifos	0.29	Yes	0.0034
Coumaphos	0.34	Yes	0.0020
Dieldrin	0.02	No	0.8118

**P*-values calculated using LM for the macrocyclic lactones and GLM for all other acaricides. A significant negative value for the slope indicates dose-dependent feeding inhibition in ticks and a significant positive value indicates a dose-dependent increase in feeding.

The partial engorged weight attained by ticks feeding on blood dosed with 10, 1, or 0.1 µg/ml abamectin, ivermectin, or doramectin was significantly smaller than the weight attained by ticks feeding on the placebo (Table 21). With moxidectin, a significantly smaller engorged weight versus the placebo was recorded only at 1 and 10 µg/ml, and ticks feeding on blood dosed with 0.01 µg/ml moxidectin attained a partial engorged weight significantly higher than the placebo (Table 21). Depending on the ML, the partial engorged weight attained by *I. ricinus* females feeding on blood dosed with 1 or 10 µg/ml of a ML was 48–62% or 75–79%, respectively, lower than the placebo. At the highest doses tested, both surviving ticks and dead ticks exposed to MLs attained partial engorged weights lower than the placebo (Figure 33). With increasing dose of each ML, variability in engorged weight remained constant ($P > 0.05$; Bartlett's test).

For all phenylpyrazoles tested, slopes of linear models fitted to each data set were not significant (Table 20); the partial engorged weight attained by ticks

Table 21. Partial engorged weight attained by *I. ricinus* females feeding on blood dosed with different systemic acaricides or placebo

Mean partial engorged weight attained (95% CI) [n]				
	Dose (µg/ml)			
Acaricide	0.01	0.1	1.0	10.0
Placebo (N = 54)	9.8 (8.6–11.0)			
Abamectin (N = 195)	10.2 (8.9–11.5) [54]	6.4*** (5.6–7.2) [47]	3.8*** (3.4–4.2) [46]	2.1*** (1.9–2.4) [48]
Ivermectin (N = 95)	8.2 (7.1–9.3) [29]	7.2* (6.2–8.2) [19]	3.7*** (3.1–4.4) [19]	2.4*** (2.1–2.7) [28]
Doramectin (N = 124)	8.6 (6.5–10.7) [31]	5.6*** (4.6–6.6) [35]	5.1*** (4.1–6.0) [28]	2.4*** (2.0–2.8) [30]
Moxidectin (N = 103)	15.9*** (13.4–18.4) [25]	10.3 (8.4–12.1) [26]	4.4*** (3.6–5.2) [25]	2.5*** (2.1–2.9) [27]
Fipronil (N = 176)	10.9 (9.0–12.9) [43]	16.3** (12.4–20.3) [52]	10.7 (7.3–14.1) [37]	11.3 (7.7–14.9) [44]
Ethiprole (N = 91)	12.5 (10.6–14.3) [27]	11.6 (9.4–13.8) [25]	10.0 (5.7–14.4) [20]	10.2 (5.8–14.5) [19]
Pyriprole (N = 107)	9.9 (7.9–11.9) [25]	11.3 (8.4–14.1) [27]	12.4 (7.7–17.1) [25]	10.8 (5.7–15.9) [30]
Fluralaner (N = 103)	9.4 (8.0–10.7) [27]	10.8 (7.6–14.1) [31]	10.4 (7.2–13.7) [24]	11.6 (7.8–15.5) [21]
Chlorpyrifos (N = 195)	11.4 (10.0–12.8) [51]	14.2* (11.2–17.3) [50]	33.4*** (27.7–39.1) [49]	24.9*** (20.5–29.3) [45]
Coumaphos (N = 99)	10.8 (9.4–12.3) [27]	8.9 (7.5–10.2) [24]	29.2*** (23.4–35.0) [26]	17.7*** (13.1–22.3) [22]
Dieldrin (N = 102)	8.2 (6.8–9.5) [25]	10.3 (8.5–12.1) [26]	9.4 (7.4–11.4) [25]	8.6 (6.7–10.5) [26]

All values are in mg; asterisks denote significant difference versus placebo

did not significantly decrease in response to increasing dose for any phenylpyrazole tested (Figure 32). No significant differences between the partial engorged weight attained by ticks feeding on different doses of phenylpyrazoles and the placebo were detected, except for 0.1 µg/ml fipronil (Figure 32), where ticks attained a partial engorged weight 66% higher than the placebo (Table 21). In general, the majority of the ticks that died after exposure to a phenylpyrazole attained a partial engorged

weight smaller than the placebo, but some surviving and dead ticks were frequently substantially heavier than the placebo (Figure 33). Variability in engorged weight of phenylpyrazole-exposed ticks increased significantly with increasing acaricide dose ($P < 0.0001$; Bartlett's test).

A linear model fitted to the fluralaner dataset was not significant; the distribution of the engorged weight data for fluralaner-exposed ticks closely resembled the distribution of phenylpyrazole-exposed ticks. No significant differences were detected between the partial engorged weight attained by ticks feeding on blood dosed with fluralaner at different doses or the placebo

For the organophosphates chlorpyrifos and coumaphos, linear models fitted to the data were significant and positive (Table 21), i.e. the partial engorged weight attained by ticks increased with increasing dose of organophosphate in blood. Variability in engorged weight for organophosphate-exposed ticks increased with increasing dose ($P < 0.0001$; Bartlett's test). *Ixodes ricinus* females exposed to 1 or 10 µg/ml of an organophosphate in this assay attained an engorged weight 100–109% or 58–87%, respectively, higher than the placebo (Figure 32). At 0.1 µg/ml in blood, coumaphos had no effect on the partial engorged weight attained by feeding ticks, whereas chlorpyrifos significantly increased the partial engorged weight compared with the placebo, despite having no effect on tick mortality at this dose. After exposure to an organophosphate in blood, the partial engorged weights attained by the ticks that died, and the ticks that survived, were both higher than the placebo (Figure 33).

Dieldrin dosed in blood in this assay had no effect on tick mortality or the partial engorged weight attained by ticks (Figure 32); dieldrin also had no effect on the variability in partial engorged weight attained at increasing doses ($P > 0.05$; Bartlett's test).

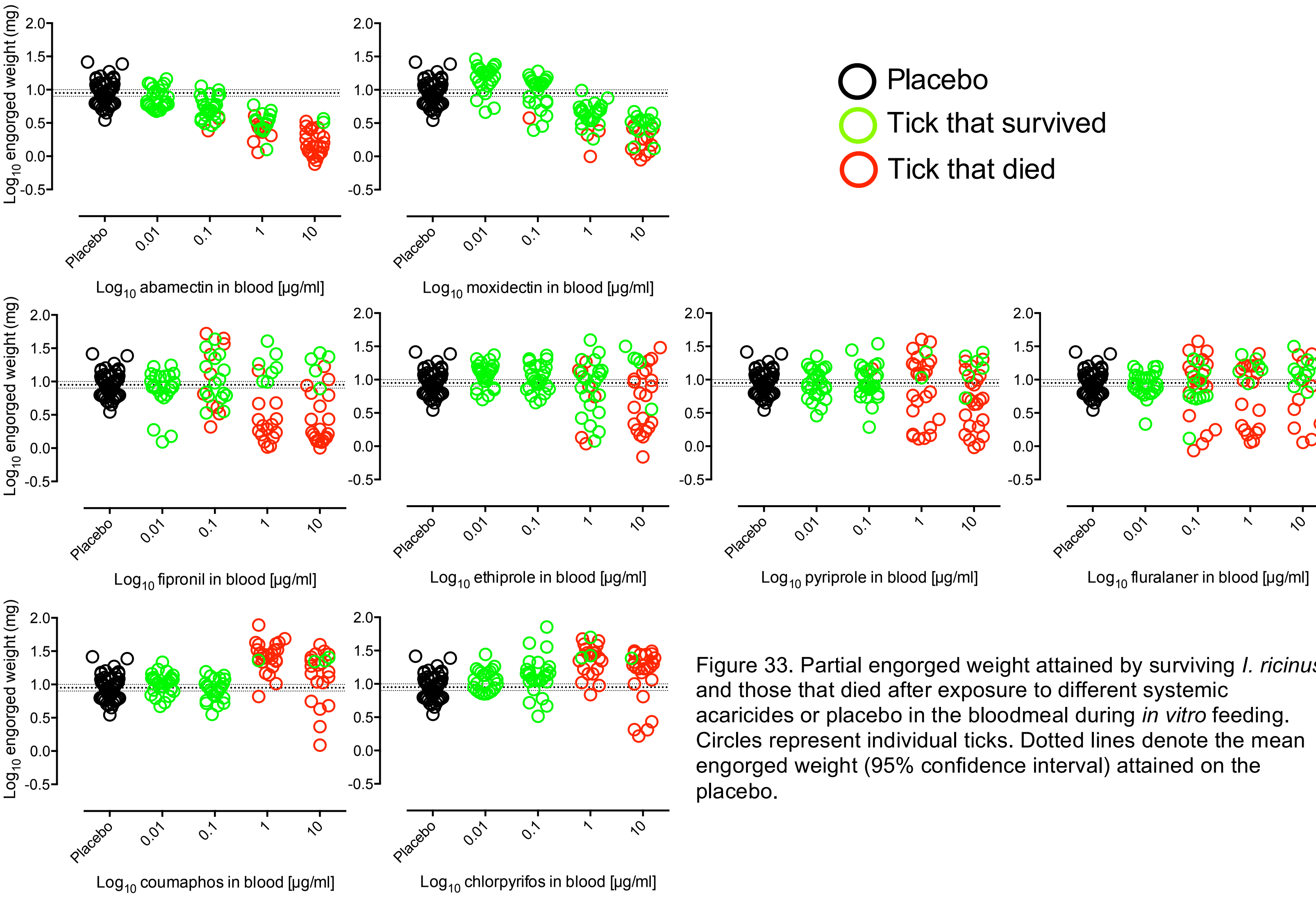

Figure 33. Partial engorged weight attained by surviving *I. ricinus* and those that died after exposure to different systemic acaricides or placebo in the bloodmeal during *in vitro* feeding. Circles represent individual ticks. Dotted lines denote the mean engorged weight (95% confidence interval) attained on the placebo.

