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Time since blood-feeding (hunger) in Ixodes ricinus ticks

measurement and consequences

Time since blood-feeding (hunger) in *Ixodes ricinus* ticks: measurement and consequences

by

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Abstract

Developing precise and practicable techniques to determine the feeding history of ticks would be an important advance for tick research. Such information could be used to help interpret studies of questing and feeding behaviour and pathogen transmission. Current approaches to determine tick feeding history involve the quantitative analysis of the tick lipid reserves. Although relatively precise, this technique is labour and time intensive and ultimately leads to destruction of the tick.

The work described in Chapter 2 aimed to evaluate a non-destructive index to estimate of time since blood feeding in *Ixodes ricinus*, using quantitative ratios derived from tick body measurements. Changes in body measurements over time were compared with lipid analysis. This showed that morphological measurements, described as the 'hunger index', appeared to have little predictive value for individual ticks as an index of time-since-feeding in relation to lipid analysis. However, it may be of value at the population level, and was used to compare cohorts of ticks collected in spring and autumn, and to make inferences on the phenology of these ticks.

The effect of time since feeding on the relationships between temperature, humidity and saturation deficit, and tick survival was evaluated in Chapter 3. There was a significant decrease in tick survival as saturation deficit increased. Mean tick survival time was 11.6 days at ≥ 3.73mmHg and ticks survived for up to 60 days at lower SD conditions (≤ 2.5mmHg). However, no clear effect of hunger on sensitivity to saturation deficit could be established experimentally, partially because ticks were highly sensitive to saturation deficit with an abrupt transition between the saturation deficits where ticks either all survived, or all died. Nevertheless, some indication of a greater sensitivity of starved ticks to saturation deficit was evident at 2.5mmHg, suggesting that in high saturation deficit conditions mortality was increased even in ticks with high lipid reserves.

Conventionally, live animals are used as blood-hosts to rear ticks *in vitro*, however several ethical and welfare issues have been raised with this practice. A suitable alternative would be to develop an artificial feeding system which excludes the use of live animals for tick rearing for experimental purposes. In Chapter 4, an artificial feeding system was evaluated for feeding *I. ricinus* ticks. Silicone membranes made using Goldbeater's skin were prepared and used to evaluate the optimum conditions for *I. ricinus* feeding. The results demonstrate that good levels of probing and attachment to the membrane was achieved and tick mortality significantly decreased at 37 °C and 70% RH in the ticks maintained in a climate-controlled incubator. However, s.

In Chapter 5, feeding and walking assays were used to assess the changes in tick behaviour as starvation progressed. In the feeding assay, the results showed a significant increase in tick attachment to the feeding membrane with starvation; after 6 weeks of starvation over 70% of the nymphs were probing or attached to the membrane within the first hour. In the walking assay, there was a significant decrease in tick activity with starvation and, as hunger increased, ticks appeared less likely to walk across a filter paper ring, impregnated with an essential oil which was considered likely to act as a repellent.

In the final Chapter, the data are discussed generally in the context of climate change, modelling tick populations and the impact of starvation on tick behaviour.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original, except where indicated by special reference in the text, and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED: MUNACHIMSO I UDOBI

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The Dynamics of Feeding in Hard Ticks

1.1 General Introduction

Ticks are obligate haematophagous ectoparasites of reptiles, birds, amphibians and mammals including humans during one or all of the phases of their life cycle. Ticks belong to the class Arachnida, sub-class Acari, Order Parasitiformes and sub-order Ixodida (Maddison and Schulz, 2007). They are placed within the sub-class Acari along with mites but can be differentiated from mites by the presence of a sensory Haller's organ located on the first pair of legs and the toothed hypostome in the anterior portion of their mouthparts. They are differentiated from insects and other arthropods by the presence of a fourth pair of legs (except in larval stages), their two pairs of mouth-associated appendages (chelicera and pedipalps) and the absence of antennae or wings (Hillyard, 1996). They are currently classified into three families (Fig 1.1): the Argasidae (soft ticks) comprising 5 genera and approximately 170 species, the Ixodidae (hard ticks), comprising 14 genera and approximately 680 species, and the monotypic family Nuttalliedae. The Nuttalliedae are not well studied, and only a single species, *Nuttalliella namaqua* has been described. It is found in scattered semi-arid areas in Namaqualand, Cape Province, South Africa and in crevices of granite boulders in a higher rainfall area of Tanzania (Oliver, 1989).

The Ixodidae (hard ticks) is the dominant tick family with respect to number of species and their medical and veterinary importance. These species are arranged in two major groups based on the position of the anal groove in the adult tick; the Prostriata, where the anal groove extends anterior to the anus and the Metastriata, where the anal groove is located posterior to or surrounding the anus (Oliver, 1989). Hard ticks are so called because they possess a hard integument, and a plate-like shield called the scutum on their dorsal surface which is absent in soft ticks. They are also easily identified by their visible mouthparts and normally feed only once during each of the three parasitic life cycle stages (larva, nymph and adult). Soft ticks lack the scutum and have less obvious mouthparts than hard ticks. They feed repeatedly, taking only small amounts of blood each time (Cupp, 1991).

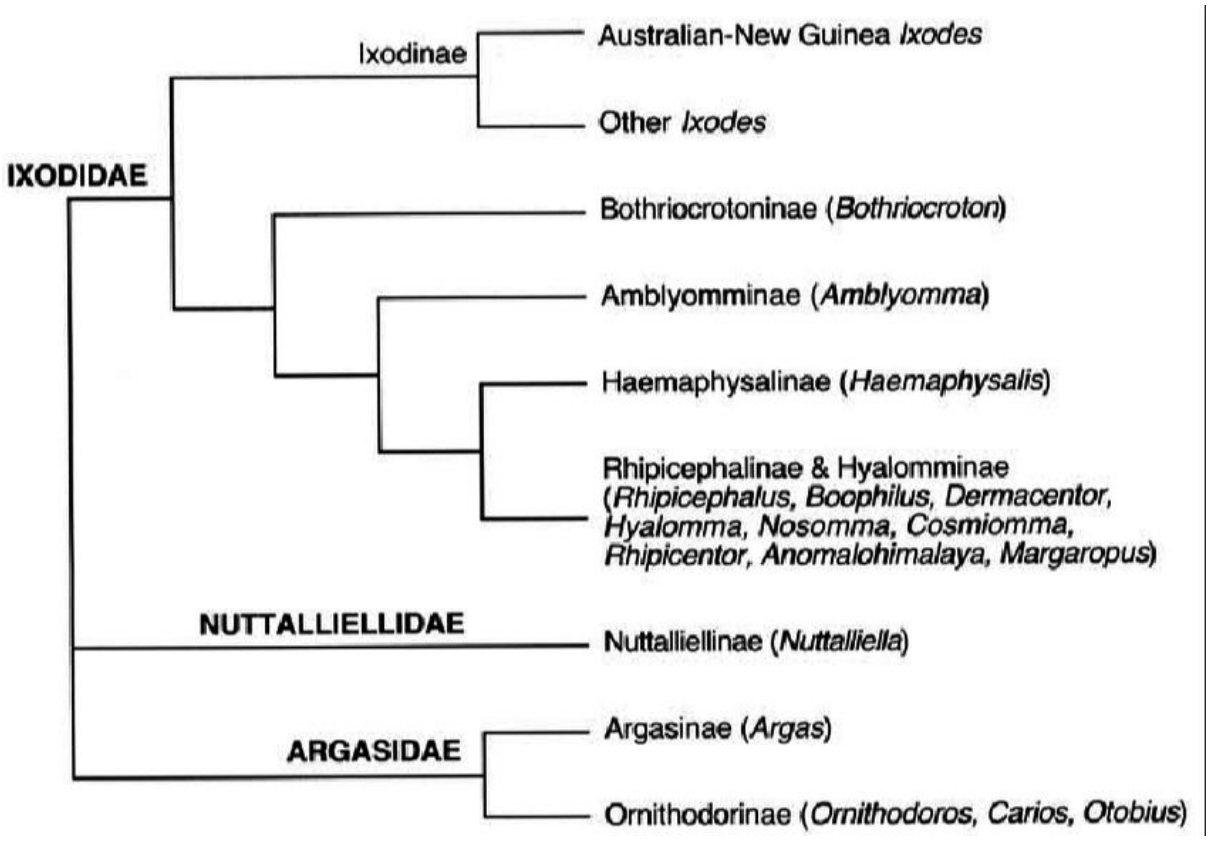


Fig 1.1 Phylogeny of the families and subfamilies of the sub order Ixodida. Image taken from Barker and Murrell (2004).

1.2 Economic Importance of Ticks

The global importance of ticks is particularly high as they have been reported to be one of the most important vectors of human and livestock diseases, second only to mosquitoes (Jongejan and Uilenberg, 2004). They transmit a wide range of pathogens including viruses, bacteria, and protozoans. This is of particular economic importance in livestock, but also has serious public health implications especially in the northern hemisphere where Lyme borreliosis and other zoonotic tick-borne illnesses of viral origins cause major morbidity and mortality in humans (Parola and Raoult, 2001). The annual costs associated with these losses and the need for control in both animals and humans worldwide amounts to billions of dollars (Sonenshine, 1991). For instance, Kivaria (2006) estimated that direct economic losses associated with ticks and tick-borne

disease in Tanzania was up to $364m annually, including an estimated mortality of 1.3 million cattle. A similar study estimated upward of $308,000 in annual costs due to ticks and TBDs in Uganda (Ocaido *et al.,* 2009). Tick-borne diseases have been ranked as one of the most important constraints to livestock keeping in parts of southern Africa (Malak *et al.,* 2012) and, in many cases, tick-borne pathogens are zoonotic and a direct threat to human health. Economic studies in rural Uganda have demonstrated that endemic cattle disease adversely affects productivity, which in turn affects household income, labour and ultimately food security; endemic bovine parasitic disease, including tick-borne disease, was shown to reduce draft cattle output by 20.9 % and potential household income from the use of draft oxen by 32.2 % (Okello *et al.*, 2015). In Tanzania, 97.3% of cattle were found to be infested with ticks (Lynen *et al.,* 2007). In 2003, the estimated annual costs and losses due of Heartwater, caused by *Ehrlichia ruminantium*, which affects all ruminants, were estimated to be $31.6m in South Africa (Brown *et al.,* 2013), $3.7m in Zambia, $3m in Mozambique, $2.9m in Tanzania, $1.9m in Swaziland (Minjauw & Mcleod, 2003) and $5.6m in Zimbabwe (Mukhebi *et al.*, 1999). It was calculated that 76% of this was due to acaricide costs, and 18% due to milk loss (Mukhebi *et al.,* 1999). Furthermore, *Theileria parva* (East Coast Fever), primarily spread by the tick *R. appendiculatus*, was estimated to cost Eastern, Central, and Southern Africa $168m in 1992, equating to the loss of around 1.1 million cattle (Mukhebi *et al.,* 1992).

In domestic ruminants, the most important diseases transmitted by ticks include babesiosis, theilerioses, anaplasmosis and cowdriosis (Jongejan and Uilenberg, 2004). In most cases, infected animals develop and retain immunity to these pathogens following recovery such that a prevalence of up to 100% may be recorded in affected herds despite the absence of clinical infections (Jongejan and Uilenberg, 2004). Pets, especially dogs, are hugely affected by tick-borne diseases, most of which are highly pathogenic to these animals. Several *Babesia spp* and *Erhlichia canis* often produce fatal infections in affected dogs in both tropical and sub-tropical regions. Tourists travelling to warmer regions in company of their dogs predispose their animals to (sub)tropical tick-borne diseases which only become clinically apparent after they return home (Brown and Prescott*.,* 2008). In temperate regions, the increasing incidence of emerging and re-emerging tick-borne zoonosis poses a constant risk to public health. Conversion of arable land into habitat suitable for maintaining large deer populations (a primary host for *Ixodes ricinus* and *Ixodes scapularis*) in urban centres has been associated with a sharp increase in number of ticks in these regions (Böhm *et al.,* 2007; Gassner *et al.,* 2016).

Besides serving as vectors for pathogens, there are several other ways in which ticks cause harm to their hosts. Ticks are pool feeders. They create a feeding blood pool in the dermis by attaching themselves to the host's skin using their chelicera and toothed hypostome to cut through tissues (Labuda and Nuttall, 2008). Their biting activity could also cause irritation to the host and damage of the skin, reducing the value of hides and skins for manufacture of leather. Some of these biting injuries also predispose the host to abscesses following secondary bacterial infection (Jongejan and Uilenberg, 2004). Marked loss of weight and morbidity has also been reported in affected animals. Some tick species, e.g *Dermacentor andersoni* and *Ixodes rubicundus* release paralysing neurotoxins during blood feeding which can result in fatalities in adult cattle in Canada and USA or a severe form of paralysis in sheep in southern Africa (Edlow *et al.,* 2008). Depending on the species, adult female ticks can also ingest between 0.5 to 2ml of blood from their host during a blood meal, and in cases of high intensity infestation, this could result in clinical anaemia in affected animals (Koch and Sauer, 1984; Jonnson, 2006).

1.3 Feeding and Feeding Behaviour in Hard Ticks

Ticks are divided into 2 categories based on their requirement of a blood meal before gametogenesis. Metastriate (Ixodidae) ticks require a blood meal before gametogenesis while Prostriate (Ixodidae) and Argasid ticks do not (Hamilton, 1992). Mating in metastriate ticks occurs on the host whereas in prostriate and argasid ticks, it occurs off the host (Hamilton, 1992).

Both metastriate and prostriate hard ticks require days to weeks to complete their feeding. This is a much longer duration when compared with other blood-feeding arthropods. These arachnids do not have strong legs or appendages to hold onto the host skin or hairs. To overcome this limitation, these ticks have strong hypostomal structures in the mouthparts which help them to pierce the host skin, remain firmly attached to the host and prevent them from becoming detached by the mechanical perturbations of the host such as grooming and scratching (Ritcher *et al.,* 2013).

Attraction, arrest of movement, aggregation, probing/attachment, feeding/engorgement and reproduction in ticks are induced by a combination of host kairomones and tick pheromones (Waladde *et al.,* 1996). The stimuli which activate the ticks or attract them to the host are divided into physical and chemical stimuli. Stimuli, such as the heat emanating from the host's body and vibrations caused by the movement of the host are included in physical stimuli. Chemical stimuli are the chemicals which are produced either by the animals or the ticks. The semiochemicals which

originate from the host animals to attract ticks are known as kairomones. Those secreted by the ticks to attract other ticks, induce physiological changes for feeding or initiate mating are known as pheromones. Kairomones include general odours produced by the host e.g CO_2 and other gases such as rumen gases in cattle (Waladde, 1996). Crude host semiochemicals can be obtained by washing areas around the tick attachment sites by using organic solvents like hexane or dichloromethane (Waladde, 1996). Bunnel *et al.* (2011) reported that the secretion of kairomones that attract other ticks can depend upon the health status of the host. They observed that *I. hexagonus* ticks were more attracted towards sick hedgehogs as compared to the healthy ones. This may indicate that healthy animals are infested with fewer ticks as compared to the sick ones.

Pheromones have been reported to play a vital role in the intraspecific communication in ticks. Other senses such as visual, tactile and auditory senses are not seen to be as important for tick communication (Hamilton, 1992). Ticks possess three kinds of pheromones: Sex pheromones, Assembly pheromones (AP) and Attraction/Aggregation/Attachment pheromones (AAAP). Some functional overlap occurs between these groups such that AAAP and assembly pheromones may also act as sex pheromones (Hamilton, 1992). There are variations in the pheromone systems between various tick species with limited knowledge in this area. There are at least three sex pheromones in ixodid ticks namely attractant sex pheromone, mounting sex pheromone and genital sex pheromone (Hamilton, 1992). The first two are common whereas the third one is has only been demonstrated in *D. variabilis* and *D. andersoni* (Sonenshine *et al.*, 1982).

Questing in ticks is the act of searching for a host and it has been observed in all stages of the tick life cycle (Mejlon and Jaenson, 1997). Because of differences in their susceptibility to desiccation, adult and nymphal ticks usually climb and quest at similar times of year but the more desiccation-susceptible larvae must swap periodically between climbing and questing activities, retreating to the matt layer of the soil when humidity becomes limiting (Danielova *et al.,* 2006; Tomkins *et al.,* 2014). In Ixodid tick species, questing is a largely passive activity, but in species of *Hyalomma*, periods of questing are regularly interspersed with periodic rest which helps preserve energy reserves and help prevent desiccation (Estrada-Pena *et al.,* 2006). Resting ticks are seen with inwardly bent legs, a position they are able to maintain for relatively long periods.

There are two strategies employed by ticks for questing. The first is the 'ambush' strategy where a tick climbs to the top of vegetation, extends their front legs and wait for a passing host (Mejlon and Jaenson, 1997). The second is the 'hunter' strategy where the tick actively pursues a potential blood host and attaches to it (Parola and Raoult, 2001). The first pair of appendages in ticks contains a sensory organ called 'Haller's organ', which have both heat and chemoreceptors,

allowing ticks to sense temperature changes, olfactory input (efficient at detecting specific lactones or phenol-based chemicals present in mammal pheromones and hair), humidity and carbon dioxide (Carr *et al.,* 2017). These features play an important role in the detection of prospective hosts by the tick.

Questing, however, is critical to tick survival and disease transmission, however, it is metabolically costly to the tick as the tick expends energy during this process and runs the risk of dehydration while receiving no nourishment (Dobson and Randolph, 2011). It is also at this stage of the tick life cycle that environmental factors directly affect tick survival, in some cases by forcing ticks to retreat to refugia in unfavourable conditions thus limiting host interaction frequency (Perret, 2008). It has been suggested that under optimal conditions, larvae can survive off host for up to 8 to 9 months (Leonovich, 2004) and nymph for about 4 months, before starving to death (Randolph and Storey, 1999). However, this is likely to differ between individuals with different levels of nutritional reserves, geographical location, temperature and humidity (Randolph and Storey, 1999).

1.4 Life Cycle of Hard Ticks

The tick life cycle comprises four stages: egg, larvae, nymph and adults. Species of hard tick family undergo one out of three different life cycles, involving one-host, two-host or three-hosts. During the one-host cycle, the ticks usually remain on the same host for the duration of the larval, nymphal and adult stages, only leaving the host prior to laying eggs. In the two-host life cycle, the larval to nymphal stages occur on the first host, but the tick leaves the host between the nymphal and adult stages to find a second host. The second host may be the same individual as the first host, the same species, or even a second species. Most ticks of public health importance undergo the three-host life cycle and include members of the genera *Ixodes*, *Amblyomma*, *Dermacentor* and *Rhipicephalus*. Here, the tick leaves the host after each of the different life cycle stages has fed and each developmental stage feeds on a different host (Földvári, 2016). During feeding, the males attach and reattach to the host, mating in between. In contrast, the adult female attaches once and remains attached to the host until engorged with blood. Following engorgement, the tick drops to the ground and lays several thousands of eggs over a period of about 30 days and then dies. The eggs develop to become larvae, which moult to become nymphs after their first blood meal. The nymphs further moult to become adults after their second blood meal, all feeding occurring on a separate host (Fig 1.2).

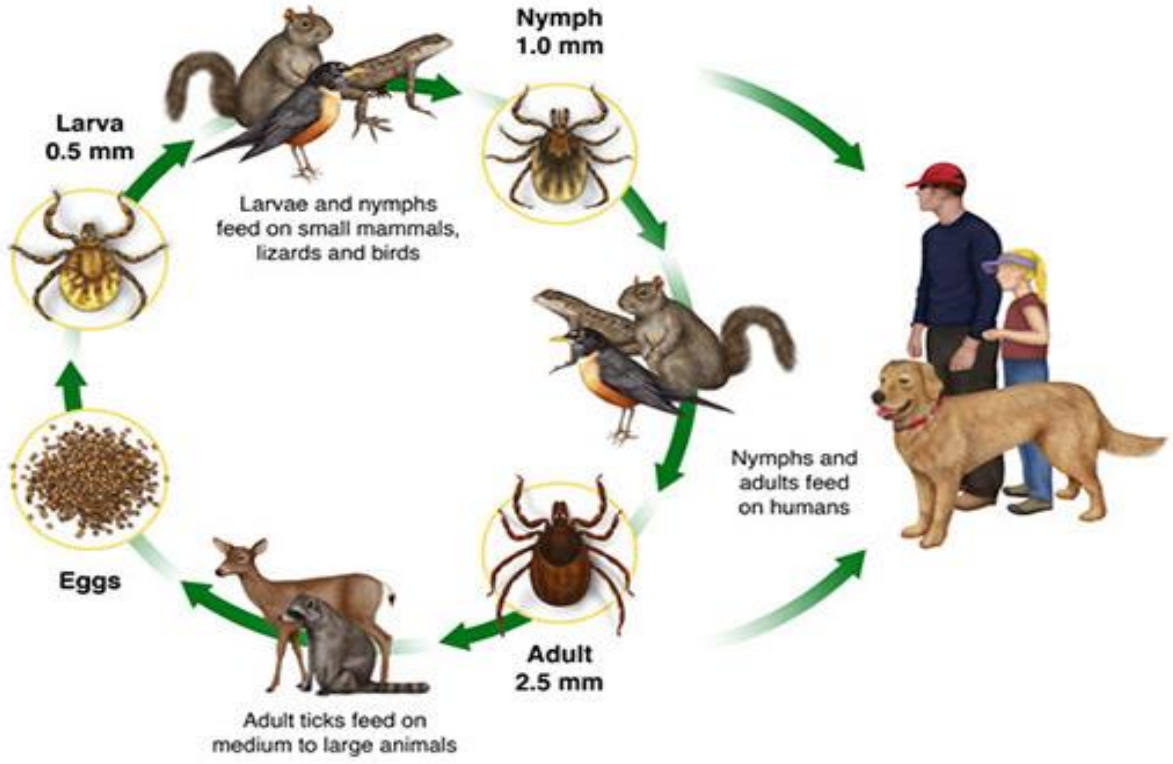


Fig 1.2 The life cycle of hard ticks. TickSense (2015)

1.5 Adaptations for Feeding in Hard Ticks

1.5.1 Tick Mouthparts

The body section carrying the tick mouthparts is described as the gnathosoma, incorporating the chelicerae, palps and the hypostome, which arise from the basis capitulum, within which are the shafts of chelicerae, pharynx and the salivary ducts (kemp *et al.,* 1982). The ventral hypostome is a plate-like structure with a central groove, armed ventrally with a row of spines or denticles. The hypostome is surrounded by a pair of chelicerae, often having hook-like barbs (Arthur, 1951; Ritcher *et al.,* 2013. The hypostome length is measured from the tip of the rostrum to the depression containing two intra-cuticular mechanoreceptors at the junction with the basis capitulum. This length varies between various instars; in *I. ricinus* measuring about 90μm in larvae, 170μm in nymphs and between 280 and 500μm in male and female adults, respectively (Krober and Guerin, 2007). A tube-like structure is formed by the dorsal groove of the hypostome

and the ventral surfaces of the chelicerae. This tube acts as a channel for the flow of food into the mouth of the tick and saliva into the host's skin. The hypostome, apart from acting as a fluid channel, also helps to stabilize and anchor the mouthparts of the tick firmly in the host skin with its denticles (Ritcher *et al.*, 2013).

The chelicerae are a pair of structures covering the hypostome dorsally. The chelicerae have a number of digits around the tip and are comprised of a retractable shaft which joins the basis capitulum with a membranous sleeve, called the 'inner cheliceral sheath' (Coons and Alberti, 1999). The chelicerae are normally retracted, leaving only the anterior digits visible. They have a hinge which aids them to flex sideways in a V-form, helping in retracting and piercing the host skin and insertion of the hypostome into the space created by this retraction. A detailed description of the feeding apparatus of *I. ricinus* is provided by Ritcher *et al.* (2013).

The pharynx is an elongate structure, tapering at both ends. It joins the oesophagus at the posterior end. The walls of pharynx are sclerotized and longitudinally ridged, forming deep folds. Anteriorly, the pharynx originates at the pharyngeal orifice and is not a continuation of the stylet (Arthur, 1946). Dilation of the pharynx is carried out by contraction of dorsal dilator muscles originating from the sub-cheliceral plate, whereas the contraction is caused by muscles crossing between the pharyngeal folds. Dilation of pharynx results in creation of a negative pressure which aids in blood sucking, whereas during the constriction of the pharynx, the buccal cavity, salivary ducts and the salivary glands act as a closed system which leads to the passage of the salivary secretions from the alveoli of the salivary glands into the duct lumen (Bertram, 1939; Arthur, 1946). The stylet closes the pharyngeal orifice during the contraction of the pharynx and prevents the reflux of blood into the wound (Arthur, 1946).

During the feeding of a tick, the blood and salivary secretion flow in alternate directions. The saliva comes from a paired salivary gland (the glands are branched, acinar, lie anterolateral and extend posteriorly). The glands have their individual ducts, which drain into a common salivarium, which opens into the food channel in front of the opening to the pharynx (Kemp, 1975). The salivary gland of ixodid ticks is complex and has 3 kinds of alveoli in females and 4 in males (Balashov, 1972; Meredith and Kaufman, 1973; Krolak *et al.*, 1982). The salivary gland of argasid ticks, on the other hand, are relatively less complex and possess only 2 types of alveoli (Roshdy and Coons,1975; El Shoura 1985; El Shoura, 1987). The alveolar morphology of salivary gland in hard ticks changes drastically during feeding as compared to the soft ticks which does not show any major change (Guirgis, 1971) and most of the fluid secretions in argasid ticks occurs via coxal

glands in contrast to the ixodid ticks where major secretions are salivary (Roshdy, 1972; Fawcett *et al.*, 1986).

1.5.2 Salivary Glands

Salivary glands serve several functions and are vital for the biological success of the tick. The salivary gland secretions of ticks during feeding have anticoagulatory, anti-inflammatory and immuno-modulatory effect on the host. When attached to the host, the salivary glands secrete various substances to circumvent the problems associated with prolonged attachment to a host. These glands also aid in concentrating the blood meals by excreting the excess water and ions into the host and as such, they are of pivotal importance during the feeding of the ticks on the host (Grigorieva and Amosova, 2008).

When the ticks are off the host, the glands aid them in absorbing moisture from the environment and therefore save the ticks from desiccation. For all these varied functions, the salivary glands have to undergo rapid structural reorganisations each time (Sauer *et al.*, 1995). The salivary glands are also the site of development of several pathogens and the route of their transmission; hence it becomes important to understand the organisation and development of these glands.

The salivary gland of female hard ticks possesses three varieties of alveoli designated as Type I, Type II and Type III. The Type I alveoli remain attached to the main salivary duct and all the cell varieties in this type of alveoli are agranular and do not change during tick feeding (Sauer *et al.,* 1995). The Type II alveoli have 6 different kinds of granular cells (designated a, b, c1-c4) and various kinds of agranular cells. This kind of alveoli undergoes a remarkable change in its cell morphology, but not in the cell number during tick feeding. Most of the granules of the cells are consumed by the end of the feeding process. In comparison to ticks secreting cement, the ones which do not secrete cement like *I. holocyclus* possess only 2 kinds of granular cells (Sauer *et al.*, 1995). The salivary glands of ticks of subfamily Ixodinae have only two types of cells in type I and II alveolus ('a' and 'b' in the former and 'd' and 'e' in the latter). This variation in cell types in the alveoli of different tick families or subfamilies indicate the variation in the composition of their salivary secretions (Grigorieva and Amosova, 2008). The type III alveoli are made of 3 types of granular cells (designated d, e and f) as well as agranular cells (Binnington, 1978; Coons and Roshdy, 1981). These alveoli are located peripherally and posteriorly on the salivary gland and undergo remarkable changes during feeding, especially the f- cells. Type III alveoli are involved in the bulk secretion of fluids during tick feeding especially in females (Sauer *et al.*, 1995). In males, which salivate less than females, the f-cells are much less active (Coons and Lamoreaux, 1986).

Type IV alveoli of male ticks has one granular cell type designated 'g', which gets filled with secretions when the males feed (Fawcett *et al.*, 1986). The males which do not feed on a host possess only type II and IV alveoli (Stone and Bennington, 1986).

The salivary glands of newly hatched larvae are small and only the salivary ducts are usually visible. The alveoli start to develop once the larvae start to feed. The alveoli types I, II and III are present in this stage, but type IV alveoli are apparently absent (Till, 1961; Chinery, 1965).

Every time a tick stage drops from the host after engorgement, the alveoli degenerate and only the salivary glands are left behind. When the next stage starts to feed after moulting, the alveoli develop again (Binnington, 1980). The salivary glands of feeding adult females increases up to 25 times in size, mass and protein content (Binnington, 1980; Shipley *et al.*, 1993). Messenger RNA is synthesized, and new proteins are produced during this phase. The mating of adult ticks is quite important for full engorgement of females. The salivary gland development is further increased after mating and the protein synthesis almost doubles after this (Selby *et al.*, 1987).

Salivary secretions of ticks contain several bioactive factors. The enzymes released into the host skin include carboxylic ester hydrolases, triacylglycerol lipase, aminopeptidase and monophenol monoxygenases and hyaluronidase as in case of *B. microplus*. Some ticks also secrete esterases and kinases (Schleger and Lincoln, 1976). There are many other kinds of enzymes which are secreted by different tick species depending on the feeding biology and behaviour. The saliva of ticks also contains various kinds of histamine blockers which help in preventing the increase in vascular permeability and inflammation with associated pain (which histamine normally does). These blockers, by reducing pain stimuli in the host, reduce their grooming instinct during the early phases of tick feeding (Chinery and Ayitey-Smith, 1977). During the later phases of engorgement, the saliva may release some histamine agonists which help in increasing plasma release into the feeding lesion and as such aid in rapid blood uptake (Chinery, 1981). Prostaglandins have been detected in the salivary secretions of several ticks. The main function of these compounds is to reduce the host immune response and inflammation and at the same time, produce a hyperaemic effect around the feeding area (Sauer *et al.,* 1993).

Male ticks also produce saliva during the mating and transfer of the spermatophore to the female. The males possess type IV alveoli having 'g' cell type, which may be producing the specific components of the saliva during this phase. The saliva helps in preventing the adhesion of the sticky spermatophores to the male or female integument (Feldman-Muhsam *et al.,* 1970).

Non-feeding ticks (i.e. the ticks in the environment) maintain their water balance through their salivary gland secretions. The alveoli type I secrete a hygroscopic fluid which help the ticks to absorb moisture from the environment (Lees 1946; Rudolph and Knulle, 1974; Needham *et al.*, 1975; Needham and Teel, 1986). The salivary glands may also aid in getting rid of the excess salts in desiccating ticks (Sigal *et al.*, 1991)

Salivary gland secretions in ticks have been found to be regulated by nervous rather than by endocrine control (Roshdy and Coons, 1975; Coons and Roshdy, 1981). Dopamine has been reported to be the neurotransmitter involved and acts on G-protein coupled receptors, resulting in activation of adenylate cyclase and formation of cAMP (Needham and Sauer, 1975; Needham and Sauer, 1979).

1.5.3 Cement

Many species of hard tick secrete a cement-like material around the feeding wound in the host skin forming a feeding cone, which helps them to cling to the host. The cement is secreted from the tick's salivary glands (Kemp *et al.*, 1982). Nosek *et al.,* (1978) argued that *I. ricinus* does not secrete any cement. Similar findings were also reported by Grigorieva and Amosova (2008) who stated that ticks of subfamily Ixodinae do not secrete any cementing material at all, thus excluding *I.ricinus*, *I. persulcatus* and *I. hexagonus* as cement secreting ticks. In contrast, however, studies by Waladde *et al.*, (1996) showed that the above-mentioned tick species secrete cement around their hypostome-chelicerae complex whereas *I. holocyclus* and *I. trianguliceps* do not produce any cement, making it hard to understand how these tick species anchor their mouthparts to the host skin. They also reported that among metastriate ticks, those with short mouth parts including *Dermacentor* spp., *Rhipicephalus* spp., *Boophilus* spp. and *Haemophasalis* spp. secrete a cement around their mouth parts, while those having long mouth parts only secrete a casing around their fully inserted hypostome-chelicerae complex.

The cement is made up of lipoproteins and glycoproteins (Arthur, 1970; Binnington, 1978). The lipids and carbohydrates form the innermost or core layer of the cement cone. The protein portion is synthesised in the 'd' and 'e' secretory cells of the type III salivary glands (Sonenshine, 1991). The cement producing cells are active at the time of tick attachment. However as feeding progresses there is a depletion of their secretions followed by atrophy of the respective cells (Waladde *et al.*, 1996).

Ixodes, *Amblyomma* and *Hyalomma* tick species have short probosces, but their mouthparts pass through the whole of the host's epidermis and reach up to the dermis. This is in contrast to *Dermacentor*, *Rhipicephalus, Boophilus* and *Haemophasalis* species, whose shorter mouth parts only reach the boundaries of the epidermis. The former have fewer cell types in their type II and III alveoli than the latter, which indicates a more complex host-tick interaction of the latter than the former (Grigorieva and Amosova, 2008).

1.6 The Feeding Process

Ticks usually spend several days on the host, engorging on its blood. Before feeding commences, the tick spends a considerable length of time exploring the host in search of a suitable site to begin feeding. The selection of a particular host and a specific site on that host is based on a blend of thermal, hygro, mechanical, olfactory and contact stimuli (Waladde and Rice, 1982; Kuhnert, 1996).

During the first few days of their attachment, hard ticks ingest small amounts of blood per unit time. During this period, they equally undergo various physiological changes including maturation of their salivary glands, synthesis of the procuticle, and production of various pheromones. During prolonged periods of their feeding, all three life-cycle stages of the tick increase in body size and weight through neosomy (Grigorieva and Amosova, 2008). Neosomy in an arthropod is a remarkable enlargement or formation of new external structures or both, resulting from the secretion of new cuticles unrelated to a moult. It is prominently seen in Acari, including all stages of hard ticks. Adult female ticks engorge fully during the last 24 hours of their feeding, ingesting about 2 to 8 times as much blood as their final weight and increase their body weight up to 100 times their initial unfed weight (Rechav *et al.,* 1994; Krober and Guerin, 2007). On the other hand, males feed intermittently and do not engorge themselves (Krober and Guerin, 2007).

The ticks of *Ixodes* species ingest whole blood containing non-lysed erythrocytes, whereas *Amblyomma* spp. ingest haemolysed blood. In the latter, lysis of erythrocytes is achieved by secreting an enzyme (haemolysin) in their saliva (Grigorieva and Amosova, 2008). The accumulation of greater volumes of blood within the tick is facilitated by secreting water and ions back into the host through specialized salivary gland cells (Kaufman, 1983; Sauer *et al.,* 1995). In contrast, argasid ticks complete their feeding within an hour. After complete feeding, their body weight increases

about 12 times their initial weight and concentration of the blood meal occurs post-feeding by excretion of the water and salts from the coxal organs (Balashov, 1972).

For successful and prolonged feeding, the tick needs to enhance bleeding, prevent inflammation and wound healing and at the same time suppress the host immune responses. For the first two days following attachment, the natural course of wound healing favours the tick. In subsequent days however, the tick needs to secrete extra protein components (vasodilators and immunomodulators) not just to maintain a constant blood supply but also to counter the host immune responses and prevent rejection (Grigorieva and Amosova, 2008). Following the onset of feeding, acini of different kinds in the salivary glands increase in size and synthesize several pharmacologically active substances involved in the feeding process including cytolytic enzymes, anticoagulants and other substances like prostaglandins (Dickinson *et al.*, 1976; Higgs *et al.,* 1976). One of the prostaglandins, PGE2, also known as dinoprostone, leads to dilation of the host's blood vessels, thereby increasing blood flow to the feeding site (Neitz and Vermeulen, 1987).

1.7 Blood Digestion in Ticks

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

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

Blood digestion in argasid ticks begins after the tick drops off from the host. The process is broadly divided into 3 phases. During the first phase, the blood meal is concentrated, and the blood cells are haemolyzed. Very little digestion occurs in this phase. The second phase involves very rapid digestion whereby the blood components are taken into the digestive epithelial cells. The third digestive phase is rather slow and helps the tick to survive starvation periods (Akov, 1982).

In ixodid ticks, feeding and digestion occurs together. Araman (1979) reported 3 different phases of feeding and digestion in hard ticks. These include the preparatory, growth and expansion phases. During the preparatory phase, there is very little feeding and digestion of blood (little protease activity), but the gut epithelium is developed in this phase. The growth phase involves vigorous feeding and digestion (peak protease activity), and the nutrients are diverted towards the expansion for the cuticle to get ready for the expansion phase. The expansion phase involves rapid feeding, but digestion is quite slow (reduced protease activity). After engorgement, the digestion of haemoglobin takes place away from the host and the nutrients are utilized for vitellogenesis and oviposition (Bogin and Hadani, 1973; Akov, 1982).

