Author:
Alasmari, Saeed M N

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Measuring and interpreting the energy budget of the tick, Ixodes ricinus

Measuring and interpreting the energy budget of the tick, *Ixodes ricinus*

By

SAEED ALASMARI

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Abstract

Precise and accessible techniques for measuring metabolic responses to environmental stress are essential to allow the likely impacts of climate and climate change on tick distribution, abundance and phenology to be predicted. Here, in Chapter 2, a series of biochemical protocols employing spectrophotometric methods are described and used to determine the entire energy budget of ticks. Protein, total lipid, neutral lipid, the total water-soluble carbohydrates and glycogen were measured in individual *Ixodes ricinus* nymphs and adults. Two key trends were identified: in adults, protein was relatively more abundant than in nymphs, whereas in nymphs, glycogen and soluble carbohydrates were more abundant than in adults, with glycogen alone composing 39% of the mass of metabolites in nymphs compared to 15 and 10% in females and males, respectively. The results demonstrate that the spectrophotometric approaches deliver relatively rapid and reliable estimates of the total energetic budget and can be used to quantify the metabolic profiles of individual ticks.

The work described in Chapter 3 aimed to investigate the effects of temperature on the rate of depletion of energy reserves by nymphal and adult *I. ricinus*. A cohort of nymphs, males and females were collected and divided into incubators at a range of temperatures. The protein, total lipid, neutral lipid, soluble carbohydrates and glycogen levels were measured over time. In nymphs, the rate of soluble carbohydrates and glycogen utilisation was higher than in males or females and the concentrations of neutral lipids were affected significantly by higher temperatures. In adults, the concentrations of protein and structural lipid (phospholipid) responded rapidly to changes in treatment time and the ambient temperature.

Nymphs and adult *I. ricinus* were sampled from the field each month from February 2018 to January 2019 and, in Chapter 4, the changes in energy source contents over the year are presented. The data suggest that there exists a well-defined cohort of relatively well-fed nymphal ticks in the early spring, most probably derived from larvae that fed the previous summer and moulted the previous autumn. They start to quest as temperatures rise sufficiently in spring to permit activity. Those that are unable to find a blood-meal continue questing but gradually exhaust their reserves. By mid-summer the only larvae left questing are close to starvation. For females, the population at the start of the year is composed of a cohort that started questing early in the year that were relatively hungry; they may have been derived from nymphs that fed relatively early the previous year moulted to become adults that were unable to feed, so overwintered and started questing early the following year in an already resource depleted state. By April, the cohort of questing females has now been joined by individuals that moulted to become adults late the previous year or early in the year and are in relatively well resourced. However, those that do not feed gradually start to exhaust their resources. In the second half of the year, females with high levels of metabolic resource appear – presumably from nymphs that early in the year of the study, digested their blood meals, moulted to become adults and are now re-joining the questing population. In males the patterns of changing energy sources were much less distinct than seen in nymphs or females.

In the final Chapter, the data are discussed generally, and further applications of the spectrophotometric techniques presented here are considered.

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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.

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Alasmari, S., and Wall, R. (2020). Metabolic rate and resource depletion in the tick *Ixodes ricinus* in response to temperature. *Experimental and Applied Acarology*, (In Press).

Chapter 1

Ticks, energy budgets and behaviour

1.1 Introduction

Ticks are obligate hematophagous ectoparasites of both domestic and wild animals as well as humans; they are distributed from the arctic to the tropical regions worldwide (Guglielmone et al., 2010). Many species are known to act as vectors of a wide range of protozoal, bacterial, viral and rickettsial pathogens (Bowman and Nuttall, 2008). Despite continual efforts to tackle tick infestations, the pathogens they transmit remain a potent threat to animal and human health all over the world (de la Fuente et al., 2008). During the off-host phase of their life ticks are particularly susceptible to environmental conditions and they are, therefore, of particular interest from the standpoint of climate change owing to their high levels of dependence on abiotic conditions for their rates of development, survival and reproduction (Vassallo et al., 2000; Estrada-Peña et al., 2004). Research suggests that vector-borne diseases are likely to increase in prevalence and distribution owing to climate change in the future. Hence, to better predict these changes, it is important to understand the impacts of temperature and humidity on development and activity patterns (Ogden, 2016).

The sheep tick, *Ixodes ricinus* is the principal vector of a wide range of pathogens which include louping-ill virus, tick-borne encephalitis (TBE), *Babesia*, *Rickettsia* and *Anaplasma* species as well as *Borrelia burgdorferi* s.l., which cause Lyme borreliosis (Shuman, 2010). In recent decades, this tick species is believed to have increased in abundance and shown range expansion (Scharlemann et al., 2008); corresponding higher incidences of tick-borne disease

by this species have also been partly attributed to climate change (Tälleklint & Jaenson, 1998). It is believed that ongoing climate change will continue to further affect *I. ricinus* as well as the epidemiology of the pathogens it transmits (Vial, 2009; Medlock et al., 2013). Hence, this species forms the primary subject of the work described in this thesis.

1.2 Tick biology, geography and pathogen transmission

Ticks can be divided into two main families: Ixodidae (hard) ticks and Argasidae (soft) ticks with at least 869 sub-species that have been recorded (Guglielmone et al., 2010), with notable differences in both morphology and ecology (Bowman and Nuttall, 2008). Hard ticks generally proliferate in open environments and exhibit seasonal variation in their numbers, whereas soft ticks inhabit sheltered niches and often do not usually demonstrate such strong seasonal variation in abundance (Bowman and Nuttall, 2008). Both require multiple hosts; generally in hard ticks one for each stage of the life cycle, and usually a different and specific animal host species. While the lifespan of hard ticks is typically in the range from a few months up to three or four years, soft ticks can live up to ten years as they tend to be much more resistant to low nutrient levels or even starvation conditions (Parola and Raoult, 2001). Moreover, the feeding habits of these two tick families are very different. For hard ticks, blood-feeding at each life-cycle stage takes several days and results from a one-point, firm attachment to the host. However, soft ticks undergo a multitude of attach-feed-detach-reattach cycles within each stage, with feeding taking place on a faster timescale, ranging from minutes to hours (Parola and Raoult, 2001).Ticks from both families transmit a wide range of pathogens.

In terms of global significance for human health, key hard tick species include *I. ricinus, I. scapularis* and *I. persulcatus* (Fig. 1.1). They predominantly inhabit forests and thickets in areas of high air humidity, feed on mammals (for example, dogs and deer), birds and reptiles with a high affinity towards humans (Parola and Raoult, 2001).