4.4.3 Time-lapse video recordings of tick feeding behavior on the placebo and in response to acaricides

During observations of video recordings, it was evident that ticks attach to the membrane in two ways: The majority of ticks clearly walked along the membrane and attached at the junction between the membrane and feeding unit wall. These ticks evidently attached to the membrane while standing on it. Other ticks attached to the membrane from the feeding unit glass wall, i.e. they walked down the wall of the feeding unit and attached to the membrane while standing on the wall. This is made apparent by the fact that these ticks are essentially upside down (ventral side facing the camera) during feeding. Ticks feeding upside-down facilitated observations of ventral gut contractions and defecation during *in vitro* feeding; ticks feeding dorsal side up facilitated observations of dorsal gut contractions.

Ticks feeding on blood treated with the placebo fed with their legs extended. Leg movements during feeding on the placebo consisted of involuntary leg twitches, and voluntary leg movements during blood sucking and readjustment at the attachment site. Tick defecation normally followed ventral muscle contractions; feces were excreted at a rate of once every 2–4 minutes during the 48–72 hours feeding period. Feces pellets solidified within 1–2 minutes after being expelled from the tick and formed long chains several times longer than the tick itself (Figure 34). For ticks feeding dorsal side up, dorsal gut contractions were clearly visible and of varying intensity and frequency; no discernable rhythm of bloodsucking was apparent. During video playback of *in vitro* feeding at speed 30X, grooves in the dorsal alloscutum were dynamic, appearing and disappearing, apparently due to changes in internal pressure during sucking and salivation. After 72 hours of feeding, grooves in the alloscutum were still visible as the tick synthesized new cuticle in order to prepare for the bloodmeal. Due to the accumulation of feces, which obstructed the view of ticks, it was not possible to measure the change in the size of the tick alloscutum over time during *in vitro* feeding on blood treated with the placebo. The median area of the tick alloscutum for ticks feeding on the placebo at the beginning of the experiment and after 22

hours, respectively, was 1.98 mm^2 and 2.86 mm^2, i.e. for *I. ricinus* females feeding *in vitro* during the 48 – 70 hour time period, the area of the alloscutum increased 0.88 mm^2 (Figure 35).

4.4.4 Video records of *I. ricinus* feeding on blood treated with abamectin

In actively feeding I. ricinus females introduced to blood dosed with 10 µg/ml abamectin (n = 9), dorsal muscle contractions in ticks stopped after just five to ten minutes of feeding and ticks appeared paralyzed. Fifteen to thirty minutes later, half of the attached ticks exhibited a brief period of uncontrolled leg movements (ULM), before seizing movement again. The other half of the attached ticks remained paralyzed throughout the 48 – 70 hour period. Feces production stopped immediately after ticks were exposed to abamectin (Figure 34). The legs of ticks curled under the tick body over time and

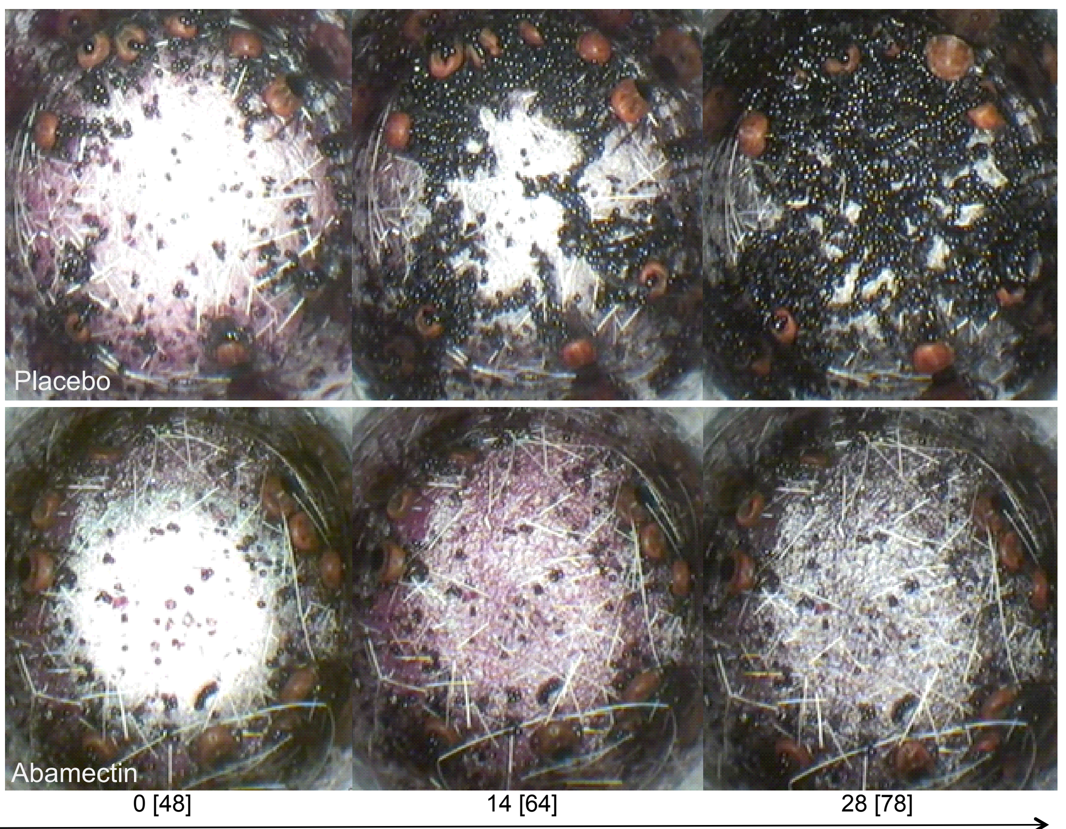

Figure 34. Video records of ticks feeding *in vitro* on blood treated with a placebo (top) and 10 µg/ml abamectin (bottom). **Click here to view the video.**