1.8 Tick Feeding and Water Balance

Maintenance of body water is critical to ticks as they have large surface-to-volume ratios and spend most part of their life cycle away from the host in varied environmental conditions (Needham and Teel, 1986).

Unfed, feeding and fed ticks have different mechanisms for maintaining their water balance. Unfed ticks have to prevent dehydration, which they do either by preventing water loss or gaining water from moist air (by their salivary gland activity or through the cuticle). Feeding ticks need to prevent overhydration because of the water they gain from the blood. They accomplish this by regurgitating the excess water back into the host on which they are feeding (Knulle and Rudolph, 1982). Engorged ticks are usually incapable of absorbing water from humid air.

Reducing their metabolic rate is one of the several ways by which ticks maintain their osmotic balance (Hair *et. al.* 1975). The exchange of water takes place mainly through the cuticle (Lees, 1946). The cuticle is the principal barrier against water loss in ticks and it covers the tracheae, oesophagus, ducts and the rectum. The cuticle is composed of two layers; the outer thin Epicuticle

and the inner thick Procuticle. The epicuticle further has 4 layers and one among these is a lipid layer, which has important waterproofing properties (Lees, 1946). The tracheal system in ticks is guarded by spiracular valves which limit water loss (Hackman, 1982). Some tick stages can absorb environmental moisture through their saliva. Apart from these, ticks usually avoid dry environments (Rudolph and Knulle, 1974; Needham *et al.*, 1975; Needham and Teel, 1986; Hillyard, 1989).

Ticks can tolerate changes in the composition of the haemolymph, which serves as the main water reserve in ticks (Hsu and Sauer, 1975). Some tropical ticks can tolerate the loss of water up to 50% of their body weight (Lees, 1969). It is because of this varied ability to tolerate water loss that ticks inhabit varied geographical areas of earth. In nature, the distribution of ticks with respect to the environmental humidity depends upon their integumental waterproofing (Knulle and Rudolf, 1982). The tick needs to maintain its body water above a threshold humidity known as Critical equilibrium humidity. The value of critical equilibrium humidity varies among different tick species and ranges between 75-94% RH. There appears to be a drastic variation among species to resistance to water losses at sub-equilibrium states (Knulle and Rudolf, 1982).

During non-feeding periods, ticks utilize an active uptake method for absorbing water from a sub-saturated atmosphere. For the most part, water and energy-deficient ticks absorb water whenever the relative humidity in environment increases either because of diurnal or weather changes or when they move from drier to more humid microhabitats (Knulle and Rudolf, 1982).

1.9 Ticks in the Environment

In three host ticks such as *Ixodes* spp, *Amblyomma* spp, or *Dermacentor* spp*,* each instar requires a blood meal from a different host before development can progress to the next stage. Finding a suitable host could take from a few to several months, hence prolonged starvation is a key feature of the life cycle of these ticks.

Questing ticks are strongly influenced by moisture content of the air (Perret *et al.,* 2000). Ticks possess a high surface area to volume ratio which makes them susceptible to dehydration during the time they spend off-host (Hillyard, 1996). They possess a waxy coating on their cuticle which helps increase water conservation and minimize water loss. Also, waste products are excreted in the form of uric acid or guanine to also minimize water loss. They obtain water through blood-feeding from mammalian hosts and through absorption of environmental vapour through

their spiracles. Closing the spiracles can also aid in body water conservation by limiting air movement (Garris and Popham, 1990; Yoder *et al.,* 2006).

A relative humidity at ground level of 85% or higher and a low saturation deficit are required to allow hydration levels to be maintained (Randoph *et al.,* 2002). *In vitro* studies have found that although relative humidity levels of 80% are optimal for survival of unfed ticks and the process of oviposition, 85% RH is optimal for fed ticks (Milne, 1944). Generally, ticks experience water loss if humidity levels are below these values. To regulate water loss, the duration and height at which ticks quest will vary in response to ambient humidity, since humidity is greater closer to the ground mat layer. However, as long as they can return to an area of higher humidity to rehydrate, ticks are able to tolerate periods of time spent in drier climatic conditions (Boyard *et al.,* 2008). Higher moisture levels at ground level are particularly important to enable ticks to rehydrate and maintain optimal water content. Hence, areas receiving medium to high rainfall levels with ample undergrowth have higher tick abundance as they have greater potential of retaining moisture within the soil, enabling high humidity levels to persist even when rainfall is low (Ogden *et al.,* 2006).

Temperature is also an important factor influencing the dynamics and distribution of questing tick populations. Tick infestation intensity in sheep flocks has been reported to correlate with temperature, with infestation levels becoming more apparent above 7 °C which is regarded as the temperature threshold at which adult and nymphal *I. ricinus* ticks are active (Macleod, 1936; Perret *et al.,* 2000). However, active ticks have been found in temperatures as low as 2 °C (Hubalek *et al.,* 2003). Higher temperatures also speed up the rate of tick development and this is thought to be responsible for the observed variation in the ratio of tick life stages from one season to another (Randolph, 2004; Perret, 2008).

The presence, depth and quality of a mat layer above the soil is also an important factor in maintaining the microclimatic conditions required for tick survival on pasture, and also is a determinant of the intensity of tick infestation (Milne, 1948). Both living and decomposing plant material contribute to this mat, which undergoes gradual decomposition and covers the ground (Milne, 1944). The nature of the underlying rock, soil type and quality of drainage in an area influences the variety of plant life able to grow, consequently affecting the thickness and quality of the mat layer produced (Milne, 1950). This layer helps to retain moisture and provides high relative humidity at ground level which increases tick survival. This is useful not only during the dry, warm summer months, but it has been suggested that bacterial activity associated with this layer may buffer the colder winter temperatures, thereby increasing the probability of winter tick survival

(Milne, 1950). For instance, for *I. ricinus*, it has been reported that the depth of the mat layer correlates positively with tick abundance and is largely dependent on the nature and species of plant material present (Milne, 1948). Tick abundance is found to be greatest where pasture quality is poor and grazing is 'rough' since poorly drained, damp, marshy areas provide higher levels of humidity (MacLeod, 1934; Milne, 1948). Seasonal alterations in weather conditions are also thought to cause annual variations in mat thickness. In the UK for instance, as temperature drops during the autumn months and deciduous trees drop their leaves, many spring and summer flowering plants die, topping up the mat layer (Greenfield, 2011).

1.10 Aims of the Study

It is the blood-feeding behaviour of ticks that highlights their importance as arthropod vectors because majority of pathogens affecting both humans and animals alike are transmitted during this activity. The overarching aim of this thesis was to investigate the physiological and behavioural changes associated with the hunger cycle between feeding episodes in *I. ricinus*, and to use this data to deduce the possible impact of starvation on tick survival and phenology. To achieve this, the first specific objective was to measure changes in tick morphometrics associated with starvation and correlate with changes in lipid content. This was to determine whether external body measurements can be used as a proxy for time since the last blood-meal (Chapter 2). Subsequently, the use of morphological measurements was also used in Chapter 5 to investigate the behaviour of ticks at higher levels of nutritional stress. The second main objective was to determine whether ticks that are nutritionally deprived are more susceptible to mortality mediated by temperature or humidity stress (Chapter 3). The third key objective was to evaluate an alternative artificial feeding system for rearing and maintaining *I. ricinus* ticks in the laboratory (Chapter 4) so that behaviour studies requiring estimation of feeding history of a tick at different intervals over a period could be carried out.

- Chapter 2 -

Inferring the feeding history of *Ixodes ricinus* ticks from lipid and body measurements

2.1 Introduction

The life-cycle of three-host ticks, as described in Chapter 1, is characterized by long periods off-host, with the on-host period lasting only a few to several days, equivalent to less than 5 % of the entire life span (Sonenshine, 2005; Umemiya-Shirafuji *et al.*, 2010). To help ticks to survive this inter-feed interval and unpredictable availability of food, they have a relatively low rate of metabolism – similar amongst the arthropods to scorpions, another sit-and-wait predator (Benton, 1992; Pimenta *et al.,* 2019; Segev *et al.,* 2020). During the lengthy off host period, ticks rely on the stored metabolic reserves derived from the previous blood-meal for homeostasis and development. Digestive products are stored largely as lipid in the tick body (Alasmari & Wall, 2021). Previous studies have reported that 8.4% of the body mass (BM) in nymphs was composed of lipids and also, that male ticks had higher lipid composition (10% BM) than females (5% BM) (Alasmari and Wall*.,* 2020). Lipids play an important role in tick metabolic processes, as a source of energy, for egg development and for maintaining internal cell membrane structures.

A thorough understanding of the complex internal and external stimuli that affect the various aspects of tick behaviours may contribute to explaining their observed phenology. However, to be able to do this, a critical problem is the ability to determine exactly when each individual tick last fed so that the make-up of the overall population can be determined in terms of feeding cohorts (Randolph *et al.,* 2002; Abdullah *et al.,* 2018). It has been suggested that stored lipid is a good indicator of the tick's energy reserves and has been reported to deplete progressively and predictably between blood meals (Alasmari and Wall., 2020).

Measurement of the amount of lipid and the rate of depletion are considered to be good indicators of the feeding history of an individual tick (Abdullah *et al.*, 2018*;* Alasmari and Wall*,* 2020). Early attempts to quantitatively measure the lipid content of ticks used a gravimetric method

(Randolph *et al.,* 2012). This involved weighing and dipping ticks in 3 consecutive washings of chloroform for 72 h and then reweighing. The lipid values obtained from the study were used to explain the seasonal dynamics of *I. ricinus* across the different seasons of the year. However, Abdullah *et al.* (2018) argued that this approach was likely to be inaccurate given the imprecision in weighing individual ticks and proposed a new approach using a spectrophotometric method based on a microquantity colorimetric sulfophosphovanillan method (SPV). This was adapted from a method first proposed by Van Handel (1985) for use in mosquitoes. This technique was based on the principle that unsaturated lipids react with sulphuric acid to produce a carbonium ion; vanillin reacts with phosphoric acid to produce an aromatic phosphate; the carbonium ion then reacts with the activated carbonyl group of phospho-vanillin to produce a charged, coloured complex that is stabilised by resonance and absorbs light maximally at about 525nm (Knight *et al.,* 1972; Johnson *et al.,* 1977; Gray, 1987). This method was shown to allow the lipid content for individual ticks to be estimated with accuracy. Using this technique, Abdullah *et al.* (2018) showed that the median lipid value in nymphs was 12.2μg, with values ranging between a minimum of 1.77μg and a maximum of 40.8μg in tick samples collected in early summer. Their study also showed that the estimated lipid reserves of the ticks was sufficient to allow survival without feeding for up to 100 to 250 days at 15 °C, depending on whether they had fed the previous autumn or the same year, respectively. This technique was shown to be highly sensitive and able to detect microquantities of lipid in individual ticks and was preferable to the gravimetric method. However, while it is relatively precise and easy to undertake, this technique ultimately does lead to destruction of the tick. This becomes a problem when attempting to design studies to consider changes in the behaviour of progressively more hungry ticks over time. Therefore, some non-destructive index of time-since-feeding would be a valuable research tool.

Uspensky *et. al.* (2006) proposed a method for determining the feeding history of a tick within an instar through a series of morphological measurements, which he termed "Physiological age index" (PAI). This works on the basis that when ticks feed and engorge with blood, the scutum of the tick remains a constant size as it is hard and sclerotised. The alloscutum however, expands as the tick feeds. Over the initial period of rapid blood meal digestion, the alloscutum declines rapidly in size. Subsequently, as the tick uses up its resources, the alloscutum continues to slowly decrease further in size over time. Hence the ratio of the sclerotised scutum to the unsclerotised alloscutum, should give an index of time since the last blood-meal. This proposal was validated in the study by Uspensky *et. al.* (2006) in which body measurements of female *Ixodes persulcatus*, including the length and width of the scutum and alloscutum, were carried out and their ratio used to derive the PAI for each individual tick. Their PAI measurements were found to correlate with

an age-grade scale developed by Balashov (1961). Balashov's scale considered histological changes during starvation and used such biomarkers such as gut caecal distention, haemoglobin and haematin content in digestive cells and the guanine content in malpighian tubules to categorise ticks into 4 classes: post-moulting development, beginning, main and final period (4) of active life. Uspensky concluded that this effectively gives an independent morphological measure of starvation over a life cycle stage and provides useful confirmation of the feeding history.

Pool *et al.* (2017) applied a similar body measurement ratio index, which they termed the 'Morphometric Age Ratio' to estimate the energy reserves in unfed *Ixodes scapularis*. In this study they also undertook the measurement of the lipid content of the tick, estimated by chloroform extraction, and they reported a significant correlation between Morphometric Age Ratio and lipid content. Another study by Springer *et al.* (2022) also applied the Morphometric Age Ratio to determine the relationship between tick physiological age and *Borrelia* infections in *Ixodes ricinus* nymphs. The study concluded that *Borrelia* infection intensity, as determined by probe-based quantitative real time PCR, declined significantly with morphometric age. Despite the supporting studies by Springer *et al.* (2022) and Pool *et al.* (2017) estimation of time since blood-feeding by measurement of body size ratios is not widely used, and further examination and verification of its accuracy is required, particularly in *I. ricinus*.

The aim of the current study, therefore, was to further explore this non-invasive morphological approach to determine whether morphological measurements do change predictably in association with starvation and if so, can they be applied to field samples to give and indicator of the feeding history of *I. ricinus* ticks. The anatomical PAI index described by Uspensky *et al.* (2006), here described as a "Hunger Index", was to be calibrated by comparison with lipid depletion, since as described above, the latter is a known precise and accurate measure of time since feeding.

2.2 Materials and Methods

2.2.1 Study material

A batch of about 200 nymphs was collected in October 2020. A second batch of approximately 500 *I. ricinus* nymphs were collected in February 2021. These will be described as the 'autumn' and 'spring' batches' respectively. The ticks were collected from field sites at Dolbury Warren and Ashton Court respectively. Both sites lie to the west of the city of Bristol; the former is a site is managed by the Avon Wildlife Trust and is grazed by a range of wild and domestic

animals while the latter is a semi-urban park which contains managed herds of red and fallow deer and unmanaged population of wild roe deer (Jennett *et al.,* 2013). Tick collection was carried out using the blanket dragging technique as described by Jaenson *et al.* (2006). A $1m^2$ piece of white cloth was attached to a 1.3m long bamboo pole and dragged slowly in a straight line for 10 metres. After this, the blankets were immediately turned over and the number of ticks which had attached to the underside of the blanket were collected with forceps into a sample container.

Collected nymphs were stored in 50 ml plastic vials and transported to the laboratory where 200 ticks each from the autumn and spring cohort were immediately frozen at -20 °C prior to processing. 300 ticks from the spring cohort were subsequently transferred individually into labelled 0.5ml Eppendorf tubes. Each tube was sealed with a piece of netting fastened with a rubber band. The Eppendorf tubes were placed in a humidified incubator (Versatile Environment Test Chamber, PHC Corporation, Japan) at 15 °C and 80% Relative humidity (RH). This environment was adequate to ensure good levels of survival of the ticks during the study. Immediately following collection (Week 1) and subsequently every 2 weeks over a period of nine weeks, about 50 nymphs selected at random were removed for morphological measurement and about 30 of these were subsequently used for lipid analysis, as described below.

2.2.2 Tick body measurements

Morphological features of the nymphs were measured (to the nearest micrometer) using a binocular microscope (Leica M212, Wetzlar, Germany) with the aid of a calibrated graticule (Table 2.1; Fig. 2.1). The objective was to measure a representative range of characters to allow the surface area of the alloscutal and scutal components of the body to be calculated.

Table 2.1: Anatomical characters of *Ixodes ricinus* nymphs measured in the study and their description, plus indices calculated from combinations of these characters.

Anatomical parameter (unit)	Acronym	Description
Scutum length (mm)	**SL**	The midline distance from the anterior edge to the posterior tip of the scutum
Scutum width (mm)	**SW**	The lateral distance at the widest point of the scutum
Body length (mm)	**BL**	The midline distance from the anterior edge of the scutum to the posterior tip of the opisthosoma
Body width (mm)	**BW**	The alloscutum width immediately after the posterior tip of the scutum
Alloscutum length (mm)	**AL**	The midline distance from the posterior tip of the scutum to the posterior tip of the opisthosoma
Scutal Index (mm^2)	**SI**	The multiplication of the scutal length by its width [SL*SW]
Body Index (mm^2)	**BI**	The multiplication of the body length by its width [BL*BW]
Alloscutum Index (mm^2)	**AI**	The difference between the tick body index and the scutal index [BI – SI]
Hunger Index	**HI**	The ratio Alloscutum Index/Scutal Index [AI/SI]

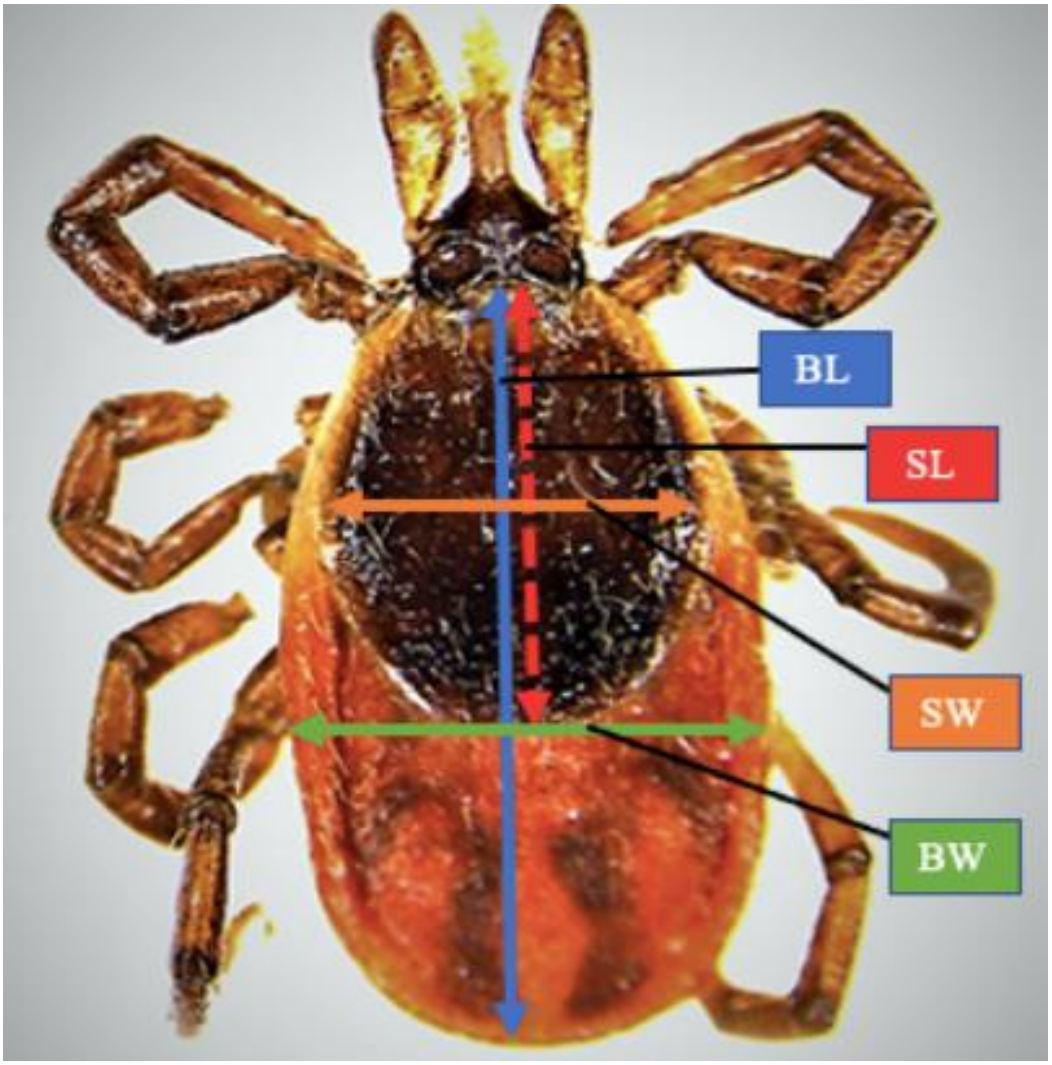


Fig. 2.1 The location of the measurements needed to calculate the Hunger index, as annotated on a dorsal view of a female adult *Ixodes ricinus* tick (Toledo, 2020). BW = Body width, SW = Scutal width, BL = Body length, SL = Scutal length

2.2.3 Measurement of tick lipid

The lipid content of each individual tick was quantified using a vanillin assay, as described by Abdullah *et al.* (2018). Every two weeks, approximately 30 ticks, selected at random, that had been used for body measurements and transferred into labelled 0.5ml Eppendorf tubes were placed in an oven (Heratherm Oven, Thermo Scientific, UK) and dried at 70 °C for 12 h and then weighed using an ultrasensitive microbalance (Sartorius-ME5, Göttingen, Germany). Each individually dried and weighed tick was placed in a clean glass test tube and crushed with a glass

rod in 0.5ml of a 1:1 chloroform-methanol mixture made up by adding equal parts of chloroform and methanol (Sigma-Aldrich, Gillingham, UK). Tick debris which had accumulated on the side of the tube were included by gently rocking the tube, after which 0.50ml of the mixture was carefully transferred into a second clean glass tube. The glass tubes were then placed in a heating block (LSE Digital Dry Bath, Corning, USA) at 100 °C in a fume cupboard to evaporate the solvent. After evaporation, 0.1ml of 98% sulphuric acid (VWR International, Leicestershire, UK) was added and the tube was heated again for 10 min at the same temperature. The tube was then left to cool, after which 2.4 ml of vanillin reagent (Acros Organics, New Jersey, USA) was added to make a total volume of 2.5ml. After adding vanillin to the mixture, a reddish colour develops within 5 min. The absorbance of each tube was read within 5 to 10 min in a spectrophotometer (Biochrome, Biowave II, Cambridge, UK) at 525 nm against a blank which had been subjected to the same procedure as above, but without the tick material present, and the lipid content of each individual tick was read directly from a calibration curve. The calibration curve of absorbance against known lipid concentration was created to allow spectrophotometric values obtained with the tick samples to be related to lipid concentration (Fig. 2.2). The curve was obtained using analytical grade standard soybean oil (0.917g/ml) (Sigma-Aldrich, Gillingham, UK) diluted in chloroform-methanol (1:1). The mixture was subjected to the same procedure as described above.

2.2.4 Data analysis

Lipid values were not normally distributed, so non-parametric tests were used. Kruskal-Wallis tests were used to examine differences in lipid concentration at different starvation time points; Kolgomorov-Smirnov tests were used to compare lipid distributions and Mann-Whitney U-tests were used to examine differences in the median lipid concentration and Hunger Index between Spring and Autumn cohorts. A Spearman's rank correlation test was carried out to examine the relationship between hunger index and lipid content. Body measurement data were normally distributed, so to describe the relationship between the linear anatomical parameters of the tick with starvation, General linear models (GLM) were used. Bonferroni *post-hoc* tests were then used to determine the differences between the pair-wise comparisons of the group means of the tick batches from different starvation time points.

All means are reported ± SEM and all medians with their range or inter-quartile range. All statistical analyses were carried out using IBM SPSS Statistics for Windows (Version 28, IBM Corp, Armonk, N.Y, U.S.A)

2.3 Results

2.3.1 Standard curve

The known concentrations of soybean oil in chloroform: methanol gave a highly significant linear relationship with absorbance (Fig. 2.2) allowing it to be used to calculate the lipid concentrations in ticks from their absorbance values.

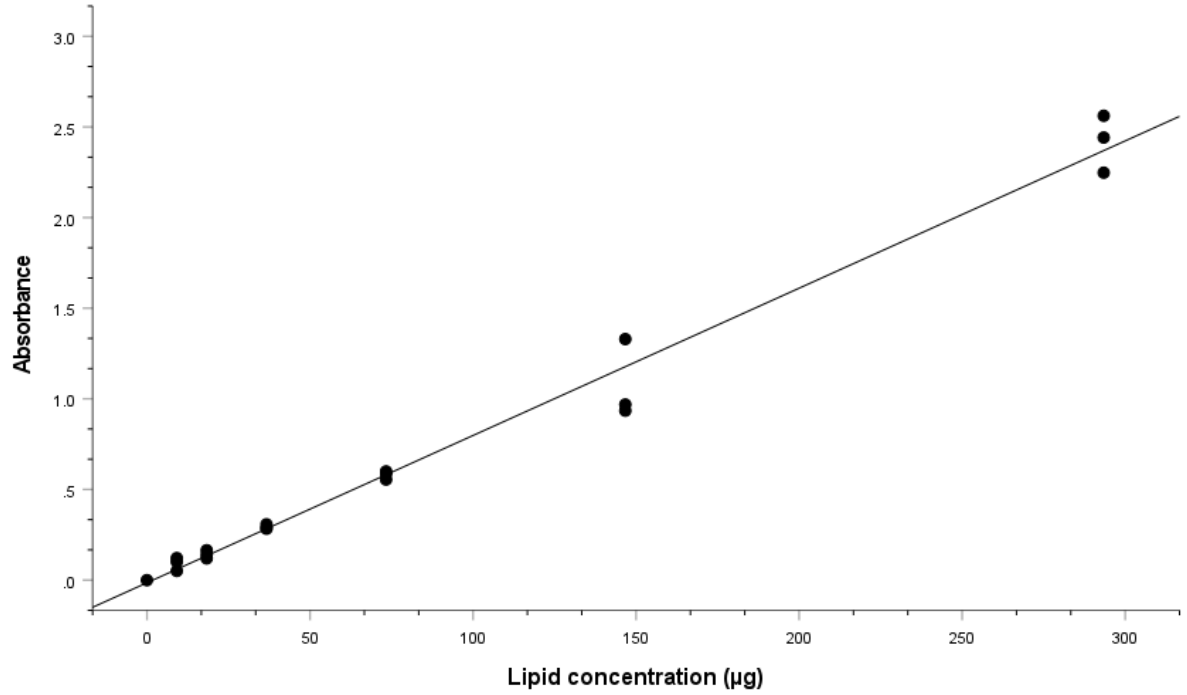


Fig. 2.2 Spectrophotometric absorbance of known concentrations of soybean oil (µg) at 525nm. Linear regression fitted: Absorbance=0.0081*lipid concentration, F = 163.29, P < 0.001, R^2 = 0.988. y = 0.0081x.

2.3.2 Change in tick lipid concentration over time

There was a significant difference in tick lipid concentration at the different starvation time points (Kruskal-Wallis, H = 74.9, P = < 0.001, df = 4; Fig 2.3). Lipid initially declined relatively quickly over the first 5 weeks from a median of 23.58 µg (range of 30.37µg) in week 1 to reach a median of 5.19 µg by week 7 (range 33.59 µg) after which there was no further significant decline, giving an overall decline of approximately 22%. By the final week of the study (week 9), 50% of the examined ticks had lipid values of between 0.12 µg and 12.6 µg.

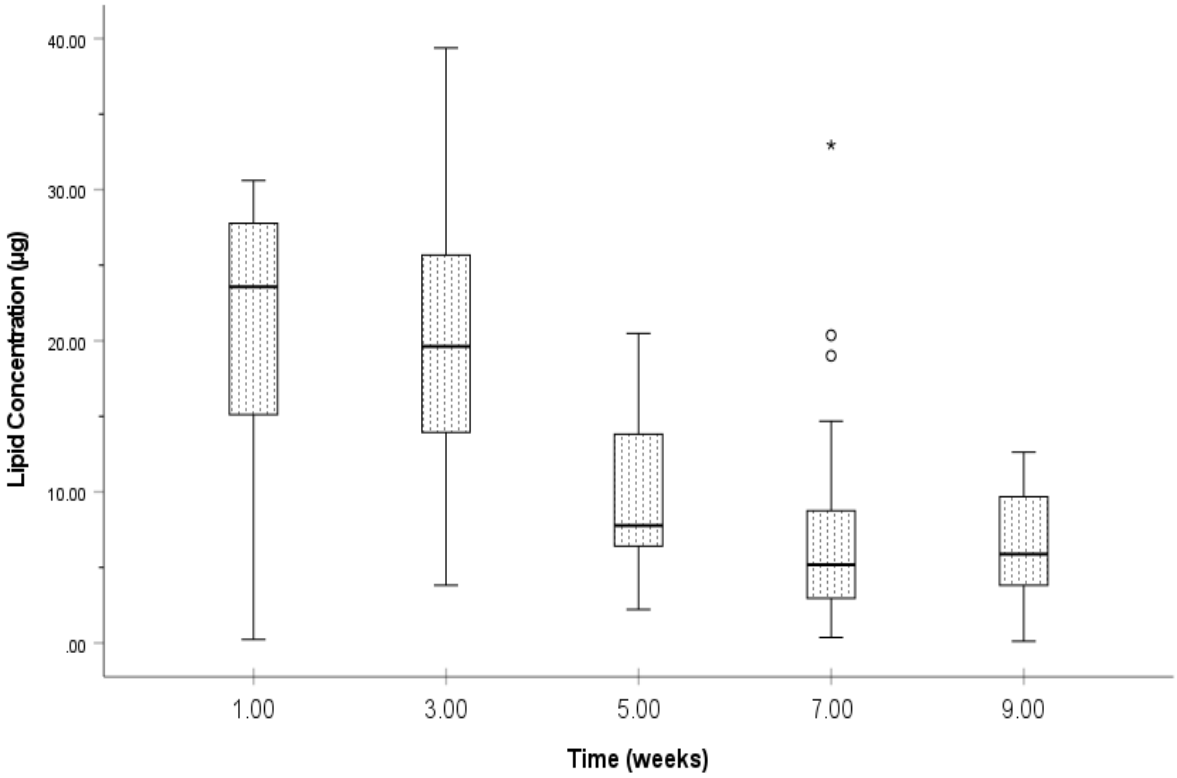


Fig 2.3 Median lipid values (µg) of individual *Ixodes ricinus* nymphs collected at a single time point and progressively starved for 9 weeks in a cooled incubator at 15 ºC and 80% RH, measured by spectrophotometric analysis at different time intervals. Horizontal line = median, box = interquartile range, whiskers = minimum and maximum, * ° = outliers, n = 30.

2.3.3 Frequency distributions of lipid

At week 1, the skewness of the lipid concentration in the nymphs was -1.16 and the kurtosis was 0.72, indicating that the distribution was negatively skewed relative to a normal distribution (Fig 2.4). At weeks 3 and 5, the skewness of the lipid concentration in the nymphs was 0.35 and 0.76 and the kurtosis was -0.35 and -0.35, respectively (Fig 2.5; 2.6). This indicates that both distributions approximated a normal distribution. At week 7, the skewness of the lipid concentration was found to be 2.22 and the kurtosis was 6.1, indicating that the distribution was now more highly positively skewed (Fig 2.7). At week 9, the skewness and kurtosis of the lipid concentration was found to be 0.03 and -0.84 respectively, again indicating that the distribution was comparable to a normal distribution (Fig 2.8).

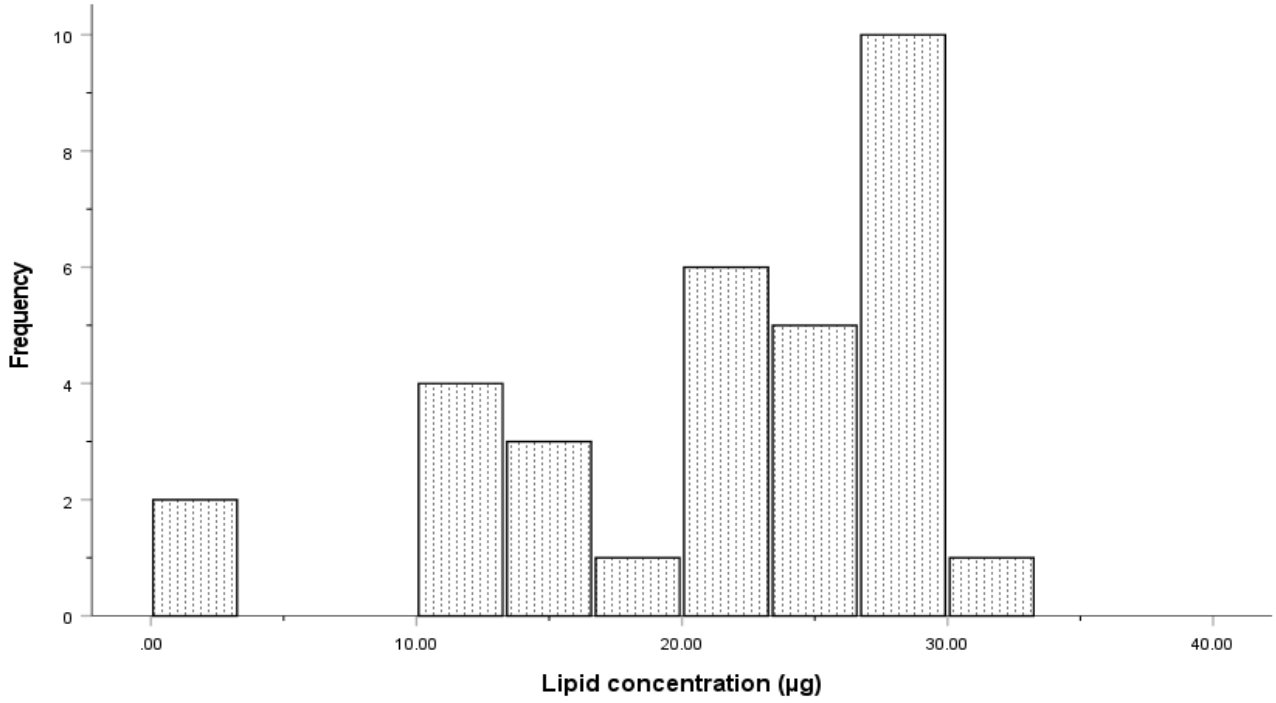


Fig 2.4 The frequency distribution of observed lipid values (µg), at week 1 measured by spectrophotometric analysis in a sample of 32 nymphal *Ixodes ricinus* collected in February 2021 in 10 µg lipid classes, n = 30.

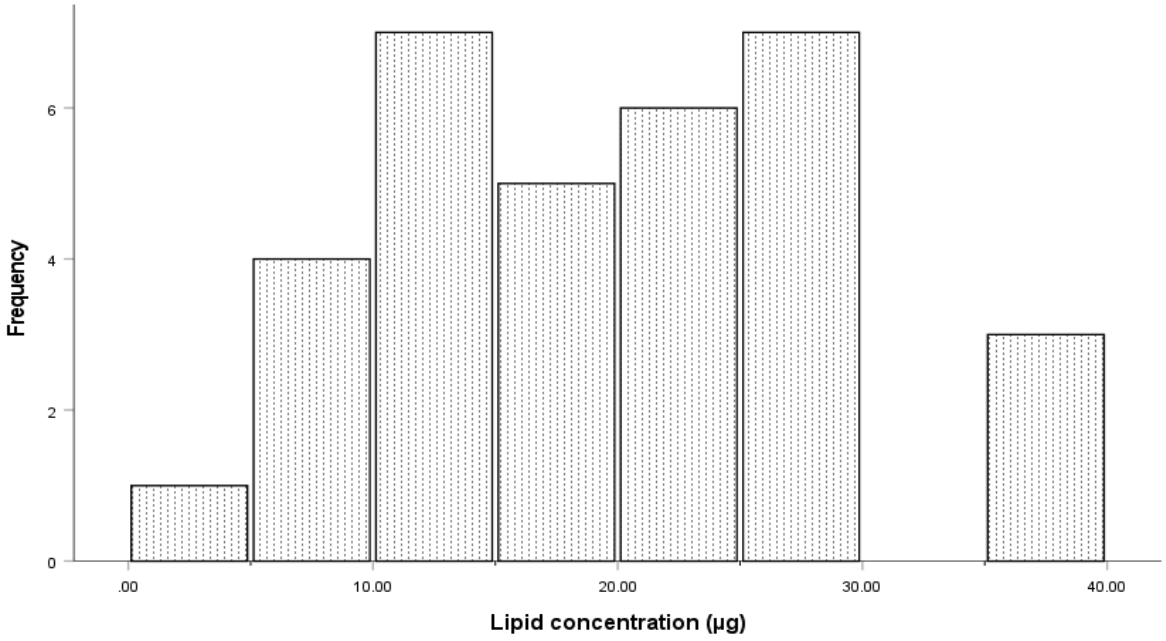


Fig 2.5 The frequency distribution of observed lipid values (µg), at week 3 measured by spectrophotometric analysis in a sample of 33 nymphal *Ixodes ricinus* collected in February 2021 in 10 µg lipid classes, n = 30.