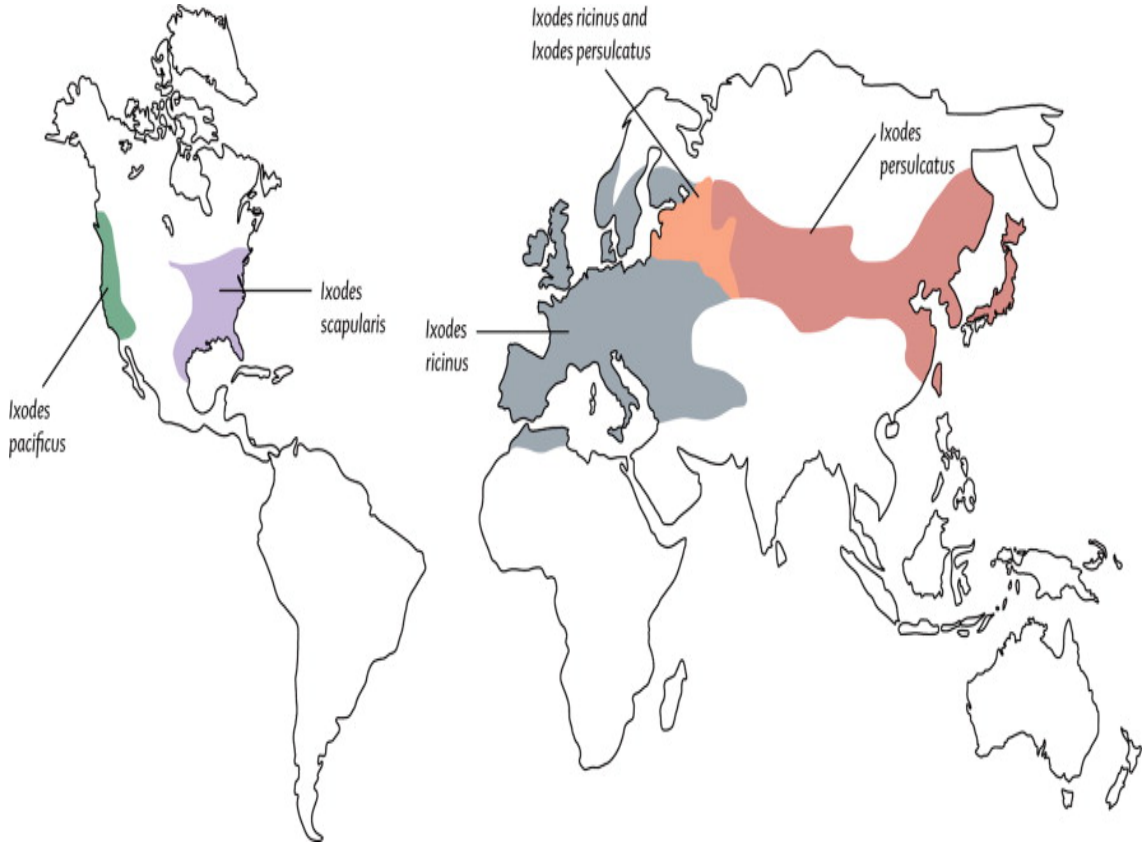

Figure 1. 1 Geographical distribution map of four species of *Ixodes* ticks that carry *Borrelia burgdorferi* s.*l.* which causes Lyme borreliosis. Image reproduced from (Stanek et al., 2012).

1.2.1 The tick life cycle

The majority of hard ticks have three motile instars in their lifecycle, with each stage (larva, nymph and adult) attaching and feeding on blood from a different host (Apanaskevich and Oliver, 2013). Subsequently, the replete tick detaches and drops from the host before seeking a safe area to digest and metabolise the key blood nutrients, prior to moulting to the next life-cycle stage followed by questing and eventual host attachment. Tick mating usually occurs on the host, with adult males feeding only briefly, whereas females feed and engorge for longer periods of time before laying between a few hundred to thousands of eggs (Parola and Raoult,

2001). Figure 1.2 shows the life cycle for *I. ricinus* with typical hosts outlined in boxes (but also humans due to high affinity).

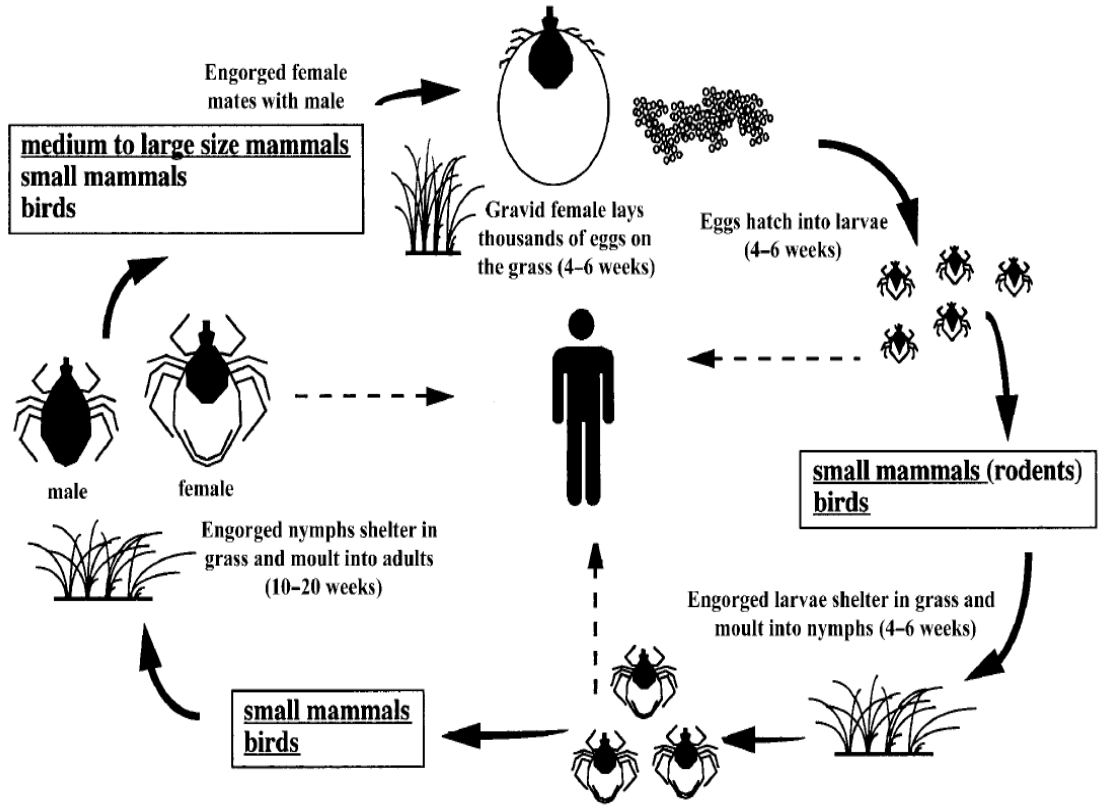

Figure 1. 2 Depiction of the different stages and hosts involved in the life cycle of the European sheep tick (*Ixodes ricinus*). Image reproduced from Parola and Raoult (2001).

The full life cycle is usually complete within 2-3 years but can be extended depending on ambient conditions such as air humidity (water content), temperature and light levels. Hard ticks are highly sensitive to desiccation and generally show highest rates of survival in warm areas with high humidity. They spend most of their time unattached and hence are seasonally active, responding to a multitude of chemical and physical stimuli to identify a suitable host at a particular stage of their life cycle. Although some tick species feed on the same species at each stage (e.g. *Rhipicephalus sanguineus*) others, such as *I. ricinus*, attach to a different host

species at each stage. The process of feeding (ingestion of blood) from the host entails a series of alternating salivation periods (pathogens are transferred in this stage), blood ingestion and regurgitation until the tick is fully engorged - at which point it detaches and drops from the host (Pfäffle et al., 2013).

When temperature and humidity conditions are suitable, ticks are able to quest for as long as their energy reserves last, until a host is found, although they may have to retreat to the moist layer of vegetation during this period in order to prevent desiccation. In areas of high humidity, seasonal tick activity may continue for as long as several months, whereas in drier areas, energy depletion and water loss is greater and, as a result, the period of activity is shorter and may last only a few weeks (Dantas-Torres et al., 2012). Within populations of *I. ricinus* the seasonal patterns of activity differ regionally, based on the climate and habitat of the region as well as the weather patterns in a given year (Gray et al., 2009). The activity pattern is also impacted by life-cycle stage, since there are different thresholds for desiccation and temperature-dependent activity; larvae stay close to the moist ground feeding on small rodents, whereas nymphs and adults are more desiccation resistant and can survive and quest for longer periods of time in summer compared to larvae (Lindgren et al., 2006).