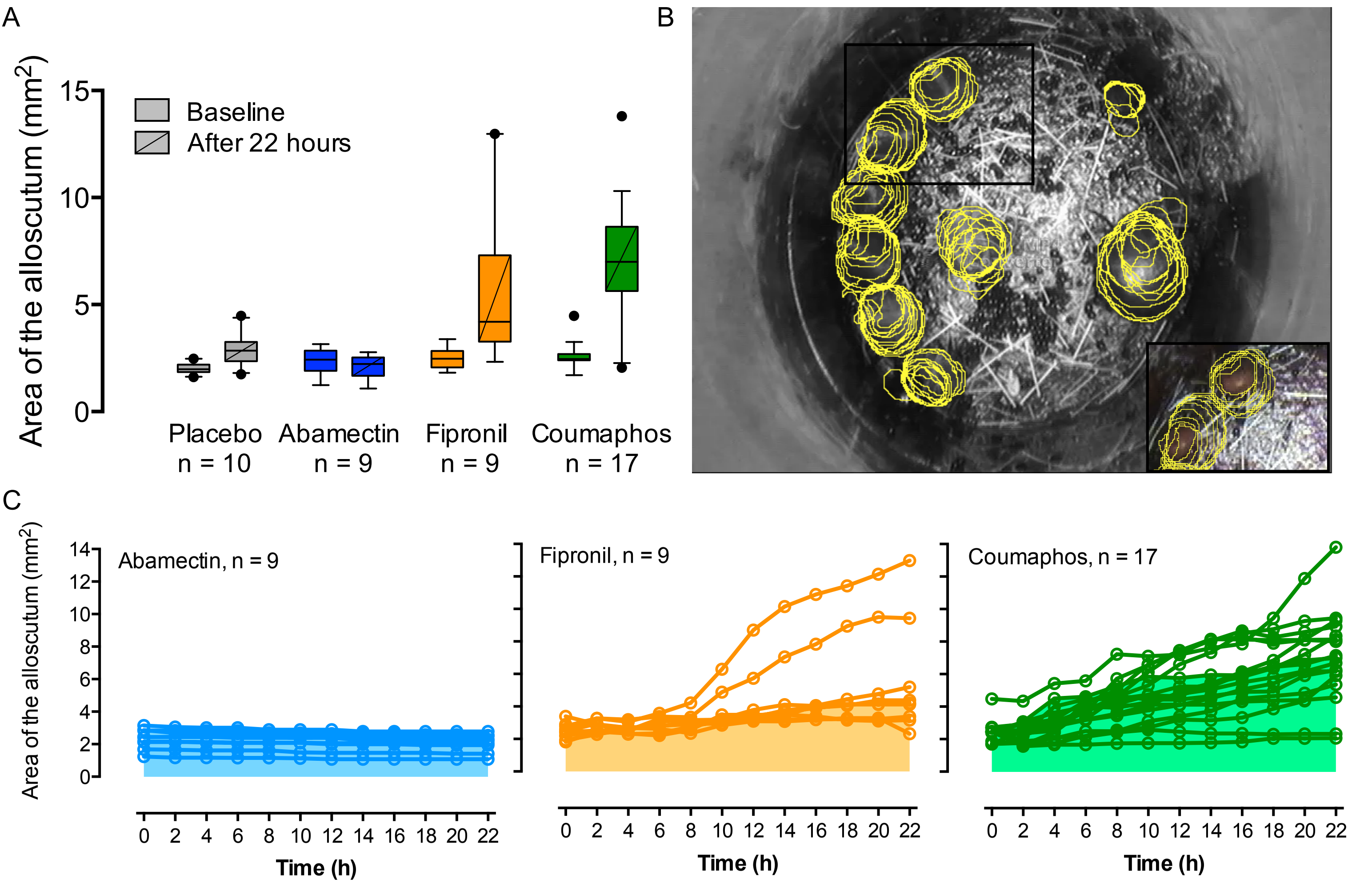

Figure 35. The size of the *I. ririncus* alloscutum (mm^2) over time during *in vitro* feeding on blood treated with placebo, abamectin, fipronil, or coumaphos. A. The size of the alloscutum at baseline (40 h) and after +22 hours; box plots show the median, interquartile range, and 10-90th percentile. B. Screenshot showing ticks at the beginning (bottom right window) and end of experiment with the contours fitted around tick alloscutums at successive two hour intervals (yellow rings). C Plots showing the change in the size of the tick alloscutum over time. The shaded region in each figure denotes the median area (mm^2) at each time point.

extended straight out in others. Depressions in the dorsal alloscutum enlarged over time and ticks appeared to lose weight (Figure 34); the size of the alloscutum of *I. ricinus* females decreased over time during exposure to abamectin (Figure 35).

4.4.5 Video records of *I. ricinus* feeding on blood treated with fipronil

Ixodes ricinus females (n = 18) exposed to fipronil (10 µg/ml) in the bloodmeal showed symptoms of poisoning, characterized by rapid ULM, after 29 minutes (median) of exposure (Figure 36). The ULM in fipronil-exposed ticks

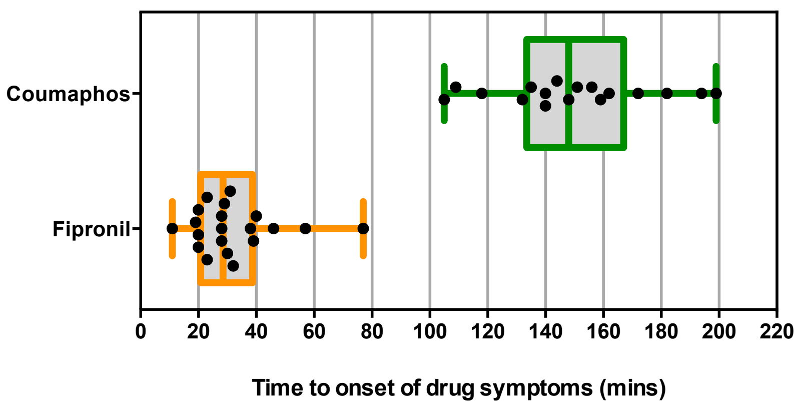

Figure 36. Comparison of time to onset of symptoms of poisoning in *I. ricinus* females exposed to fipronil and coumaphos in blood (10 ug/ml) during *in vitro* feeding. Box plots show the median, interquartile range, and range with onset of symptoms for individual ticks superimposed on each plot. Ticks exposed to fipronil showed symptoms of poisoning (median: 29 m) sooner than ticks exposed to coumaphos (median: 148 m) ($P < 0.0001$, Mann-Whitney U-test). The variability in the time to onset of symptoms for fipronil-exposed ticks was lower than coumaphos-exposed ticks ($P = 0.01$, F-test).

started with the two front legs and moved posteriorly until all legs moved uncontrollably. Feces production stopped immediately following the onset of ULM (Figure 37). After 2–20 minutes of ULM, the legs of fipronil-exposed ticks typically curled under the tick body and then ULM transitioned to a brief period of acute shutters or cramping. Over the next approximately eight hours, ticks then appeared to lose weight, as indicated by the formation of growing depressions in the dorsal alloscutum. Following this eight-hour period, ticks then began to increase in size, but this was highly variable (Figure 35). After 22 hours, ticks exposed to fipronil were generally slightly larger than they were at the beginning of the experiment, and larger than the placebo after 22 hours (Figure 35)

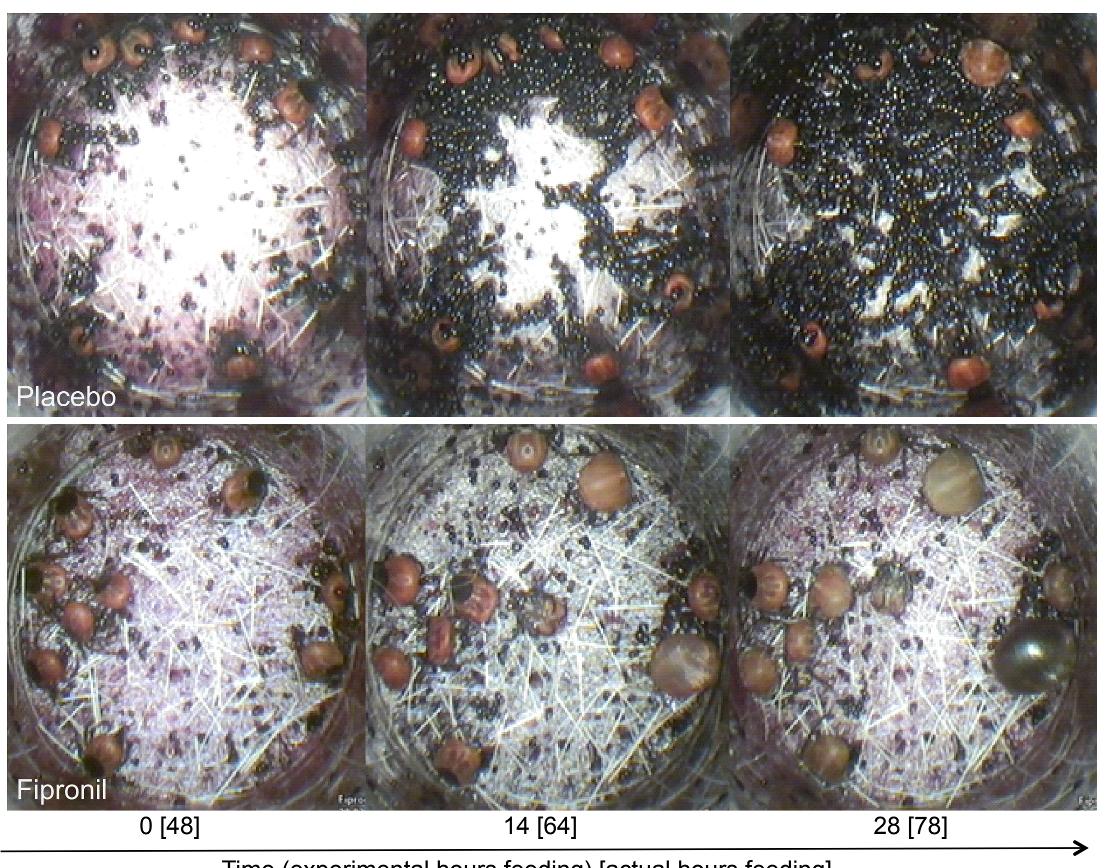

Figure 37. Video records of ticks feeding *in vitro* on blood treated with a placebo (top) and 10 ug/ml fipronil (bottom). **Click here to view the video.**

4.4.6 Video records of *I. ricinus* feeding on blood treated with coumaphos

Ixodes ricinus females (n = 18) exposed to coumaphos (10 µg/ml) in blood presented symptoms of acaricide poisoning, characterized by rapid ULM, after 148 minutes (median) of feeding (Figure 36). Compared with fipronil (median 29 minutes), coumaphos required a significantly longer exposure time before symptoms of poisoning appeared (Figure 36). Variability in the exposure time needed before symptoms appeared was also greater for coumaphos compared with fipronil (Figure 36). Systemic poisoning with coumaphos in *I. ricinus* was associated with rapid and uncontrolled ULM at onset that started with the two front legs and moved posterior until all legs were moving uncontrollably. Ticks continued to produce feces two to three hours after symptoms of poisoning appeared and then stopped producing feces (Figure 38). There was no major delay between the onset of symptoms of coumaphos poisoning and increased body expansion, as observed for fipronil (Figure 35); after approximately two

hours of exposure to coumaphos, the area of the tick alloscutum increased in size steadily at a rate of 0.25 mm^2 per hour (Figure 35). Dorsal folds in the alloscutum were not visible after just 7–11 hours of feeding and it appeared that the expansion of the alloscutum was occurring faster than the tick was able to synthesize new cuticle (Figure 38). After 10 hours, the median area of the alloscutum had doubled in size (Figure 35). The cuticle of coumaphos-exposed ticks frequently turned black and could be easily punctured with forceps if not handled carefully (Figure 38).