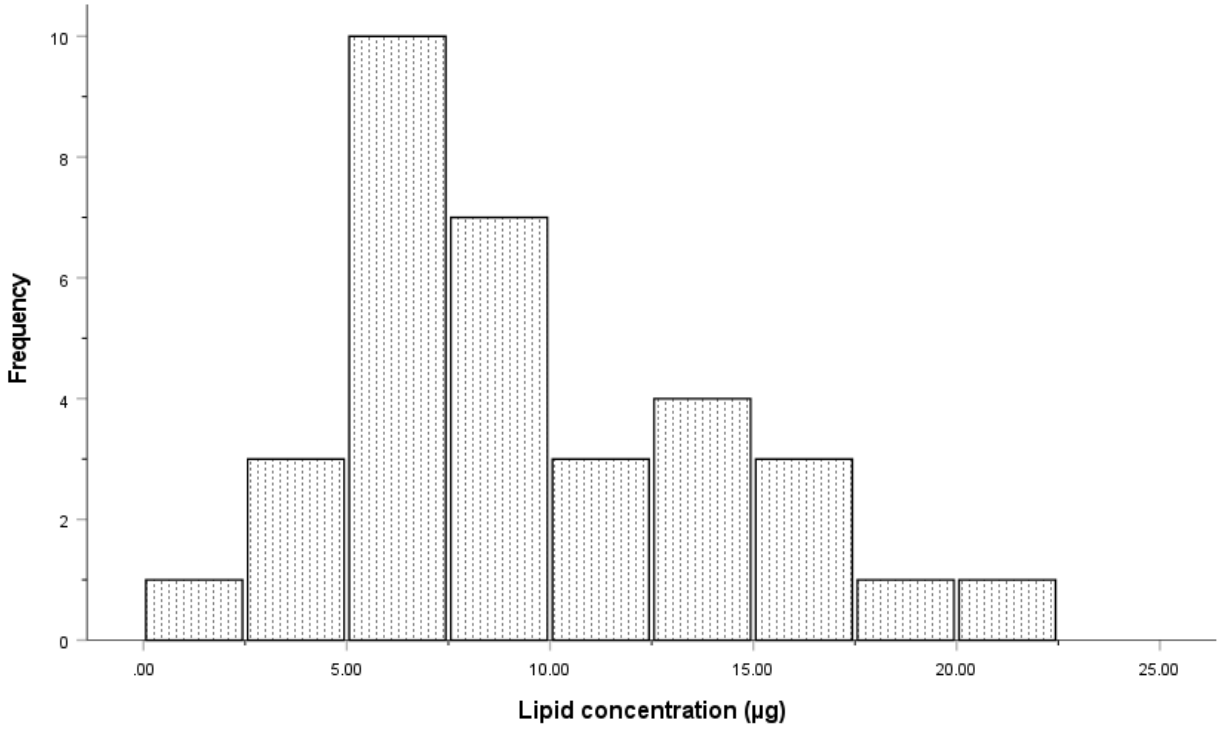


Fig 2.6 The frequency distribution of observed lipid values (µg) at week 5 measured by spectrophotometric analysis in a sample of 33 nymphal *Ixodes ricinus* collected in February 2021 in 5 µg lipid classes, n = 30.

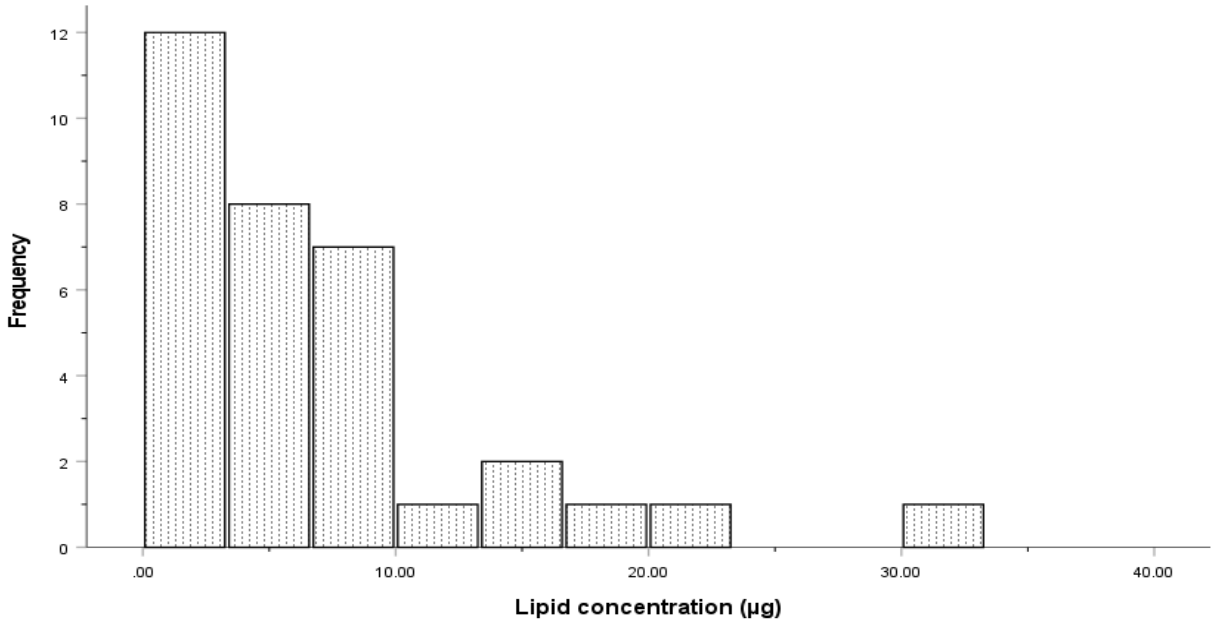


Fig 2.7 The frequency distribution of observed lipid values (µg) at week 7 measured by spectrophotometric analysis in a sample of 33 nymphal *Ixodes ricinus* collected in February 2021 in 10 µg lipid classes., n = 30.

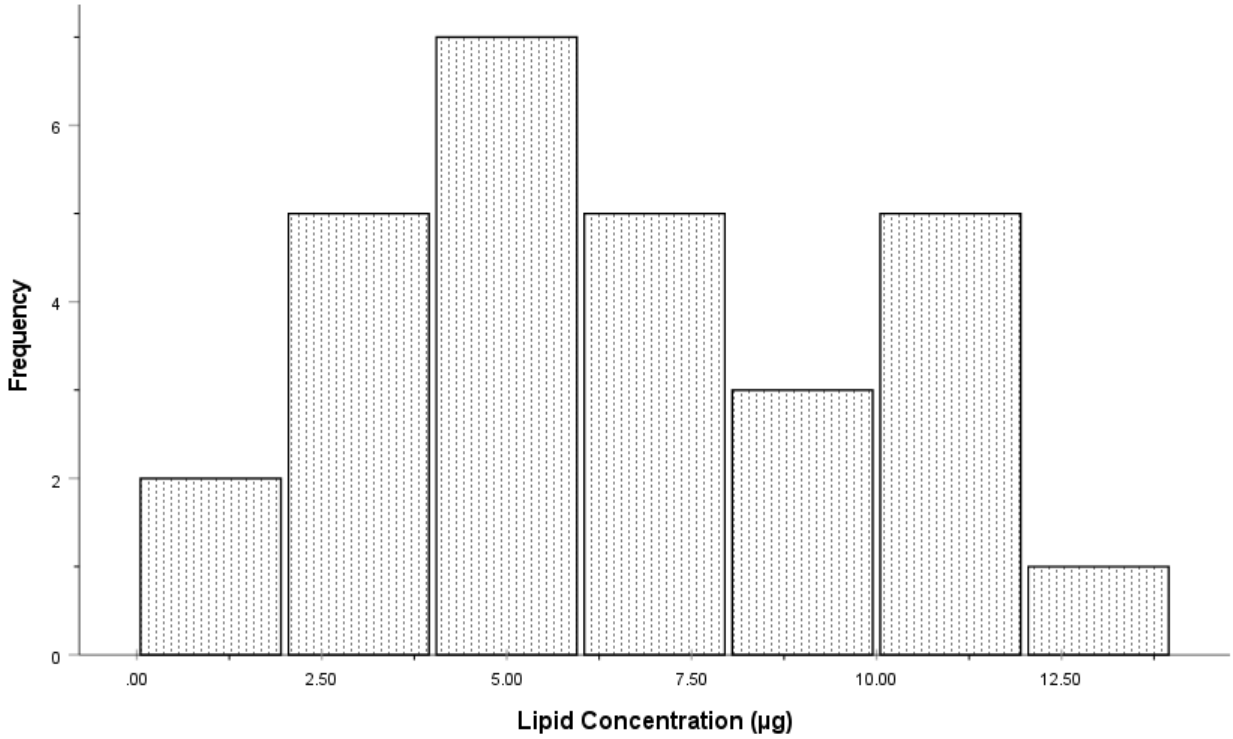


Fig 2.8 The frequency distribution of observed lipid values (µg) at week 9 measured by spectrophotometric analysis in a sample of 28 nymphal *Ixodes ricinus* collected in February 2021 in 2.5 µg lipid classes, n = 30.

2.3.4 Changes in body measurements over time

Overall, there was a significant change in mean body weight of ticks with starvation (GLM: $F_{4, 159} = 19.99$, $R^2 = 0.34$, $P < 0.001$). At week 1, the mean body weight was 148.5 (± 6.05) µg. As starvation progressed, there was no change in body weight until week 9 when the mean weight fell to 80.8 ± 5.74 µg, a decrease of 54.4 % (Fig 2.9).

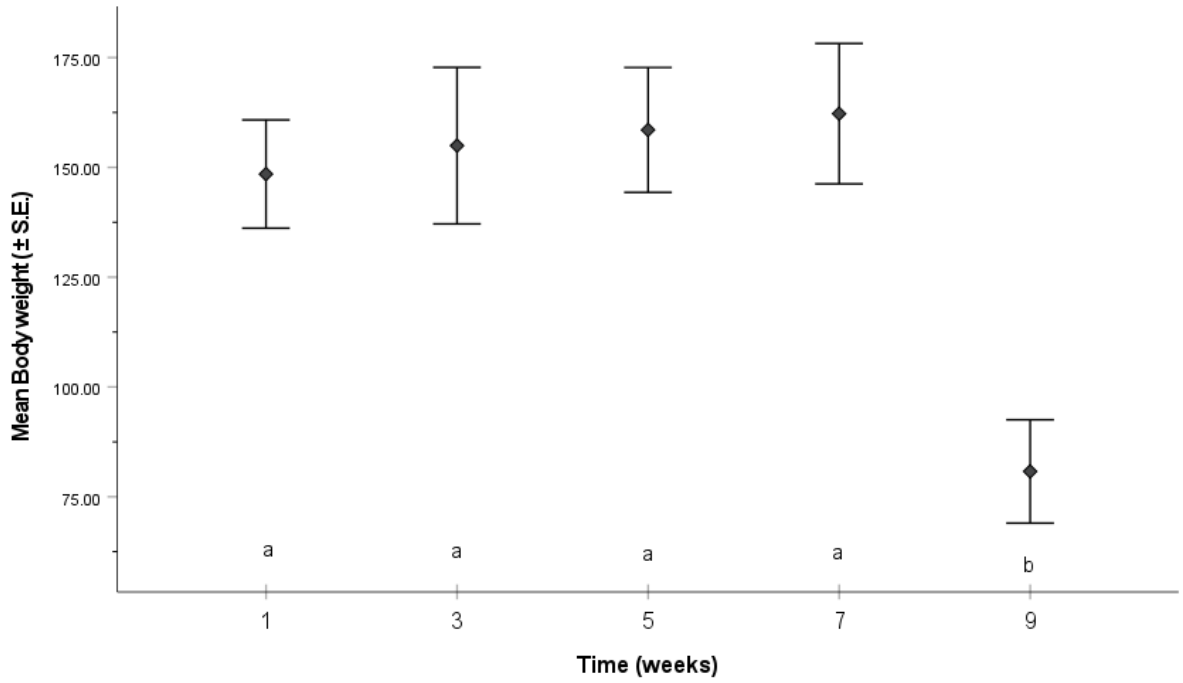


Fig 2.9 The mean body weight (± S.E) for groups of nymphal *Ixodes ricinus*. Individual nymphs were collected at a single time point and progressively starved for 9 weeks in a cooled incubator at 15 °C and 80% RH and weighed at different time intervals. Lower case letters above x-axis denote statistically homogenous groups ($P < 0.05$) as determined by Bonferroni multiple comparison *post-hoc* tests, n = 50.

There was no significant change in scutum length, scutum width, scutal index and body length with starvation. However, the body width (BW), Body Index (BI), Alloscutum length (AL), Alloscutum index (AI) and Hunger Index (HI) of the ticks decreased significantly with time as starvation progressed (Table 2.2). BW and AL of the ticks decreased from a mean of 0.72 (± 0.05) and 0.51 (± 0.06mm) respectively in week 1, to 0.68 (± 0.06) and 0.47 (± 0.07) mm in week 9 respectively, an overall decrease of approximately 5.6% and 7.84% for each parameter, respectively (Fig 2.10; 2.12).

BI is derived from the product of the body length of each individual tick and its width. An overall decrease of 5.06% was recorded in this parameter across 9 weeks of the study. In the first week of the study, a mean BI value of 0.79 (± 0.01) mm^2 was recorded. Each week of the observations, the values decreased but not significantly until week 9 when the BI of ticks fell sharply (Fig 2.11). AI also declined over time, but more gradually than BI. At week 1, a mean of

0.48 (± 0.07) mm^2 was recorded and an overall decrease of 16.7% was observed by the end of the study. At week 9, 50% of observed ticks had AI values between a minimum of 0.27 to 0.56 mm^2, within a range of 0.37 mm^2 (Fig 2.13).

Table 2.2 Regression statistics for the relationship between tick body measurements and time (weeks) for nymphal *Ixodes ricinus* collected at a single time point and starved for 9 weeks in a cooled incubator maintained at 15 °C and 80% RH, with R^2 and statistical significance (P).

Body Measurement	**F**	**P**	**R^2**
Scutum length	0.379	0.824	0.006
Scutum width	2.195	0.070	0.036
Body length	2.217	0.068	0.036
Body width	4.306	0.002*	0.068
Alloscutum length	3.176	0.014*	0.051
Scutal Index	1.449	0.219	0.024
Body Index	4.040	0.003*	0.064
Alloscutum Index	6.345	< 0.001*	0.097
Hunger Index	8.076	< 0.001*	0.121

*Indicates statistically significant ($P < 0.05$) values

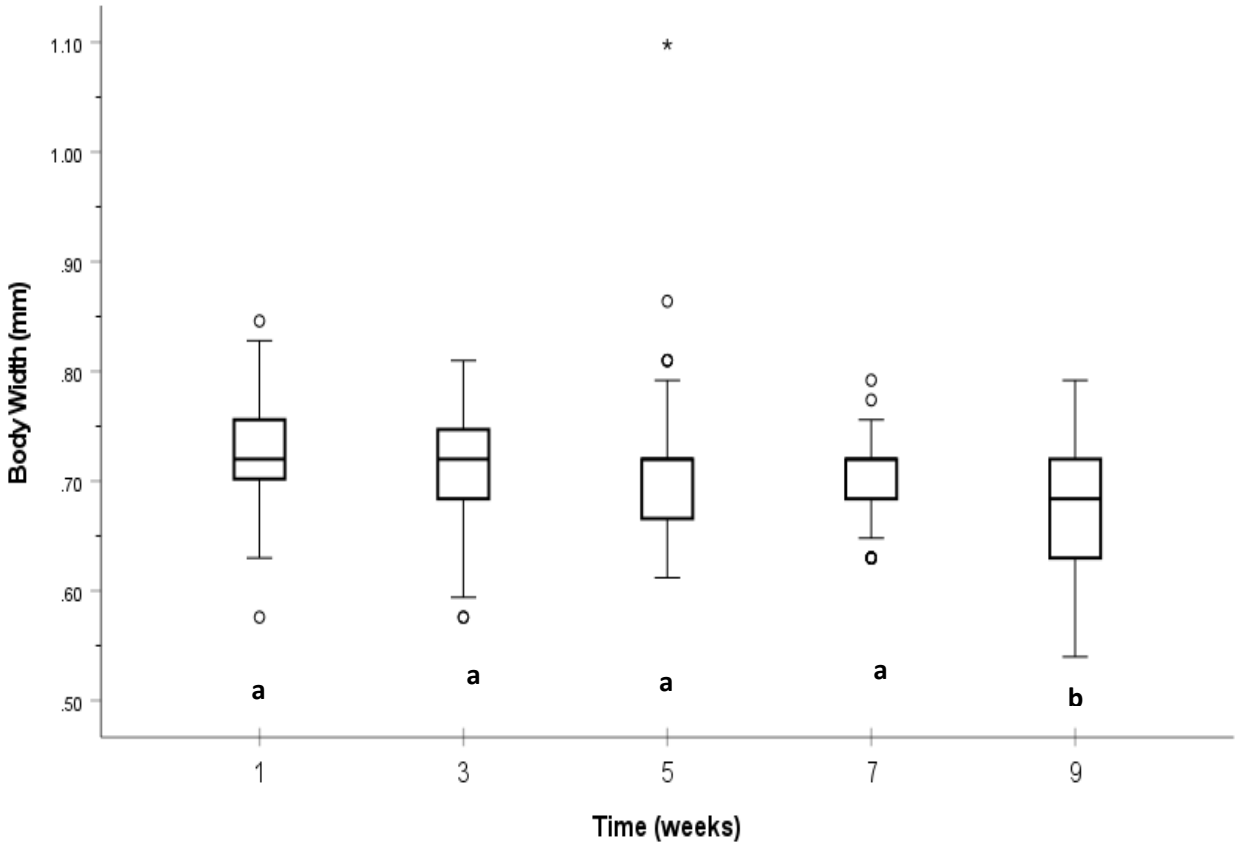


Fig 2.10 The body width of nymphal *Ixodes ricinus* collected from the field at a single time point and starved for 9 weeks in a cooled incubator at 15 °C and 80% RH and measured at different time intervals. Lower case letters above x-axis denote statistically homogenous groups ($P < 0.05$) as determined by Bonferroni multiple comparison *post-hoc* tests. Middle line = mean, box = mean ± SD, y min = mean – 3 SD, y max = mean + 3 SD, outliers = * °, n = 50.

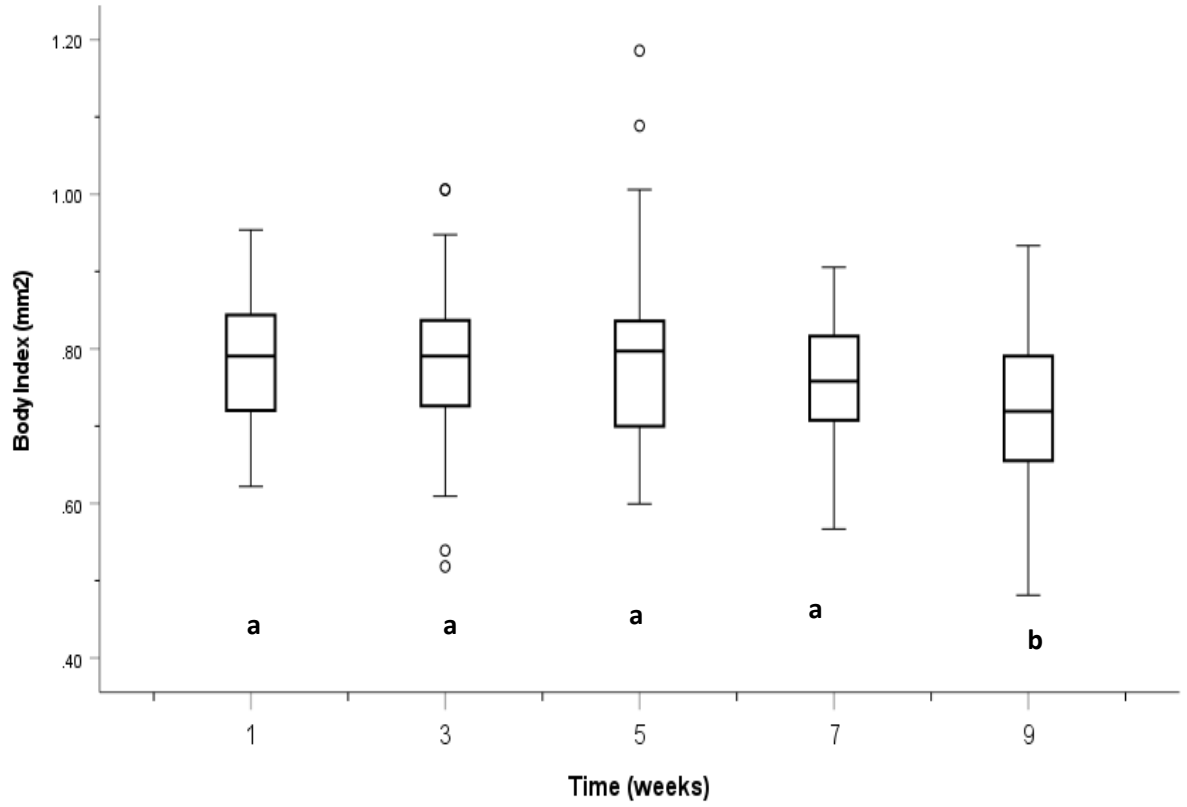


Fig 2.11 The Body index (product of the body length and width) of nymphal *Ixodes ricinus* collected from the field at a single time point and starved for 9 weeks in a cooled incubator at 15 °C and 80% RH and measured at different time intervals. Lower case letters above x-axis denote statistically homogenous groups ($P < 0.05$) as determined by Bonferroni multiple comparison *post-hoc* tests. Middle line = mean, box = mean ± SD, y min = mean – 3 SD, y max = mean + 3 SD, outliers = °, n = 50.

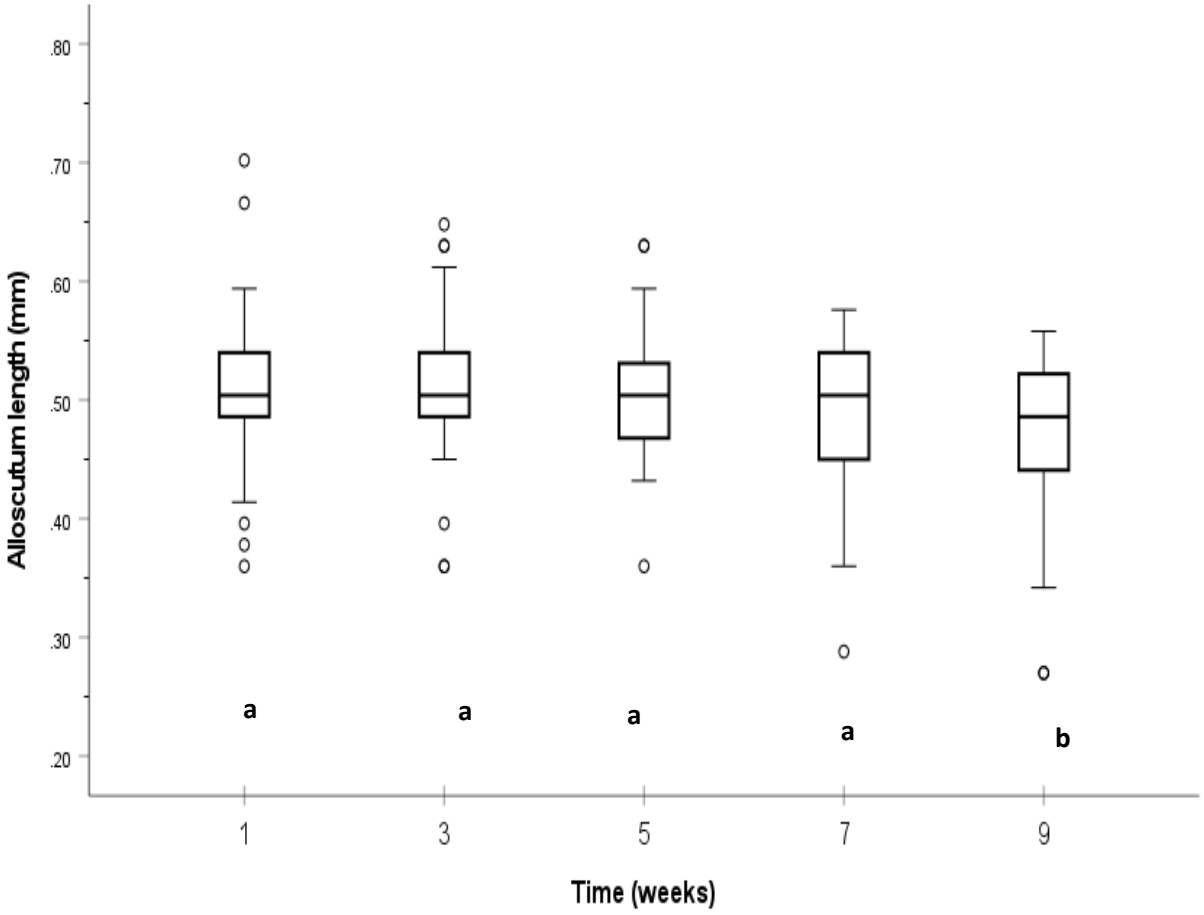


Fig 2.12 The Alloscutum length of nymphal *Ixodes ricinus* collected from the field at a single time point and starved for 9 weeks in a cooled incubator at 15 °C and 80% RH and measured at different time intervals. Lower case letters above x-axis denote statistically homogenous groups ($P < 0.05$) as determined by Bonferroni multiple comparison *post-hoc* tests. Middle line = mean, box = mean ± SD, y min = mean – 3 SD, y max = mean + 3 SD, outliers = °, n = 50.

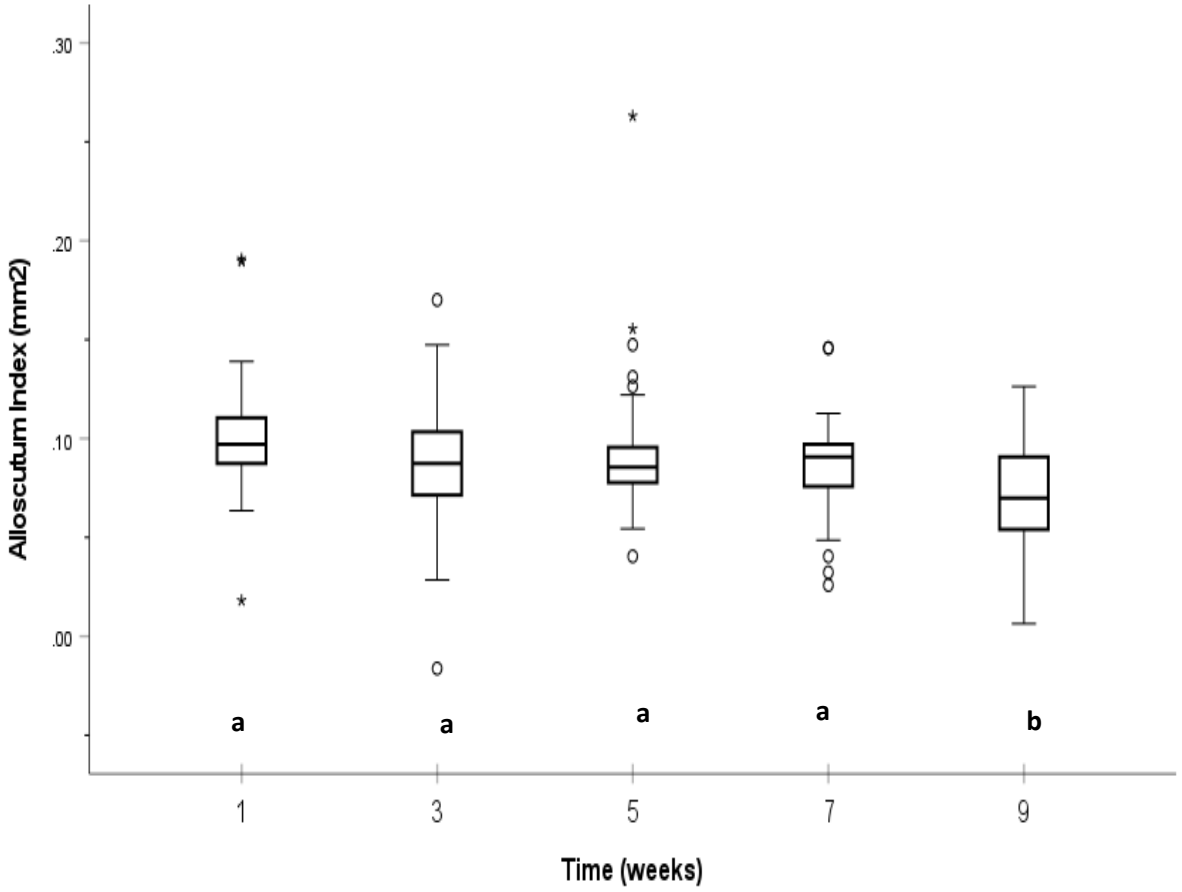


Fig 2.13 The Alloscutum index of nymphal *Ixodes ricinus* collected from the field at a single time point and starved for 9 weeks in a cooled incubator at 15 °C and 80% RH and measured at different time intervals. Lower case letters above x-axis denote statistically homogenous groups ($P < 0.05$) as determined by Bonferroni multiple comparison *post-hoc* tests. Middle line = mean, box = mean ± SD, y min = mean – 3 SD, y max = mean + 3 SD, outliers = * °, n = 50.

There was a significant decline in hunger index over time (GLM: $F_{4,239} = 8.08$; $R^2 = 0.12$, $P < 0.001$) which changed from a mean of 2.60 (± 0.30) in week 1 to 2.28 (±0.25) in week 9, an overall decline of 11.54% (Fig 2.14). Post-hoc tests showed that tick batches from week 1 had significantly higher HI values when compared with weeks 3 and 9 respectively (1:3: $t = 0.16$, $P = 0.036$; 1:9: $t = 0.32$, $P = < 0.001$). All other comparisons were not significant (Fig 2.14).

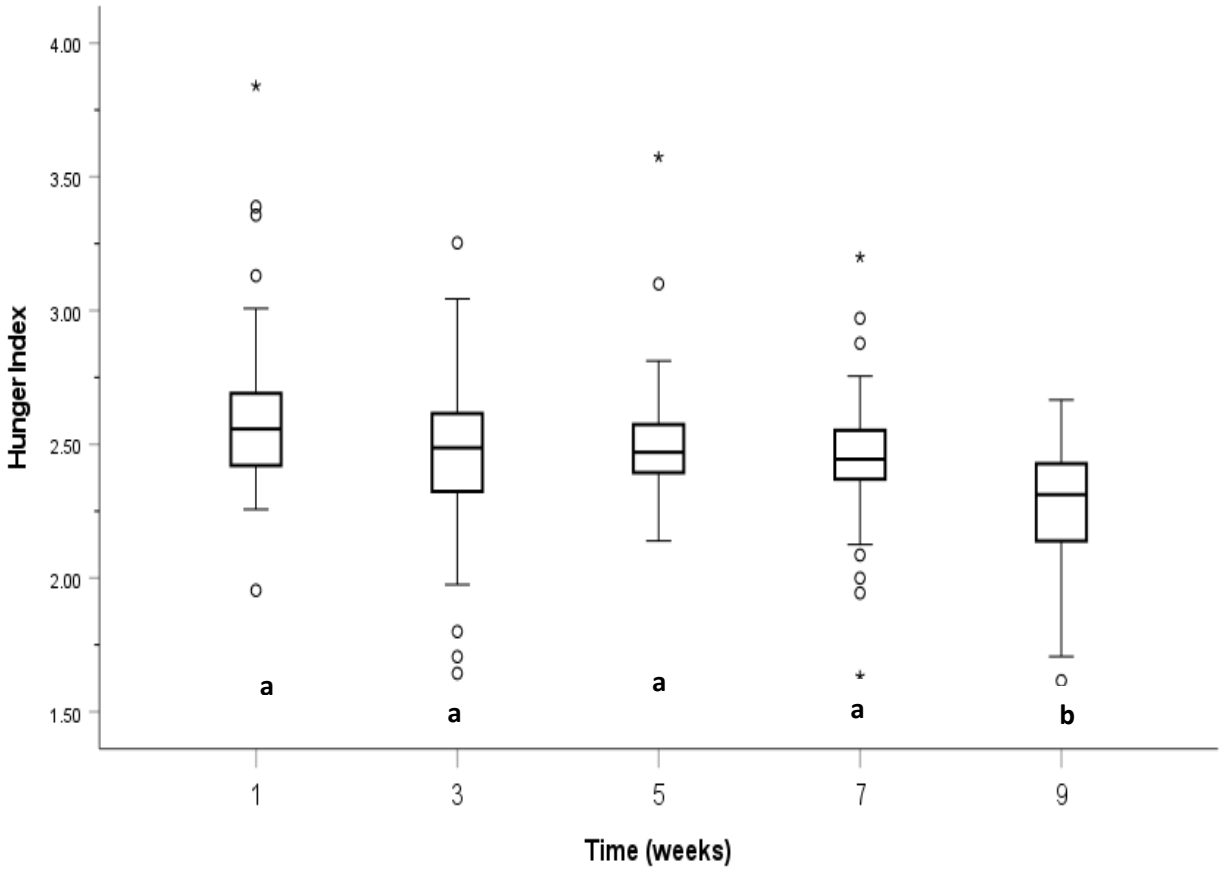


Fig 2.14 The hunger Index (ratio of the alloscutum Index/scutal index) of nymphal *Ixodes ricinus* collected from the field at a single time point and starved for 9 weeks in a cooled incubator at 15 °C and 80% RH and measured at different time intervals. Lower case letters above x-axis denote statistically homogenous groups ($P < 0.05$) as determined by Bonferroni multiple comparison *post-hoc* tests. Middle line = mean, box = mean ± SD, y min = mean – 3 SD, y max = mean + 3 SD, outliers = * °, n = 50.

2.3.5 Hunger Index frequency distributions

At week 1, the skewness of the distribution of hunger index in observed nymphs was found to be 1.83 and the kurtosis was 2.15 indicating that the distribution was positively skewed. (Fig 2.15). At week 3, the skewness was -0.34 while the kurtosis was 1.20, indicating that the values were relatively normally distributed (Fig 2.16). At week 5, the skewness of the distribution was found to be 2.46 and the kurtosis was 9.74 indicating a positive skew (Fig 2.17). At weeks 7 and 9, the skewness and kurtosis were - 0.28, and 3.21(Fig 2.18) and -0.80 and 0.56 (Fig 2.19) respectively, indicating that the distributions were relatively normally distributed.

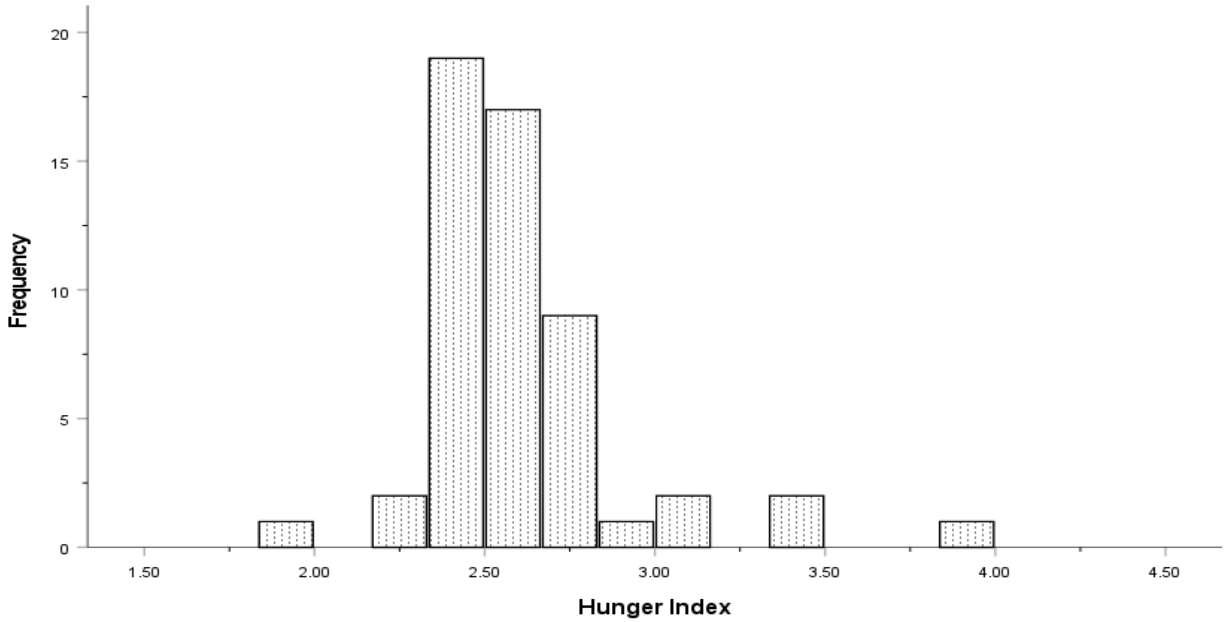


Fig 2.15 The frequency distribution of observed Hunger Index values at week 1 derived from measured linear anatomical measurements in a sample of 54 nymphal *Ixodes ricinus* collected in February 2021 at a single time point and starved for 10 weeks in a cooled incubator at 15 °C and 80% RH, n = 50.