The population structure of tick hosts in different habitats can impact the prevalence of pathogens like *Borrelia* and *Anaplasma* owing to differences in host competence (Hancock et al., 2011). Variation in the diversity and abundance of hosts has been suggested to be a vital determinant of the prevalence of tick-borne pathogens (Gilbert, 2010). The prevalence of infected ticks may be lower at sites with higher tick-host biodiversity. However, the abundance of tick-borne pathogens may not only vary spatially, but also temporally, owing to changing densities of tick hosts.

Ticks are unusual among arthropod parasites in the duration of their feeding, and they show a range of adaptations to facilitate the extended period of intimate contact with the host. Through

salivary secretions they biologically modulate the host's ability to defend itself (Tran and Waller, 2013). When they feed, they undergo major structural change; when they engorge they can increase their body mass by 140 times in nymphs and 47 in adults, which is a metabolically demanding physical process (Lighton and Fielden, 1995). They also expend energy to maintain water balance which is critical to the feeding and digestion process (Herrmann et al., 2013). The metabolic processes associated with feeding are particular importance since they affect all other aspects of tick development and survival (Lighton and Fielden, 1995).

1.3 The influence of the environment and climate change

Understanding the factors that limit the distribution and abundance of ticks is essential for understanding and predicting disease occurrence and emergence (Hancock et al., 2011, Rogers and Randolph, 2006). Ticks are relatively sensitive to climate variations and it has been argued by some authors that climate is probably the biggest determinant of tick abundance and behaviour in a particular region (Cumming, 2002; McCoy et al., 2013). Based on the different climate change scenarios, a rise in global habitat suitability of 1–9 million km^2 has been predicted over the next 100 years for amost tick species in the UK and Africa (Cumming and Van Vuuren, 2006). However, indirect effects of climate may act through changes in vegetation which may be an important modifier of microclimate, influencing tick survival, furthermore, as outlined above, the abundance of specific host types may also be important and affected by habitat.

Numerous studies report expansions in the global distribution of ticks and their associated pathogens associated with changes in climate (Estrada-Peña et al., 2006). The expansion of *I. ricinus* northward has been well-documented (Jore et al., 2011). However, ticks may also disappear from some places as conditions of temperature and humidity become unsuitable. Climate change in European regions with a Mediterranean climate might be expected to lead

to the disappearance of *I. ricinus* in such regions owing to increased moisture stress (Estrada-Peña et al., 2006). However, it is unclear whether colonization and extinction probabilities with regard to climatic conditions are equivalent for all tick species. Some species may be able to gradually adapt to changes in environmental conditions. Moreover, the potential for successful translocations of ticks into other countries is also likely to increase as climate changes.

Apart from changes in temperature and humidity, other factors may also be important in determining tick range changes; The reforestation of some landscapes and fragmentation of others can result in variation in microclimatic conditions (Foley et al., 2005, Gray et al., 2009), which can lead to changes in the diversity and abundance of ticks and their principal hosts. Changes in host populations could have a significant effect on the distribution of ticks at varying scales (Jonsson, 2006). For instance, an increase in the population of reservoir host species or amplifying host species could lead to increased tick density and the prevalence of pathogens that they transmit (LoGiudice et al., 2003; LoGiudice et al., 2008; Gilbert, 2010).

1.4 Energetic challenges for ticks

Nutrient metabolism is intimately associated with homeostasis, growth and reproduction and appropriate responses to the environment; it helps maintain an organism's energetic integrity. For this reason, understanding the dynamic energy metabolism of ticks, as well as their utilization of nutrients, may help explain tick physiological responses to environmental change (Woods et al., 2003; McMillan et al., 2005; Coracini et al. 2007). Furthermore, it may also help effective drug and vaccine development for the control of these vectors (Merino et al., 2013). However, while existing literature provides some information on metabolic events during both immature and adult stage of ticks (Kamaraju and Subbarao, 2002; Chandra et al., 2008) the detailed studies about their energy requirements are lacking.

Tick nutrition is derived from the blood-meal and sufficient must be obtained to allow larvae and nymphs to undergo development to the next life-cycle stage, and eventually initiate repeated questing until another host is located. For adults, accumulated metabolic reserves must also provide the resources for reproduction (Arrese & Soulages, 2010). The longer ticks can survive between blood-meals, the higher the chance of encountering a new host. However, the rate of energy source depletion is strongly affected by temperature and precise measurement of the rates of depletion may allow insights into the effects of climate on tick activity and the more exact determination of feeding patterns (Costamagna and Landis, 2004; Terblanche and Chown, 2007).

In many arthropod parasites the content of nutritional reserves derived from the bloodmeal can be used to infer the time since they last fed and hence their physiological age. This understanding has allowed the age-structure of field populations of blood feeding flies to be determined, and age correlated with activity and life-history (Bursell and Taylor, 1980, Chambers and Klowden, 1990). In ticks, understanding of physiological age is particularly important because patterns of pathogen infection and infectiousness are thought to be influenced by tick physiological age and nutritional status (Balashov, 1962, Uspensky et al., 2006). Subolesin (SUB) is important for tick feeding and reproduction and for pathogen infection, for instance, in *I. scapularis* during blood feeding and at high temperatures when SUB hsp20, and hsp70 is over-expressed. Hence, the SUB response in ticks is an important component of immunity against minimising pathogen infection (Busby et al., 2012). Furthermore, susceptibility to acaricides is also thought to increase with tick age (Uspensky, 1995). On the other hand, stress responses such as heat shocks, elevated temperatures and other conditions like pathogen infection and toxicity are also known to play a role in influencing tick mortality and growth (Tutar and Tutar, 2010).

1.5 Metabolism and tick phenology

Given the difficulty of inferring the effects of climate on ticks through measurement of seasonal patterns of abundance alone, novel approaches that are able to detect climate-mediated impacts directly will be of value. One approach is through the analysis of metabolic rate and nutritional resources (Arrese & Soulages, 2010). Given that the rate of energy source depletion is strongly affected by temperature and precise measurement of the rates of depletion may allow insights into the effects of climate on tick activity and the more exact determination of feeding patterns. Studies of metabolism and energy balance have been carried out on a range of arthropods at different lifecycle stages and/or activity levels, a range of approaches (Foray et al., 2012, Olson et al., 2000, Phillips et al., 2018, Schilman, 2017), but primarily using histological or respiratory rate-based analysis. For example, (Lighton and Fielden, 1995) measured metabolic rates of adult ticks in standardised flow chambers using gas analysers to assess CO_2 respiratory quotients. Ivanova et al. (2014) used histological examination to categorise ixodid ticks into feeding stages. Such approaches are relatively imprecise and interpretation could be especially challenging for regions with variable seasonal weather patterns (Gray, 2008). Hence there is considerable scope for further advancements in assessing tick nutritional status and examining its relationships with tick phenology, age and behaviour.

One feasible approach to analysing the feeding history of ticks more precisely is by evaluating lipid reserves (Walker, 2001). The stored lipid of ticks is largely derived from the blood on which they feed (Uspensky, 1995), although some may be synthesised from carbohydrate. They utilize thisto support their survival between ingesting blood meals, questing activities and reproduction (Pool et al., 2017). Therefore, measuring lipid allows the identification of tick (both individual and cohort) feeding history. Lipid reserves are known to decline predictably with time since their most recent meal (Abdullah et al., 2018). These studies have shown that in south western England a cohort of newly-moulted nymphs usually appears in autumn and early spring; these nymphs are relatively rich in lipid. The lipid values of nymphs decline

steadily over the year as they attempt to feed and those that fail to do so eventually starve (Abdullah et al., 2018). Hence, the more extensive analysis of lipid reserves could provide the way for a greater understanding of some variation in seasonal activity patterns observed within tick populations. However, in most cases, the measurement of lipid has relied on gravimetric methods, but such approaches are relatively inaccurate especially when applied to individual ticks (Mirth and Riddiford, 2007; Guglielmone et al., 2010). Consideration of lipid alone also only gives a view of one aspect of the way ticks partition and utilise metabolic reserves over time as they seek to obtain a further blood meal. Determination of the full metabolic profile will give a more comprehensive overview and, given that the profile may vary between life cycle stages, may give more sensitive insight into the behaviour of the entire feeding cohort. To allow this, the first requirement is to identify reliable biochemical approaches to measuring a full metabolic profile in ticks.