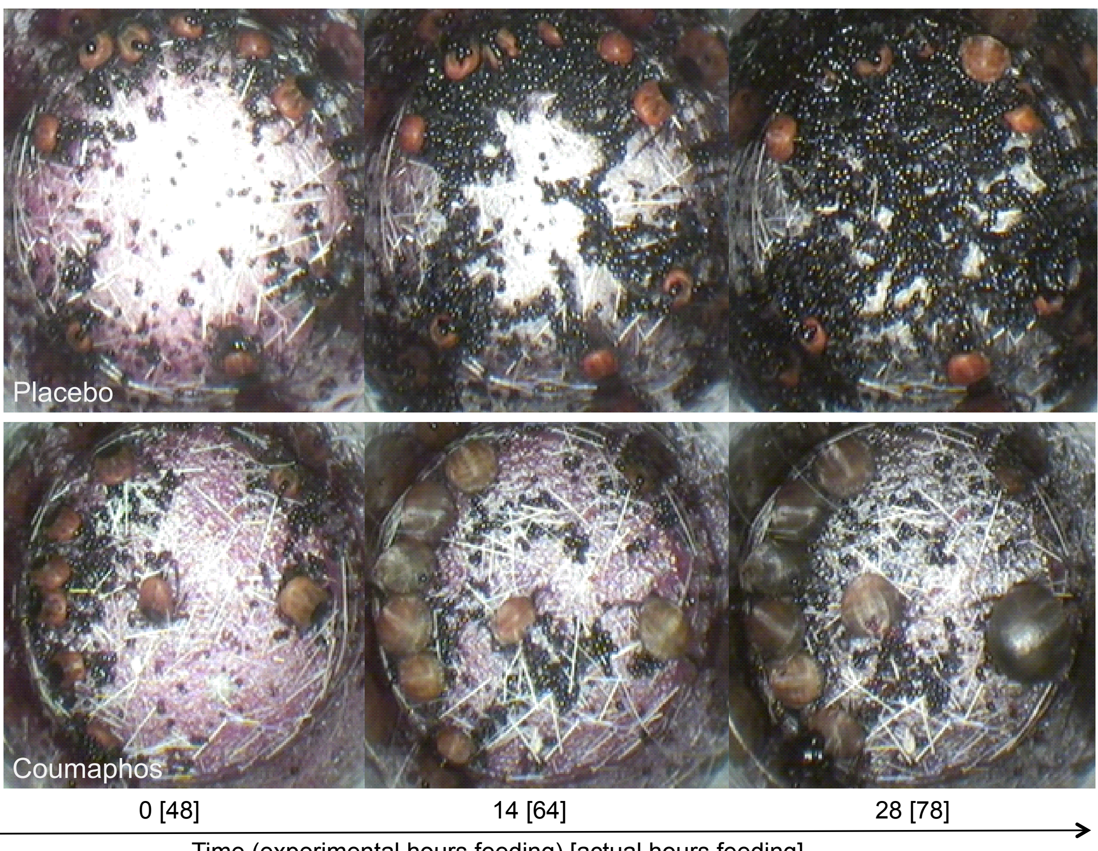

Figure 38. Video records of ticks feeding *in vitro* on blood treated with a placebo (top) and 10 ug/ml coumaphos (bottom). **Click here to view the video.**

4.5 Discussion

4.5.1 Macrocyclic Lactones

As a class, the macrocyclic lactones (MLs) were the most effective inhibitors of *I. ricinus* feeding in this study. Even in ticks exposed to the least effective moxidectin where mean mortality reached only 58% at 10 ug/ml, nearly all of these ticks, dead and surviving, still attained partial engorged weights just 25% of placebo ticks. The organophosphates, in contrast, increased feeding in ticks, and although the phenylpyrazoles were effective inhibitors of tick feeding at 10 µg/ml , some surviving ticks exposed to phenylpyrazoles attained partial engorged weights higher than ticks fed on the placebo. The strongest conclusion from the ML data, therefore, is that as a class, the MLs are clearly effective in their ability to inhibit tick feeding *in vitro*, as demonstrated across four different MLs, and compared with phenylpyrazole and organophosphate acaricides tested in the same assay.

Within the macrocyclic lactones, abamectin had the greatest effect on *I. ricinus* mortality in the assay, although the LD_{50} of abamectin was not significantly different from ivermectin. The rank order of the MLs tested in terms of efficacy against *I. ricinus* females was: abamectin > ivermectin > eprinomectin > doramectin > moxidectin. Eprinomectin was less active than abamectin, but did not differ from ivermectin and these three molecules were each at least ten times more active systemically than moxidectin and doramectin. In this assay, the MLs tested can be divided into two groups with regard to systemic efficacy: group 1 with abamectin, ivermectin, and eprinomectin with LD_{50}-values in the range 0.4–1.1 µg/ml , and group 2 with doramectin and moxidectin with LD_{50}-values higher than 10 µg/ml.

In addition to having the lowest efficacy of the MLs, moxidectin was the least effective inhibitor of tick feeding. All MLs tested, except for moxidectin, significantly inhibited tick feeding from 10–0.1 µg/ml in blood. In addition to having an undetectable effect on *I. ricinus* feeding at 0.1 µg/ml , moxidectin was the only ML where *I. ricinus* attained a partial engorged weight significantly higher

than placebo fed ticks at a dose of 0.01 µg/ml. Moxidectin's low efficacy and comparatively poor inhibition of tick feeding relative the other MLs combine to strongly indicate that moxidectin was the least active ML in this assay.

The similarities and differences in activity between the MLs were generally consistent with their structural relatedness as molecules. Abamectin, ivermectin, eprinomectin and doramectin belong to the avermectins, a family of MLs originating from fermentation products of the soil bacterium *Streptomyces avermitilis* (Prichard et al. 2012). Abamectin was the first avermectin synthesized and is a 9:1 mixture of the fermentation products avermectin B1a and avermectin B1b, respectively (Prichard et al. 2012). Ivermectin is derived from abamectin differing only in a methylene group at the C26 position (Campbell 1989; Prichard et al. 2012). Eprinomectin was discovered amongst ivermectin analogues and the main component is avermectin B1 possessing a terminal oleandrose moiety (Prichard et al. 2012). Doramectin is unique in that it originates from mutational biosynthesis with *S. avermitilis*, but structurally it is similar to abamectin, differing only in a cyclohexyl group at the C25 position (Prichard et al. 2012).

Moxidectin belongs to a sub-family of the MLs distinct from the avermectins known as the milbemycins. The milbemycins are derived from fermentation products of the soil bacterium *Streptomyces cyaneogriseus*. Structurally, the milbemycins have the same 16-member macrocyclic lactone backbone as the avermectins, but are unglycosylated, lacking a disaccharide group at the C13 position. Within the milbemycins, moxidectin possesses a substituted olefinic side chain at the C25 position and a methoxime moiety at the C23 position (Prichard et al. 2012).

The similar range of activity observed between abamectin, ivermectin, and eprinomectin in the assay employed here is in good agreement with the structural similarity of these three molecules. The lack of efficacy measured for doramectin is puzzling given its structural similarity to abamectin, but despite its decreased effect on tick mortality, doramectin still interestingly inhibited feeding down to 0.1 µg/ml , consistent with its avermectin sister molecules. Moxidectin's unique

combination of poor efficacy and poor inhibition of tick feeding is consistent with its unique structure and milbemycin origin.

It is tempting to hypothesize that moxidectin's decreased activity in this assay is related to the loss of the disaccharide group in the milbemycins. However before this hypothesis could be proposed with confidence, supporting data is needed on the effects of other milbemycins on *I. ricinus* mortality and feeding inhibition using this assay. In addition, the experiment where partial engorged weight attained by ticks exposed to moxidectin was tested was not repeated. Nevertheless, the effect of moxidectin on *I. ricinus* mortality was tested independently twice, and mortality even at the highest dose tested, 10 μg/ml , never approached 100%. The data for moxidectin therefore confidently show that moxidectin was the least effective of the MLs in terms of its effect on tick mortality, and preliminary data suggest that it is also the least effective inhibitor of tick feeding.

It is important to note that moxidectin has been shown to be as effective or more effective than ivermectin for the treatment of *Rhipicephalus microplus* infestations on cattle (Davey et al. 2011). Nevertheless recent studies, particularly *in vitro*, support the differences in activity detected between the avermectins and moxidectin in this assay. For example, in the nematode *Caenorhabditis elegans,* pharyngeal pumping is strongly inhibited by the MLs, however moxidectin (80 nM) requires a 16-fold higher dose *in vitro* than ivermectin (5 nM) to obtain a similar level of paralysis (Ardelli et al. 2009). In a membrane-based *in vitro* blood feeding assay, ivermectin and eprinomectin had roughly the same effects on *Anopheles gambiae* mortality (LC_{50}-values of 22.4 and 23.6 ng/ml, respectively), whereas moxidectin was 125-fold less effective (LC_{50} of 2789 ng/ml) (Butters et al. 2012). The similarities and differences in activity between the MLs reported for *A. gambiae* mosquitoes (Butters et al. 2012) and for ticks, in this study, are especially noteworthy. In both studies, the avermectins ivermectin and eprinomectin had comparable activity and the milbemycin moxidectin was at least an order of magnitude or two orders of magnitude less active. Such big differences in activity between the avermectins and moxidectin, *in vitro*, have led some researchers to hypothesize previously that moxidectin may have lower binding affinity for the glutamate-gated chloride channel (Glu-Cl) receptor, or

possibly a lower concentration of moxidectin arrives at the Glu-Cl receptor in the parasites in question (Prichard et al. 2012). This hypothesis is supported by recent receptor affinity binding studies with the MLs and the Glu-Cl receptor (Prichard et al. 2012), however conflicting results are reported in different studies and the body of this work was carried out mainly in nematodes. More research is needed to compare the affinities of the MLs for the Glu-Cl receptor, particularly in ticks.