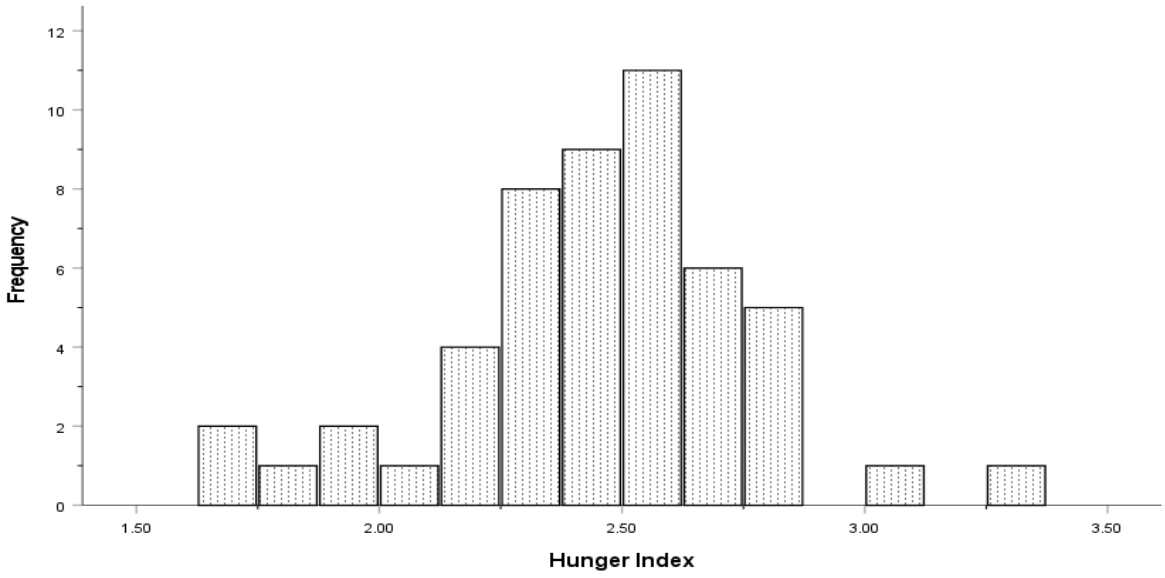


Fig 2.16 The frequency distribution of observed Hunger Index values at week 3 derived from measured linear anatomical measurements in a sample of 54 nymphal *Ixodes ricinus* collected in February 2021 at a single time point and starved for 10 weeks in a cooled incubator at 15 °C and 80% RH, n = 50.

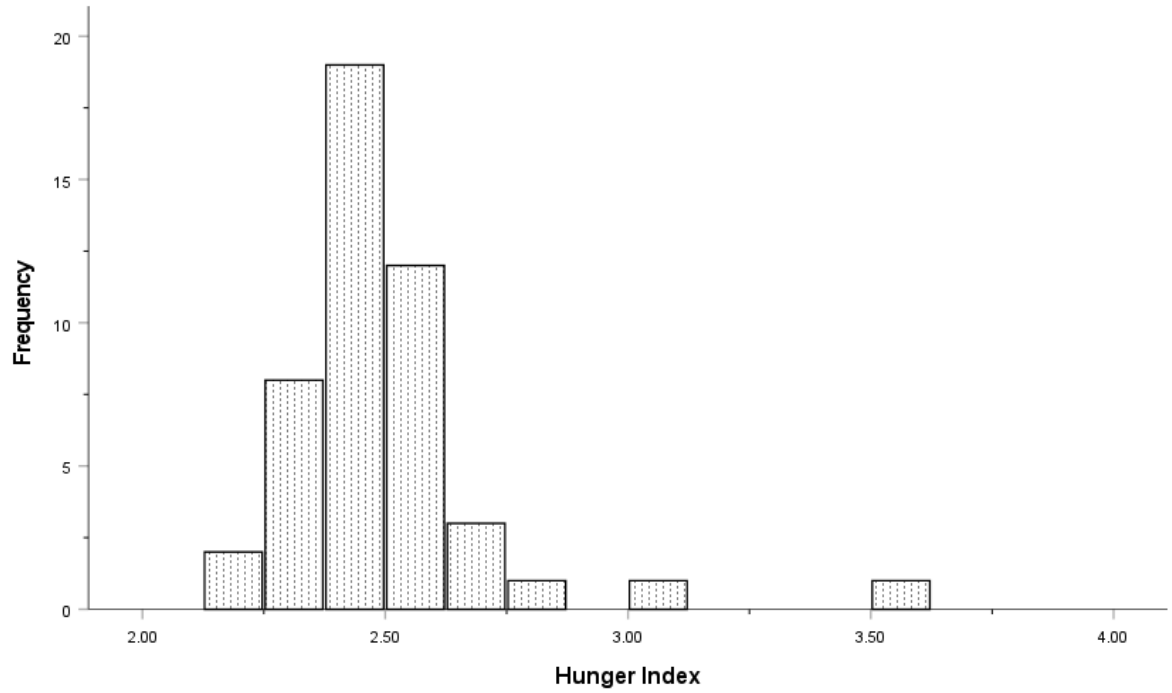


Fig 2.17 The frequency distribution of observed Hunger Index values at week 5 derived from measured linear anatomical measurements in a sample of 54 nymphal *Ixodes ricinus* collected in February 2021 at a single time point and starved for 10 weeks in a cooled incubator at 15 °C and 80% RH, n = 50.

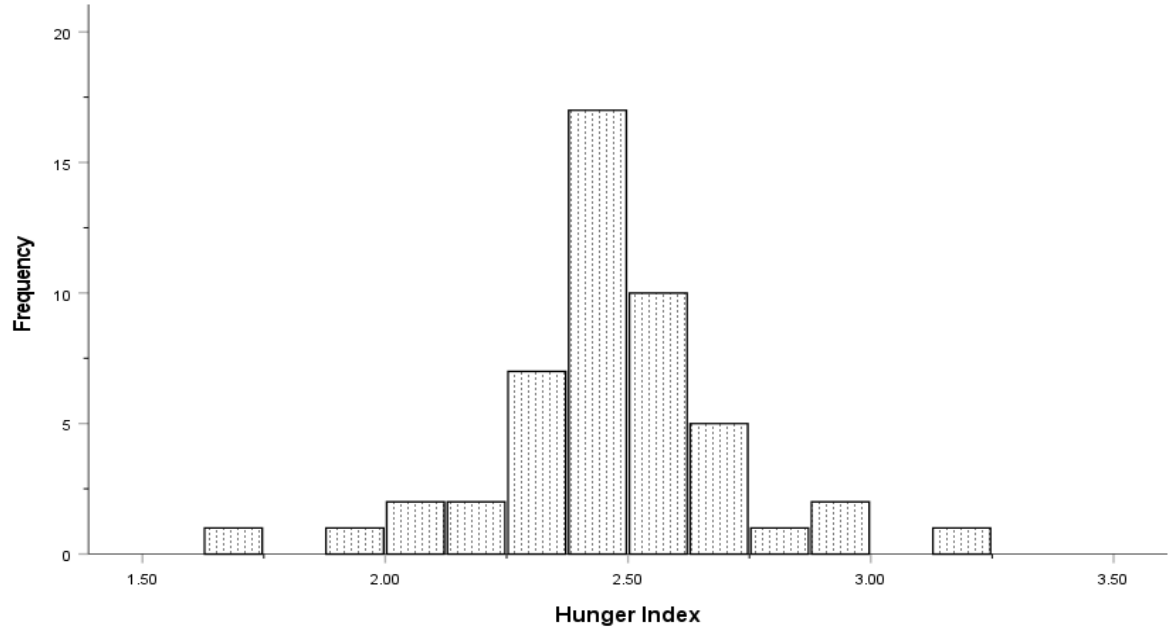


Fig 2.18 The frequency distribution of observed Hunger Index values at week 7 derived from measured linear anatomical measurements in a sample of 54 nymphal *Ixodes ricinus* collected in February 2021 at a single time point and starved for 10 weeks in a cooled incubator at 15 °C and 80% RH, n = 50.

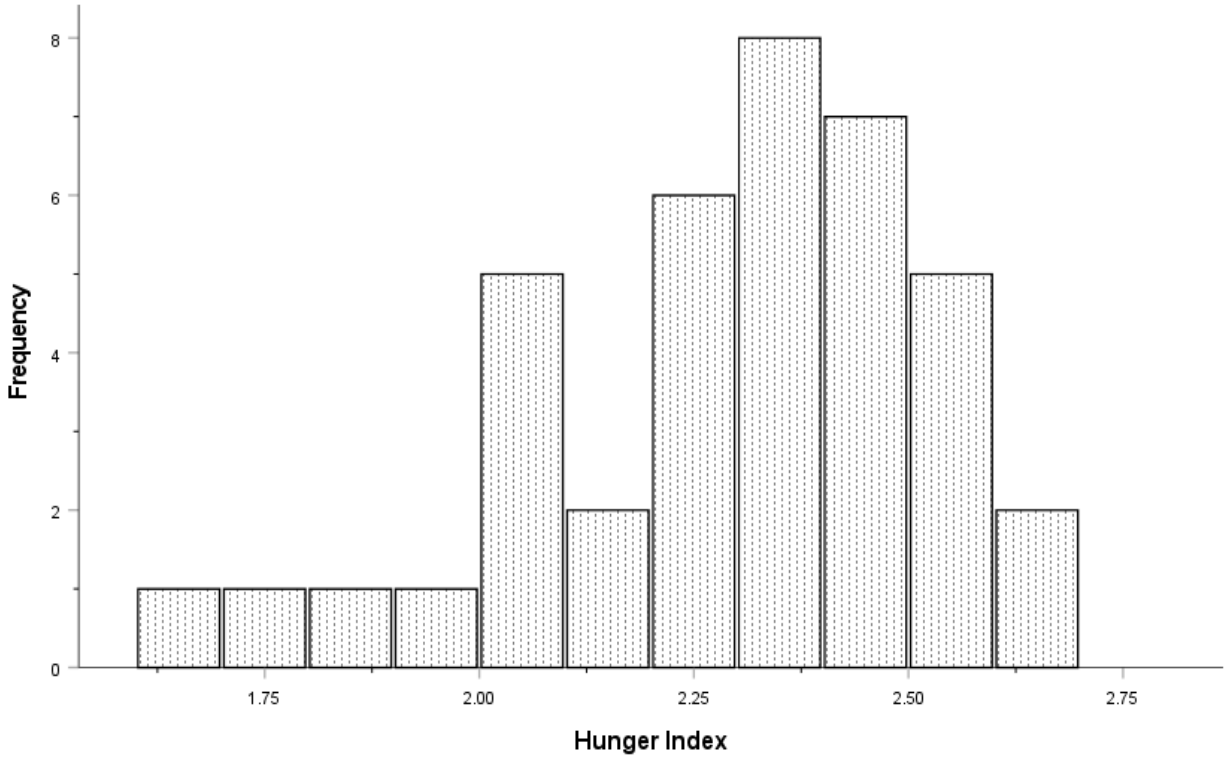


Fig 2.19 The frequency distribution of observed Hunger Index values at week 9 derived from measured linear anatomical measurements in a sample of 54 nymphal *Ixodes ricinus* collected in February 2021 at a single time point and starved for 10 weeks in a cooled incubator at 15 °C and 80% RH, n = 50.

2.3.6 Relationship between tick body measurements with lipid concentration

There were no significant correlations between lipid content of the sampled ticks and scutum length, scutum width, body length, body width, alloscutum length, body index, scutal index, alloscutum index or body weight (Table 2.3). However, there was a weak, but significant positive correlation between Hunger Index and lipid concentration (Table 2.3; Fig 2.20).

Table 2.3 Spearman's rank correlation statistics for the relationship between tick body measurements and Lipid (µg) for nymphal *Ixodes ricinus* collected at a single time point and starved for 9 weeks in a cooled incubator maintained at 15 °C and 80% RH, with correlation coefficient (R_s) and significance of the correlation (P*)*

Body Measurement	R_s	P
SL	-0.08	0.292
SW	0.02	0.845
BL	0.05	0.511
BW	0.16	0.051
AL	0.10	0.192
BI	0.09	0.244
SI	-0.04	0.584
AI	0.15	0.064
Body Weight	0.15	0.064
HI	0.20	0.01*

*Indicates statistically significant ($P < 0.05$) values

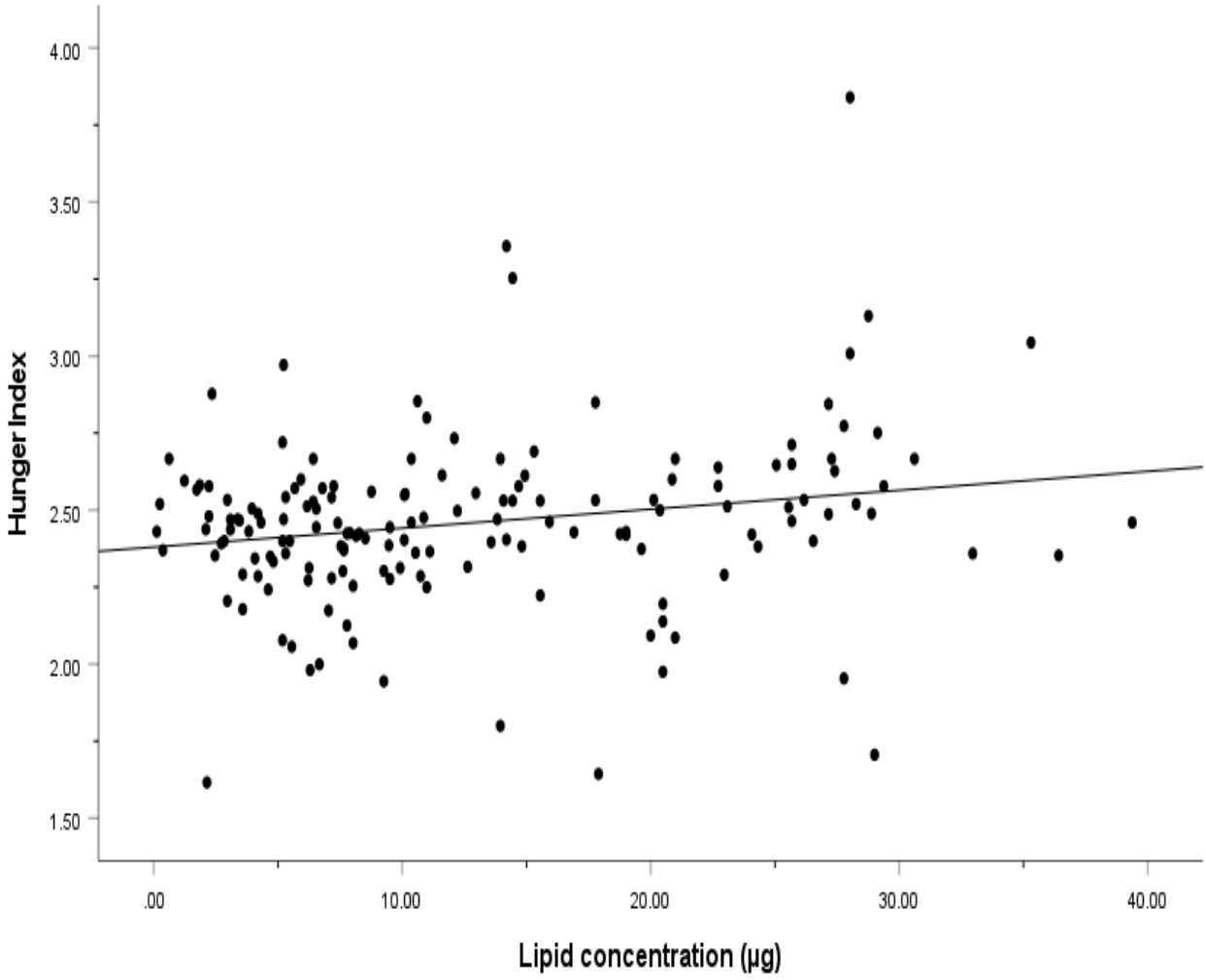


Fig 2.20 The Hunger Index of nymphal *Ixodes ricinus* at different lipid concentrations collected from the field at a single time point and starved for 9 weeks in a cooled incubator at 15 °C and 80% RH (linear regression fitted: y = 2.38 + 6.14E-3*x), n = 30.

2.3.7 Hunger Index and lipid concentration in spring and autumn

The autumn cohort of ticks had a mean hunger index of 1.61 (± 0.27), and the spring cohort had a mean hunger index of 1.46 (± 0.15). The frequency distribution of the hunger index of ticks collected in spring was not skewed (skewness = 0.49). For the autumn collected ticks, the frequency distribution was positively skewed and had more outliers when compared to a normal distribution (skewness = 1.35, kurtosis = 2.72). A Kolmogorov-Smirnov test showed there was a significant difference in the distribution of the hunger index of ticks between the autumn and spring cohorts ($Z = 2.45$, $df = 198$, $P < 0.001$).

For the ticks collected in the autumn, the median lipid content was 23.1 μg, (range 26.6 μg) between a minimum value of 9.30 μg and a maximum value of 35.90 μg. The frequency distribution of lipid content of the autumn cohort was not skewed (skewness = 0.01) (Fig 2.21). For the ticks collected in spring, the median lipid content was 18.8 μg, (range 33.85 μg) between a minimum value of 7.11 μg and a maximum value of 40.96 μg. The frequency distribution showed the lipid contents of the spring cohort displayed a moderate positive skew (skewness = 0.67, df = 198) (Fig 2.24). The lipid values for the spring cohort appeared to follow a bimodal distribution, showing the spring cohort consisted of both well fed and starving nymphs. A Kolmogorov-Smirnov test showed there was a significant difference in the distribution of the lipid content of ticks between the autumn and spring cohort ($Z = 2.45$, df = 198, $P < 0.001$). A Mann Whitney U-test showed the lipid content of the autumn cohort was significantly higher than that of the spring cohort ($U = 15389$, $P < 0.001$), giving evidence that the autumn cohort consisted of a greater proportion of well-fed ticks than the spring cohort

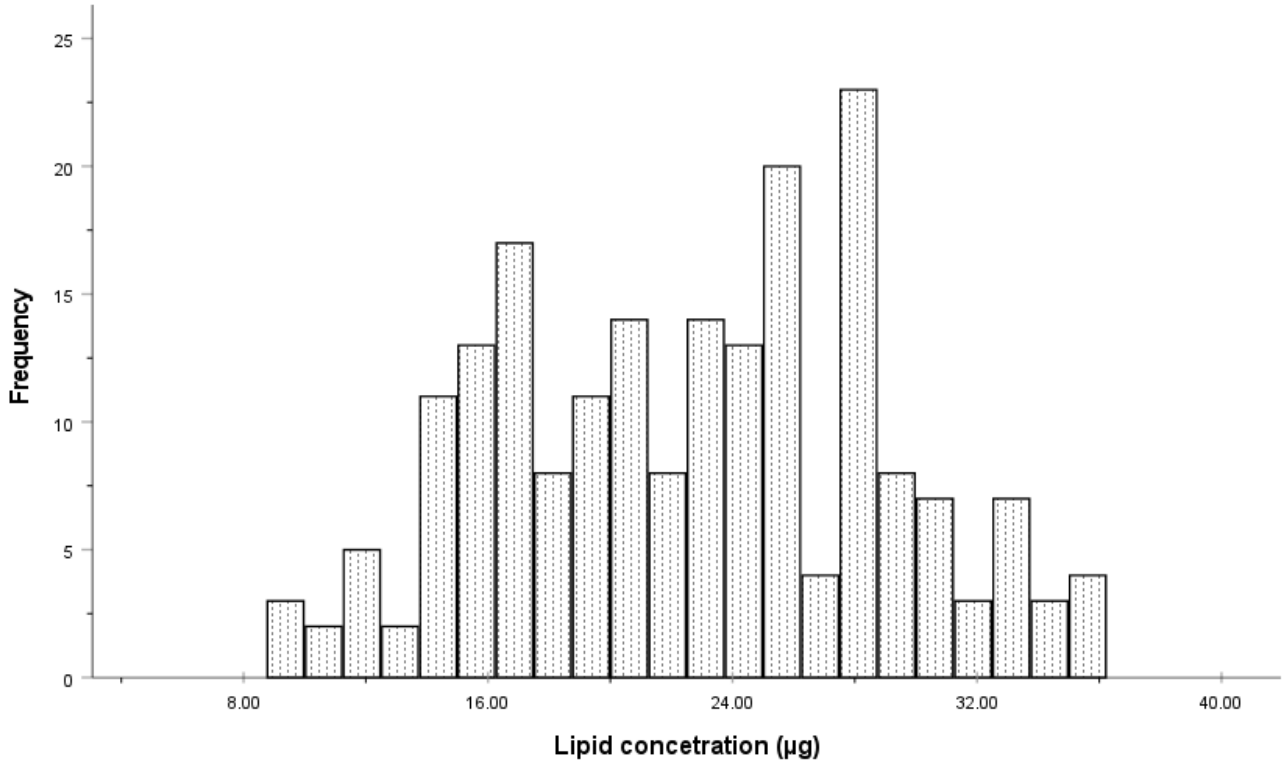


Fig 2.21 The frequency distribution of the lipid concentration (µg) of an autumn cohort of *Ixodes ricinus* nymphs collected by blanket dragging in October 2020 at Dolbury Warren, n = 200.

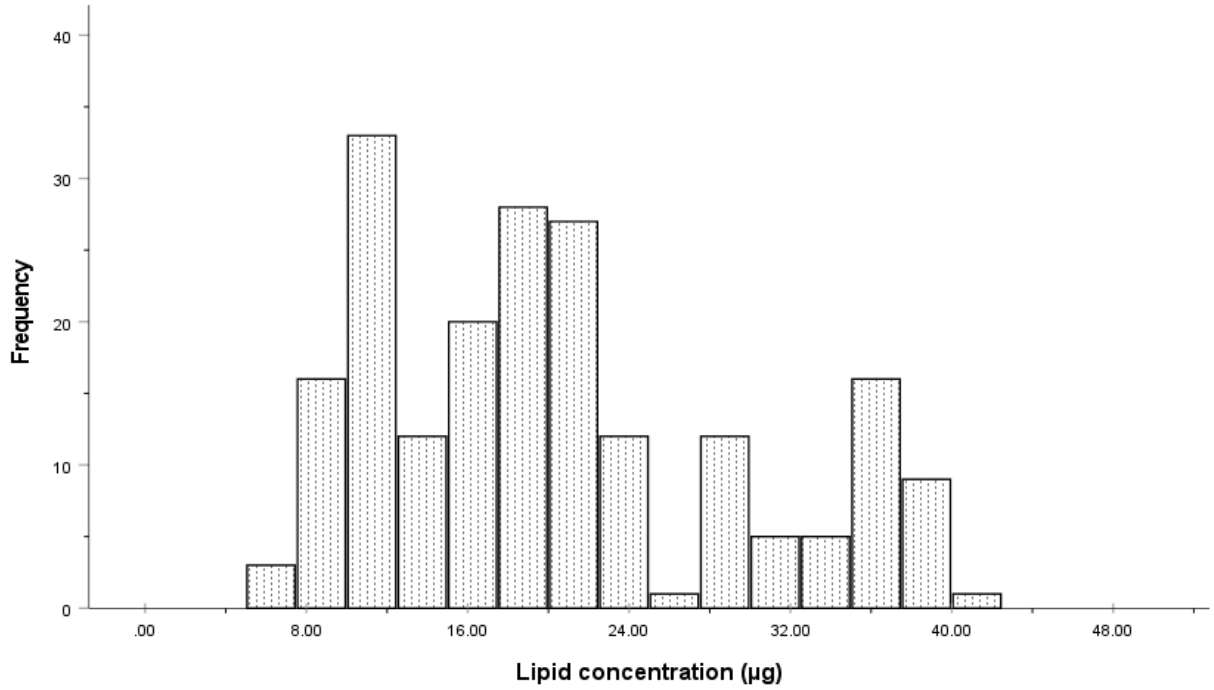


Fig 2.22 The frequency distribution of the lipid concentration (µg) of a spring cohort of *Ixodes ricinus* nymphs collected by blanket dragging in February 2021 at Ashton Court Estate, n = 200.

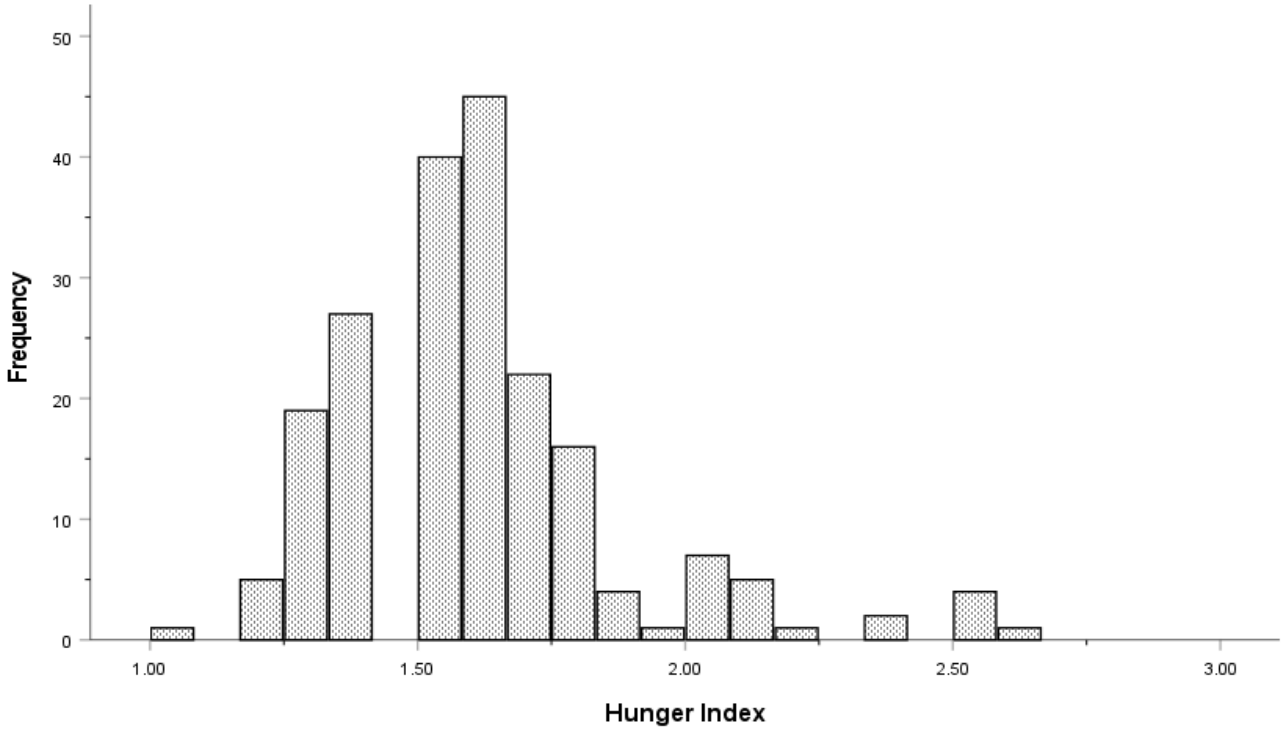


Fig 2.23 The frequency distribution of the hunger index of an autumn cohort of *Ixodes ricinus* nymphs collected by blanket dragging in October 2020 at Dolbury Warren, n = 200.

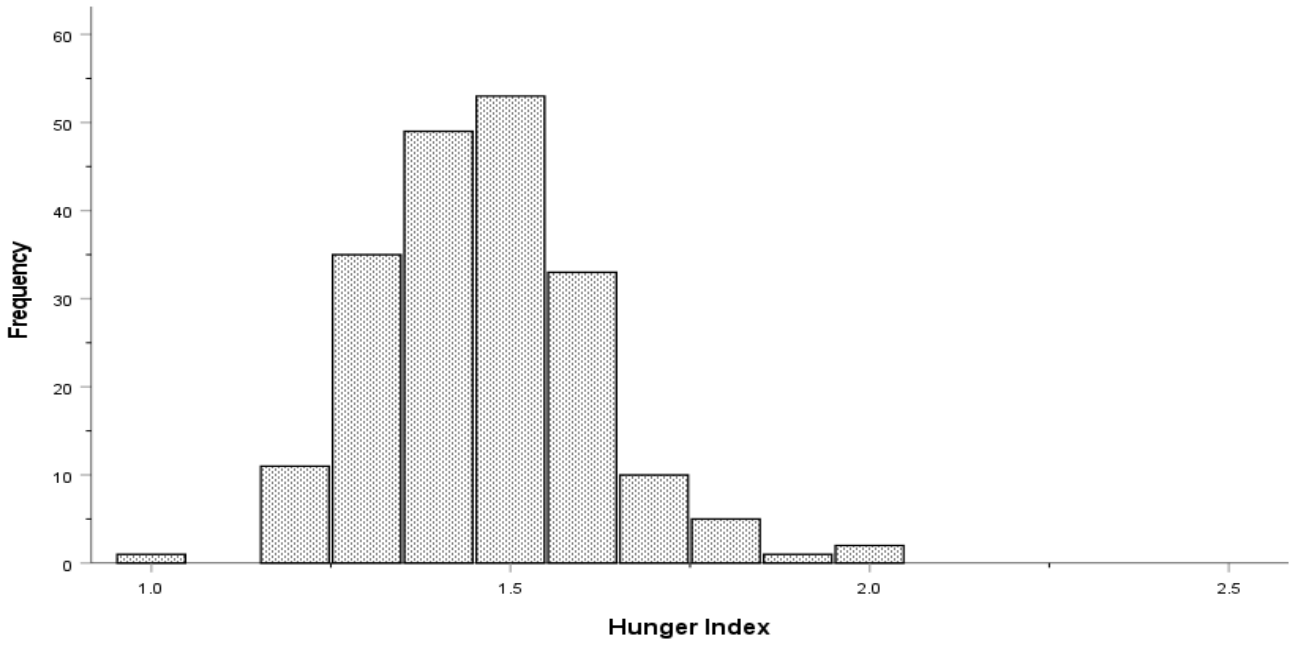


Fig 2.24 The frequency distribution of the hunger index of a spring cohort of *Ixodes ricinus* nymphs collected by blanket dragging in February 2021 at Ashton Court Estate, n = 200.

2.4 Discussion

To address the question of the impact of starvation on tick lipid values and linear anatomical parameters, collected nymphs were maintained in a controlled environment akin to climate conditions in their most active states, the spring and autumn. The saturation deficit in spring and autumn has been reported to vary between 1.5 to 2.5mmHg, with an average of 2.5mmHg (Sands *et al.*, 2021). Hence, 15 °C and 80 %RH, representing a SD of 2.5mmHg, were used in this study and this was adequate to ensure survival of the nymphs throughout the study period. Similar studies have reported that *I. ricinus* nymphs were able to survive for up to 3 months with minimal mortalities in similar temperature and humidity conditions (Hermann and Gern, 2014; Medlock *et al.*, 2014). Biweekly tick body measurements and lipid analysis of randomly selected nymphs were carried out throughout the study.

As expected, the scutum length (SL) and width (SW) remained stable as starvation progressed (Jensen and Kauffman, 2003; Uspensky *et al.*, 2006). This finding also validates the application of the scutum index (being the product of the scutum length and width of each individual tick) as the index for the immovable or fixed part of the alloscutum in the formula for calculating the hunger index (HI) as a possible predictor for the feeding history of an individual tick.

The body length (BL) and width (BW) were also considered when measuring tick body parameters. According to previous studies, these measurements reflected the changing parts of the alloscutum where the differences in the tick's external anatomy due to hunger or starvation were observed. In the present study, there was no significant change in the body length with time. This was different from the findings of other researchers who reported a strong correlation between body length and time of tick feeding (Balashov, 1998; Yeh *et al.,* 1995; Uspensky *et al.*, 2006). In the present study, unfed nymphs of *I. ricinus* were used while unfed adult *I. persulcatus* were used in Uspensky's study. The instar and species difference could have accounted for the difference in the results for the scutum length, suggesting that the anatomical characteristics of ixodid ticks may be species specific. However, this is considered unlikely.

The body width (BW) of the ticks and the body index (BI), which was a product of the body length and width, however, decreased significantly with starvation possibly because of depletion of the tick's body reserves with increasing hunger. Furthermore, the alloscutum length (AL) being the measure of the midline distance from the posterior tip of the scutum to the opisthsoma was observed to also decrease significantly with starvation.

The body weight of the ticks was also measured over the course of starvation and in the study, was observed to decrease significantly with starvation. A similar decrease in dry body mass with starvation was reported by Rosendale *et al.* (2018) The size of the structures in unfed ticks have been reported to vary over a wide range (Uspensky, 1995). Tick body size has also been reported to depend on the quality of the previous blood meal, which is influenced by several factors including host species, immunological status of the host, sex etc (Jensen and Kauffmann, 2003). Body size is also considered to have a major effect on hunger tolerance in arthropods because smaller individuals have both less body reserves and a higher metabolic rate. This could lead to earlier expression of the behavioural consequences of starvation (Gergs and Jager, 2014; Scharf, 2016). The findings from this study show that starvation appears to have a significant impact on the body size of *I. ricinus* nymphs.

The Hunger Index value which was derived from the ratio of alloscutum index and scutum index decreased significantly with starvation. This was expected since the alloscutum index was observed to gradually reduce in value while the scutum index remained constant. This agreed with the findings of Uspensky *et al.* (2006) with respect to the physiological age index in their study, also derived from the same variables. It is important to highlight that in this study, batches of about 50 nymphs were selected randomly at defined intervals to establish these observed changes, and this was suitable to make conclusions about the changes in tick body measurements with starvation at population level. Perhaps, repeated observation of a specific individual over time could lend more credence to the HI values recorded in this study or highlight peculiar changes with individual ticks which may have been masked by extrapolating results from a batch. This was however outside the scope of the current study but opens up an area for further research in the future.

The frequency distribution of hunger index values was also observed biweekly throughout the study period. In the first week of the study, the distribution was observed to be positively skewed with a greater number of ticks having high HI values. This was expected as, considering the data for the lipid concentration which would be discussed subsequently, the results showed that a greater percentage of ticks in the selected batch recorded higher lipid values than low values. As starvation progressed, the distribution of hunger index values tended towards a normal distribution, albeit with more outliers by the 9th week of the study increasing the variance around the mean of the distribution by the end of the study.

The results showed a clear trend that the lipid concentration of the ticks decreased with starvation and agrees with the findings of several other studies (Randolph *et al.*, 2002; Abdullah *et al.*, 2018; Alasmari and Wall, 2020). Evaluating the frequency distribution showed that the sampled

batch at week 1 had larger number of ticks with high lipid values at the beginning of the study. Within the first five weeks of the study, the lipid values became more normally distributed around the mean, suggesting that most of the ticks with extremely low values had either been removed for analysis, or more likely had died. By the 7th week of the study, ticks having lipid values less than 20 μg were predominant, with only a few outliers with lipid content above 20 μg present. By the 9th week of the study, the impact of starvation on tick lipid concentration was highlighted with most of the sampled ticks having lipid values below 15 μg and recording a median of 7.68 μg. This would most likely be due to depletion of reserves as they by expended energy on maintaining metabolic processes such as walking etc (Alasmari and Wall, 2020). It was also observed that physical activity e.g walking was seen to decrease with starvation, although this was not quantified. At the beginning of the study, it was not uncommon to find ticks actively moving about within the starving tubes during routine observations. Activity levels however decreased with starvation and by the 9th week of the study, the ticks were almost immobile and needed to be prodded to elicit physical activity.