1.6 Aims of this study

The overarching aim of the work described in this thesis was to provide tools to allow a better understanding of metabolic resources in *I. ricinus* ticks, and that would allow future researchers to assess the likely impacts of climate and climate change on tick survival and behaviour. To achieve this the first specific aim of the work was to evaluate the use of the range of spectrophotometric techniques for quantifying protein, lipid, total water-soluble carbohydrate and glycogen resources in individual ticks in the nymphal and adult life-cycle stages (Chapter 2). The second aim was to assess the rate of use of these energy sources at a range of temperatures (Chapter 3) so that finally (Chapter 4) the pattern of resource use in field populations could be explained.

Chapter 2

Determining the total energy budget of the tick *Ixodes ricinus*

Summary
Precise and accessible techniques for measuring metabolic responses to environmental stress are essential to allow the likely impacts of climate and climate change on tick distribution, abundance and phenology to be predicted. A more detailed understanding of the metabolic profile of ticks may also help the complex responses to pathogen infection and effects on transmission to be evaluated. Here, a series of biochemical protocols employing spectrophotometric methods are used to determine the entire energy budget of ticks. Protein, total lipid, neutral lipid, soluble carbohydrates and glycogen were measured in individual *Ixodes ricinus* nymphs and adults. Two key trends were identified: in adults, protein was relatively more abundant than in nymphs, whereas in nymphs, glycogen and soluble carbohydrates were more abundant than in adults, with glycogen alone composing 39% of the mass of metabolites in nymphs compared to 15 and 10% in females and males, respectively. The methods used were able to successfully separate neutral lipids from the polar phospholipids and the importance of distinguishing stored from structural lipid in estimates of lipid reserves is emphasised. The results demonstrate that the spectrophotometric approaches deliver relatively rapid and reliable estimates of the total energetic budget and can be used to quantify the metabolic profiles of individual ticks, demonstrating their suitability for use in ecological and epidemiological studies.

2.1 Introduction

For multi-host ixodid ticks, the blood meal obtained by each lifecycle stage must provide the resource required for development and/or reproduction (Sonenshine and Roe, 2014). Between feeding events, ticks spend the majority of their lives off the host where they need to survive adverse environmental conditions (Needham and Teel, 1991). To facilitate survival during these extended inter-feed intervals and then ensure that sufficient stored resources are available

to allow repeated questing until a host is located, ixodid ticks have a metabolic rate which is typically 13% below that of most arthropods (Lighton and Fielden, 1995). Given this intermittent 'high-risk' sit-and-wait feeding strategy, understanding how ticks partition resources between maintenance, development, reproduction and storage for future requirements is of intrinsic physiological interest but their measurement can also allow insight into the feeding history and ecology of cohorts of field-derived ticks (Abdullah et al., 2018, Randolph et al., 2002) and can allow predictions about the likely impact of changes in rate determining environmental factors such as temperature and humidity (Randolph et al., 2002, Rosendale et al., 2017).

The main immediate source of energy for ticks is glucose, which is stored in a hydrated polymeric form as glycogen. Glycogen serves as an energy source to support the post-feeding stage but is rapidly depleted (Moraes et al., 2007). Metabolism of glycogen involves its breakdown to glucose with resultant energy release. Carbohydrate can be used to replenish glycogen reserves so that energy is available for activity. Lipids also play key roles in tick metabolism, both as an energy source and structurally in cell membranes, in hormones and for egg development (Kluck et al., 2018). Lipid is stored primarily the form of triglycerides in adipocytes the main fat body cell and additionally as cytoplasmic lipid droplets. Storage is mostly regulated by hormones in response to physiological demands; in insects marked decreases in lipid content have been associated with key growth phases (Arrese and Soulages, 2010). Lipids measured in total (stored lipids such as triglyceride plus structural lipids present in cell membranes) are commonly used as proxy for stored lipids to give an indication of energy reserves (Abdullah et al., 2018). However, only the stored lipids are functionally valuable as an energy reserve and consideration of total lipid alone may be misleading because the dominant triglyceride component fluctuates considerably with developmental stage, sex and even season and may vary independently of other lipid classes, such as diglyceride,

monoglyceride, sterols, sterol esters, free fatty acids and phospholipids (Angelo et al., 2013). Hence, accurate distinction between structural polar lipids and total lipids may be important. Finally, proteins are important and may act as a long-term energy reserve (Williams et al., 1986) and are structurally essential for muscle, cuticle synthesis, hormones, enzymes, carrier proteins (Kluck et al., 2018), in females for the synthesis of egg yolk (Tatchell, 1971, Xavier et al., 2019) and in males for sperm and gonadal proteins that trigger engorgement in females (Weiss and Kaufman, 2004). Glycogen resynthesis by protein degradation through the gluconeogenesis pathway has been demonstrated in ticks, notably at the end of the embryonic period (Martins et al., 2018).

The measurement of energy budgets and the factors affecting metabolic rate have been well studied in insects (Chown et al., 2004) and approaches using spectrophotometric techniques, as developed by (Van Handel, 1985a,b) have been used widely for such measurements (Raubenheimer et al., 2009), for example allowing insect nutrient levels to be related to egg load or longevity (Otronen, 1995), or to allow explanations of insect population dynamics and behavioural ecology in terms of resource availability (Pelosse et al., 2007). Such studies have looked, in particular, at the measurement of lipids, but the analytical approaches have also been extended to allow the estimation of glycogen and free sugars. These analytical methods are used increasingly because they are relatively inexpensive, rapid and precise. However, although applied extensively in insects, these techniques have rarely been used in ticks (Abdullah et al., 2018).

The aim of the work described in the Chapter, therefore, was to evaluate the use of the range of spectrophotometric techniques for quantifying protein, carbohydrate, lipid and glycogen resources in ticks in the nymphal and adult life-cycle stages. A further aim was to explore the separate quantification of neutral lipids and structural polar phospholipids to allow more exact quantification of the contribution of lipids to energy reserves. In particular, the work aimed to

determine whether several energy sources could be determined for an individual tick. To explore this, two analytical approaches were compared. The first applied multiple biochemical measurements to individual ticks, whereas the second, used separate ticks for the measurement of complimentary groups of energy sources.

2.2 Material and methods

2.2.1 Sample collection

Ixodes ricinus ticks (nymphs, males and females) were collected every 2 weeks from the field between March to May in 2017 by blanket-dragging. The field site was a semi-urban park (51°26'49.2"N, 2°38'01.9"W) to the west of the city of Bristol in southwest England, containing managed herds of red and fallow deer and a population of wild roe deer and where ticks are abundant (Jennett et al., 2013).The ticks collected were identified to species, sex and lifecycle stage using standard keys (Arthur, 1963, Hillyard, 1996, Walker et al., 2003). They were weighed using an ultrasensitive microbalance (Sartorius-ME5, Goettingen, Germany) to the nearest microgram and then stored at -20 °C for a maximum of 8 weeks prior to analysis. The ticks collected were divided at random into two groups based on the analytical approach to be used. In the first group, referred to as the 'individual-analysis' group, a total of 60 ticks were used, 30 for protein analysis and 30 for the measurement of all other energy sources in each specimen. In the second, referred to as the 'multi-analysis' group, in total 90 ticks were used, 30 for protein analysis, 30 for both free sugars and glycogen and 30 for total lipids and neutral lipids. In all cases, each group of 30 ticks consisted of 10 nymphs, 10 males and 10 females.