Finally, any discussion on differences in activity between systemic acaricides requires a discussion of acaricide pharmacokinetics. Systemic acaricides dosed in the blood of vertebrates *in vivo* are subject to enzymatic breakdown, excretion, absorption, and a range of other factors affecting the fate and distribution of the drug in the host (Prichard et al. 2012). The sum of these factors describes the pharmacokinetic profile of an acaricide molecule, and it is well known that pharmacokinetic properties of acaricide molecules differ greatly between acaricides, even within the same compound class. The pharmacokinetic properties of acaricides, particularly bioavailability, in turn, often greatly influence the efficacy of the molecule against target parasites *in vivo*. Fat tissue, for example, is well known to act as a reservoir for lipophilic molecules in hosts, and increased lipophilicity of acaricide molecules is often strongly associated with increased acaricidal activity (Prichard et al. 2012). The partition coefficient, a measure of a molecule's lipophilicity (logP), is higher in moxidectin (logP 6) compared with ivermectin (logP 4.8); in mean residence time studies in hosts, oral moxidectin is detected in the plasma of dogs for over 25 days compared with just 3 days for ivermectin (Al-Azzam et al. 2007).

The possibility that moxidectin has lower binding affinity for the tick Glu-CL receptor seems plausible given moxidectin's increased bioavailability, which may compensate in the *in vivo* situation for low binding affinity. Moreover, if the efficacy of moxidectin depends on its pharmacokinetic profile, it follows that moxidectin would perform especially poorly in the *in vitro* assay employed here, as indeed observed, since bioavailability is less likely to influence efficacy *in vitro* (Butters et al. 2012). The efficacy and feeding inhibition data in this study therefore appear to support the hypothesis that moxidectin has lower binding

affinity for the Glu-Cl receptor in ticks compared with abamectin and the other avermectins tested.

It is important to note that the physicochemical properties of the compounds tested in this study have not been discussed until this point. It is well known that physicochemical properties (e.g. solubility and stability) affect the toxicity of insecticides/acaricides (Harris 1972; Mcintosh 1995). The solubility of higher doses of compounds in bovine blood in this assay (e.g. at 37° C), and the different interactions between the compounds and blood consituents, as well as the interactions between the compounds and the stainless steel wells of the 60-well plate, are factors that should be studied in more detail in the future for their effects on systemic toxicity to ticks *in vitro*.

The macrocyclic lactones are agonists of the inhibitory Glu-Cl channel, and opening of the Glu-Cl in ML poisoned invertebrates is permanent leading to paralysis and death (Pitterna 2011). While maximum mortality from ML poisoning is usually reached after two to four days in insects, the onset of muscle paralysis is rapid (Pitterna 2011). Feeding inhibition in the form of strongly reduced partial engorged weights attained by ticks treated with MLs in the *in vitro* feeding assay used here is consistent with the inhibitory mode of action of the MLs, causing paralysis of muscles involved in tick feeding. The observations from real-time video recordings of *I. ricinus* feeding *in vitro* on blood dosed with 10 µg/ml abamectin also supports this conclusion (see below).

Blood sucking is an intense procedure in ticks controlled by strong anterior pharyngeal dilator muscles (PDMs). During initiation of blood sucking, the PDMs contract and pull apart the walls of the pharyngeal cavity (Balashov 1972). During expansion of this cavity, negative pressure is built up at the anterior base of the pharynx and this pressure is transmitted down to the posterior end of the preoral cavity where blood is then drawn rapidly into the tick. To move the blood into the midgut, the tick relaxes the PDMs and then contracts a group of circular muscles at the anterior end of the pharyngeal cavity. These circular muscles close the cavity behind the bloodmeal and force the bloodmeal into the midgut (Balashov 1972). In real-time video recordings of *I. ricinus* feeding *in vitro*, dorsal muscle

contractions and intense and alternating changes in internal pressure were clearly visible, consistent with sucking and movement of the bloodmeal within the tick. It is important to note that some of these muscle contractions were likely also associated with salivation. In actively feeding *I. ricinus* exposed to blood dosed with 10 µg/ml abamectin, all dorsal muscle contractions apparently associated with bloodsucking or salivation stopped after just 5–10 minutes of feeding. This rapid paralysis of muscle contractions observed in video recordings of *I. ricinus* exposed to abamectin is consistent with the inhibitory mode of action of the MLs and consistent with the measured reduction in partial engorged weight attained by *I. ricinus*.

Others have tested the effects of abamectin on tick development and engorgement. In partially engorged ticks detached from cattle, Friesen et al. (2003) showed that pre-fed *A. hebraeum* females injected with abamectin showed signs of dorso-ventral muscle paralysis. *Amblyomma americanum* and *Dermacentor variabilis* adults feeding on cattle treated systemically with ivermectin showed reduced partial engorged weights versus controls (Wilson et al. 1991). While engorged weight attained by ticks exposed to acaricides is a common measure used to quantify tick feeding inhibition, it is important to note that engorged weight is a relatively indirect measure of feeding inhibition and may not necessarily measure feeding inhibition, at all. To the best of my knowledge, a clear distinction has never been made as to whether feeding inhibition is a true effect of the avermectins in ticks, or if feeding inhibition by the avermectins is an indirect result of mortality. In other words, ticks exposed to an acaricide during feeding may simply die after exposure to the acaricide during feeding, resulting in reduced engorged weights compared with placebo ticks, giving the impression that feeding was inhibited, but how can this be confirmed? It's possible, for example, that a tick might briefly increase feeding before death after exposure to an acaricide, but this would not be detected by measuring engorged weight, alone, since the engorged weight would still be less than the placebo and interpreted as reduced. To fill this gap in the literature, this study directly observed the inhibitory mode of action of the avermectins on actively feeding ticks using video recordings of ticks feeding *in vitro* on acaricide-treated blood, in combination with tick engorged weight data as a function of acaricide dose. To

the best of my knowledge, this is the first report demonstrating that feeding inhibition is a true effect of the avermectins in ticks; I demonstrate a dose-dependent inhibitory effect of the MLs on the partial engorged weight attained by *I. ricinus* exposed to MLs in the bloodmeal, and then using time-lapse video recordings of ticks feeding in real-time on blood treated with abamectin, I show that reduced engorged weight results from a paralyzing effect, including paralysis of dorsal muscle contractions 5–10 minutes after exposure to 10 µg/ml abamectin in the bloodmeal.

In summary, the *in vitro* feeding assay for ticks employed in this study proved sensitive enough to detect differences in acaricide effects on tick feeding behaviour both between the MLs and other compound classes, and within the MLs, themselves. As a class, the MLs were by far the most effective inhibitors of tick feeding, inhibiting the partial engorged weight attained by up to 75% at 10 µg/ml. Within the MLs, abamectin, ivermectin, and eprinomectin had similar effects on tick mortality (LD_{50}-value in the range 0.4–1.1 µg/ml) whereas moxidectin was at least an order of magnitude less effective. A similar pattern was observed between the avermectins and moxidectin with regard to feeding inhibition. The similarities and differences in activity are in good agreement with the structural relationships shared between theses acaricide molecules, and in agreement with the activities of these molecules reported in other parasite species. These results demonstrate that biologically relevant results are obtained using the *in vitro* assay, and that by measuring the effects of acaricides on tick engorged weight, in combination with the mortality data, and video recordings, a comprehensive data set is obtained that provides a good picture of a molecule’s target-site affinity and mode of action.

4.5.2 Phenylpyrazoles

With respect to the effect on tick mortality, the phenylpyrazoles represent some of the most active acaricides tested in the *in vitro* assay. The LD_{50} calculated for fipronil was six and 10-fold lower than the two best MLs tested, abamectin and ivermectin, respectively, and fipronil was by far the most active acaricide tested in the assay. Unexpectedly, however, a significant negative dose-dependent effect

on *I. ricinus* bloodfeeding was not detected for fipronil or two other phenylpyrazoles in this assay, as was strongly detected for each of the four MLs tested. Therefore, although fipronil was highly effective in terms of its effect on tick mortality, the effect of fipronil on tick feeding was not necessarily inhibitory. These data draw further attention to the idea that a careful distinction needs to be made between the direct effects of acaricides on tick feeding and the indirect effects of acaricides on tick feeding resulting from mortality.