To evaluate the possible application of tick body measurements as predictors for the feeding history or hunger status of a tick, the interaction between the linear parameters measured in the study and the lipid concentration of each individual tick was explored. Lipid content had been shown to decrease with starvation and in previous studies and had been established as a good predictor of feeding history of a tick (Lehane, 1991; Randolph and Storey, 1999; Pool *et al.,* 2017; Rosendale *et al.,* 2017). To achieve this, lipid analysis of each tick was carried out immediately after the different body measurements. Although the Body Width, Body Index, Alloscutum Index and Body Weight were observed to change with starvation, there was no correlation between these parameters and the lipid concentration of the ticks.

The data showed that Hunger Index values, which also declined with time, did correlated significantly with the lipid concentration of the nymphs. However, this relationship appeared to be weak and highly variable. A problem with this sort of study is that the hungriest ticks have a higher probability of dying than the least hungry, so the distribution within the sample is increasingly corrupted over time. Extending the study beyond 9 weeks was considered, but this may not have improved the levels of variability in the data set, making it harder to make predictions about the interaction of the two variables. Increasing the sample size would have been desirable to reduce this variation.

The results also showed that ticks collected during the autumn season of peak abundance had significantly higher lipid values than ticks collected in the spring. This suggested that most of the questing population of nymphs collected in autumn would have depleted very little of their

lipid reserves at the point they were collected. Based on the current explanation of the dynamics behind the seasonal activity pattern of ticks involving two separate subpopulations each year with different mechanisms of diapause for over-wintering (Gray, 1991), the higher lipid values recorded in autumn collected nymphs in this study represented nymphs which fed as larvae in the spring earlier that year, moulted to the nymphal stage over the summer and were just beginning their questing activity in the autumn. This was likely driven by the inhibition of post-moulting development or inefficient levels of questing behaviour seen during unsuitable or unfavourable times of the year, such as during the high temperatures or high saturation deficits of the summer.

The distribution of lipid content of the autumn population of ticks used in this study was consistent with the findings of previous studies. Both Randolph *et al.* (2002) and Abdullah *et al.* (2018) carried out monthly lipid analysis, albeit using different methods, for a two-year period, illustrating that ticks collected in September/October had significantly higher lipid values than those collected at any other time of the year. Contrary to these findings, Walker (2001) who also analysed the lipid content of questing ticks over three years found that nymphs collected in the autumn did not have significantly higher lipid values when compared with ticks collected in the spring. However, these results could have been influenced by the difference in methodology for tick lipid estimation; here the gut and Malpighian tubes of the sampled ticks were dissected, and any lipid present was stained. Lipid content was then ranked based on the proportion of the Malpighian tubules and gut that was stained. This was a relatively subjective method of analysis, hence the quantitative methods referenced earlier likely produced a more accurate result.

There was a significant difference in the hunger Index (HI) between ticks collected in the spring and autumn. The autumn ticks had a higher Hunger Index value than ticks collected in the spring. A higher Hunder Index value implied that these ticks were better fed than the spring collected ticks. This result corroborated the finding of higher lipid reserves observed in autumn collected ticks when compared with those collected in the spring.

The data from this study also adds clarity to the debate over the feeding history of the nymphs questing in spring. A bimodal distribution was observed when the distribution of the lipid content of spring-collected ticks was considered. A considerable number of ticks in the sampled population had low lipid values between around 8 μg and 20 μg, while a smaller portion of ticks possessing higher lipid reserves at around 40 μg, were observed. This bimodal distribution demonstrates the presence of two separate feeding cohorts in the spring cohort of questing nymphs comprised of both nymphs that fed as larvae the previous autumn/winter, and nymphs that fed as larvae in the spring/summer. For the first group, these larvae fed before the critical

daylight threshold for diapause and subsequently moulted into the nymphal instar over the summer. They would then have begun questing as nymphs in the autumn but been unsuccessful in finding a host. As a result, questing activity would have continued past this period into the following spring, at which point they were collected. Given the length of time since their previous blood meal, their lipid reserves would have been considerably depleted. This group therefore represent the portion of the spring cohort with lower lipid values in the distribution. The second group (with higher lipid values in the distribution) represent nymphs which had recently fed as larvae the previous autumn, entered into morphogenetic diapause after the critical daylight threshold and emerged as new questing nymphs in the spring at which point they were collected. This would explain the higher lipid reserves in this group of ticks as seen in the distribution. These results therefore support the existence of two separate feeding cohorts of nymphs in the population, one which enters the questing population in the autumn, and the other in the spring. These cohorts appear to be separated by morphogenetic diapause. Other studies have arrived at a similar conclusion about the population dynamics of *I. ricinus* ticks (Gray, 1991; Walker, 2001; Randolph *et al.,* 2002; Gray *et al.,* 2016; Abdullah *et al.,* 2018).

In conclusion, the results of the study indicate that overall, tick lipid values decreased significantly with duration of starvation. The analysis supports the findings from similar studies which reported that to date, the most reliable indicator of time since last feeding is the quantitative measurement of lipid (Abdullah *et al*., 2018; Alasmari and Wall, 2020; 2021). The results also demonstrate a weak positive correlation between lipid concentration and Hunger Index in nymphs. These findings suggest that Hunger Index, depending on the context of the investigation and possibly used in conjunction with lipid analysis, may be used as a proxy for time since last feeding and to predict feeding history of ticks at present, but this would require considerable caution. Its usefulness may be improved in some circumstances, for example by repeated monitoring of individual ticks over time.

- Chapter 3 -

Effects of hunger and saturation deficit on the survival of *Ixodes ricinus*

3.1 Introduction

Humidity and temperature are particularly important determinants of tick survival for two reasons. First, *I. ricinus* ticks possess a high surface area to volume ratio which makes them susceptible to dehydration during the period they spend off-host, particularly while active during questing. During questing, the tick loses moisture and so must descend the vegetation into the mat layer to rehydrate; descent into mat layer reduces the chances of encountering a host. They obtain water through blood feeding but also through absorption from environmental vapour through their spiracles (Randolph *et al.,* 1999). Second, survival is also dependent on the availability of metabolic resources, particularly lipid, the depletion of which is highly temperature dependent (Alasmari & Wall, 2021). The interaction of these two variables is usually characterised as the saturation deficit (SD), which is defined as a measure of the drying power of the air. SD has been reported to limit duration of questing (Perret *et al.,* 2000; 2004; Burri *et al.*, 2007), but comprehensive analysis of their combined effects over time are lacking, particularly in relation to changing levels of resource availability (Lees,1946; Milne, 1950; Hermann and Gern, 2010; Wongnak *et al.,* 2022). A key question, therefore is: are increasingly resource depleted ticks more or less affected by climatic conditions?

Studies to address this question are important because changes in temperature and humidity associated with climate changes will be expected to have a major impact on *I. ricinus* phenology either as a direct result of the effects of abiotic factors, or indirectly due to climate-induced changes in vegetation patterns which may alter potential host population density in certain areas (MacLeod, 1935; Milne, 1949; McCoy *et al.*, 2013). The overarching aims of the work described in this Chapter therefore were to assess the impact and interaction of the abiotic factors,

temperature and humidity, on tick survival, particularly in relation to changes in resource availability (hunger).

3.2 Materials and Methods

3.2.1 Tick Collection

Ixodes ricinus nymphs were collected from the Ashton Court Estate by blanket dragging, as described in Chapter 2. Collected ticks were then transported to the laboratory where they were placed individually in labelled 0.5ml Eppendorf tubes closed by cloth mesh held in place by an elastic band and used as described below.

3.2.2 Effects of Saturation deficit on *Ixodes ricinus* survival

Different saturation deficit conditions used for the study were created within glass desiccators (Fisher Scientific) by placing them into a humidified incubator (MLR-35IH, Sanyo, Panasonic, Loughborough, UK) set at a specific temperature. Five different temperature ranges were used in the study to produce the respective saturation deficits within the desiccator: 4°C, 15°C, 20°C, 25°C and 30°C (Table 3.2). In addition, each desiccator contained a specific concentration of potassium hydroxide (KOH) solution for the purpose of producing a known relative humidity (RH) within the chamber. As described by Solomon (1951), the RH was achieved by altering the quantity of KOH added to deionized water to make up a 100ml KOH solution (Table 3.1).

Table 3.1: The amount of potassium hydroxide (g) made up to 100ml with deionised water inside a sealed glass desiccator to create a range of different relative humidity conditions.

KOH (g)	Relative Humidity (%)
5	97
20	85
30	75
50	55
80	25

The resulting solution was poured into the bottom of the desiccator. Two tubes containing six nymphs each were placed into each desiccator on a wire mesh above the liquid. The relative humidity in each desiccator was measured with an EasyLog USB data logger (Lascar Electronics, UK). Saturation Deficit (SD) gives an integrated measure of the drying power of the atmosphere and was calculated using the following formula:

$$SD = (1 - RH/100) \times 4.9463 \times e^{(0.0621 \times T)},$$

where SD represents Saturation Deficit in millimetres of mercury, RH represents Relative Humidity in percent and T represents Temperature (°C) (Randolph *et al.,* 1999; Perret *et al.,* 2000; Knap *et al.,* 2009).

Table 3.2: The temperature (°C), relative humidity (%) and the resultant saturation deficit inside the sealed glass desiccators containing the ticks.

Temperature (°C)	**Relative Humidity (%)**	**Saturation Deficit (mmHg)**
4	97	0.15
15	97	0.31
20	97	0.41
25	97	0.56
30	97	0.77
4	85	0.82
4	75	1.38
15	85	1.63
20	85	2.21
4	55	2.51
15	75	2.74
25	85	3.01
20	75	3.73
20	25	3.84
30	85	4.11
15	55	4.98
25	75	5.08
20	55	6.76
30	75	6.94
15	25	7.62
25	55	9.21
20	25	10.35
30	55	12.59
25	25	14.1
30	25	19.28

For the initial studies of the effect of saturation deficit on tick survival, after being collected from the field, ticks were stored initially at 7°C and then moved to their designated temperature/humidity chamber within 24 h of collection. Ticks were then inspected within each desiccator. Any mortality was recorded daily for the first two weeks of the study, and subsequently every other day untill day 60, when the study was terminated.

The rim of the desiccator was covered by a fine layer of petroleum jelly (Vaseline®, Sigma-Aldrich, UK) to form a seal and, when checking the ticks, the lid was replaced over the jar as quickly as possible after tubes were removed to ensure minimal changes to internal humidity. Ticks were considered dead after a persistent lack of movement over a 5-minute period when the ticks were placed on a flat surface and gently prodded with forceps. Where ticks survived for longer than two weeks, the KOH solution was changed every 2 weeks to ensure humidity stayed at desired level throughout the study.

3.2.3 Effect of hunger on survival of *Ixodes ricinus* ticks at different saturation deficits

To examine the effects of hunger on survival at a range of different saturation deficits, a second cohort of nymphal ticks was collected from the field site. These ticks were placed in a humidified incubator (PHCBI Versatile Environment Test Chamber, PHC Corporation, Japan) at 15 °C and 80 % RH (SD 2.51 mmHg). This environment was adequate to ensure good levels of survival of the ticks throughout the study. Then, after 1, 4 and 8 weeks respectively, 30 ticks were removed, and 6 ticks were placed individually into one of 5 desiccator jars at different saturation deficits of 0.64, 2.51, 5.03, 7.54, 10.5 mmHg. Ticks exposed to SD 2.51 mmHg can be regarded as a control group for the study, since this most closely replicated the conditions under which they had been held immediately after collection from the field. The ticks were checked daily to allow the mortality to be recorded. Once the tick was considered dead (as described above), linear body measurements were immediately recorded, and the lipid content was measured using the vanillin assay as described in Chapter 2. Inspection was continued for 60 days after the ticks were placed into their experimental saturation deficit conditions.

3.2.4 Data Analysis

Data were tested for normality and parametric or non-parametric tests, used accordingly with means or medians presented graphically. A general linear model was used to describe the relationship between saturation deficit, starvation (tick lipid) and tick survival (time to median tick

death in each experimental group). Bonferroni *post-hoc* tests were used to analyse the differences between the pair-wise comparisons of the group means of the tick batches from different starvation time points (groups). Least squares regression was used to fit linear or polynomial lines to significant relationships between survival and temperature, humidity or saturation deficit. Data analysis was performed with the statistical software IBM SPSS Statistics version 28.

3.3 Results

3.3.1 Survival of *Ixodes ricinus* nymphs at different temperature and humidity

Overall, as temperatures increased and relative humidity decreased, tick survival declined, as demonstrated by the significant interaction between temperature and relative humidity in their effect on tick survival (time to median tick death) (GLM $R^2 = 0.72$, $F_{8, 49} = 12.9$, $P < 0.001$). At 4 °C, ticks died in less than 10 days, this pattern persisted until the relative humidity was above 80%, when they lived for the full extent of the study. A similar result was observed at 15 and 20 °C. At 25 °C, the ticks were unable to live beyond 10 days even at 97% relative humidity. At 30 °C, even at the highest humidity, the ticks did not live beyond 5 days (Figs 3.2 a–e).

At 25% RH, ticks survived for a maximum of 5 days even at the lowest temperatures (4 °C). At 20 °C and higher, the ticks died relatively quickly at this humidity, within a period of 2 to 3 days. Similar results were observed at 55, 75 and 85% RH. However, at 97% RH, ticks were observed to survive up to 60 days at the lowest temperature (4°C). As temperature increased, tick mortality increased with ticks at 30 °C living for a maximum of 5 days at this humidity (Fig 3.3 a – e).

The results showed a significant positive effect of saturation deficit (SD) on time to median tick death observed in study groups (GLM: $R^2 = 0.98$, $F_{24, 49} = 160.74$, $P < 0.001$). The data showed that 80% of ticks survived up to a maximum of 4 days in SD 3.73mmHg and above, with highest mortalities occurring on day 2. At lower SDs (≤ 0.5mmHg), ticks were able to survive up to 60 days (Fig 3.4 a – e).

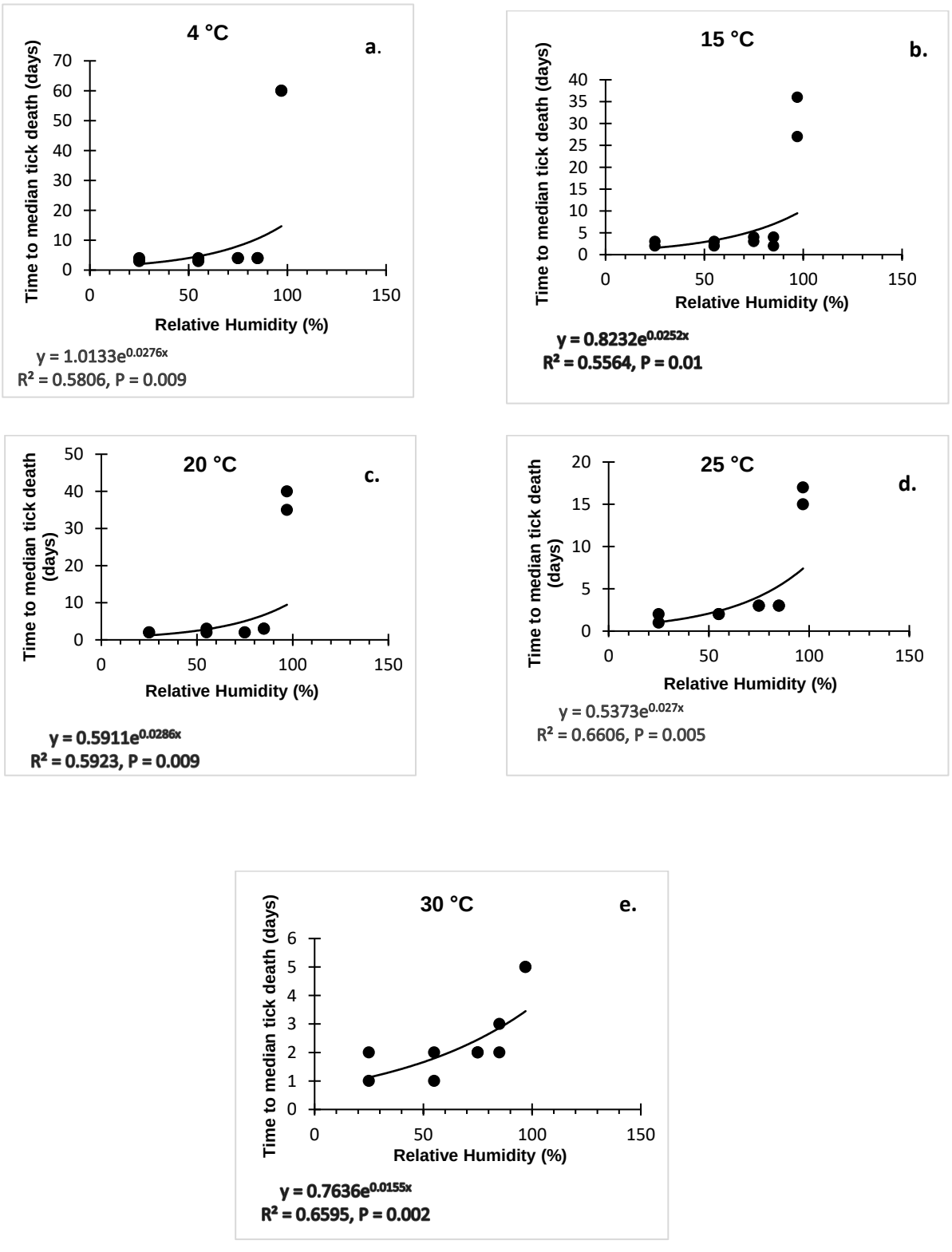
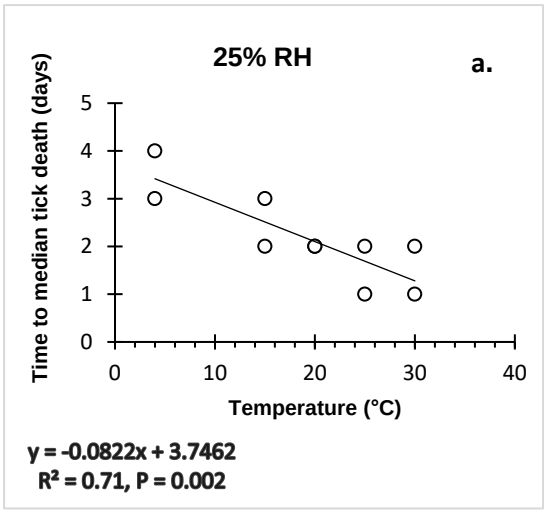
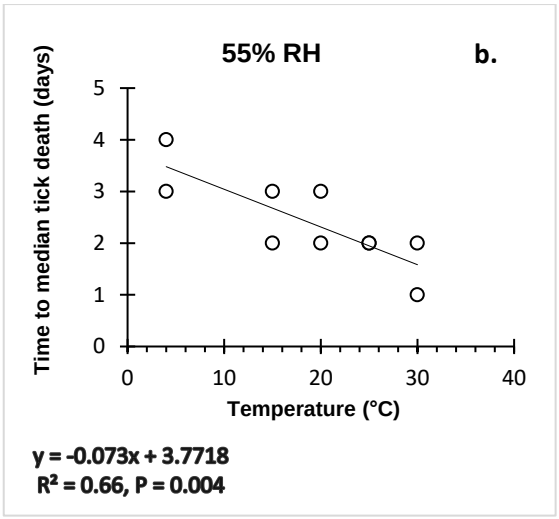
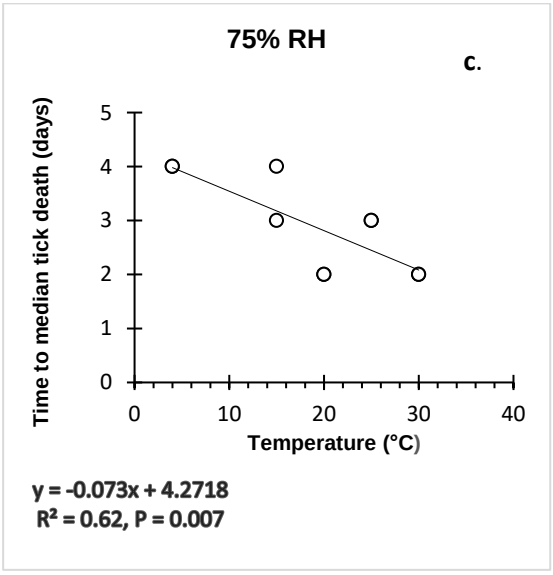
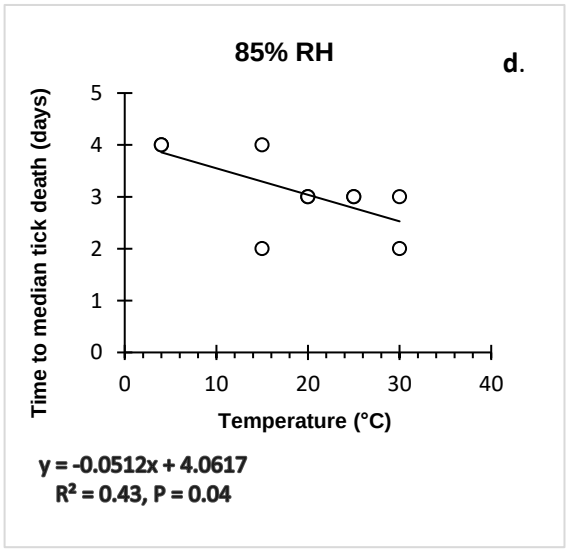


Figure 3.2 (a – e) The time to median tick death (days) observed at different relative humidity conditions in groups of nymphal *Ixodes ricinus* placed in glass desiccators maintained at 4 °C, 15 °C, 20 °C, 25 °C and 30 ºC. Exponential regression line fitted









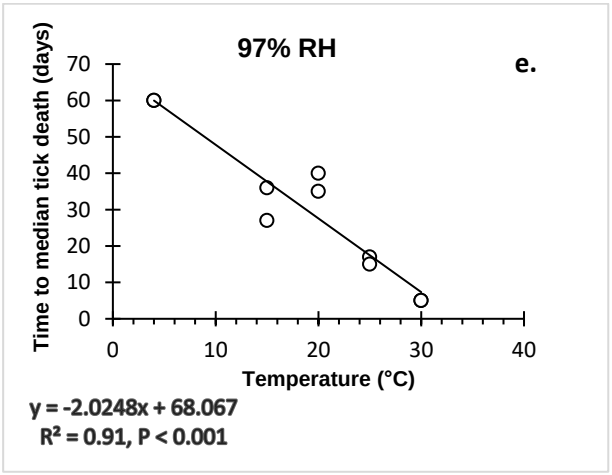


Figure 3.3 (a – e) The time to median tick death (days) observed at different temperatures in groups of nymphal *Ixodes ricinus* placed in glass desiccators maintained at 25%, 55%, 75%, 85% and 97% Relative Humidity. Linear regression line fitted

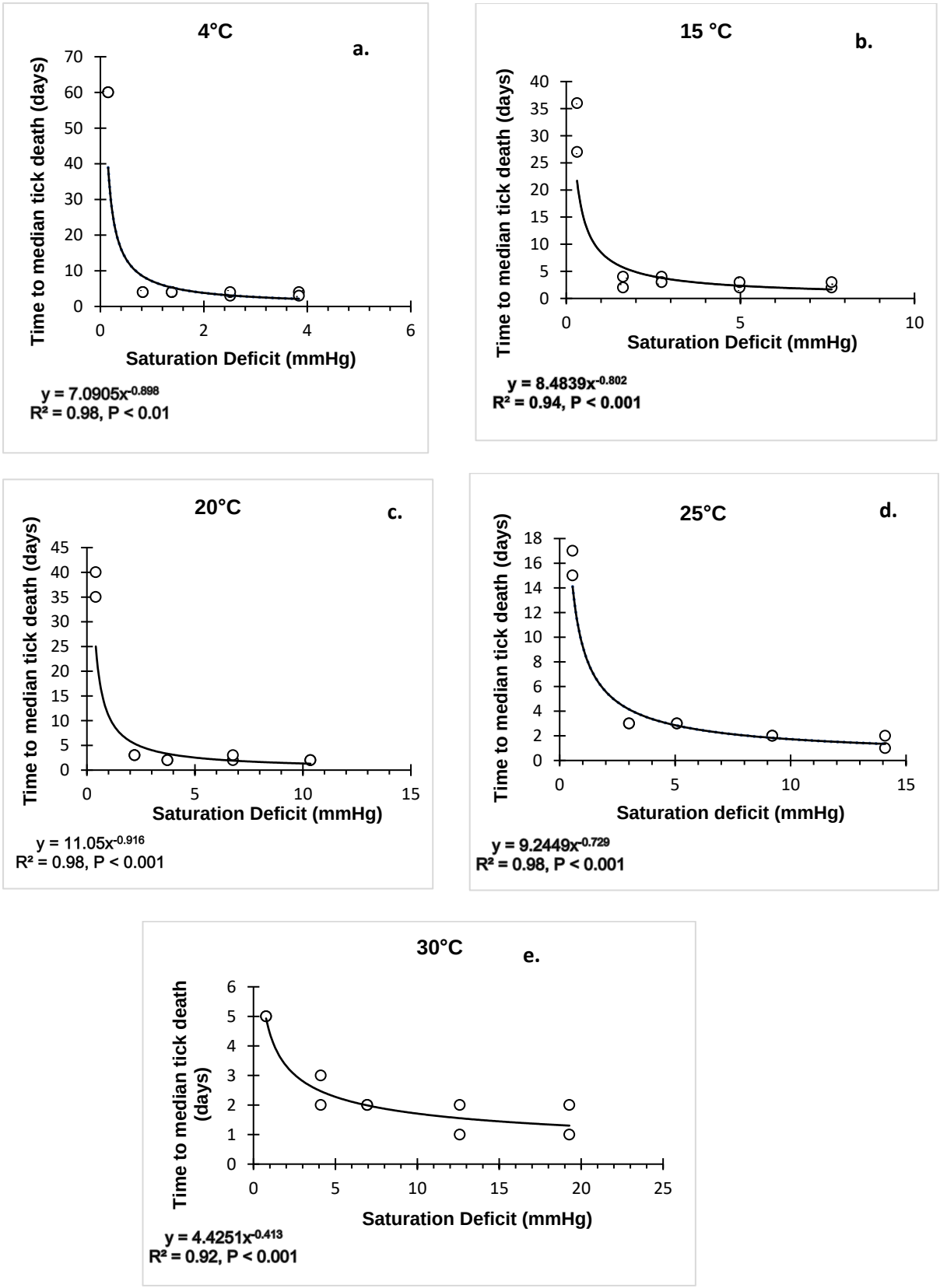


Figure 3.4 (a – e) The time to median tick death in groups of *Ixodes ricinus* nymphs exposed to varying saturation deficit conditions produced in glass desiccators using potassium hydroxide (KOH) solution observed at 4 °C, 15 °C, 20 °C, 25 °C. Power regression line fitted

3.3.2 Effect of starvation on tick survival at different saturation deficits

3.3.2.1 Change in tick survival with starvation at different saturation deficits

Tick batches maintained at saturation deficits of 7.5 and 10 mmHg had significantly higher mortality rates than the ticks at 2.5 mmHg (pairwise comparisons with Bonferroni *post hoc* test: SD 2.5 vs SD 7.5: $t = -15.7$, $P < 0.001$; SD 2.5 vs SD 10.0: $t = -46.3$, $P < 0.001$). 82.2% of the nymphs at a SD of 0.6 mmHg survived for the full duration of the experiment. Survival at the lower SD was not significantly different from SD 2.5 mmHg ($P > 0.05$). But, there was no significant interaction between saturation deficit and starvation time on tick survival (Fig 3.5; GLM $F_{8,88} = 1.006$, $P = 0.439$) showing that, overall, starvation did not affect their response to SD. A significant difference was observed in the group held at a SD of 2.5 (GLM: $R^2 = 0.77$, $F_{14,88} = 17.6$, $P < 0.001$) but since this pattern was not seen in any of the other groups this is unlikely to be a biologically meaningful result.

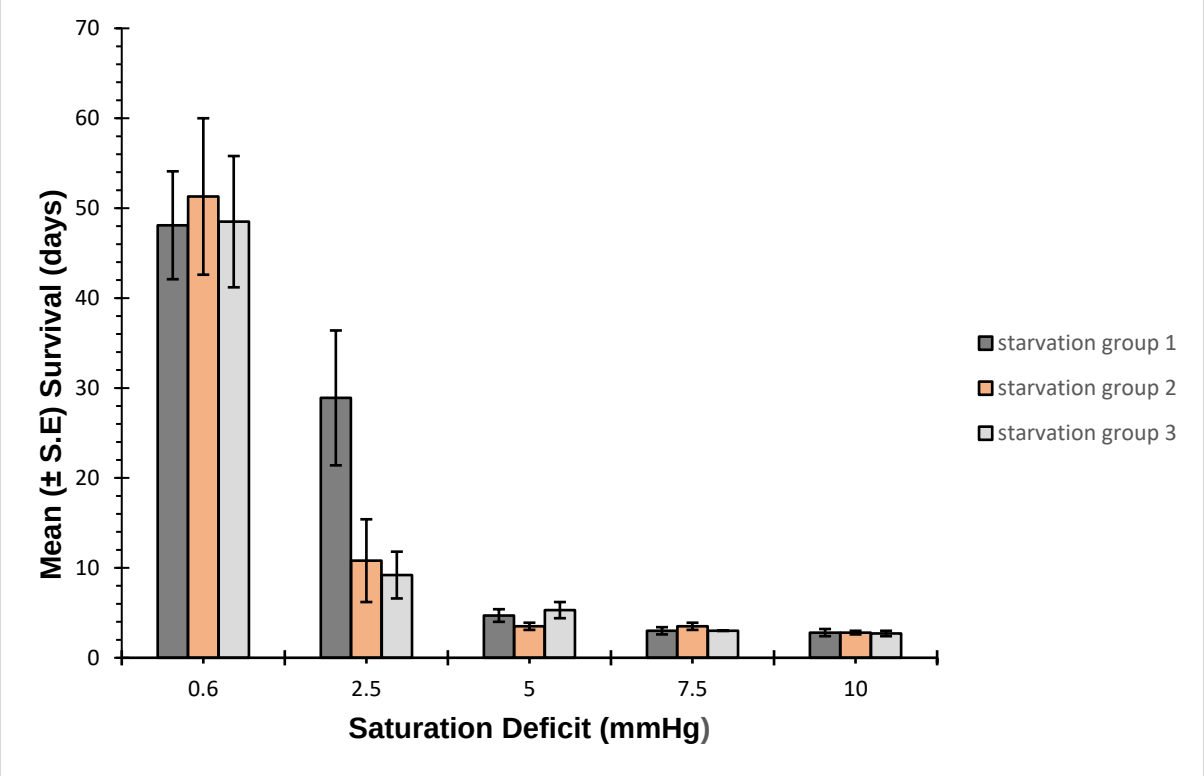


Fig 3.5 The mean survival (days) ± standard error of nymphal *Ixodes ricinus* exposed to a range of Saturation Deficit conditions (mmHg) after starvation for 1, 4 or 8 weeks. Error bars indicate 95% confidence intervals, n = 30.

3.3.2.2 Change in tick lipid concentration with starvation at different saturation deficits

As expected, ticks that had been starved for longer had lower lipid concentrations; Bonferroni *post host* tests showed that lipid values in ticks starved for 8 weeks were significantly lower than ticks starved for 1 or 4 weeks ($t = -12.2$, $P < 0.001$; $t = -9.73$, $P < 0.001$, respectively) (Fig 3.6). In some ticks, SD appeared to have significant effects on the lipid values, for example in the ticks starved for only 1 week, lipid values were lower in the SD 0.6mmHg ticks than in the SD 10.0mmHg group, but this was not the case in the ticks starved for 4 or 8 weeks. However, overall, there was no significant, interaction between starvation time and SD in their effect on the lipid content of observed ticks ($F_{8,88} = 1.16$, $P = 0.34$) showing that starvation had no consistent effect on the response to SD.

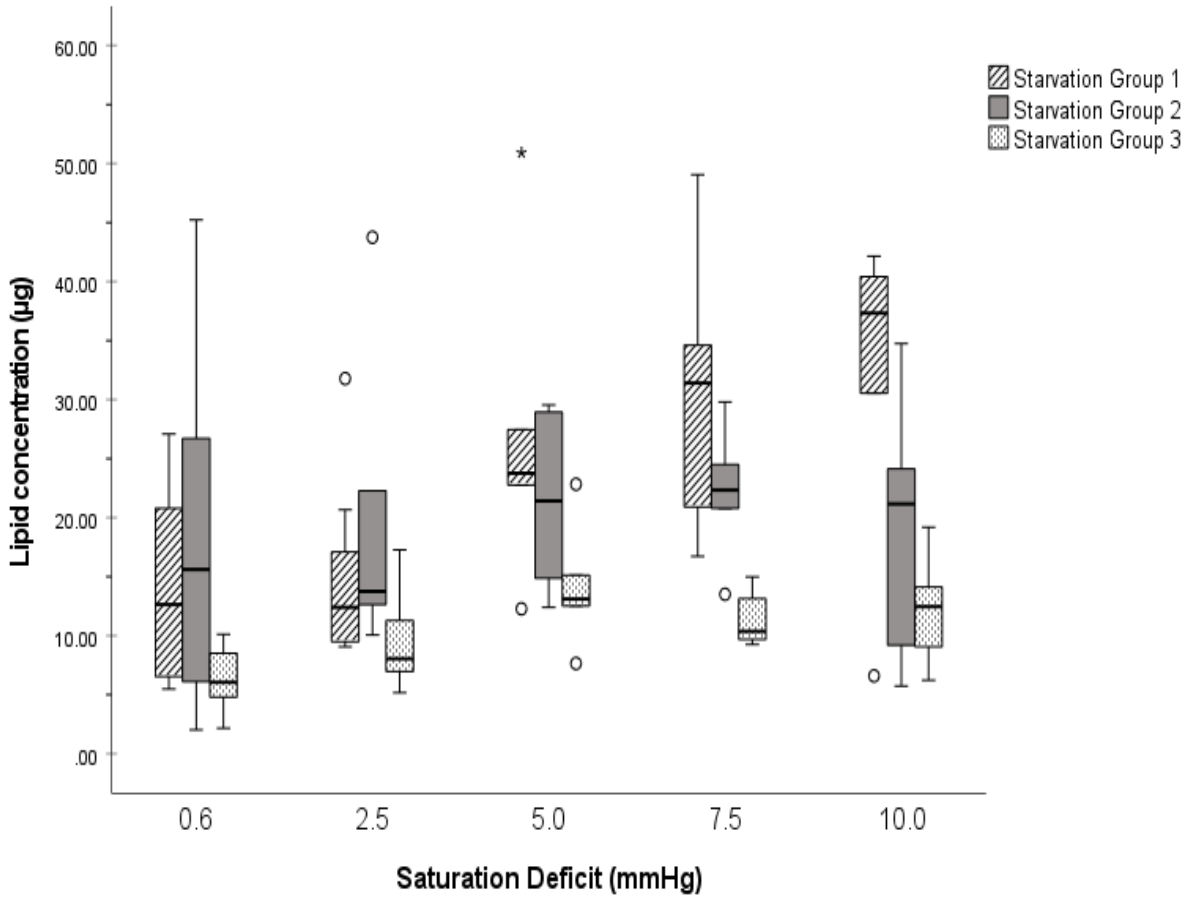


Fig 3.6 The median lipid concentration (µg) of *Ixodes ricinus* nymphs exposed to a range of different saturation deficit conditions (mmHg) after starvation for 1, 4 or 8 weeks. Horizontal line = median, box = interquartile range, whiskers = minimum and maximum, * ° = outliers, n = 30.

3.3.2.3 Change in tick hunger index with starvation at different saturation deficits

There was no significant difference in the Hunger Index of ticks maintained at different saturation deficits or at different starvation times (GLM: $R^2 = 0.19$, $F_{14,88} = 1.26$, $P = 0.25$; Fig 3.7).