2.2.2 Individual analysis ticks

Protein was measured following the method described by (Bradford, 1976). Ticks were first placed individually into a clean borosilicate tube (12 ml) and crushed using a clean glass rod.

This was followed by the addition of 1500 µl of phosphate buffer solution to extract the protein (100 mM of monopotassium phosphate, 1 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of dithiothreitol (DTT), pH 7.4). Thereafter, the homogenate was placed on ice prior to processing (about 1 min). For nymphs, 1000 µl was transferred to a cuvette and mixed with 1000 µl of Bradford reagent (Sigma, Dorset, UK). For males and females, 50 µl was transferred to a cuvette and mixed with 1500 µl of Bradford reagent. Individual cuvettes were incubated at room temperature for 5 min and then the absorbance value was immediately measured at a wavelength of 595 nm using a Biochrome spectrophotometer (Biowave II, Cambridge, UK). The procedure is based on the formation of a protein-dye complex; coomassie dye in the Bradford reagent binds with proteins and results in a change in colour from brown to blue leading to a shift in the absorption maximum from 465 to 595 nm.

For analysis of other energy sources, a single individual tick was placed into a clean borosilicate tube and crushed, as above. To dissolve all water-soluble carbohydrates and the total fats, 200 µl of 2% sodium sulphate solution (VWR International, Leicestershire, UK), and 1500 µl of a chloroform/methanol mixture (1: 2 vol/vol) were added to each tube. This mixture was then transferred into a 2 ml Eppendorf tube and centrifuged (Centrifuge 5418R; Eppendorf Lutterworth, UK) for 15 min at 180 ***g*** and 4 °C. For total lipid, neutral lipid and the total water-soluble carbohydrates analysis, the supernatant was removed into a new tube; and the pellets containing glycogen were retained for further analysis.

Beginning with soluble carbohydrates determination, the method of (Van Handel, 1965) was used. 200 µl of the supernatant from individual samples were moved into a borosilicate tube and placed in a water bath at 90 °C for 40 s to evaporate the solvent to about 20 µl. Thereafter, 1 ml of freshly prepared anthrone reagent (Sigma, Dorset, UK) (1.42 g/l in 70% sulphuric acid; VWR International) was added to each sample and incubated for 15 min at 25 °C. Subsequently, the tubes were heated for 15 min at 90 °C and then cooled at room temperature

for 15 min, after which the samples were placed in microcuvette and finally read in a spectrophotometer set at 625 nm, to determine the total water-soluble carbohydrates. Blue green coloration indicates the binding of anthrone to sugar.

For glycogen determination, pellets were washed on two occasions using 400 µl of 80% methanol to remove sodium sulphate. Vigorous vortexing was followed by centrifugation, for 5 min at 180 ***g*** at 4 °C. Once the supernatant was eliminated, 1 ml of fresh anthrone reagent was added and the mixture was incubated at 90 °C for 15 min. Each sample was cooled on ice to end the reaction and filtered on low-protein binding membranes of 0.45 µm diameter (Fisher Scientific, Leicestershire, UK). Finally, the absorbance was read in a spectrophotometer at 625 nm to quantify the presence of water-soluble carbohydrates.

The total lipids were quantified using a vanillin assay. For this, 200 µl of the supernatant was added into a new borosilicate tube and placed into a heating block at 90 °C until total evaporation was achieved. 40 µl of 95% sulphuric acid (VWR International, Leicestershire, UK), was added to the mixture and heated at 90 °C for 2 min and then cooled in ice. 960 µl of freshly prepared 1.2 g/l vanillin reagent (Fisher Scientific, Leicestershire, UK) in 68% phosphoric acid (Sigma, Dorset, UK) was added and followed by incubation at room temperature for 15 min. The absorbance was read in a spectrophotometer at 525 nm (Van Handel, 1985b). Finally, the neutral lipid content was measured by placing 500 µl of the supernatant in a new tube, which was heated at 90 °C to evaporate off the solvents. One 1 ml of chloroform was added into each tube to re-solubilize the fats. 200 mg of dry silicic acid (Sigma) was added to each sample. Thereafter, all samples were mixed and then centrifuged at 180 ***g*** and 4 °C for 10 min to remove polar lipids in the silicic acid. From the final supernatant 200 µl was pipetted off and removed into new tube. Again, the absorbance was read in a spectrophotometer at 525 nm (Van Handel, 1985b).

2.2.3 Multi-analysis ticks

For multi-analysis, 30 ticks were used for protein analysis, as described above. Thirty ticks were used for the analysis of soluble carbohydrates and glycogen only and a further 30 were used only for the analysis of lipids, following the procedures described above.

2.2.4 Standard curves

Standard curves of absorbance against known metabolite contents were created to allow spectrophotometric values to be related to metabolite contents. Care was taken to ensure linearity within the various contents and that they started below the lowest sample value and ended beyond the highest. For protein concentration a standard curve was generated with a dilution series of bovine serum albumin (BSA, Sigma, Dorset, UK), treated as described above (Table 2.1; Fig. 2.1). For the total water-soluble carbohydrates and glycogen, a standard curve was generated using glucose (≥99.5%, D-(+)-Glucose is the open chain form of D-glucose. It is a D-glucose and an aldehydo-glucose. It is an enantiomer of an aldehydo-L-glucose) at a range of dilutions (Table 2.2; Fig. 2.2). For lipids a standard curve was generated using glycerol trioleate (≥99%, derived from glycerol. It is composed of three oleic acid units and is an unsaturated triglyceride) at various dilutions, (Table 2.3; Fig. 2.3). Standard curves were systematically repeated using serial dilutions, to produce a mean standard curve; this was done to account for variability that might occur at different times, possibly as a result of slight differences in temperature, the incubation time, or even the quality of the reagent used.

Table 2. 1 Dilution series using a concentration reference for protein. The stock solution contained 1 mg/mL of BSA.

Protein (BSA) concentration (µl/µg)	Stock solution BSA (µl)	Buffer solution (µl)
0	0	1500
0.52	0.781	1499.2
1.04	1.562	1498.4
2.08	3.125	1496.8
4.16	6.25	1493.75
8.33	12.5	1487.5
16.66	25	1475
33.33	50	1450