The challenge associated with interpreting feeding inhibition in ticks due to systemic acaricides becomes clearer when one looks at the engorged weight data broken down in terms of the weights of surviving and dead ticks exposed to acaricide: the majority of ticks exposed to the highest doses of fipronil died (indicated by red circles) and fed less than on the placebo, as would be expected for the most potent acaricide tested against *I. ricinus* in this study. Some ticks, mainly surviving ticks, confusingly attained engorged weights higher than the placebo, but the mean engorged weight is not significantly different from the placebo. Initially, these data might be interpreted as inhibition of feeding and the higher engorged weights attributed to variability or unexplainable factors associated with the *in vitro* situation. It is only in comparison to, or in the context of, the macrocyclic lactones that it becomes clear that the phenylpyrazoles are not effective inhibitors of tick feeding *in vitro*.

Dead ticks exposed to abamectin, as well as ticks that survived, were clearly smaller than the placebo. Moreover, the standard deviations of the engorged weight data for ticks exposed to abamectin were ± 4.94, 2.73, 1.48, and 0.9 mg at 0.01, 0.1, 1, and 10 µg/ml in blood, and therefore both the partial engorged weight and standard deviation decreased with increasing dose of abamectin. In other words, the potency of the paralyzing effect of abamectin increased with increasing dose. In response to increasing dose of abamectin, tick mortality increased, engorged weight decreased, and variability in engorged weight decreased. This is the expected result of a molecule acting solely by paralysis, the paralyzing effect being higher with increasing dose. In contrast, for fipronil, standard deviations were 6.25, 14.16, 10.27, and 11.90 at 0.01, 0.1, 1 and 10 µg/ml in blood. Therefore, in response to fipronil, variability in engorged weight

initially increased with increasing dose and then stayed relatively high even at the highest doses tested, showing that even though mean engorged weight decreased with increasing dose of fipronil, fipronil had an excitatory or stimulatory effect on tick feeding; in the case of fipronil, the stimulatory effect on feeding increased with increasing dose, although at the highest doses, many ticks clearly died before attaining higher weights. The data indicate an effect whereby the phenylpyrazoles stimulate or increase feeding in ticks while killing them. This clearly accounts for the increased variability in the partial engorged weight data in ticks exposed to fipronil, and also accounts for the lower slope of the engorged weight data compared with the MLs.

This conclusion clarifies that the variability in the engorged weight data observed for the phenylpyrazoles is not unexplainable variability associated with the assay, but is a signature of the phenylplyrazoles' stimulatory mode of action. Importantly, it is clear that the MLs and phenylpyrazoles had distinct effects on the distribution of the engorged weight data. This unique signature appears to be another useful predictor of acaricide mode of action (Figure 32). As previously mentioned for abamectin, in response to increasing dose of abamectin, the partial engorged weight attained by ticks decreased, the slope was relatively sharp, and variability in engorged weight decreased with increasing dose. This result is wholly consistent with an inhibitory Glu-Cl receptor agonist, which the avermectins are known to be. The distribution of the engorged weight data for fipronil is also consistent with fipronil's molecular mode of action: The slope of the tick engorged weight data in response to fipronil decreased with increasing dose, but variability was relatively high and the slope was not significantly negative. The phenylpyrazoles are known as convulsants, blocking the γ-aminobutyric acid (GABA)-Cl channel, causing overexcitation of the nervous system and death. The variability in the engorged weight data, apparently caused by increased feeding in some ticks in response to fipronil, is consistent with a molecule causing overexcitation leading to increased feeding and thus increased variability in engorged weight.

Characterizing the distribution of the partial engorged weight data of ticks exposed to different systemic acaricides might be useful in the future as a

reference tool for determining the mode of action of molecules with unknown modes of action. Fluralaner, an isoxazoline molecule, is a new acaricide for tick control with a unique mode of action (Gassel et al. 2014). It is known already that fluralaner blocks both the GABA-Cl and Glu-Cl channels in ticks, however its acaricidal activity is mainly attributable to its blocking effect on the GABA-Cl channel (Gassel et al. 2014). Fluralaner, therefore, is essentially similar to the phenylpyrazoles in its mode of action. In the *in vitro* assay employed here, the effect of fluralaner on the distribution of the partial engorged weight data was very similar to the phenylpyrazoles and the assay correctly predicted the mode of action of fluralaner (Figure 32).

While the distribution of the tick engorged weight data for different acaricides is useful for predicting mode of action of a product, data recorded at individual concentrations in this assay also have predictive value. The data at 0.1 µg/ml fipronil are particularly interesting in that this was the only concentration where the partial engorged weight was detected as significantly different from the placebo among the concentrations tested for the three different phenylpyrazoles. Both ethiprole and pyriprole had negligible effects on feeding at 0.1 µg/ml and were also less active than fipronil in terms of mortality. It makes sense that the most active acaricide tested in terms of tick mortality, fipronil, would also have an effect on engorged weight at the lowest dose. Combined, these data strongly show that fipronil was the most active phenylpyrazole tested. In addition, the data at 0.1 µg/ml fipronil provide support for an effect of the phenylpyrazoles, whereby tick mortality is achieved in the phenylpyrazoles by increasing feeding. If the phenylpyrazoles indeed increase feeding in ticks, it follows that at lower doses, we might expect to see less mortality and larger ticks. In other words, there might be lower doses of the phenylpyrazoles where feeding is stimulated, but where the concentration is not high enough to cause mortality in the assay or to stop feeding. This dose appears to be close to 0.1 µg/ml for fipronil in this assay. Interestingly, no other acaricide tested stimulated feeding at concentrations where significant mortality was not also achieved. This result indicates the potency of fipronil; the effect of fipronil on partial engorged weight at 0.1 µg/ml, combined with fipronil's lower LD_{50}-value, suggests fipronil may have greater affinity for the GABA-Cl channel relative to ethiprole and pyriprole.

Although unexpected, the increased feeding effect recorded for fipronil *in vitro* on *I. ricinus* females here is consistent with the mode of action of the phenylpyrazoles. As previously mentioned, in contrast to the MLs, which cause paralysis, the phenylpyrazoles are antagonists of inhibitory GABA-Cl channels, causing overstimulation of the tick nervous system, and are generally known as convulsants. In real-time video recordings of *I. ricinus* feeding *in vitro* on blood dosed with 10 µg/ml fipronil, the excitatory effect of fipronil on tick feeding was confirmed. After roughly 30 minutes, ticks exposed to fipronil began to move their legs uncontrollably, and after a delay (~ 8 h), alternating changes in internal pressure apparently associated with bloodsucking and movement of the bloodmeal inside the tick were clearly visible and some ticks rapidly increased in size in response to 10 µg/ml fipronil. It was not possible to quantify an increase in the rate of blood sucking in response to fipronil, however the increase in body size over time was quantified using video recordings. These data could not be compared to ticks feeding on the placebo because large amounts of feces obscured the view of ticks in the placebo, but in comparison to ticks exposed to abamectin, it was clear that fipronil-exposed ticks increased in size whereas abamectin exposed ticks decreased in size (Figure 35). The fipronil experiment where tick body size was measured was repeated and similar results were obtained in this experiment, but the video file was damaged and the data for fipronil presented are therefore from one experiment.

In summary, fipronil was by far the most active systemic acaricide tested against *I. ricinus* in this study and the LD_{50} value for fipronil was about ten-times lower than for ivermectin. In addition, fipronil, and the phenylpyrazoles as a class, increased feeding in some ticks, as demonstrated by higher partial engorged weights attained by some ticks and increased variability in the partial engorged weight attained by ticks exposed to fipronil versus the MLs. This conclusion was confirmed in video recordings of *I. ricinus* females feeding in real-time on blood dosed with 10 µg/ml fipronil where an increase in body size over time was measured in some ticks, clearly showing that feeding was increased in some ticks exposed to fipronil. This conclusion is consistent with the excitatory mode of action of the fipronil molecule (GABA-Cl channel blocker); however, as far as I am aware, this is the first study demonstrating that the phenylpyrazoles, as

systemic acaricides, have the potential to increase feeding in ticks, *in vitro*, when presented in the bloodmeal. The unique distribution of the partial engorged weight data for ticks exposed to fipronil (0.01–10 µg/ml), characterized by higher variability and a lower negative slope, was very similar across the class and distinct versus the MLs, showing that the unique distribution of the tick engorged weight data recorded for acaricides in this assay is predictive of mode of action.

4.5.3 Organophosphates

The systemic efficacy of two organophosphates, coumaphos and chlorpyrifos, were tested against *I. ricinus* in the *in vitro* feeding assay and both organophosphates had nearly identical effects on tick mortality. Both molecules were highly effective at the highest doses tested of 1 and 10 µg/ml, reaching close to 100% mortality; however, the organophosphates displayed no activity at the lowest doses tested, making it difficult to accurately calculate LD_{50}-values for these molecules. I can only estimate that the LD_{50}-value of coumaphos and chlorpyrifos in this assay was in the range 0.1–1 µg/ml , possibly making them more effective than the avermectins in terms of mortality, but less effective than fipronil against *I. ricinus* in the *in vitro* feeding assay.