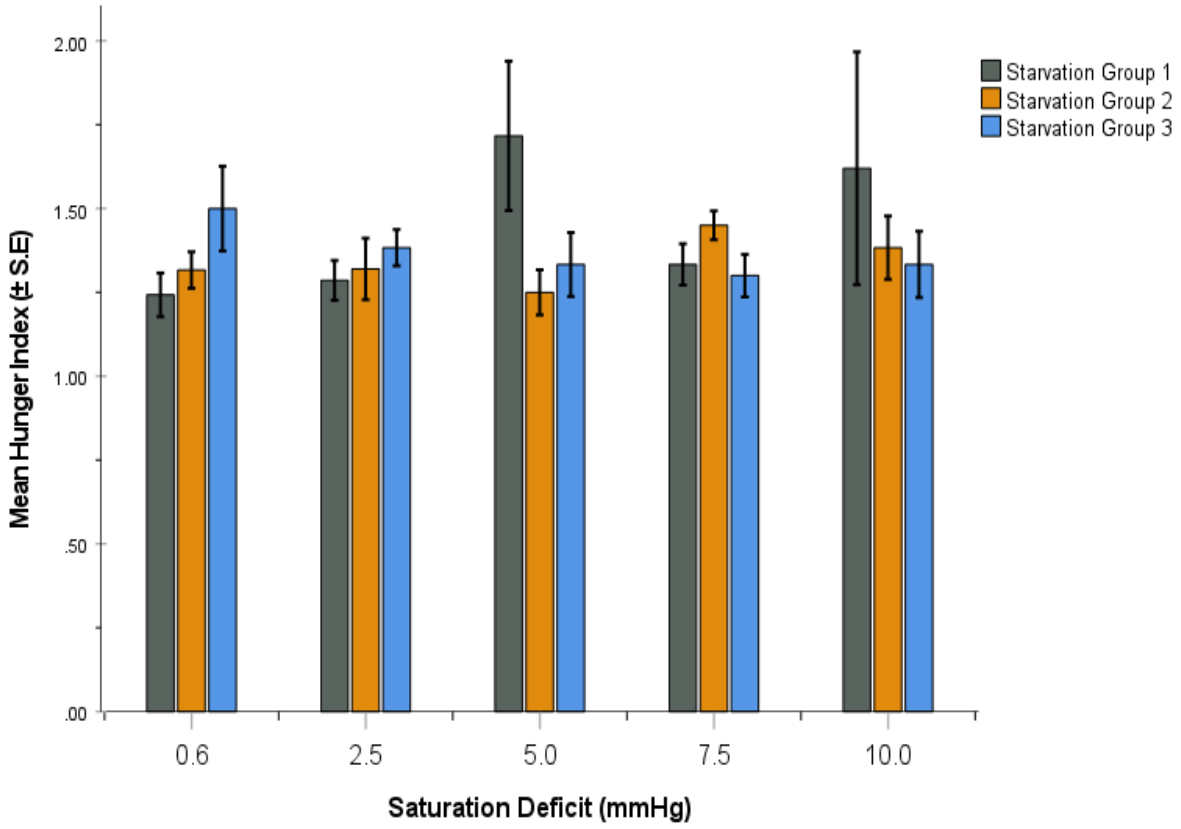


Fig 3.7 The mean ± standard error Hunger Index of *Ixodes ricinus* nymphs held at a range of different saturation deficit conditions after starvation for 1, 4 or 8 weeks. Error bars indicate 95% confidence intervals, n = 30.

3. 4 Discussion

Understanding the impact of temperature and humidity, and their interaction through Saturation Deficit, on the physiological performance and survival of invertebrate vectors, both in the laboratory and in the field, is an important step toward predicting the effects of climate change on their population dynamics and public health (Koussoroplis *et al.,* 2017). According to the report by UKCP09, the UK climate is projected to change significantly within the next 50 years, even under the medium emission scenario, with an increase of up to 5.4°C in maximum temperatures in parts of Southern England and 2.8°C in parts of Northern Britain in the summer. Winter temperatures are also projected to increase between 1.5°C and 2.5°C across the country. There is also a projected decrease in relative humidity by around -9% in parts of southern England in the summer and in the winter, by a few percentage points in other parts of the country. Summer precipitation is also projected to decrease everywhere by about 10-20%. In the winter, slight increases in precipitation of around 10%, are projected across the country. It is believed that beyond 2100, global warming will continue even if anthropogenic emissions cease. If the mean global warming reaches 2°C, the entirety of the UK would face minimum warming of 1-2 °C throughout the year. Winter days would be warmer by 1-1.5°C (Lowe *et al.,* 2018).

These anticipated changes in climate are likely to alter the abundance and activity of arthropod vectors, and consequently, vector-borne disease, in a variety of ways such as reducing the direct effects of cold on mortality rates during winter, shortening parasite life cycles and lengthening seasons for vector activity (Ogden *et al.,* 2014). Occurrence of earlier spring would also be expected to advance the seasonal activity of vectors where they are already endemic (Levi *et al.,* 2015). Aspects of tick behaviour, disease transmission and host responses are likely to be strongly affected, depending upon the tick species in question, the location at which populations are established, and its environmental requirements (Dantas-Torres, 2015).

The aim of the studies described here, therefore, was to determine whether *I. ricinus* ticks that were nutritionally deprived were more susceptible to mortality mediated by temperature and humidity stress. Saturation deficit was further used to integrate the measure of both variables (temperature and humidity) to produce a single value which reflected the drying power of the air. Saturation deficit (SD) has been reported to be a more sensitive indicator of the moisture content of the air than relative humidity, as it more clearly reflects the impact of the variation in atmospheric moisture content due to temperature change than relative humidity does (Anderson, 1936).

In the first study, the impact of temperature, humidity and saturation deficit on tick survival was elucidated by observing field collected *I. ricinus* nymphs exposed to different temperature and humidity conditions *in vitro*. As temperature increased and relative humidity decreased, tick survival declined and *vice versa*. When the relative humidity was low (25%), 100% mortality was recorded at day 5 even at the lowest temperature. However, ticks exposed to 97%RH at 4°C lived to the full extent of the study (60 days). On the other hand, when temperature was low (4°C), the ticks were unable to survive beyond 10 days until the relative humidity was above 85%. At ≥ 25°C even at 97% RH, ticks died withing 5 to 10 days following exposure to these conditions. This agreed with the findings of other studies that *I. ricinus* nymphs were highly sensitive to desiccation (Sonenshine, 1991; Herman and Gern, 2014). Overall, the results showed a negative non-linear relationship between saturation deficit and tick mortality. Higher SDs from 3.7mmHg and above caused a progressive increase in tick mortality with the nymphs surviving only for a maximum of 2 days at the highest SD considered (19mmHg). At ≤1mmHg, mortality rate was low with nymphs surviving up to 60 days at 0.6mmHg. These results demonstrate the high degree of sensitivity of *I. ricinus* to SD and highlight the narrow threshold of SDs below which *I. ricinus* survival is high.

The high mortality rate recorded at medium to high saturation deficits was most likely due to increased rate of water loss from the ticks. Several studies have pointed out the fact that the biggest challenge for ticks living in a relatively dry environment is the ability to maintain water balance (MacLeod, 1935; Perret *et al.,* 2000). Similar findings were reported in a study carried out by Herrmann and Gern (2014) to determine whether infection by *Borrelia burgdoferi* influenced *I. ricinus* survival under thermohygrometric stress. Their results showed no effect of infection, and that the most important factor which determined tick survival was SD; as SD increased survival decreased in infected and uninfected ticks. As described in Chapter 1, active water vapour uptake from subsaturated air appears to be the most important way for ticks to compensate for water loss, especially when unfed (Knülle and Rudolph, 1982). During this process, the agranular alveoli type I salivary glands produce a secretion of unknown composition that is extruded onto the external mouthparts and that absorbs atmospheric water vapour when the ambient relative humidity is above 80 to 90 % (McMullen *et al.,* 1976; Needham and Coons, 1984; Kahl and Knülle, 1988; Kahl *et al.,* 1990; Gaede and Knülle, 1997). Subsequently, the water-enriched oral secretion is swallowed thereby achieving a net water gain. This mechanism is very effective, providing substantial amounts of net water gain within hours or days, and has a comparatively low energy demand on the ticks (Macleod, 1935; Fielden and Lighton, 1996). In contrast, Ogden and Lindsay (2016) suggested that in the field, ticks are usually able to mitigate the effects of abiotic factors such as temperature and

humidity through behavioural avoidance, and that tick population dynamics are more strongly affected by habitat and host density or availability.

In the second study, the effect of saturation deficit on the survival of starved *I. ricinus* nymphs was investigated. The rationale for this study was to determine whether ticks at different stages of the hunger cycle, might be more susceptible to temperature and humidity stress, as this would affect their seasonal questing behaviour and survival. While as expected ticks starved for longer had significantly lower lipid values, and SD itself had a significant effect on survival, overall, there was no consistent effect of starvation on the impact of SD on survival. It would be expected therefore that ticks will be equally susceptible to the effects of high temperature or low humidity at all times of year.

There was no significant change in hunger index with starvation in this study. It could be that the duration of the study was too short to reflect any obvious changes in tick morphometry due to starvation. In addition, no significant effect of SD on hunger index was observed. As highlighted in Chapter 2, changes in tick anatomy consequent upon starvation especially in the nymphal stage are expected to be slight, highlighting the conclusion of Chapter 2 that body morphology is not a particularly sensitive way to determine time since the last blood meal.

A key difference between ticks and dipteran vectors is the speed at which their populations and pathogen transmission cycles can respond to short term changes in weather. Dipterans respond rapidly to increase in air temperature because their life cycles are short and immature stages are affected directly. Cycles of tick-borne pathogen transmission, on the other hand, respond more slowly to external changes because their duration is determined by the long development rates and slow metabolic rates of the ticks. The longevity of tick cycles and behavioural use of microclimate in habitat refuges serve to smooth out short-term fluctuations in air temperatures and this means that short term variations in climate have limited impact on their populations (Ogden *et al.,* 2004). However, when these conditions are prolonged or significantly altered, marked changes are likely to be observed.

It has been suggested that the abundance and distribution of *I. ricinus* ticks have increased over the past 20 years across the UK. This has been attributed to changing climate patterns resulting in drier warmer summers and wetter colder winter temperatures (Jaenson *et al.,* 2012). As a result, ticks have been observed to begin questing earlier than before. In addition, the observed poleward expansion of *I. ricinus* at extremes of altitude and latitude has been associated with warmer climate (Leighton *et al.,* 2012). This has also been facilitated by this species being a woodland habitat generalist and by the south to north dispersal routes followed by migratory birds

(Ogden *et al.,* 2008; Hasle *et al.,* 2011). Since *I. ricinus* require a SD of at least 0.6mmHg to survive during its off-host periods, as confirmed here, they are therefore restricted to areas where rainfall and associated habitat maintain these conditions (Medlock *et al.,* 2005). Changes in climate in the region that result in milder winters and warmer springs would allow ticks to begin questing earlier in the year, but periods of excessive heat and dryness in the spring or summer will cause ticks to cease questing activity.

The main limitation encountered during the study was the inability to carry out fieldwork and the closure of labs following the lockdown during the COVID-19 pandemic in 2020, during the second study. The batch of ticks used for the study was limited to those collected before the imposition of the lockdown. The result was the small sample size of nymphs used to evaluate the effect of starvation on tick survival at the different starvation deficits per desiccator. Greater replication would have been desirable and several other environmental factors, such as photoperiod and soil temperature, which are also known to affect tick activity and mortality rates might have also been considered (Schulz *et al.,* 2014; Földvari, 2016; Gray *et al.,* 2016; Cat *et al.,* 2017). Further studies are therefore needed to investigate the impact of these factors on survival of hungry ticks in different climate change scenarios.

- Chapter 4 -

Evaluating an artificial system for *Ixodes ricinus* feeding

4.1 Introduction

The routine production of high-quality laboratory-reared ticks could make an important contribution to studies of their biology, control and role as vectors of pathogens (Sonenshine 1993). To produce such research material, in many parts of the world, ticks are maintained in the laboratory using a combination of hosts which include rats, guinea pigs or smaller-sized rodents for immature stages while rabbits, dogs or other medium to large animals are used for the adult stages (Bonnet and Liu, 2012). Depending on the instar to be fed, a host is usually selected and anesthetized. The area where the ticks would be retained on the host is shaved using motor clippers. The back is usually used in guinea-pigs, while the ear is used in rabbits especially where ear bags are used. After the hair is shaved, 2 – 3 retaining cells are then fixed to the animal's skin using non-irritating glue. Larvae, nymph and adult stages are usually maintained separately, each in a retaining cell consisting of only one life cycle stage (Jones *et al.,* 1988). After the area is prepared, the ticks are immobilized by placing them on ice for about 5 minutes. The larvae or nymphs are then transferred to a Petri-dish using a fine brush pasted on the animal. Approximately 200-400 larvae or 50-100 nymphs are usually placed in each retaining cell. The cells are then covered with a nylon mesh, trimmed to size. In the case of the adult ticks, equal numbers of males and females are introduced into each retaining cell (to facilitate mating) before the nylon mesh is sealed. Fewer numbers of adults (about 10 to 15) are used in each retaining cell. As soon as the ticks attach, the host animal is placed in a cage on a rack surrounded by an oil 'moat' to prevent escape of ticks (Jones *et al.,* 1988). Retaining cells are then monitored daily until the ticks are engorged. This usually takes between 5 to 7 days in larvae and nymphs and about 8 to 14 days in adult ticks (Sonenshine, 1993). After the ticks engorge, they are removed for storage.

The use of natural hosts for feeding and maintaining ticks in the laboratory provides the best natural conditions to facilitate feeding. However, acquisition, housing, and handling of these animal hosts can be complicated and expensive (Kuhnert, 1996), especially given the host

specificity of some tick species (Waladde *et al.,* 1991). This is mainly because ticks require blood feeding multiple times throughout their life cycle and most of the rearing procedures are time intensive (Sonenshine, 1993; Bouchard and Wikel, 2005). In addition, ethical concerns associated with the use of live animals for experimental purposes, the advocacy for the 3R principles (reduction, refinement, replacement) and other administrative requirements further complicate the process. Furthermore, physical harm to these laboratory hosts from tick feeding procedures could result in skin inflammation, anaemia and interference with their grooming behaviour when subjecting them to such mechanical restraints as the Elizabethan collar used to prevent the animal from licking or scratching its head or neck after the tick rearing apparatus has been affixed (Waladde *et al.,* 1991). As a result, there is considerable interest in the search for suitable alternative measures for tick rearing and colonization.

Recent advances in the development of alternative *in vitro* feeding methods such as capillary tube feeding or the use of membrane-based feeding systems, have shown promise for the replacement of the use of living animals for the maintenance of tick colonies for research purposes (Tajeri *et al*., 2016). This advancement will facilitate studies geared towards understanding the dynamics of the relationship between haemoparasites and their tick vectors, as well as various aspects of tick behaviour. These systems will also make it possible to carry out studies where several parameters influencing tick physiology or biology could be studied, which were hitherto difficult to control in living animal systems (Krobër and Guerin, 2007).

Many creative approaches to induce blood-feeding have been examined in the laboratory, mostly based on the original design of the Rutledge feeder (Wade, 1976; Costa-da-Silva, 2013). One method is the capillary feeding technique which involves placing a micro-capillary tube over the mouth parts of a properly secured semi-engorged tick. The capillary tube is filled with blood which is either manually defibrinated or contains added anticoagulant. The process is quite slow and takes several hours to complete, but even then, the ticks are not replete (Broadwater *et al*., 2002; Billeter *et al*., 2012). Capillary feeding has been employed by in many studies, particularly to infect ticks with pathogens (Nuttall and Hindle, 1913; Chabaud, 1950; Burgdorfer, 1957; Purnell and Joyner, 1967; Jones *et al*., 1988; Waladde *et al*., 1996; Burkot *et al*., 2001). However, it has been suggested that semi-engorged ticks are better candidates for capillary feeding compared to unfed ones (Abel, 2004; Rangel *et al*., 2008). Hence, capillary feeding is usually supplemented by initial feeding on natural hosts (Kuhnert, 1996).

Membrane feeding is another method which has been attempted for feeding ticks *in vitro* (Kuhnert, 1996). Here, the procedure involves use of feeding chambers with various kinds of

membranes usually made of natural or synthetic materials. For instance, natural membranes such as the air sac of embryonated chicken eggs, bat wing, the skin of bovine animals or biodegradable glue-impregnated Baudruche membrane have all been used previously with variable success (Pierce and Pierce, 1956; Youdeowei and Mango, 1975; Kemp *et al.*, 1975; Waladde *et al.*, 1991; Voigt *et al.,* 1993). More recently, synthetic membranes made of silicone have been developed and adapted for different tick species by several researchers (Habedank and Hiepe, 1993; Kuhnert *et al.*, 1998; Krobër and Guerin, 2007; Fourie *et al.,* 2013; Tajeri *et al.*, 2016; Krull *et al.,* 2017).

Most of the feeders developed for ticks are composed of a heating element (for blood warming), a blood reservoir, a feeding chamber, and an artificial membrane surface simulating vertebrate skin. The ticks are usually fed on the blood placed into the feeding chamber. Blood from a number of animal species may be used served, but it is most usually from larger domestic animals like cattle, sheep or horses (Waladde *et al.,* 1979; Stone *et al.*, 1983; Kuhnert *et al.,* 1995; Krober and Guerin, 2007; Tajeri and Ramzi, 2011). The thickness of the membrane produced depends on the species and life-cycle stage of the tick to be fed. It is usually determined by the length of the mouth parts of the tick especially the hypostome-chelicerae complex, which also varies with the instar of the tick (Waladde *et al.*, 1996).

During membrane feeding of ticks, all the salivary secretions flow into and accumulate in the blood meal on which the ticks are fed, and this will result in the rapid deterioration of the blood in feeding chamber. This happens more rapidly when many ticks are engorging simultaneously, necessitating the replacement of the blood in the chamber every 8 to 12 hours (Waladde *et al.*, 1996).

The aim of the study described in this chapter was to evaluate a silicone membrane-based feeding system for *I. ricinus* nymphs in the laboratory. The study largely focussed on the tick feeding units descried by Krober and Guerin (2007), with some modifications.

4.2 Materials and Methods

4.2.1 Tick collection

For the feeding experiments, 360 *I. ricinus* nymphs were collected into 50ml plastic tubes from Ashton Court Estate by the blanket dragging technique, as described in Chapter 2, and immediately transferred to the laboratory for storage at 7 °C until required. The ticks were acclimatized in the laboratory at room temperature for 3 h prior to the commencement of the

study, before being assigned at random into the respective artificial tick feeding chambers used for the study.

4.2.2 Feeding Assay Preparation

Artificial tick feeding chambers were prepared following the description of Krobër and Guerin (2007), with slight modifications. The feeding chamber setup comprised a transparent tube, artificial silicone membranes and a 6-well tissue culture plate (Fig 4.1). Each tube was made of acrylic (28mm diameter, 2mm wall thickness and 45mm high). A ring was fitted around each tube 4 mm from the base to limit the depth to which the chamber sank into the wells of the 6-well tissue culture plate which served as a blood reservoir for the feeding apparatus. A stopper was placed about 2 cm from the base of the tube to prevent ticks from wandering up and down the walls of the acrylic tube and away from the silicone membrane (Fig.4.2).

Silicone membranes were prepared using RTV-1 Elastosil E4 Silicone rubber (Wacker Chemie, Germany) with a Shore A hardness of 16 degrees. 30 g of the glue was mixed with toluene solvent (BDH Chemicals, England) inside a glass beaker at a ratio of 4:1. 30% Silicone oil (DC 200, viscosity ~10 mPa, Fluka, Switzerland) was also added to the mixture to render the resulting membrane softer. The mixture was then evenly spread over Goldbeaters skin (Preservation Equipment Ltd, UK) using a silicone rubber spreader. The resulting silicone impregnated membrane was then fixed to clingfilm using sticky tape and allowed to polymerise and dry overnight. Membranes with thickness of between 40-70 μm were used for feeding. Subsequently, the membranes were attached to the acrylic glass tubes using Elastosil E4 silicone glue and allowed to dry for at least 3 h. Excess membrane was cut flush with the tube when dry. The permeability of each membrane attached to the feeding chamber was then tested with normal saline solution. Only membranes that did not permit the entry of saline after 20 min were used in experiments. The feeding chambers were then surface sterilized with 70% ethanol before use.

Blood

Fresh bovine blood was collected from the abattoir at the Langford School of Veterinary Science site, and directly supplemented with 4% w/v of sodium citrate (FastChem Chemical Supplies, UK) and 4g/L glucose (Fisher Scientific, UK) to stabilize the erythrocytes. The blood was then stored at 4 ℃ for up to 1 week. Just before use in the study, 10^{-3} mM of the phagostimulant adenosine triphosphate (Acros Organics, Japan) was added to the blood.

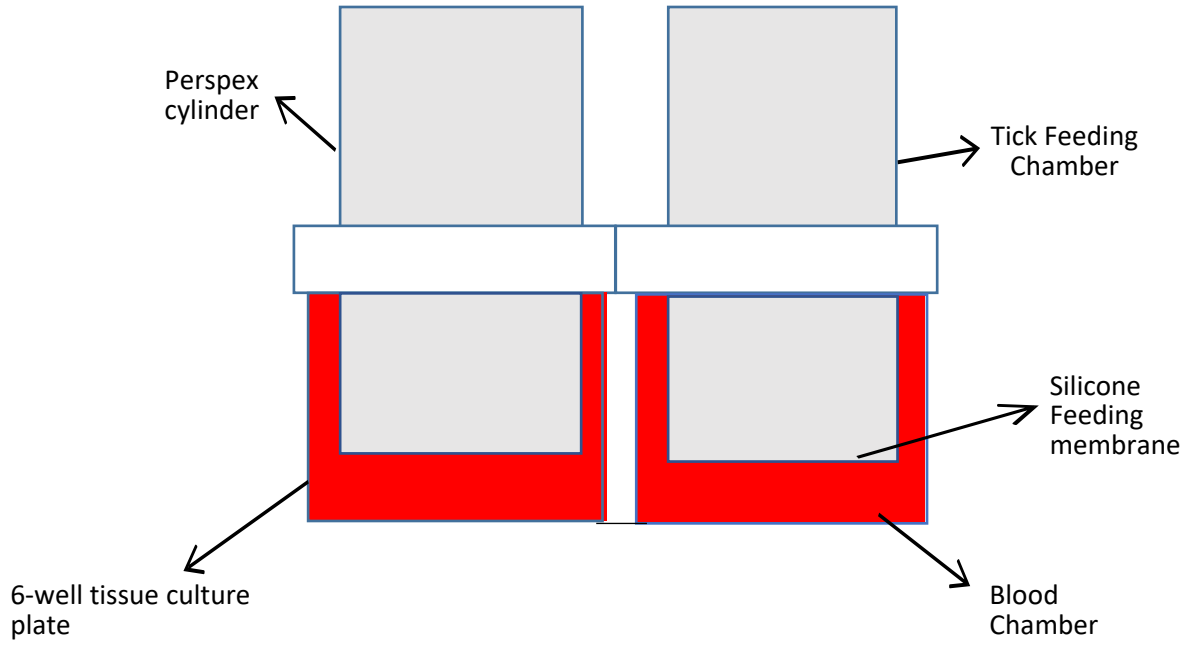


Fig 4.1. Schematic diagram of the tick feeding assay

Feeding/Attachment stimuli

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

4.2.3 Evaluating the tick feeding assay for *Ixodes ricinus*

Twenty (20) randomly selected *I. ricinus* nymphs were added to each of 6 feeding chambers. The ticks were lightly covered with a 1 cm deep layer of cow hair, which had been cut to about 5 mm in length, and the feeding chamber was sealed with a perforated stopper secured with cloth netting. Following this, 3ml of blood was added into the 6-well tissue culture plates and warmed up to 37 °C in an incubator. The feeding chambers were randomly assigned to one of two groups.

Group 1 feeding chambers were placed into a humidified incubator (Prem Mich Insect Chambers, MicroClima-Series, Snijders Labs, Netherlands) set at 37 ℃, 70% RH and 16:8 h L:D regime throughout the study. Group 2 feeding chambers were placed on a hotplate (Minitübe GmbH, Germany) maintained at 37 °C on the laboratory bench at room temperature (approximately 21 °C, at an average humidity of 58% RH) but not controlled. For the main study, the ticks were inspected at 0, 3, 6 and 12 h after set-up, and subsequently every 24 h till the end of the study (96 h). The number of ticks attached to and feeding through the membrane per chamber at each inspection was recorded. The numbers of living and dead ticks were also recorded. Blood was replaced every 12 h. During each blood change, the artificial membranes were rinsed with saline warmed up to 37 ℃. The temperature and relative humidity of the incubator was also monitored daily (37 °C and 70% RH). The study was carried out in triplicate.

4.2.4 Data Analysis

In batches maintained either in the incubator (Group 1) or on the hot-plate (Group 2), a t-test for independent samples was used to compare the number of ticks which attached to the membrane and the number of ticks alive. All means are reported ± SEM. All statistical analyses were carried out using IBM SPSS Statistics for Windows (Version 28, IBM Corp, Armonk, N.Y, U.S.A).

4.3 Results

4.3.1 Evaluating the tick feeding assay for *Ixodes ricinus*

There was a significant difference in tick attachment rate to the artificial feeding membrane between the group maintained in the incubator [Group 1] or at room temperature on a hotplate [Group 2] (t (46) = 5.24, P < 0.001). At 24 h, 93.3% of nymphs maintained in the incubator had attached to the membrane and begun feeding. This contrasted with 10% recorded in the second group at the same time point. In the group in the incubator, the ticks remained attached and continued blood feeding through the membrane at a similar rate at the different time points observed throughout the study. In contrast, the majority of the ticks in the group maintained on the laboratory bench on a hotplate did not attach to the feeding membrane, and, for those that did attach initially, 100% detachment had occurred by 72 h (Fig 4.3). The mortality rate was also significantly higher among the ticks maintained on the hotplate compared to the incubator (t (46)

= 3.92, P < 0.001). The mean number of ticks alive at 48 h in the group maintained on the laboratory bench had declined by 68%, compared to the group in the incubator where an overall mortality of 25.5% was recorded throughout the study, with 44.7 ± 1.5 ticks still alive after 96 h in the latter treatment group (Fig 4.4).

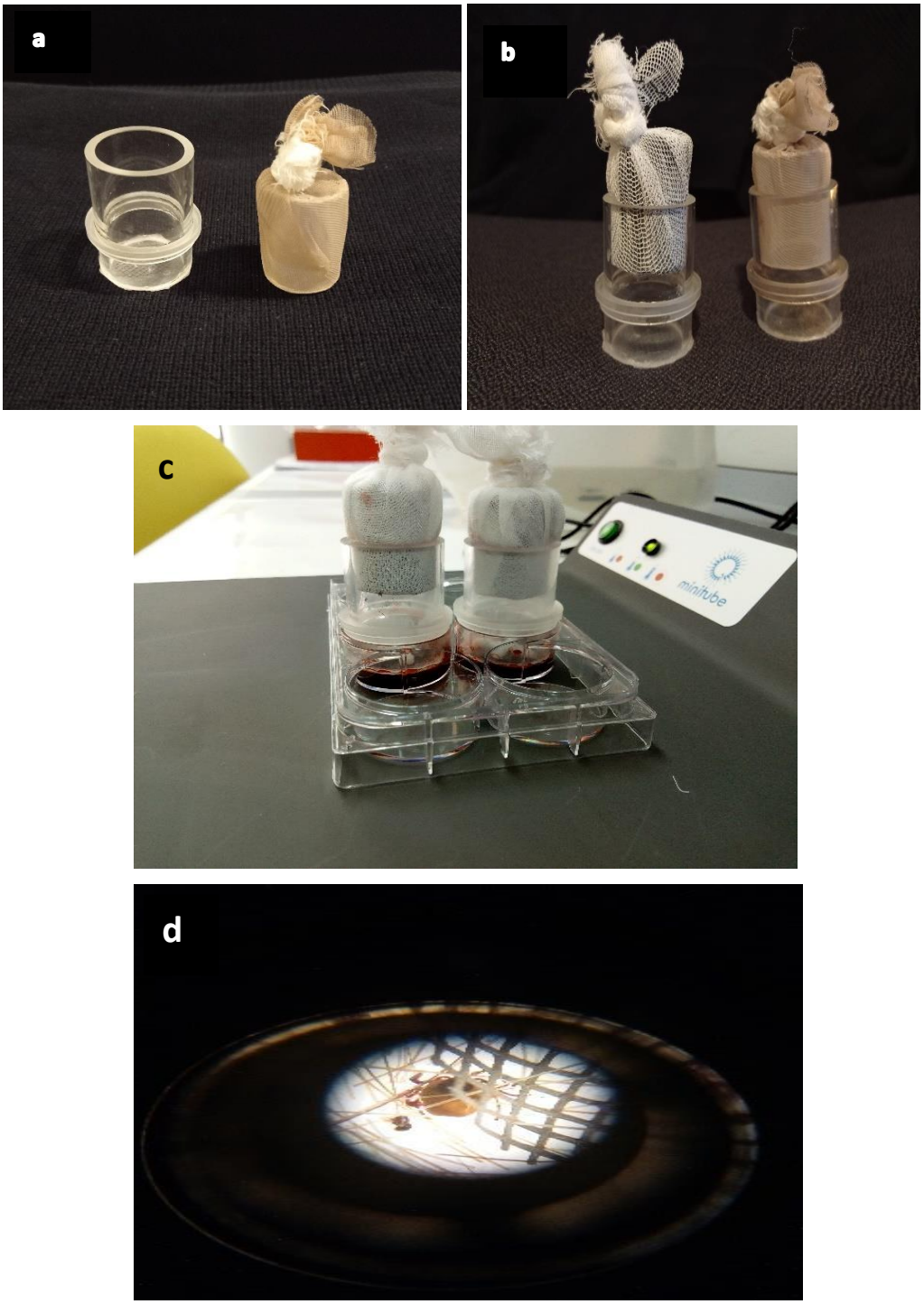


Fig 4.2. Components of the tick feeding apparatus **a**. acrylic tube and perforated stopper secured with cloth netting **b.** Assembled tube **c.** Feeding chambers in 6-well tissue culture plate containing blood placed on a hotplate **d.** Partially engorged *Ixodes ricinus* nymph

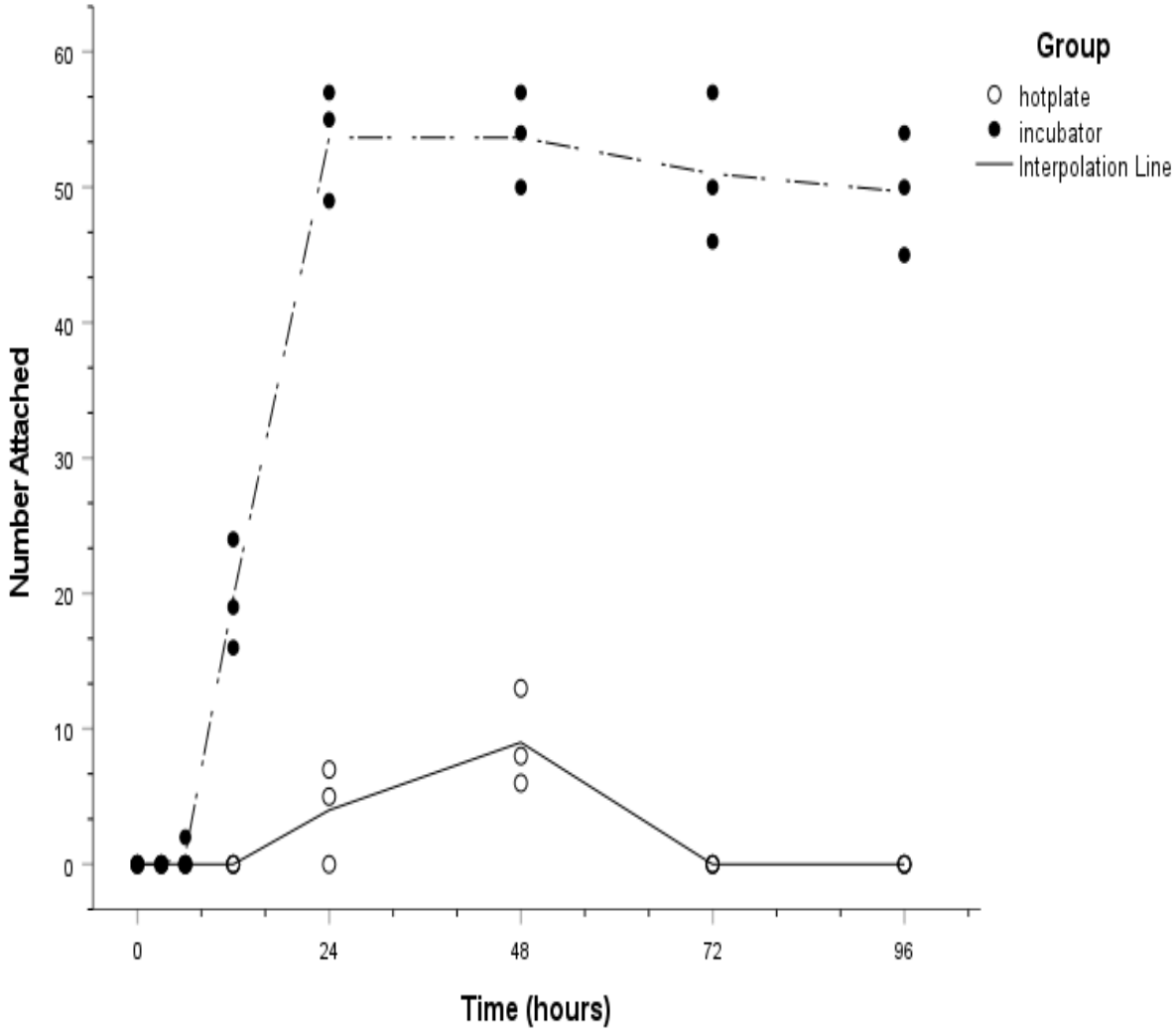


Fig 4.3 The number of non-starved nymphal *Ixodes ricinus* that were attached to an artificial feeding membrane counted at different time intervals (h) maintained either in a humidified incubator at 37 °C and 70% RH [Group 1] or on a hotplate set at 37 °C on a laboratory bench at room temperature (21 °C, 58 % RH) [Group 2]. Line plotted is the three-point moving average.

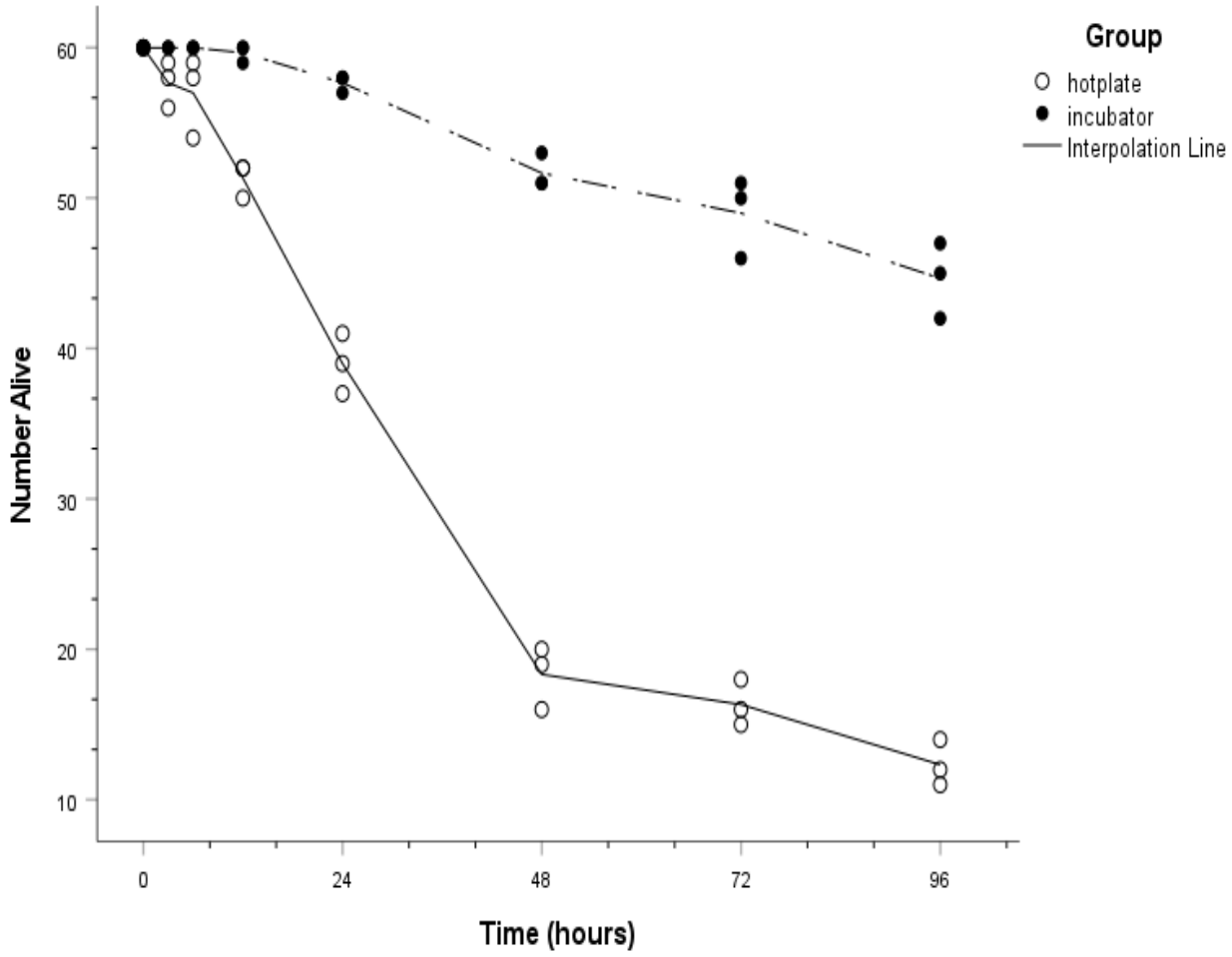


Fig 4.4 The number of non-starved nymphal *Ixodes ricinus* that were alive after exposure to an artificial feeding membrane at different time intervals (h) maintained either in a humidified incubator at 37 °C and 70% RH [Group 1] or on a hotplate set at 37 °C on a laboratory bench at room temperature (21 °C, 58 % RH) [Group 2]. Line plotted is the three-point moving average.