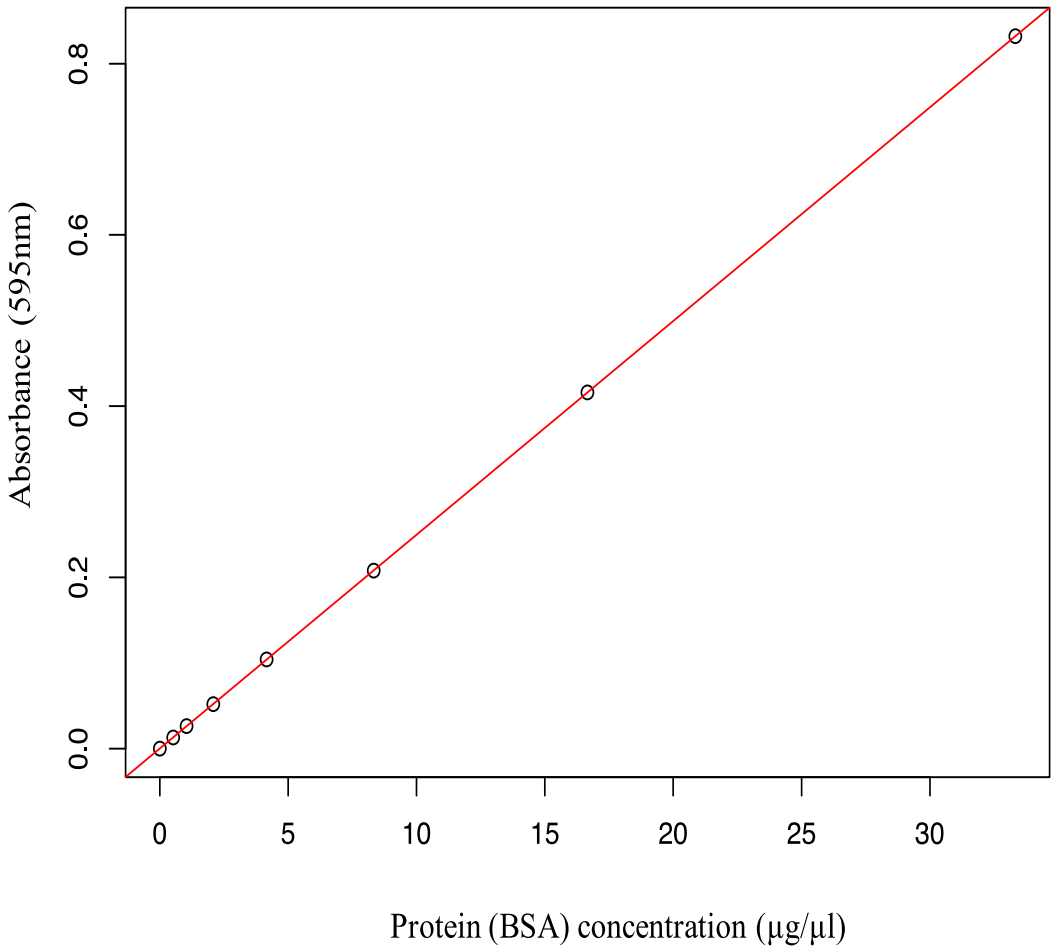

Figure 2. 1 The mean absorbance of a range concentrations of protein BSA (µg/µl) measured in a spectrophotometer at 595 nm to produce a standard curve. $R^2 = 0.99$, $F = 2438$, $P<0.001$.

Table 2. 2 A dilution series using a concentration reference for soluble carbohydrates and glycogen. The stock solution contains 1mg/mL of glucose.

Glucose concentration (µl/µg)	Stock solution (µl)	Chloroform-methanol solution (µl)
0	0	1500
0.459	0.781	1499.2
0.918	1.562	1498.4
7.35	12.5	1487.5
14.7	25	1475
29.41	50	1450
58.82	100	1400
117.64	200	1300

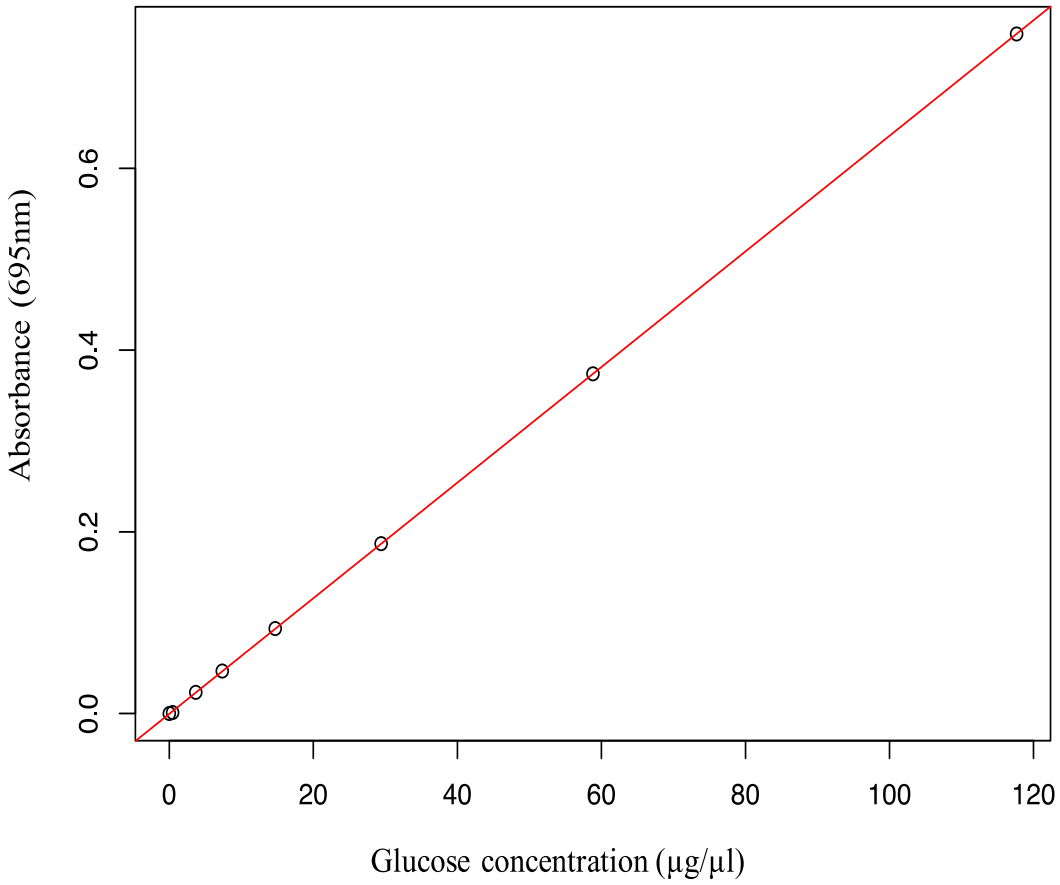

Figure 2. 2 The mean absorbance (nm) of a range of concentrations (µg/ml) of glucose measured with a Spectrophotometer at 625 nm. $R^2 = 0.99$, $F = 153.21$, $P<0.001$.

Table 2. 3 A dilution series using concentration reference for phospholipid and neutral lipid. The stock solution contains 1 mg/mL of glyceryl trioleate.

Glyceryl trioleate Concentration (µl/µg)	stock solution (µl)	Chloroform-methanol solution (µl)
0	0	1500
0.459	0.781	1499.2
0.918	1.562	1498.4
1.838	3.125	1496.8
3.67	6.25	1493.75
7.35	12.5	1487.5
14.7	25	1475
29.41	50	1450