The organophosphates, unexpectedly, were the only systemic acaricides tested that significantly increased feeding in ticks with increasing dose. The increased feeding effect of the organophosphates on pre-attached (40 hours) *I. ricinus* in this assay was significant and the mean partial engorged weight attained by ticks exposed to 1 µg/ml chlorpyrifos was 300% higher than the placebo. Direct observations of feeding ticks exposed to chlorpyrifos in time-lapse video recordings confirmed the stimulatory effect of the organophosphates on feeding (Figure 35). In response to 10 µg/ml chlorpyrifos, ticks attached for 40 hours increased in size rapidly. During the period 40 hours after attachment to day 4, representing the attachment period during which ticks were exposed to acaricides in this assay, ticks exposed to 10 µg/ml chlorpyrifos attained mean engorged weights higher than control ticks fed *in vitro* for 6 days (29 mg vs 18 mg, respectively); in other words, two days of exposure to chlorpyrifos during feeding, over days 2 and 3, made chlorpyrifos-exposed ticks on day 4 weigh more than

ticks fed on the placebo for 6 days. In a preliminary experiment, the increased feeding effect of chlorpyrifos was also observed in unfed ticks feeding *in vitro* in the assay (Appendix).

Ixodid ticks consume more than 100-times their initial weight in blood during feeding and the ixodid tick bloodmeal is one of the most remarkable physiological feats in the animal kingdom. As far as I am aware, this study is the first to demonstrate induced hyperphagia in an ixodid tick by an acaricide or any other molecule. Examples of hyperphagia in insects induced by insecticides during feeding are known in the literature. Dimethylchlordimeform, for example, presented to *Phormia regina* in water caused *P. regina* to consume extraordinary volumes of water, leading in some cases to a tripling of initial weight (Long and Murdock 1983). Although neuromodulators involved in tick salivation have been studied in detail, little is known about the neuromodulators controlling blood sucking in ticks. The organophosphates act by non-competitive binding to acetylcholinesterase, inhibiting the action of acetylcholinesterase and causing a build-up of acetylcholine in the synaptic cleft. The accumulation of acetylcholine in the synaptic cleft of organophosphate poisoned arthropods leads to overexcitation of the nervous system and death. The pronounced stimulatory effect of the organophosphates on tick feeding observed in this study suggests that acetylcholine possibly plays an important role in blood sucking in ixodid ticks. The observation that the cuticle was extremely fragile after rapid body expansion during feeding on organophosphate-treated blood may also suggest a breakdown in the feedback loop that controls blood intake as a function of cuticle availability.

5. General discussion and conclusions

Ixodid ticks are considered one of the most difficult haematophagous arthropods to feed *in vitro*. They possess an array of chemosensory adaptations, and tick host acceptance and attachment to the host are sensitive processes dependent upon the presence of a specific set of host-associated attachment stimuli (i.e. thermal, hygro, mechanical, olfactory and contact chemostimuli (Guerin et al. 2011). After attachment, the complex and prolonged feeding and engorgement requirements of ixodid ticks further complicate their feeding process. Combined, these factors significantly confound efforts to feed ixodid ticks using an artificial membrane (Kuhnert 1996; Kröber and Guerin 2007b). Mosquitoes, fleas, and bed bugs are fed *in vitro* relatively easily using Parafilm as a membrane, whereas ixodid ticks remain one of the few blood-feeding arthropods still commonly fed using semi-artificial membranes (Hatta et al. 2012) and membranes derived from whole animal skins (Bonnet et al. 2007). Indeed, few studies have fed an ixodid tick to repletion *in vitro* using a completely artificial membrane; only one study has completed the life cycle of an ixodid tick *in vitro* (Kuhnert et al. 1995); and only one study has compared the systemic efficacy of acaricides using an artificial membrane (Kröber and Guerin 2007b).

Recently, *Ixodes scapularis* females were fed to repletion using a simplified version of the silicone membrane described by Kröber and Guerin (2007b) (Andrade et al. 2014). As all artificial membranes described to date for *in vitro* feeding of ixodid ticks are custom made and comprised of several components, a major limiting factor for *in vitro* feeding methods has been the production of the membrane itself. In addition, the membrane must be produced with a suitable thickness, which is dependent upon the hypostome length of the tick species and life stage being fed. Andrade et al. (2014) showed that the silicone glue, which was previously spread manually into the cellulose rayon lens paper to a suitable thickness (Kuhnert et al. 1995; Kröber and Guerin 2007b), could be brought into solution in toluene and the lens paper dipped into this solution in order to coat the membrane with a layer of glue of a defined thickness. However, the tick attachment rate attained with this membrane was low (45%), and only a small

number of ticks were tested in the study (N = 110) (Andrade et al. 2014). This membrane has been improved significantly during the course of this study research. Importantly, it is shown here that the relationship between the concentration of silicone in solution and membrane thickness is linear. Thus, manipulating the silicone concentration facilitates controlled manipulation of membrane thickness. This advance has standardized the procedure for preparing artificial membranes for *in vitro* feeding of ixodid ticks. As the range of membrane thicknesses possible with the method (84 – 584 µm) covers the hypostome lengths of a variety of ixodid tick species and life stages, the method should facilitate development of standardized membranes for different tick species. Here, using membranes prepared with 339 gL^{-1} and 291 gL^{-1} silicone in toluene for *A. hebraeum* nymphs (N = 480) and *I. ricinus* females (N = 2,135), high rates of tick attachment (86% and 89%, respectively) were attained, comparable or superior to the attachent rates attained by Kuhnert et al. (1995) and Kröber and Guerin (2007a). However, it is important to note that the method will need to be modified in order to feed larvae of some ixodid tick species such as those with the shortest hypostomes (e.g. ticks of the *Rhipicephalus* genus). These ticks will likely require membranes less than 50 µm thick (or spaces in the membrane thinner than 50 µm, e.g. between the fibers of the cellulose rayon matrix [see Kröber and Guerin 2007a), which is thinner than the thinnest membranes possible with this method.

Using *I. ricinus* females and two different blood sources (steer and rabbits), I have shown that ticks feeding *in vitro* feed less than ticks feeding *in vivo*, as determined by the overall engorged weight attained by the ticks. There is some controversy in the literature regarding the effect of *in vitro* feeding on engorged weight in ixodid ticks and no clear concensus on the matter appears to have been reached. This is further confounded by studies that do not use the same blood source *in vitro* and *in vivo*. For example, Bonnet et al. (2007) concluded that there was no difference between the engorged weight attained by *in vitro* and *in vivo*-fed *I. ricinus* females, but used rabbit ears *in vivo* (N = 95) and bovine or ovine red blood cells *in vitro* (N = 23). In this study, the engorged weight of *I. ricinus* females fed on rabbit ears *in vivo* (N = 114) was not different compared with ticks fed *in vitro* on bovine blood (N = 215), but in a more direct comparison, and with

larger numbers of ticks, ticks fed *in vitro* on bovine blood were more than two-times smaller by weight than ticks fed *in vivo* on steer (N = 94). Other studies support the conclusion that female ticks feed less *in vitro* compared with the *in vivo* situation. For example, *A. hebraeum* and *Amblyomma variegatum* females fed *in vitro,* respectively, on defibrinated bovine blood and heparinized bovine blood also attained engorged weights more than two times lower than ticks fed *in vivo* on steer (Kuhnert et al. 1995 and Voigt et al. 1993). Interestingly, this relationship may not be true for other life stages of ixodid ticks. For example, nymphs of three different species (*A. hebraeum*, *A. variegatum*, and *Rhipicephalus appendiculatus*) have all been shown to attain similar weights *in vitro* versus the *in vivo* situation (Kuhnert et al. 1995; Voigt et al. 1993; Musyoki et al. 2004), which is at odds with the result in this study for *A. hebraeum* nymphs.

The finding that female ticks feed less *in vitro* compared to *in vivo* may have implications for the generalizability of *in vitro* studies with female ticks for questions relating to, for example, the time to transmission or acquisition of tick-borne pathogens or the effects of systemic acaricides on tick feeding. If ticks feeding *in vitro* weigh less at repletion it follows that they may feed less during the initial phases of feeding and this may effect tick-pathogen and tick-acaricide interactions. Importantly, however, I have shown in this study that this is likely not the case, as the overall engorged weight attained by *I. ricinus* females feeding *in vitro* and *in vivo* was not different after three days of feeding, indicating that ticks feeding *in vitro* and *in vivo* feed similarly during the initial part of the slow feeding phase after attachment. *In vitro* feeding studies with tick pathogens and studies testing systemic acaricides are mainly interested in demonstrating effects within 48 hours after tick attachment, well within the time period shown to be associated with similar feeding patterns for *in vitro* and *in vivo* ticks.

While feeding was similar over the first three days of feeding, the divergence in feeding after day 3 was significant. While studies in the past have noted differences between the overall engorged weights attained by ticks fed *in vitro* and *in vivo*, it has never been known what was happening leading up to engorgement. For example, *in vitro*-fed ticks may have fed similarly throughout feeding and then only consumed less blood during the final day of the rapid

feeding phase. The research in this study rules this out and shows that the differences in feeding leading to different engorged weights starts relatively early in the feeding process towards the end of the slow feeding phase after day 3.

Additional experiments, including dissections focused on the development of the midgut and other organs over time during feeding, and feeding experiments with more data points spread over the first days of feeding are needed to confirm the conclusion that feeding is similar over the first three days and to provide an explanation as to why *in vitro* fed ticks do not accelerate feeding after day 3.