4.4 Discussion

Achieving membrane feeding of hard ticks is especially difficult to standardize due to the long feeding duration of most species (Kuhnert, 1996; Krobër and Guerin, 2007). Unlike tsetse or mosquitoes, ticks are not easily fed through synthetic membranes and usually require a complex combination of living host attractants and tick pheromones to initiate the feeding process; many research groups have attempted to establish *in vitro* feeding systems for ticks with only partial success (Kemp *et al.,* 1986; Bonnet and Liu, 2012). Both kairomones and pheromones are thought to be important feeding stimuli (Waladde *et al.*, 1993). In the present study, bovine hair extract was used to provide host kairomones while tick faecal material was included in the feeding chamber to provide tick pheromones (attraction-aggregation-attachment pheromones) which have been reported to facilitate sustained tick feeding response after locating a suitable host (Sonenshine, 2006). However, although both groups contained these semiochemicals in the feeding chamber, heat cues were also noted to play a significant role in the feeding response.

Temperature sensitivity of bloodsucking arthropods such as ticks or mosquitoes is pronounced and is important in eliciting feeding, with the warmth of the potential host's body being one of the primary influences stimulating and guiding such blood feeders (Zermoglio *et al.,* 2017; Carr *et al.,* 2019). In this study, 24 h after placing the feeding chamber in the incubator at near host temperatures with adequate humidity, 93.3% of the ticks in the incubator had either attached to the artificial membrane or commenced blood feeding; this environment also gave relatively low mortality rates, whereas the use of a hot plate as the heating element in the second group yielded almost no tick attachment and high mortality rates, despite addition of all the feeding stimuli to the membrane. Clearly, the localised heat from beneath from the hotplate was less effective at stimulating feeding than the generalised heat in the incubator. In the former case, the ticks were active and were seen walking around within the tube, however probing the membrane and attachment for feeding was infrequent. However, the lack of feeding and high mortality rates recorded in this group might potentially also have been associated with the lower humidity conditions in the laboratory, whereas high humidity was maintained within the incubator. This cannot be determined from the present study and subsequent work should assess the effects of humidity on feeding behaviour. Dehydration has been reported to be a major cause of tick mortality with *I. ricinus* requiring at least 70-90% RH for survival and a saturation deficit of approximately 2.5mmHg and below to ensure survival (Baan, 2014; Rosendale *et al.,* 2017). However, the current findings do highlight a likely synergy between the effects of host kairomones, tick pheromones and microenvironment in the feeding response of *I. ricinus* ticks.

An effective *in vitro* tick feeding system would offer several advantages. These could include its use in quantifying dose effects of newly developed tick control products or for the assessment of the capacity of a surface acaricide to affect tick attachment for a blood meal. Here, after many alternatives had been investigated, the use of goldbeater's skin was found to be helpful in allowing silicone membranes of ≤ 40µm, to be produced which is significantly thinner than those that were produced with lens cleaning paper with an average thickness of 70µm as described in other studies (Krobër and Guerin, 2007; Krull *et al.,* 2017). This may have allowed the ticks to grip and begin to feed without the need for additional structural edges which are often added to *in vitro* feeding systems for attachment and were also thin enough to allow the nymphs pierce through to reach the blood. The thinner membranes produced in this study might also make it possible for tick larvae or tick species with shorter mouthparts to achieve feeding *in vitro*. However, the major problem for all *in vitro* tick feeding systems is associated with the fact that because they feed over periods of several days, the blood starts to decompose and therefore needs to be changed at regular intervals. This is labour intensive and results in disturbance to the feeding ticks. Flow-through systems, with circulating blood that can be changed from a central reservoir, are being investigated by some research groups (Böhme *et al.,* 2018) but are not yet commonly used.

In conclusion, the results showed that feeding behaviour was seen at temperatures comparable to that of a potential host (37°C). Also, controlling the tick microenvironment in the incubator decreased *I. ricinus* mortality and improved tick attachment to the artificial feeding membrane.

- Chapter 5 -

Effects of starvation on the behaviour of *Ixodes ricinus*

5.1 Introduction

Changes in behaviour or physiology over time after feeding have been reported in a wide range of arthropods. This has been particularly well-studied in blood-feeding insect vectors where the blood feeding interval and biting rate are of epidemiological importance. For example, in tsetse (*Glossina*) activity was shown to increase progressively with hunger (Jackson, 1946; Brady, 1972a, b). Here, with increasing activity, a four-phase change in behaviour over each 'hunger cycle' was proposed by Bursell (1961; 1966). In phase 1, flies are initially inactive after feeding. Following partial digestion and replenishment of the fat stores, males enter an active second phase in which they approach and follow a moving object and are sexually active. As flight activity leads to depletion of food reserves, flies enter a third phase 3, in which increasing levels of activity become redirected towards finding resting host animals, ultimately leading to probing and feeding. Phase 4 is composed of very hungry flies which are once again activated and responsive to moving objects. This was supported in laboratory studies of *Glossina morsitans morsitans* and *G. pallidipes*, where Wall (1988) showed that the activity of males increased with both age and hunger; however, after blood-feeding the behaviour that led to attempts to mate decreased consistently with increasing hunger over a period of 3 or 4 days. In both species, the duration of mating attempts did not change with age but the number of attempts declined with increasing hunger. Similar changes in activity have been reported over a hunger cycle in mosquitoes (Canyon *et al.,* 1999; Lalubin *et al.,* 2014; Price *et al.,* 2015; Joy *et al.,* 2018).

The effect of starvation on the responsiveness of ticks to host-related stimuli has not been studied in any detail, but it has been hypothesized that responsiveness may increase with starvation (Fourie *et al.,* 1993; Kilipinen and Mullens, 2004; Thomas *et al.,* 2020). However, the majority of available studies have mainly compared starved ticks with recently fed ones, or different life-cycle stages. There has been little consideration of the progressive change in behavioural responses of

ticks at a gradient of different levels of food deprivation or when they may be approaching a critical level of starvation.

Using the morphological measurement and the spectrophotometric method of lipid estimation described in Chapter 2, the aim of the study described in this chapter was to determine some of the changes in behavioural responses of *I. ricinus* ticks that might be associated with starvation. The first objective of the study was to determine whether ticks at higher levels of nutritional stress were more likely to probe on an artificial feeding membrane than ticks that were resource rich. The second objective was to examine the activity of *I. ricinus* ticks, presented with volatile cues from a botanical acaricide, with progressive starvation.

5.2 Materials and Methods

5.2.1 Change in tick feeding behaviour with starvation

To determine the effect of starvation on tick feeding behaviour, the tick feeding assay described in Chapter 4 was used. A batch of 200 *I. ricinus* nymphs were collected from the field in March 2022. These were starved over a period of 6 weeks in an incubator at 15 °C and 80% RH. Each week about 30 nymphs, selected at random, were removed and were divided into 3 groups of 10 ticks each per feeding chamber. For this study the ticks were monitored at 1-, 5- and 24-hour intervals, and the number of ticks probing and attached to the membrane at each time point was recorded. Attached ticks were removed from the tick feeding chamber into individually labelled 0.5ml Eppendorf tubes and killed by freezing. Only ticks which had not attached to the membrane at each time point were allowed to remain in the chambers until the end of the study (24 h). At the end of the observation period, all unattached ticks from each respective group were also collected into labelled tubes and killed. Linear body measurements including scutum length, scutum width, body length and body width were carried out to calculate the Hunger Index of each individual tick (as described in Chapter 2). Lipid analysis was then also carried out using the vanillin assay (as described in Chapter 2). The aim of this experimental design was to test the prediction that hungrier ticks would be more likely to probe the artificial membrane more quickly. The study was carried out in triplicate.

5.2.2 Change in tick walking activity with starvation

To obtain a further index of behavioural activity, a laboratory assay was used to examine the willingness of ticks to cross a barrier of putatively repellent chemical. It was expected that such an assay might give insight into a tick's motivational state, and it was hypothesised that hungrier ticks might be more willing to cross a barrier of repellent given their greater need to find a host.

For this, a batch of 180 *I. ricinus* nymphs were collected from the field site. The nymphs were placed individually into labelled 0.5 ml Eppendorf tubes and then progressively starved for 8 weeks in a humidified incubator (PHCBI Versatile Environment Test Chamber, PHC Corporation, Japan) at 15 °C and 80% RH. Thyme oil (Essential Oils Direct Ltd, Oldham, UK) was used as it has been described previously as being repellent to ticks (Taberi *et al.,* 2017; Goode *et al.,* 2018; Soutar, 2019). The oil was diluted using ethanol (≥ 99.8%; VWK Intl. Ltd, Lutterworth, UK) to produce a 5% solution.

An arena for the walking assay was constructed by drawing 3 concentric rings of radius 15 to 45 mm onto Whatman No.1 filter papers (Cytiva Ltd., UK) with a pencil. A separate filter paper ring, of radius 30 to 45mm matching ring 3, was carefully cut out from a second filter paper disk and transferred to a plastic petri dish. To this ring, 10 ml of 5% thyme oil was applied evenly to the point of visible saturation using a pipette. The ring was allowed to dry for 20 min before being secured over the third ring on the filter paper using petroleum jelly (Vaseline®, Sigma-Aldrich, UK) to form a seal and to prevent ticks from crawling into the space beneath the attached ring and the filter paper ring (Fig 5.1). Each arena was used only once. The control consisted of an identical setup without the thyme oil.

At week 1, 60 nymphs from the food-deprived batch were randomly selected for the study. Nymphs were divided into 3 groups of 20. Each group was gently transferred to the central ring of the respective study arena using a fine-tipped paint brush, immediately after the thyme-oil impregnated ring had been placed onto the arena. As soon as all the ticks were placed in the arena, a timer was started and run for 60 min. Observations of the distribution of the ticks were made at 5 min intervals, counting the total number of nymphs left within the arena's central ring and the number that had crossed the thyme oil impregnated filter-paper. Ticks that crossed the thyme-oil impregnated ring within the 60 min interval were immediately collected and placed in labelled 0.5ml Eppendorf tubes and the crossing time was recorded. After 60 min, the ticks that had not crossed the impregnated ring were also collected into labelled tubes. The study was repeated at 4- and 8-week starvation time points. Ticks were then killed by freezing and body measurements

made (length, width, scutum length and scutum width) followed by lipid analysis on each individual tick. The study was carried out in triplicate.

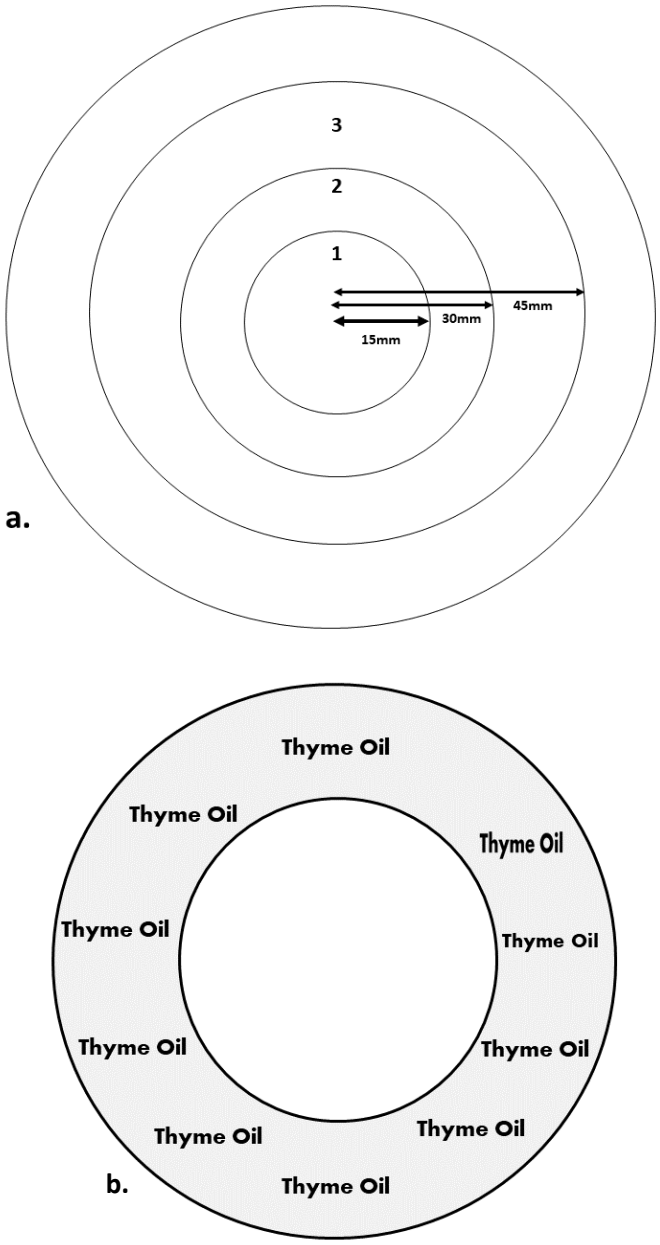


Fig 5.1. Walking assay arena **a**. Whatman no.1 filter paper with 3 concentric rings of radius 15mm to 45 mm. **b**. Oil-impregnated ring matching ring 3 of the arena.

5.2.3 Data Analysis

Regression analyses were used to examine the relationship between the number of ticks attached to the membrane and the period of starvation after 1 or 24 h of exposure to the membrane. Lipid values were not normally distributed, so a non-parametric Kruskal-Wallis test was used to determine the differences in lipid concentration at different starvation time points in the feeding and walking assays. A Mann Whitney U test was performed to compare the difference between lipid values of ticks that did or did not attach to the membrane (feeding assay). A spearman's rank correlation test was used to examine the relationship between tick lipid concentration and the time taken to cross the essential oil (5% thyme oil) impregnated ring (walking assay). To consider any relationship between Hunger index (HI) and the time of tick attachment to the membrane (feeding assay) or time taken to cross the essential oil (5% thyme oil) impregnated ring (walking assay), a parametric regression analysis was carried out.

All means are reported ± SEM and all medians with their range or interquartile range. All statistical analyses were carried out using IBM SPSS Statistics for Windows (Version 28, IBM Corp, Armonk, N.Y, U.S.A).

5.3 Results

5.3.1 Change in tick feeding behaviour with starvation

The numbers of ticks that were attached at 1, 5 or 24 h after different starvation times is plotted in Fig 5.2. The figure shows that a greater number of ticks attached more quickly in the groups that had been starved for longer. Because most ticks attached within 1 h, for example in the groups that had been starved for 6 weeks, there were no unattached ticks by 24 h after exposure to the membrane.

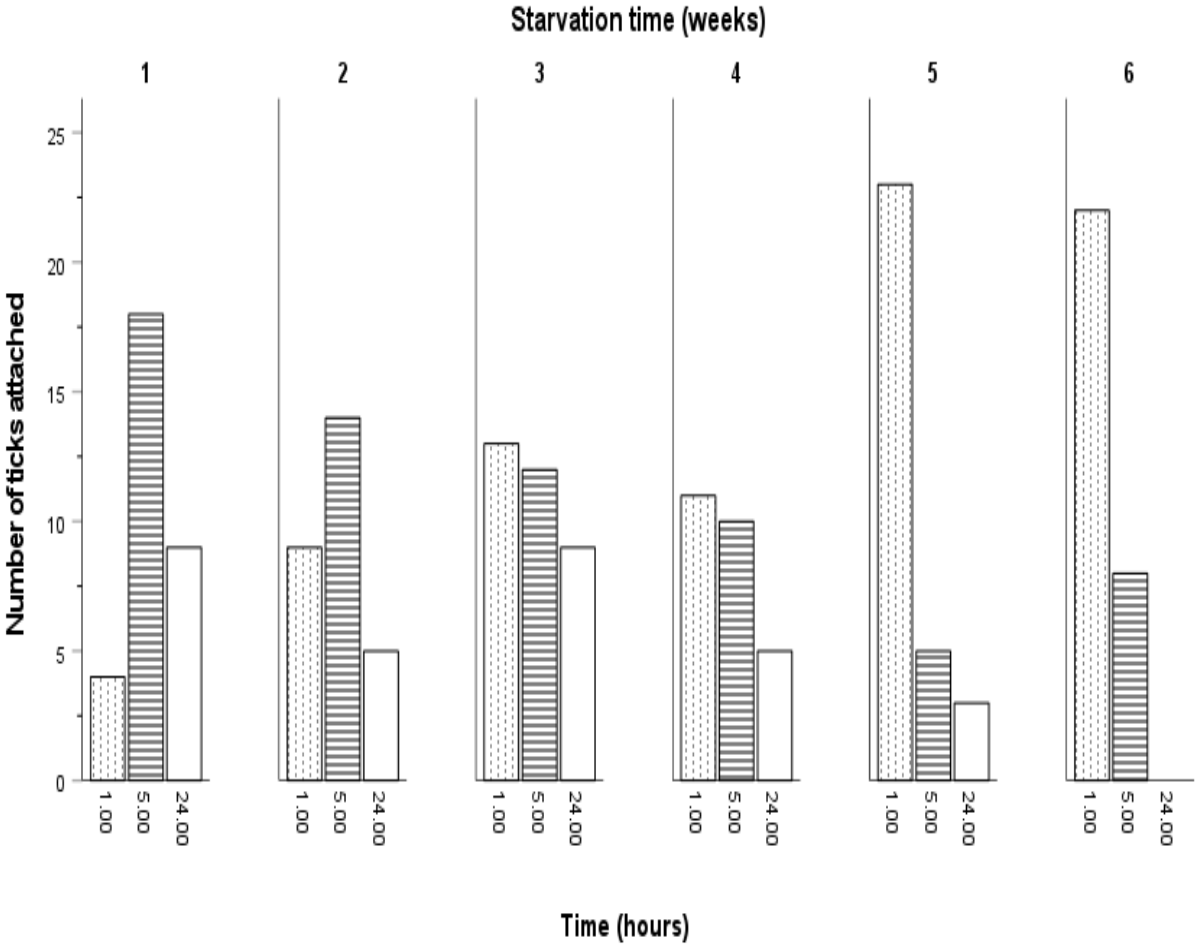


Fig 5.2 The number of starved *Ixodes ricinus* nymphs attached after 1, 5 or 24 h when exposed to an artificial feeding membrane in a humidified incubator at 37 °C and 70% RH.

After 24 h, there was a significant increase in tick attachment to the feeding membrane as starvation increased ($F_{(2,15)} = 17.51$, $P < 0.001$, $R^2 = 0.70$). Week 1 was slightly anomalously high relative to the overall pattern, with 83% attached after 24 h but subsequently 68.3% were attached after 2 weeks and this rose to 100% after 6 weeks of starvation (Fig 5.3).

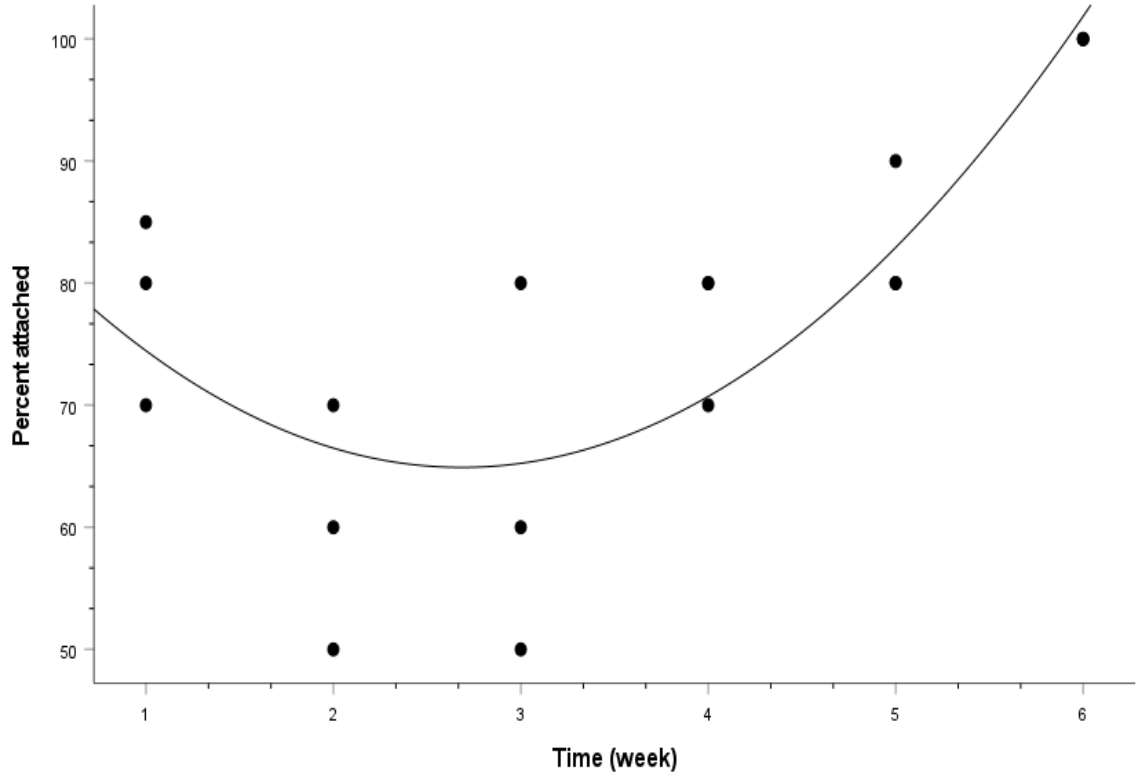


Fig 5.3 The percentage of *Ixodes ricinus* nymphs attached to an artificial feeding membrane after 24 h in a humidified incubator at 37 °C and 70% RH, after being starved for different time periods (weeks). Quadratic regression line fitted: $y = 89.17 – 18.07*x + 3.36*x^2$, $R^2 = 0.70$, $P < 0.001$, $n = 30$.

There was also a significant linear increase in the number of ticks which attached to the membrane within 1 h of exposure to the artificial membrane as starvation time increased ($F_{(5,17)} = 6.52$, $P = 0.004$, $R^2 = 0.73$). Here, tick attachment rate increased progressively from 12.9% in week 1 to 73.3% in week 6 (Fig 5.4).

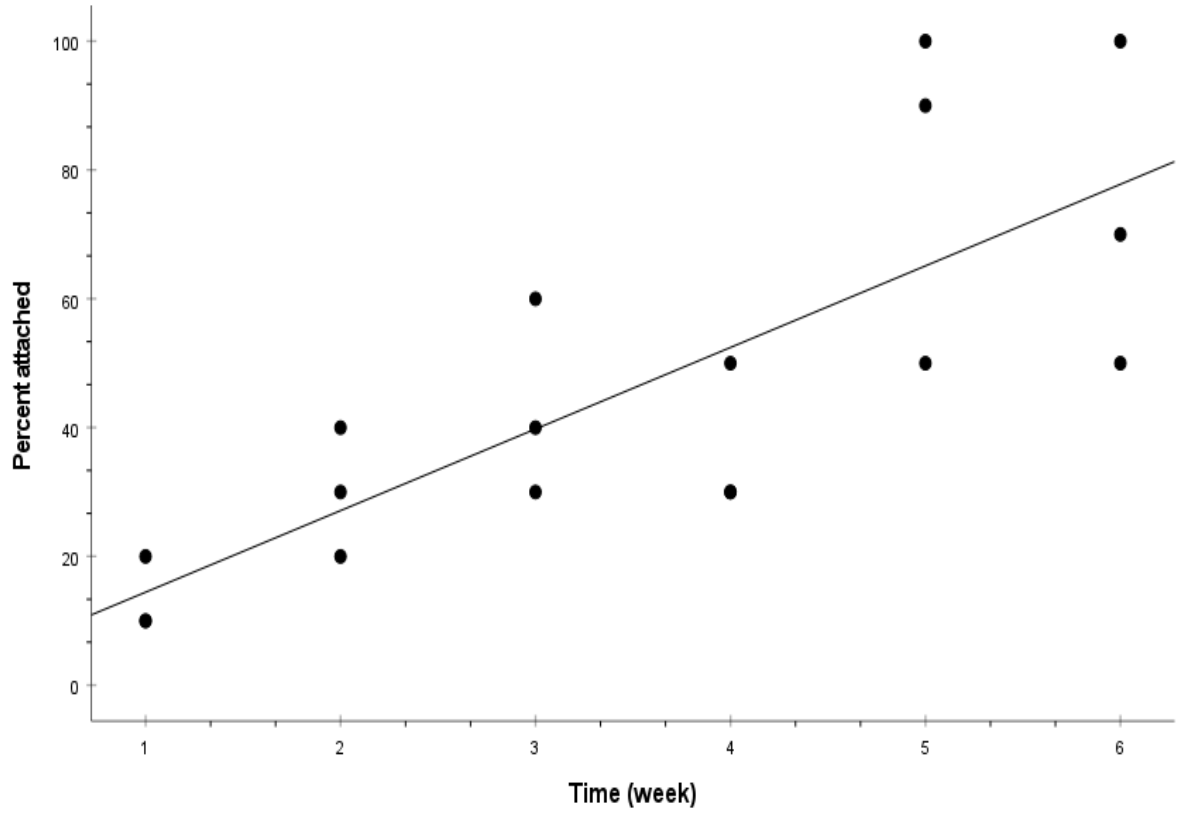


Fig 5.4 The percentage of *Ixodes ricinus* nymphs that were attached to an artificial feeding membrane after one hour in a humidified incubator at 37 °C and 70 % RH, after being starved for different time periods (weeks). Linear regression line fitted: y = 1.78 + 12.67*x, R^2 = 0.73, P = 0.004, n = 30.

Lipid

As expected, lipid concentration of the nymphs declined significantly with starvation from a median of 25.4 μg [minimum: 21.6 μg, maximum: 27.83 μg, range: 6.3 μg] in week 1, to 13.9 μg [minimum: 9.5 μg, maximum: 28.5 μg, range: 18.9 μg] in week 6 (Kruskal-Wallis: H = 68.5, P < 0.001, df = 5). As starvation progressed, lipid values appeared to decrease at a slightly faster rate in the ticks which did not attach to the membrane than in those which attached (Fig 5.5) but this difference was not significant ($F_{(4,220)}$ = 0.92, P = 0.46).

Although ticks that attached to the membrane within 1 h had slightly higher lipid values throughout the study when compared with those which attached after 24 h (Fig 5.6), a Mann-Whitney test also indicated that this was not a significant difference (Z = - 0.30, P = 0.76).

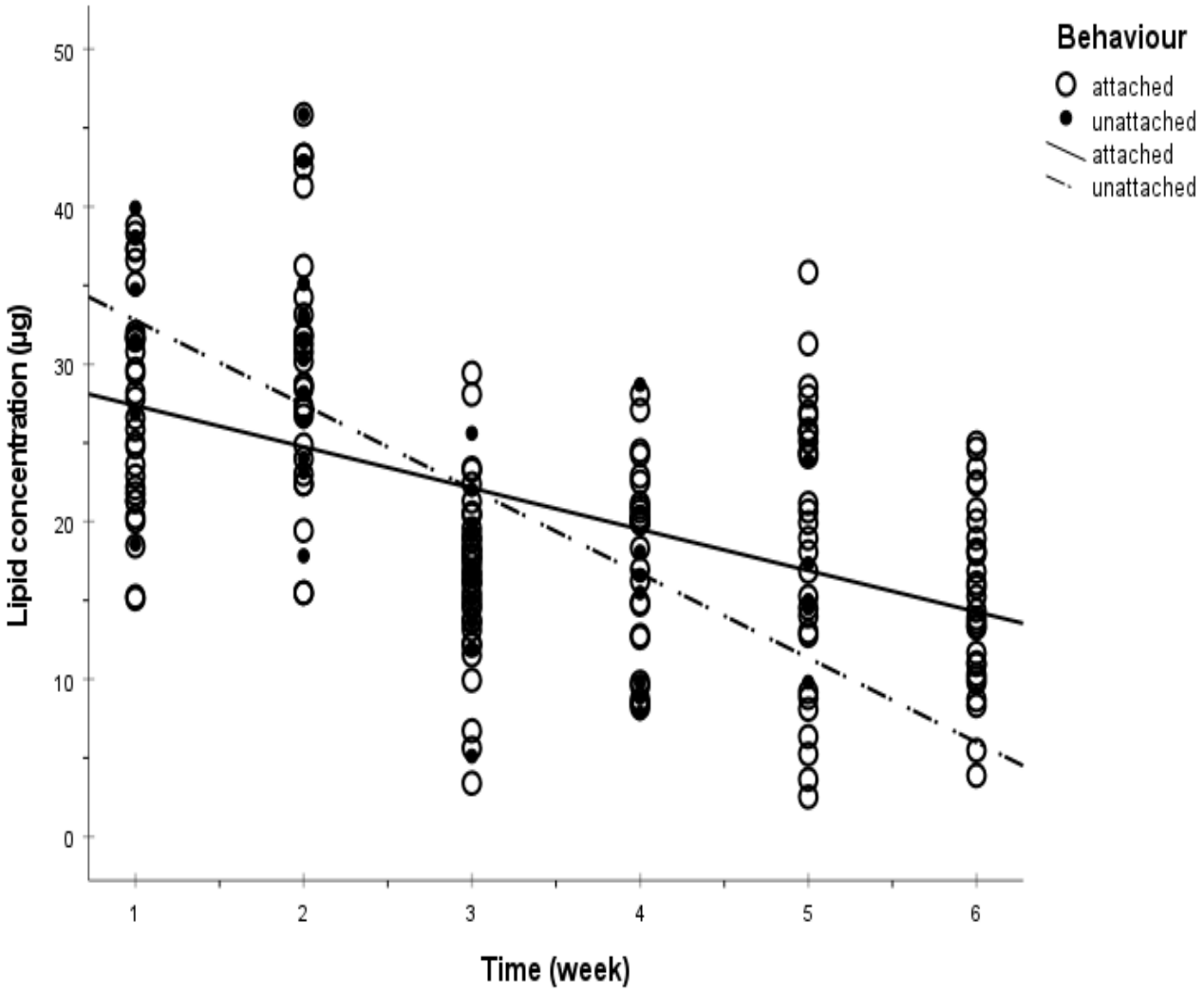


Fig 5.5 Lipid concentration (µg) recorded in progressively starved *Ixodes ricinus* nymphs which attached (open circles) or did not attach (closed circles) to an artificial feeding membrane in a humidified incubator at 37 °C and 70% RH. Linear regression line fitted: Attached: y = 30 – 2.62*x, R^2 = 0.26; Unattached: y = 38.15 – 5.36*x; R^2 = 0.43; P = 0.024, n = 30.

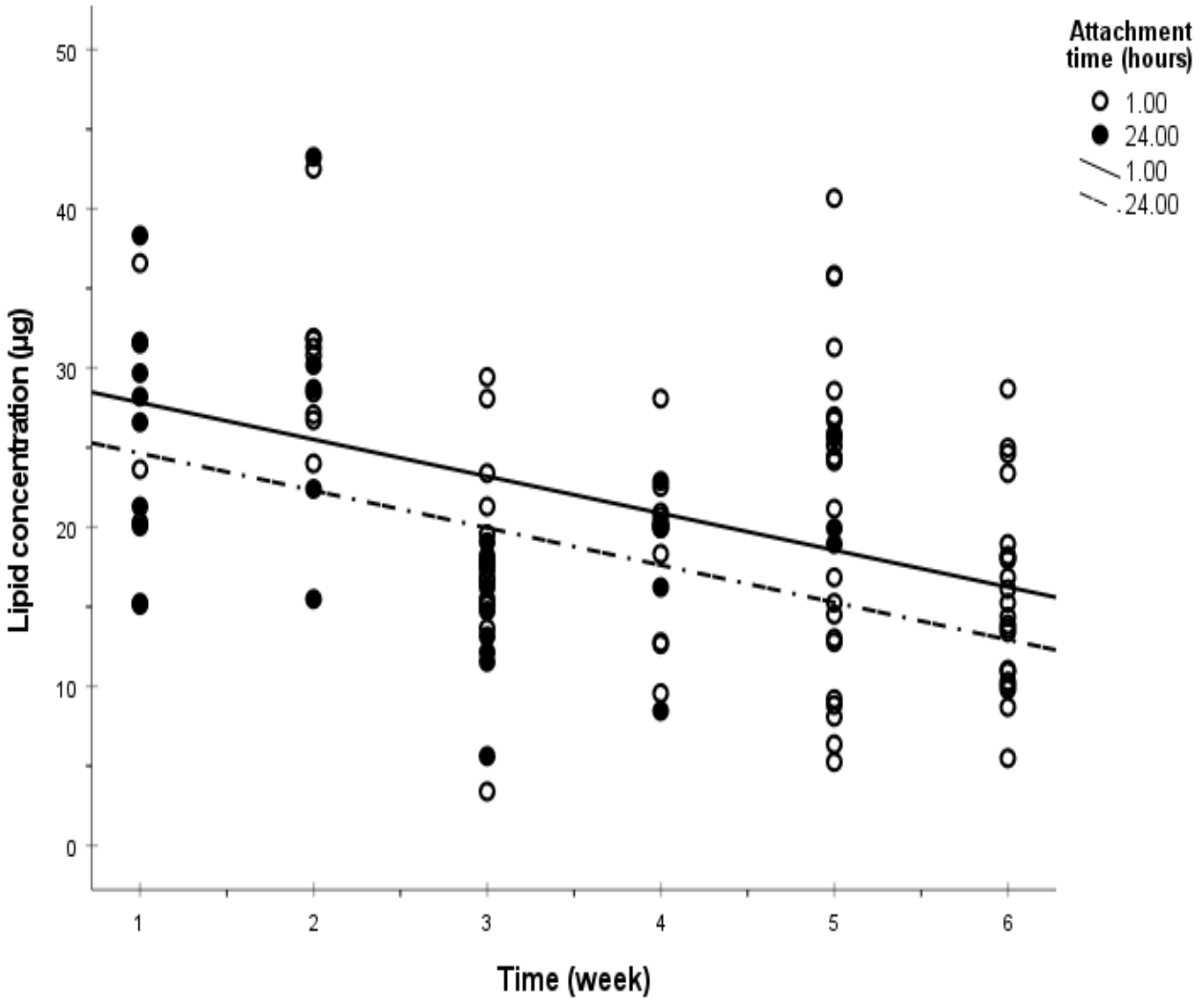


Fig 5.6 Lipid concentration (µg) recorded in progressively starved *Ixodes ricinus* nymphs attached after exposure to an artificial feeding membrane for 1 (open circles) or 24 h (closed circles), in a humidified incubator at 37°C and 70 %. Linear regression line fitted: 1 h: $y = 29.45 – 2.47*x$, $R^2 = 0.17$; 24 h: $y = 27.78 – 3.12*x$, $R^2 = 0.14$, $P < 0.001$, $n = 30$.