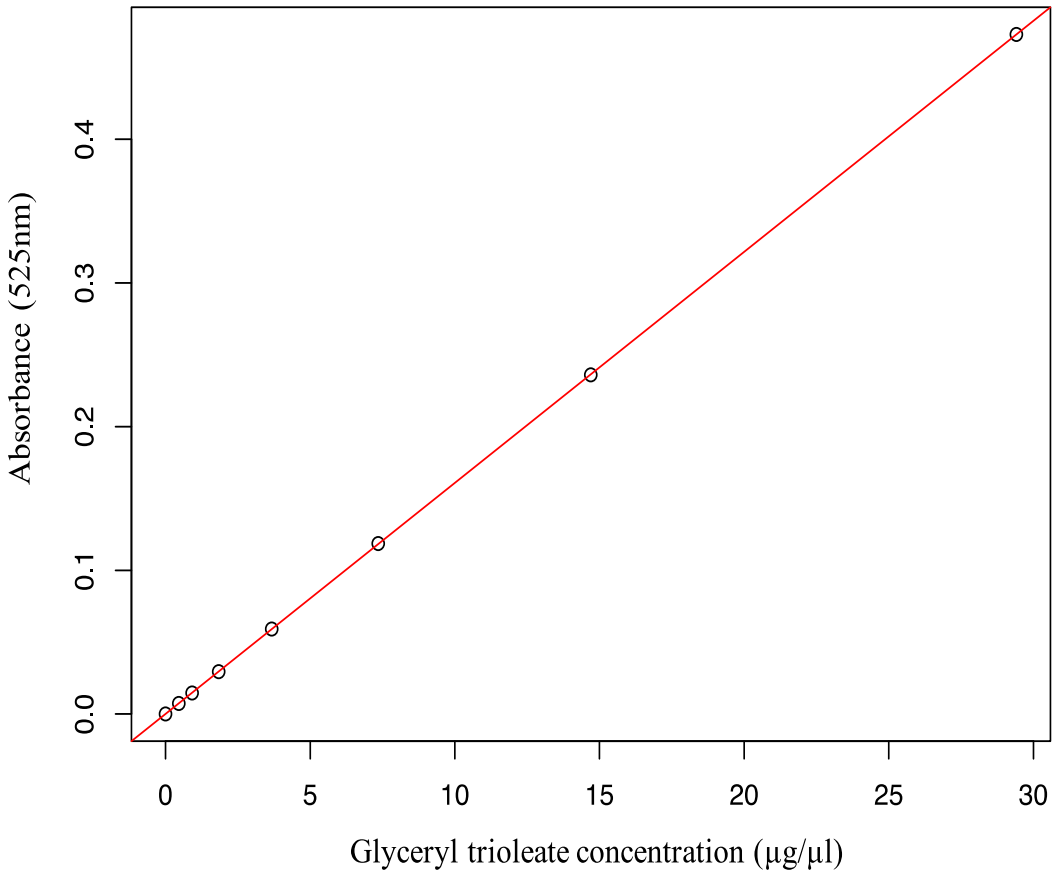

Figure 2. 3 The mean absorbance (nm) of a range of concentrations (µg/ml) of glyceryl trioleate measured with a spectrophometer at 525 nm. $R^2 = 0.99$, $F = 144.95$, $P<0.001$.

2.2.5 Statistical analysis

Generalized linear models (with a gamma distribution and inverse link) with Tukey multiple comparison post-hoc tests were used to compare the mass of different energy sources within each lifecycle stage and the masses of energy sources measured using the individual-analysis or multiple-analysis approaches and the weights of ticks included in the two analysis-groups. One-way ANOVA was used to compare tick body weights between analysis-groups. All analyses were performed with the R-Studio statistical package (R v.3.5.3, 2019, R Foundation for Statistical Computing). For graphical presentation of the energy sources data, medians are plotted with 95% confidence intervals.

2.3 Results

All the ticks collected from the field were *I. ricinus*. The mean (± SE) body mass of nymphs, males and females used in the individual analysis was 0.138 (± 0.006), 0.884 (± 0.034) and 1.649 (± 0.069) mg, whereas the means of the weights of these lifecycle stages in the multi-analysis groups were 0.130 (± 0.004), 0.843 (± 0.022) and 1.634 (± 0.006) mg, respectively. Within each lifecycle stage these weights were not significantly different from each other (nymphs: $F_{1,149} = 1.44$, $P = 0.23$; males: $F_{1,149} = 1.14$, $P = 0.29$; females: $F_{1,149} = 0.03$, $P = 0.86$). Statistical analysis showed that there were no significant differences between the individual- or multi-analysis methods in any of the metabolite contents recorded in nymphs, males or females (Table 2.4) and data for each life-cycle stage were therefore pooled for subsequent analysis.

Table 2. 4 Statistical comparison of the mass of phospholipid, neutral lipid, soluble carbohydrates and glycogen values recorded in nymphs, males and females of *Ixodes ricinus* (n = 30 in each case), where all were measured either in a single individual or where lipids and glycogen/ soluble carbohydrates were measured separately in different groups. F and P values are based on a generalised linear model (GLM).

energy sources category	Lifecycle stages	F	P
Phospholipid	Nymphs	1.90	0.28
	Males	1.03	0.35
	Females	1.73	0.24
Neutral lipids	Nymphs	0.76	0.41
	Males	1.24	0.31
	Females	0.21	0.66
Soluble carbohydrates	Nymphs	0.69	0.43
	Males	7.79	0.15
	Females	0.34	0.56
Glycogen	Nymphs	6.53	0.22
	Males	4.81	0.43
	Females	0.30	0.60

In nymphs, the metabolite contents show a significant difference between the amounts of different categories present ($F_{4,45}$ = 12.31, P<0.001). Glycogen was the most abundant metabolite with a median of 0.014 mg and was significantly more abundant than any other metabolite. Protein was the second most abundant metabolite, with a high degree of consistency between individuals. Total lipid and neutral lipid contents were low as was the median concentration of the total water-soluble carbohydrates, but the latter was notable for its very high degree of variation between individuals (Fig. 2.4).