As far as I am aware, this is the first study to quantify the LD_{50} values of systemic acaricides for ixodid ticks *in vitro*. Within the macrocyclic lactones, I have shown that abamectin has the greatest effect on *I. ricinus* mortality, although its LD_{50} was not significantly different from ivermectin. Eprinomectin was less active than abamectin but did not differ from ivermectin and these three molecules, namely abamectin, ivermectin and eprinomectin, were each at least ten times more active systemically than moxidectin and doramectin. These relationships are generally in good agreement with the structural relatedness of the molecules and the reported effects of the MLs in other blood-feeding arthropods, e.g. mosquitoes (Butters et al. 2012).

In addition to testing the effect of each molecule on tick mortality, the effect on tick feeding behaviour was quantified, as determined by the effect of the acaricide on the partial engorged weight attained by ticks. A clear inverse dose response relationship was demonstrated between increasing dose of each ML and the partial engorged weight. Consistent effects were observed across all MLs tested. At 10 µg/ml in blood, the partial engorged weight attained by *I. ricinus* females was inhibited by the MLs by up to 75% compared with the placebo. In real-time video recordings, the growth of the alloscutum of *I. ricinus* females was inhibited during *in vitro* feeding and decreased in size over time in response to 10 µg/ml abamectin in blood. Dorsoventral muscle contractions associated with blood sucking and salivation stopped after 5-10 minutes of feeding on blood dosed with 10 µg/ml abamectin. The paralyzing effects of the MLs observed in the *in vitro*

feeding assay employed this study are consistent with their mode of action as agonists of the inhibitory glutamate-gated chloride channel receptor.

The LD_{50} of fipronil, a phenylpyrazole, was six and 10-fold lower than the two most effective MLs tested, abamectin and ivermectin, respectively, and fipronil was by far the most active systemic acaricide tested in the *in vitro* feeding assay in terms of effects on tick mortality. Unexpectedly, however, a significant inverse dose-dependent effect on *I. ricinus* partial engorged weight was not detected. This effect was consistent in two other phenylypyrazoles tested *in vitro*, ethiprole and pyriprole. In other words, the data suggest that while fipronil is highly effective as a systemic acaricide for ticks feeding *in vitro*, it does not have a significant inhibitory effect on tick feeding and may increase feeding in some individual ticks. In real-time video recordings of *I. ricinus* females actively feeding on blood dosed with 10 µg/ml fipronil, rapid uncontrolled leg movements were observed in ticks after approximately 30 minutes of exposure. The effect of fipronil on the size of the tick alloscutum over time was inconsistent with some ticks ending up smaller and others growing considerably bigger than the placebo. Overall, the stimulatory effects of fipronil on tick feeding behavior *in vitro* as described in this study are consistent with the excitatory mode of action of the fipronil molecule (gamma-aminobutyric [GABA]-gated chloride channel blocker). However, as far as I am aware, this is the first study to present results suggesting that a phenylpyrazole or any acaricide introduced to ixodid ticks in a feeding situation may increase feeding in some individuals.

Finally, the organophosphates, coumaphos and chlorpyrifos, were both highly effective against *I. ricinus* females feeding *in vitro* at the highest doses tested (1 and 10 µg/ml), reaching close to 100% mortalty; however, the organophosphates displayed no activity at the lowest doses tested such that an LD_{50} could not be accurately calculated for these molecules. The organophosphates, unexpectedly, were the only systemic acaricides tested in this study that significantly increased feeding in ticks with increasing dose. In other words, a significant dose dependent effect was observed whereby the partial engorged weight attained by *I. ricinus* females feeding *in vitro* on blood dosed with an organophosphate increased with increasing dose. The effect was generally consistent between the

two organophosphates tested. The mean partial engorged weight attained by *I. ricinus* females exposed to 1 μg/ml chlorpyrifos in blood was 300% higher than the weight attained by placebo-fed ticks. As far as I am aware, this study is the first to demonstrate induced hyperphagia in an ixodid tick by an acaricide. To the best of my knowledge, the analysis of video recordings of ticks feeding *in vitro* in this study represents the first to directly quantify tick engorgement over time using video data. The organophosphates act by non-competitive binding to acetylcholinesterase, inhibiting the action of the enzyme leading to overexcitation and death. The stimulatory effect of the organophosphates on *I. ricinus* feeding *in vitro* is therefore consistent with the molecules' mode of action.

The extent to which the effects of the systemic acaricides observed on tick feeding behaviour *in vitro* in this study are applicable to the *in vivo* situation is not known. By defining the different effects of systemic acaricides on tick feeding behavior *in vitro* in this study, e.g. inhibition of tick feeding versus stimulation of tick feeding, questions can be raised regarding the optimal characteristics of a systemic acaricide for tick control. Chlorpyrifos, for example, substantially increased feeding in attached partially engorged ticks in this study and it is unclear if this effect would facilitate or increase pathogen transmission by ticks feeding *in vivo*. The possibility that fipronil, or any other antagonist of the GABA-Cl channel, could increase feeding in some partially engorged attached ticks *in vivo* cannot be ignored and further experimentation is needed to ascertain if this is the case. Additionally, even if the GABA-Cl channel antagonist was initially administered *in vivo* at the highest recommended dose, there may be a possibility that over time, as the concentration in blood decreases, a level could be reached that could increase tick feeding without killing. Experiments employing systemic acaricides over a wide range of concentrations *in vivo* are needed in the future to rule this possibility out.

At a minimum, the different effects of systemic acaricides on the partial engorged weight attained by *I. ricinus* females fed *in vitro* in this study research draws attention to the importance of measuring the size of the tick bloodmeal when quantifying the efficacy of a systemic acaricide. As ticks must attach and feed on the blood of hosts in order to be exposed to systemic acaricides, it should be

demonstrated *in vivo* that feeding is inhibited. Recently, several reports were published on the *in vivo* efficacy of the isoxazoline systemic acaricide afoxalaner against a number of different three-host tick species of companion animals (Dumont et al. 2014; Kondo et al. 2014; Mitchell et al. 2014a,b). However, none of these studies reported data on the partial engorged weight attained by ticks after attaching and feeding on pets treated with afoxalaner and therefore no comparisons could be made between the cited studies and the findings presented in this study.

From a strictly test system standpoint, the *in vitro* feeding assay for ixodid ticks employed in this study research proved sensitive enough to detect differences in the effects of acaricides on tick feeding behaviour both between and within compound classes. Similarities and differences in activity of the acaricides were in good agreement with the structural relationships and known mode of action shared between the molecules, and also in good agreement with the activities of the molecules reported in other parasite groups. Furthermore, the distribution of the tick partial engorged weight data in response to increasing acaricide dose is predictive of mode of action. Overall, the findings presented here demonstrate how biologically relevant parameters are obtained using the assay, and that by measuring the effect of an acaricide on tick partial engorged weight in combination with data on tick alloscutum size and mortality, a comprehensive data set can be obtained that provides a good picture of an acaricide’s mode of action and target-site sensitivity in the tick.

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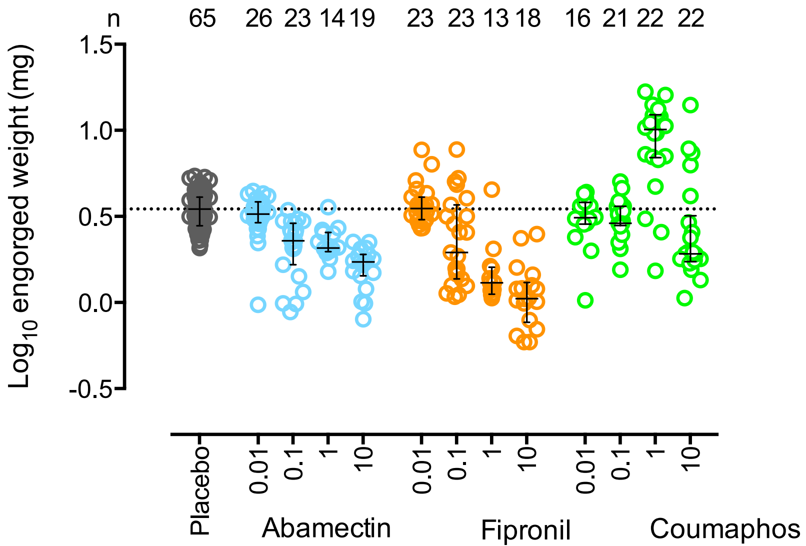
Appendix



Appendix. Median partial engorged weight (interquartile range) attained by unfed *I. ricinus* females after attachment and feeding *in vitro* on blood containing different acaricides for 48 hours. The dotted line depicts the median partial engorged weight attained by the placebo.

The effect of abamectin, fipronil, and coumaphos, respectively, on the partial engorged weight attained by unfed *Ixodes ricinus* females feeding *in vitro* was tested in this study in one experiment, and individual tick weights were recorded at all concentrations tested. In general, abamectin and fipronil inhibited feeding with increasing dose, and coumaphos increased feeding at higher doses in blood in the unfed ticks after attachment to membranes and feeding (Appendix). Although fipronil decreased feeding overall with increasing dose, the range for the relative partial engorged weight attained by ticks with reference to the placebo for fipronil was 31–222% at 0.1 µg/ml compared to 25–99% for abamectin-exposed ticks, and several individual ticks exposed to fipronil attained weights considerably larger than the placebo at this dose (Appendix).