Hunger Index

There was no significant change in hunger index with starvation in the study ($F_{(1,178)} = 1.03$, $P = 0.31$). Overall, the hunger index of the ticks decreased by 4.9% from a mean of 1.43± 0.06 at week 1 to 1.36 ±0.04 at week 6. Also, there was no relationship between hunger index and time of tick attachment to the artificial feeding membrane $F_{(9,178)} = 0.59$, $P = 0.81$) (Fig 5.7)

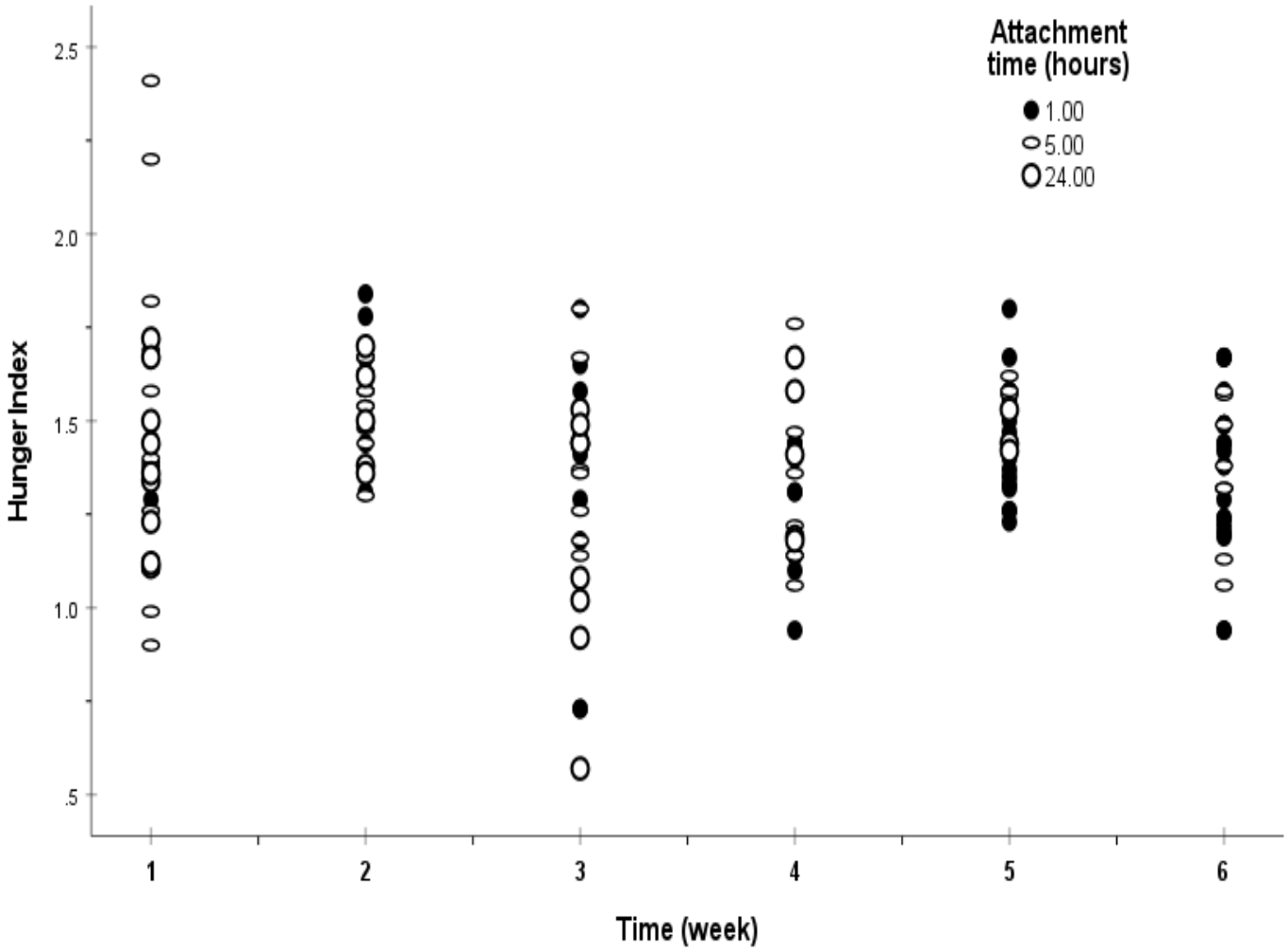


Fig 5.7 Hunger Index of *Ixodes ricinus* nymphs attached to the artificial feeding membrane following exposure to 37°C and 70% RH in a humidified incubator for 1 (solid circles), 5 (ovals) and 24 hours (open circles), observed at different starvation time points (weeks), n = 30.

5.3.2 Change in tick activity with starvation

Overall, significantly fewer of the ticks that had been starved for 4 and 8 weeks left the arena in 60 min than the ticks that had been starved for only 1 week ($F_{(2,179)} = 13.17$, $P < 0.001$). 23.3% of the ticks did not cross the oil-impregnated ring at week 1, followed by 51.7% at week 4 and 66.7% at week 8 (Fig 5.8).

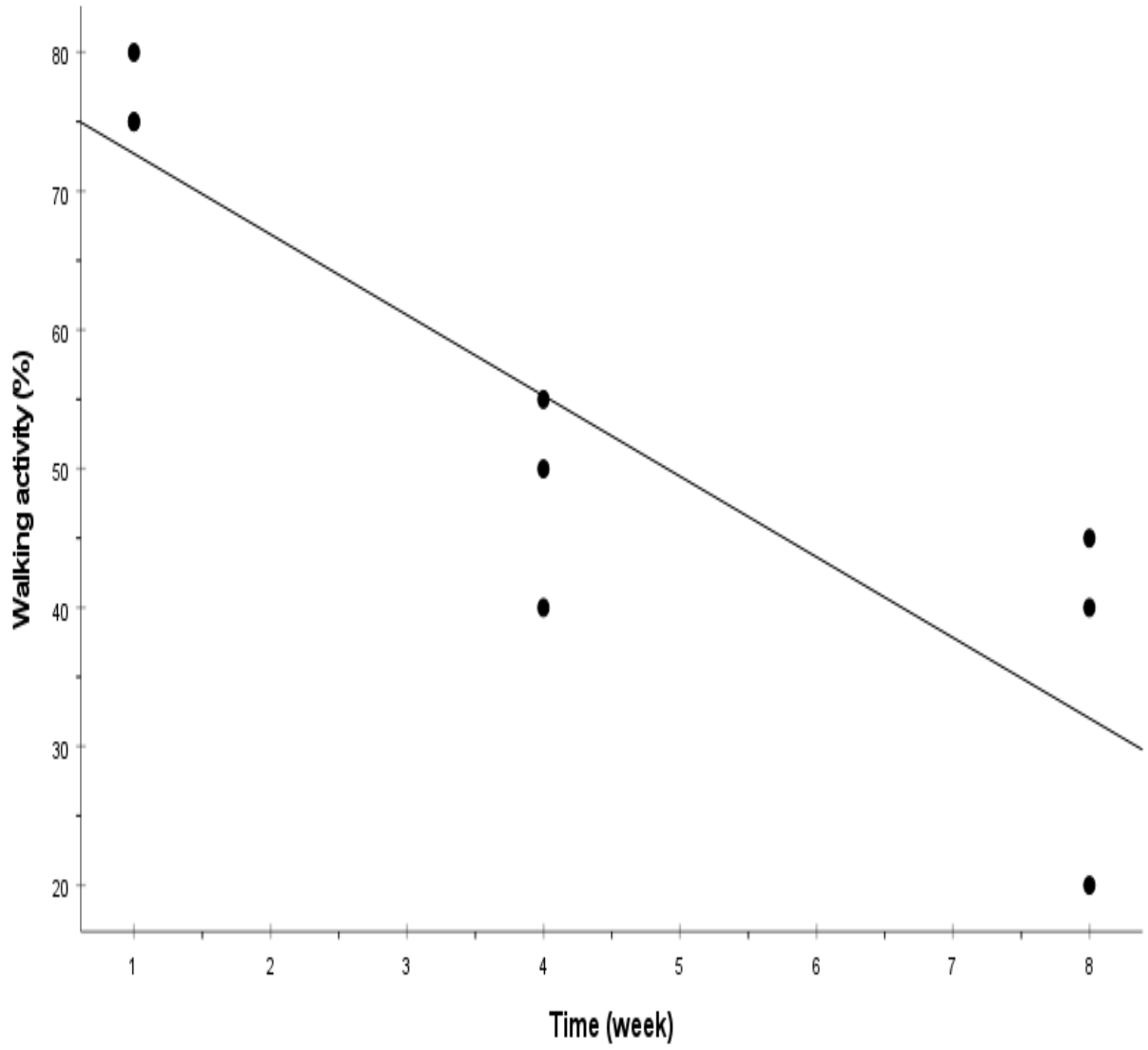


Fig 5.8 Percentage of *Ixodes ricinus* nymphs that walked across an essential oil (5% thyme oil) impregnated ring after having been starved for 1, 4 or 8 weeks in a cooled incubator maintained at 15°C and 80% RH. Linear regression line fitted: $y = 78.51 – 5.81*x$, $R^2 = 0.78$, $P = 0.002$, $n = 60$.

All the walking behaviour occurred within the first 25 minutes, such that any ticks which remained in the arena after 25 min did not subsequently cross the oil-impregnated ring by the end of the study (Fig 4.11). For those ticks that did cross the oil impregnated ring, there was a significant decrease in the time taken to cross the oil-impregnated ring as starvation progressed ($F_{(2,94)} = 3.75$, $P = 0.03$). For the ticks which crossed the oil-impregnated ring at week 1, the median to crossing was 15 mins. As starvation progressed, response time decreased to a median of 10 mins in both weeks 4 and 8. The data also show that in the ticks which starved for 4 and 8 weeks, the distribution of crossing times was highly skewed, because most ticks crossed the barrier within 10 minutes (Fig 5.9).

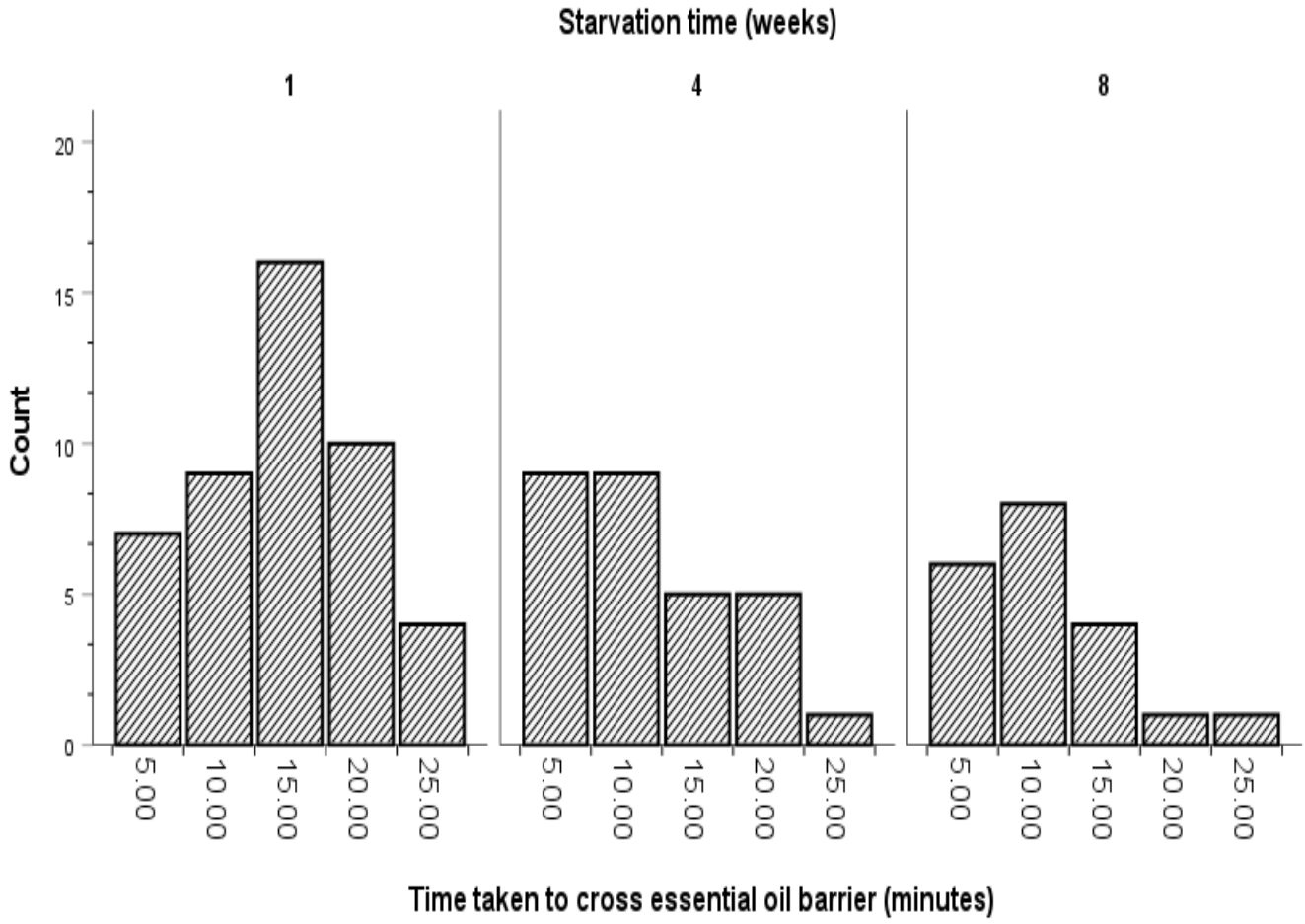


Fig 5.9 The number of *Ixodes ricinus* nymphs that walked across an essential oil (5% thyme oil) impregnated ring at 5, 10-, 15-, 20- and 25-minutes, observed at different starvation periods (1, 4 and 8 weeks)

Lipid

As expected, lipid concentration decreased significantly with starvation in the study (Kruskal-Wallis: $H = 50.22$, $P < 0.001$, $df = 2$). There were significant differences between individual lipid concentration of ticks that crossed the essential oil impregnated ring after different periods of starvation, but this was modulated by periods of starvation. After 1 week of starvation, ticks which had the lowest lipid values crossed the essential oil ring significantly more quickly than less hungry ticks ($F_{(1,45)} = 5.76$, $P = 0.02$; Fig. 5.10). However, after 4 and 8 weeks there were no longer any significant differences between the lipid values of ticks that crossed the oil-impregnated filter paper at different times ($P > 0.05$).

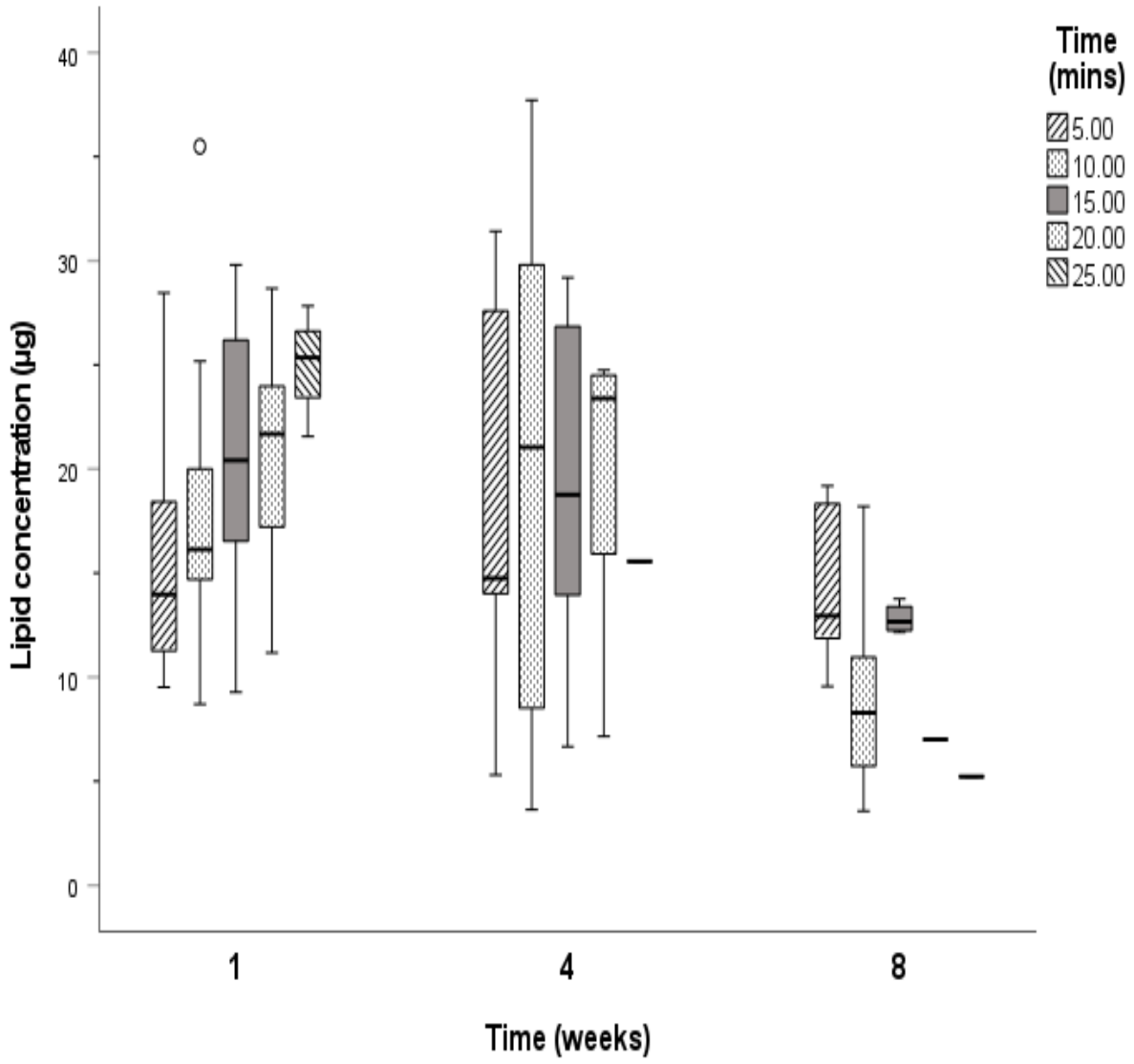


Fig 5.10 The Lipid concentration (µg) of starved *Ixodes ricinus* nymphs which crossed the essential oil (5% thyme oil) impregnated ring at 5, 10-, 15-, 20- and 25-minutes, observed at different starvation intervals (week 1, 4 and 8). Horizontal line = median, box = interquartile range, whiskers = minimum and maximum, ° = outliers, n = 60.

Hunger Index

The results showed there was no significant relationship between hunger index and duration of starvation. Also, there were no significant differences between the hunger index of ticks that crossed the oil-impregnated filter paper at different times (Fig 5.11).

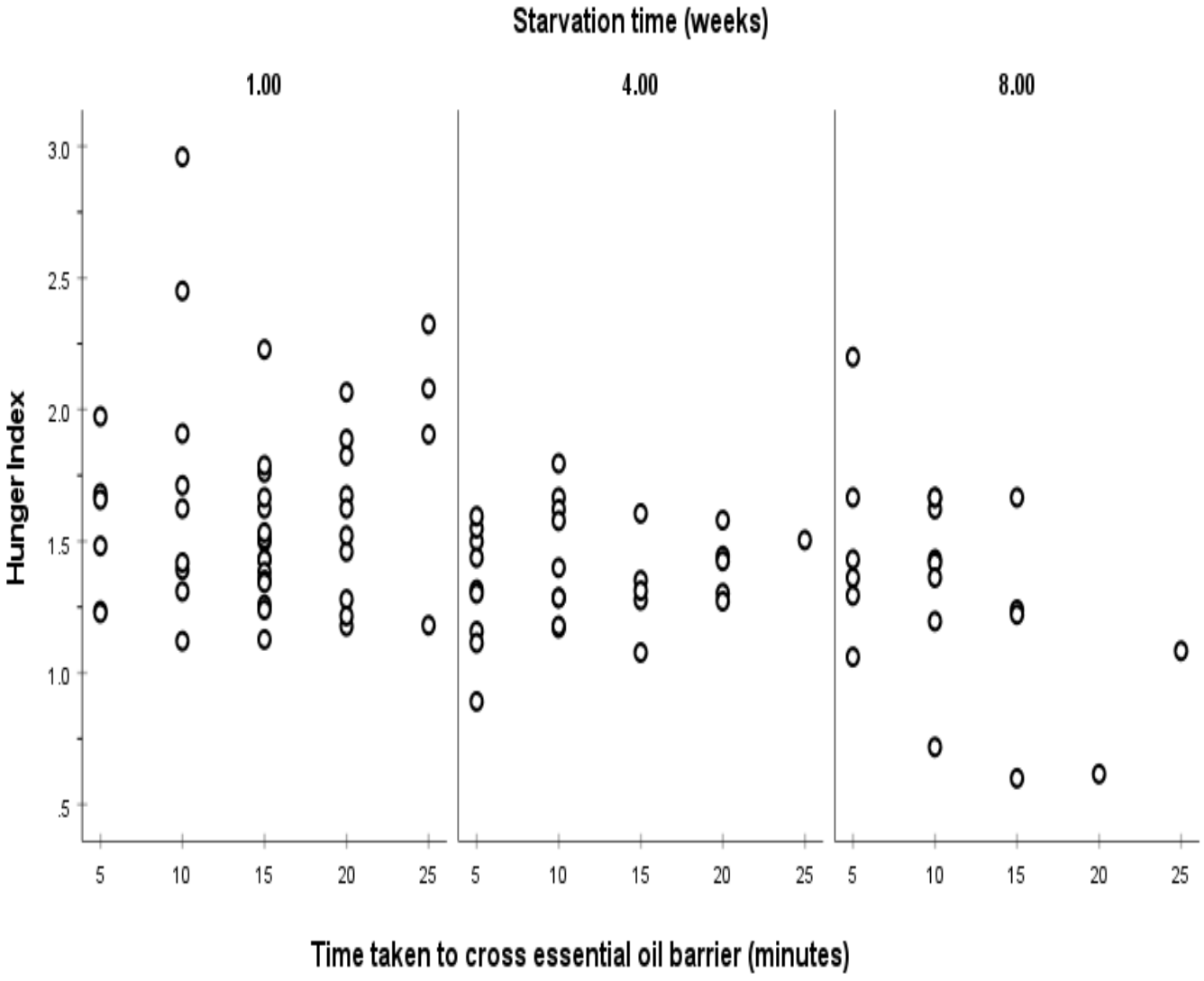


Fig 5.11 The Hunger index of starved *Ixodes ricinus* nymphs that walked across an essential oil (5% thyme oil) impregnated ring at 5, 10-, 15-, 20- and 25-minutes, observed at different starvation intervals (week 1, 4 and 8), n = 60.

5.4 Discussion

The aim of the study described in this chapter was to determine whether there were changes in the behaviour of *I. ricinus* ticks associated with starvation. Feeding and locomotory activity in response to external stimuli, which are key behavioural responses playing an important role in fitness and survival, were studied. Overall, in starved ticks, the results showed that hungrier ticks attached to the artificial feeding membrane more quickly, mostly within the first hour. The results also showed that the hungrier the ticks were, the less likely they were to walk across the essential oil impregnated ring, which was considered to be repellent. For those that crossed, the time it took to leave decreased with increasing hunger. In both studies, Hunger Index did not appear to have any significant association with feeding behaviour or walking activity of *I. ricinus* ticks, further highlighting the unsuitability of morphological measurements for quantifying starvation over the time periods examined here.

For ticks, questing for hosts, represents a trade-off of risks. The chance presence of a host brushing against an infected tick is relatively low, so extended periods of questing are required, while the risk of desiccation during this questing period is high. As a result, questing ticks might be expected to be initially limited to periods when humidity is highest and host availability most likely. But as ticks become increasingly nutritionally deprived, the optimum trade off point might be expected to change as the risk of death from starvation becomes more likely. Here the tick feeding assay was used to study the changes in tick behaviour with starvation and the results showed that there was an increase in the likelihood of ticks attaching to the membrane as starvation increased. This was particularly evident within the first hour of the study and after 6 weeks of starvation, over 70% of the nymphs were probing or attached to the membrane. Analysis of the lipid concentration of the ticks used in the study confirmed a progressive decrease in lipid values with starvation.

However, interestingly within each batch that had been starved for a specific period of time, it was the ticks with higher lipid values that appeared to respond fastest to feeding stimuli. This apparently contradictory result is difficult to explain but suggests that nested within the overall effect of an increase in the likelihood of probing and attaching to the membrane with starvation, there may be some additional underlying effect where the ticks with higher lipid values are more active. Further study would be needed to investigate this in more detail.

Ticks serve as vectors for many pathogens which cause debilitating and sometimes fatal disease in both human and animal hosts, with major economic and public health consequences (Anderson *et al.,* 2008; Estrada-Peña *et al.,* 2013; Sonenshine and Roe, 2013). To avoid infection by such microorganisms, tick bites must be prevented. One practical way is by using effective tick

repellents for personal protection. However, commercially available repellents are usually only partially effective (Schreck *et al.,* 1995). This is mainly because most available repellents, such as DEET, have been developed for protection against mosquito bites rather than for tick bites. Hence the need for continuous testing of candidate substances in a suitable bioassay to facilitate finding of new, safe tick repellents. Also, because of the growing incidence of resistance to conventional acaricides, the search for environmentally safe alternatives for managing or preventing tick infestation of humans or animals is essential. In the present study, volatile cues from an essential oil (thyme) that has been shown to act as a repellent and an acaricide was investigated; thyme oil was reported to produce approximately 90% repellence against adult ticks in several studies (Ellse and Wall, 2014; Tabari *et al.,* 2017; Goode *et al.,* 2018).

The hypothesis was that hungrier ticks might be more willing to cross the essential oil-impregnated filter paper given their need to find a host quickly. In contrast, however, the ticks appeared less likely to walk across the oil-impregnated filter paper as hunger increased. One explanation could be that the sensitivity of ticks to thyme oil increased with starvation. Unlike in flying insects where repellence implies a fly away effect due to volatile effect of the repellent molecules, repellence in ticks would be mainly due to an irritant contact effect preventing attachment or inducing the feeding animal to leave the host (Dumont *et al.,* 2015; Goode *et al.,* 2018; Soutar *et al.,* 2019). One limitation of this study, however, was that no specific host stimuli such as heat or kairomones were incorporated into the assay. It is difficult therefore to draw clear conclusions about the relationship between the behaviour observed and its meaning for ticks in the field.

In conclusion, the results of these studies provide evidence of a change in *I. ricinus* behaviour associated with starvation. Previous studies directed towards controlling ticks, tick-borne diseases or modelling tick populations due to climate change have generally not incorporated the impact of starvation on the results or outcomes of these models. This should be considered. Further studies are recommended on how starvation can affect other important behaviour in *I. ricinus* to elucidate the potential implications for human or animal health.

- Chapter 6 -

General Discussion

The distribution of vector borne pathogens, especially those transmitted by immobile, slow-feeding vectors such as ticks, is currently increasing and this poses serious implications for the welfare and health of both humans and animals which serve as hosts for these vectors (Brown *et al.,* 2005; Nieto *et al.,* 2018). Climate change and other multiple, inter-related and complex factors have been highlighted in several studies as the reason for this changing distribution (Randolph, 2008; Gray *et al.,* 2009; Gilbert *et al.,* 2014). However, determining whether changes are occurring due to climate change or other drivers can be difficult to establish because of the complexity of tick-borne disease (tbd) systems. Generally, tbd transmission rate is related to tick abundance because with greater tick density, the likelihood of the occurrence of a tick bite (consequently acquiring infection from a host) increases. Even where climate change may not directly affect the demographics of tick populations in the field, habitat and host abundance may be altered, with a ripple effect on tick density and prevalence of tick-borne infections (Gilbert, 2021). Most empirical and theoretical studies demonstrate or predict range shifts or increases in ticks and tbds, but there can be a lot of heterogeneity in such predictions (Randolph, 2002; Estrada-Pena, 2009; Gray *et al.,* 2009). Predictive models are an invaluable tool used in projecting these changes according to different climate change scenarios, however validating these models remains challenging, and estimating uncertainty in their predictions is essential. For most economically important tick species, starvation tolerance and its implications for tick response to changing climate parameters is an important but mostly neglected consideration in generating these models, as it could provide an index of the resilience of ticks and tick-borne pathogens to projected changes in climate.

In the UK and throughout Europe, *Ixodes ricinus* is the most important tick in terms of abundance, distribution and contribution to tbds (Medlock *et al.,* 2013) and its life cycle is characterised by long periods of starvation between blood meals (about 95% of the entire life span) (Sonenshine, 2005; Umemiya-Shirafuji *et al.*, 2010). During the long off-host periods, ticks depend on stored metabolic reserves accumulated from the previous blood meal to maintain homeostasis and developmental processes (Alasmari and Wall, 2021). To help ticks to survive this inter-feed interval and unpredictable availability of food, they have a relatively low rate of metabolism – similar amongst the arthropods to scorpions, another sit-and-wait predator (Benton, 1992;

Pimenta *et al.,* 2019; Segev *et al.,* 2020). This characteristic is thought to be important in the life-history strategy of Ixodid ticks. Questing is also a key activity which occurs within this period. Questing is necessary but risky and metabolically costly to the tick because it expends energy and runs the risk of dehydration because it is exposed to the elements. Failure to encounter a host to feed on during questing will, ultimately result in its death, after body reserves are completely depleted. Blanket dragging, the principal technique used to collect ticks in the field makes use of the questing response but has the drawback that only the relatively hungry portion of the tick population is collected.

During questing, water loss is one of the key challenges that affect tick behavior and survival. The interaction of temperature and humidity, reported as saturation deficit, on survival were demonstrated in the first part of Chapter 3, but the work then went on to evaluate the effects of hunger on this relationship. Starved ticks were exposed to varying saturation deficits ranging from 0.6 to 10mmHg. A clear effect of starvation on sensitivity to saturation deficit was difficult to establish experimentally, first because ticks starved for longer may be more likely to die anyway, but also because some saturation deficits examined were either too low (so ticks survived the full extent of the study regardless) or too high (so ticks died anyway whether they were starved or not), suggesting that in high saturation deficit conditions mortality was increased even in ticks with high lipid reserves. However, a threshold relationship showing some indication of a greater sensitivity of starved ticks to saturation deficit was evident at 2.5mmHg. Nevertheless, the results support those of previous studies, which found that ticks with limited energy stores had a greater susceptibility to water loss when compared to more recently fed ticks (Estrada-Pena, 2008; Hermann and Gern, 2014; Swei *et al.,* 2014). Herrmann and Gern (2013) also reported that ticks with higher lipid reserves survived exposure to low temperatures better than starved ticks with low lipid levels. This study therefore confirms that *I. ricinus* is highly sensitive to climatic conditions, with a distinct environmental niche. This suggests that as a species, it might be expected to be strongly affected by changes in climate, particularly precipitation and humidity, as well as increasing temperatures. One implication of this is that the range of expansion of *I. ricinus* into more northerly latitudes may increase as higher latitudes have been projected by CMIP5 models to record higher precipitation falls as climate changes over the next few years (Lau *et al.,* 2013; *Ziu et al.*, 2014; Behrangi *et al.,* 2016). Climate change could also alter the seasonal phenology of the different parasitic stages of the tick by interfering with the timing of tick development, oviposition, mortality or activity. This may result in range shifts in the period of larvae, nymph or adult tick abundance, leading to the occurrence of different instars on a potential host and increasing disease

transmission potential, perhaps due to non-systemic cofeeding transmission (Randolph, 1996; Kocan and de la Fuente, 2003; Nah *et al.,* 2019).

The results of this study also suggest that at the current edges of its range, climate change may make the ticks less sensitive to starvation, increasing survival and prolonging tick challenge (Estrada-Pena, 2008; Hermann and Gern, 2014; Swei *et al.,* 2014). However, any such effects are likely to be complex because of the multitude of factors that affect tick survival and behaviour in the field. Nevertheless, further work on these interactions is certainly likely to be productive and essential to gaining a comprehensive understanding of the challenge presented by ticks over their long hunger cycle, especially at the threshold temperature and saturation deficits established in this study.

The results from the study in Chapter 2 demonstrated that, as expected, the values for the scutal parameters (scutum length and width) remained constant over time, while the parameters for the alloscutum varied. The variation in the alloscutum appeared to be mainly due to changes in the body width rather than the body length, as recorded by other studies (Yeh *et al.,* 1995; Uspensky *et al.*, 2006). This is an important finding as it could provide substantive evidence for the difference in the morphometrics of different tick species possibly related to differences in genetic composition of ticks evident at species level. This study confirmed that quantitative estimation of lipid reserves is a good estimate of time-since-last-feeding in ticks, as described in other studies (Abdullah *et al.,* 2018; Alasmari and Wall, 2020). Our 'Hunger index' also shown to significantly decrease with starvation in the study showed promise as an index of time-since-feeding, however the variation in the results as well as the weak correlation between hunger index and lipid concentration (R_s = 0.2) suggests that morphological measurements and calculation of hunger index may have minimal predictive value as an index of time-since-feeding in *I. ricinus* ticks. This relative to that of lipid measurement, and a higher sample size would certainly improve the confidence in its estimation. The sample sizes used and replication that was possible, was the minimum adequate but the COVID19 pandemic and consequent lockdowns during which laboratory and field work activities were interrupted for several months, interfered with the timing of tick sample collections from the field, as well as the number of ticks which could be collected. The results from this thesis suggest that, although hunger index may have other applications, such as for classifying ticks into different feeding cohorts, as used in Uspensky's (1999) study, or as used here to compare the difference between cohorts of *I. ricinus* ticks collected either in the autumn or in the spring, it may not be a reliable non-invasive measure for determining the feeding history in *I. ricinus* for use in behaviour studies. This may have been because the rate of occurrence of obvious changes in tick morphometry due to starvation occurs at a slow rate with considerable

individual variation, and its measurement is inherently less precise than that of lipid measurement. It has also been suggested that there may be a decrease in metabolic rate in *I. ricinus* with starvation (Alasmari and Wall, 2020) which could affect the rate of morphological change. To further clarify the rate of morphological change, it would be useful in future work to generate a reference curve by feeding individual ticks to repletion and then starving them over time, where repeated measures of body parameters from the same individual are recorded over time-to-death to produce a referral graph of changes in tick morphometry over the course of life.

Conventionally, live animals such as cattle, dogs or laboratory animals are commonly used as hosts to maintain artificial tick colonies in the laboratory (Hadani *et al.,* 1969; Sonenshine, 1999; Allan, 2013). However, this has raised several ethical and welfare issues including physical harm to the animals, development of conditions such as anaemia, inflammation exacerbated by restrictive conditions which the animal may be subjected to which may interfere with normal grooming behaviour, not to mention the high maintenance costs associated with keeping these animals or the near impossibility of acquiring permits or endorsements for such studies. To avoid these issues, the solution is to develop viable *in vitro* feeding systems without the use of live animals. Many research groups have attempted to do this, with variable success. Here in Chapter 4, an artificial feeding system, comprising a feeding chamber and a silicone membrane, was evaluated for tick feeding *in vitro*, after applying some modifications from similar apparatus developed by other studies (Krober and Guerin, 2001; Krull *et al.,* 2007; Militzer *et al.,* 2021). A major challenge is that achieving membrane feeding of hard ticks is especially difficult to accomplish due to long feeding duration of most tick species (Waladde *et al.,* 1996; Kuhnert, 1996; Krobër and Guerin, 2007) in addition to the complex combination of kairomones required to initiate and sustain the feeding process (Kemp *et al.,* 1986; Bonnet and Liu, 2012). The work described here was able to get good levels of probing and attachment to the membrane but feeding to repletion was harder to achieve possibly due to the fact that the blood needs to be changed every 12 hours to avoid bacterial contamination. Prolonged feeding would require frequent blood changes (every 12 h) and treating the membrane with antibacterial and antifungal agents to prevent infection. This is a critical problem that needs to be resolved to allow ticks to be fed *in vitro* in experimentally useful numbers. Nevertheless, by recording probing and attachment the assay developed here was used successfully to study the response of starved ticks to feeding stimuli in Chapter 5. Perhaps, an automation of the artificial tick feeding system, especially focused on automated blood changes would reduce the need to interrupt feeding ticks within the chambers and allow constant feeding to repletion, enabling the goal to be achieved. This presents opportunities for further investigation.

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