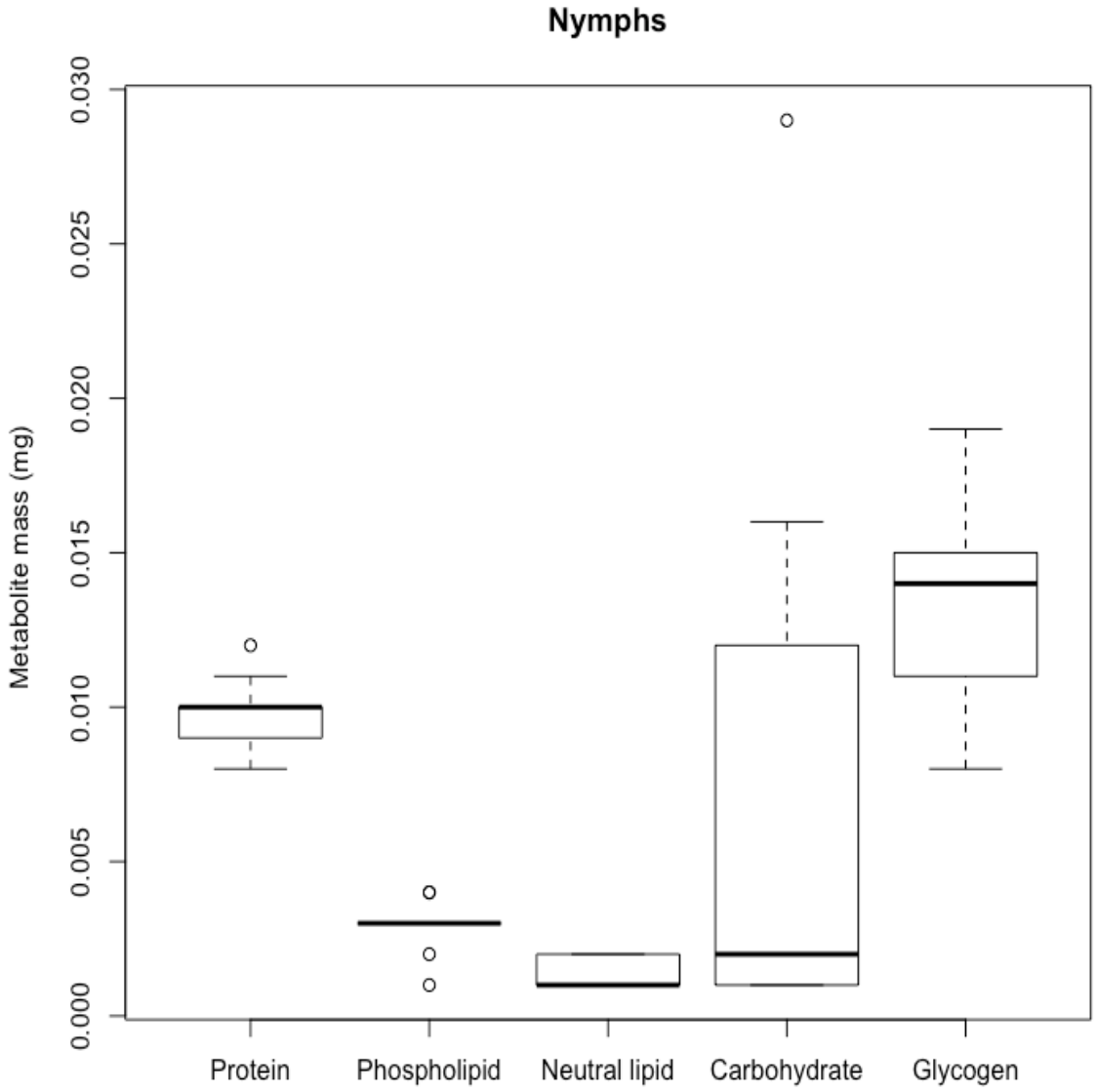

Figure 2. 4 The median mass of five metabolites (mg) in *Ixodes ricinus* nymphs with 95% confidence interval (dashed lines) and lower and upper quartiles (box).

In adults, there were significant differences in the amounts of the different categories of nutrient present, in males ($F_{4,45}$ = 172, P<0.001**)** and females ($F_{4,45}$ = 196.8, P<0.001). Males had high median values for protein, at 0.056 mg, which was significantly greater than other metabolites, which had concentrations that were not significantly different from each other. The degree of variation around the median values was consistently relatively small (Fig. 2.5). Females also had concentrations of protein which were relatively high and similar to those of males (Fig. 2.6). They had low concentrations of lipid, both total lipid and neutral lipid, but

they had concentrations of the total water-soluble carbohydrates and glycogen at around 0.3 mg, which were considerably greater than those seen in males (Fig. 2.5).

Consideration of the amounts of each metabolite present as a percentage, to allow for comparison between lifecycle stages, shows that the greatest differences in energy source contents between nymphal and adult stages were in protein and glycogen (Fig. 2,7). In nymphs, glycogen accounted for 39% of the metabolites measured, whereas protein accounted for 25%. However, in males and females the protein values were at 60 and 53%, respectively, whereas the glycogen contents were lower at 12 and 19%, respectively. Other energy sources were relatively similar between lifecycle stages. Notably lipid was consistently the least abundant metabolite.

The methods used were able successfully to separate neutral lipids from the polar lipids; about 94.6% of polar lipid was extracted from the standardized phospholipid solution. In nymphs, 8.4% of the mass was composed of lipids of which neutral lipids represented 45.2%. In adults, total lipid composed about 10 and 5% of the total body mass in males and females, respectively, whereas neutral lipids composed about 85% of the lipid fraction in males and about 96% in females (Fig. 2.7). This method could be further adapted for use with very small arthropods, using small volumes in 96-well microplates, (Cheng et al., 2011, Williams et al., 2011, Foray et al., 2012, Lee, 2019). It would be expected that the sensitivity when using microplates would remain high. Fluorescence analysis has long been used to measure the emission spectra of fluorophores binding to samples excited by wavelengths for highly specific molecular quantification (Alquicer et al., 2009, Foray et al., 2012).

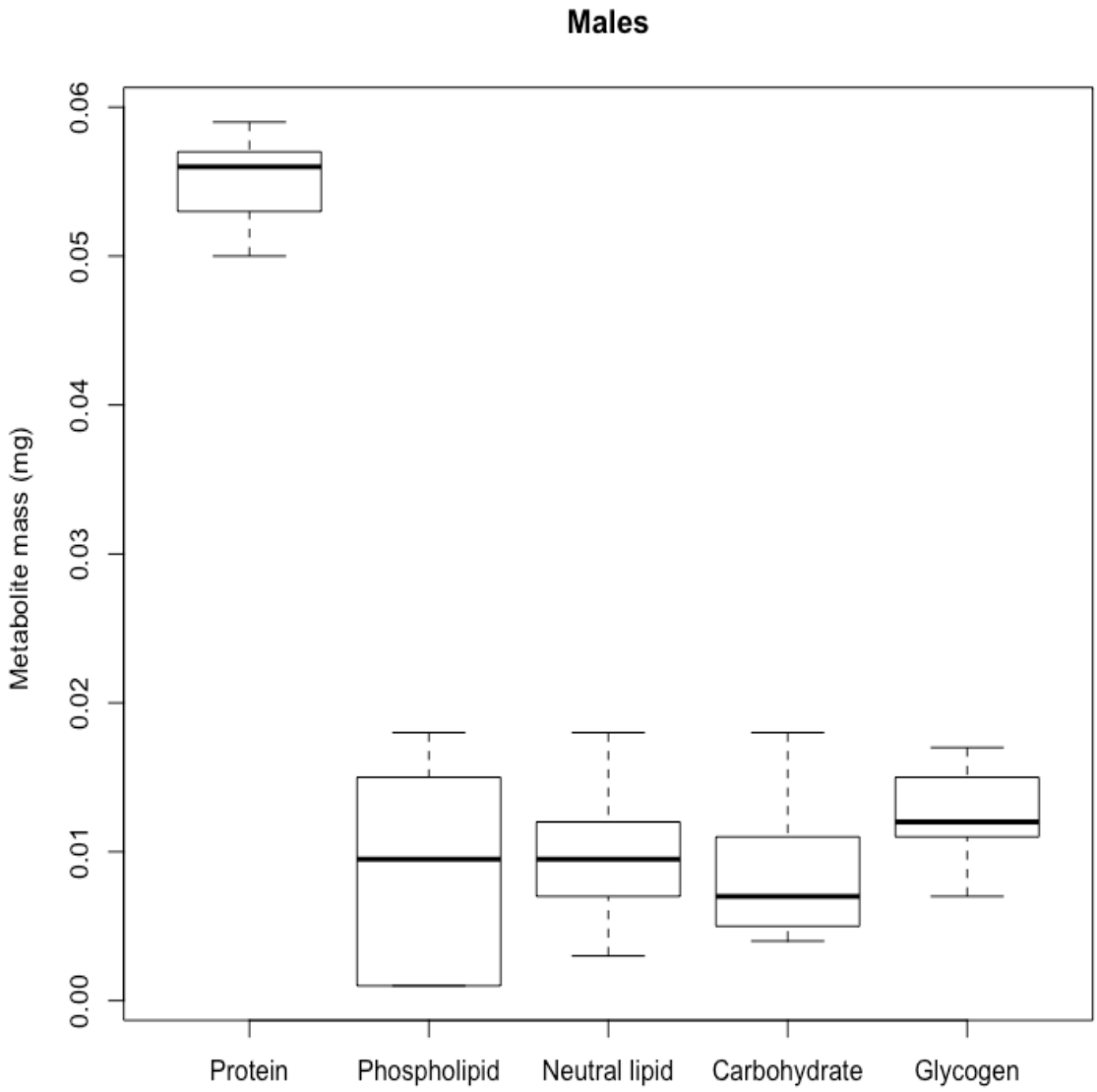

Figure 2. 5 The median mass of five metabolites (mg) in *Ixodes ricinus* males with 95% confidence interval (dashed lines) and lower and upper quartiles (box).

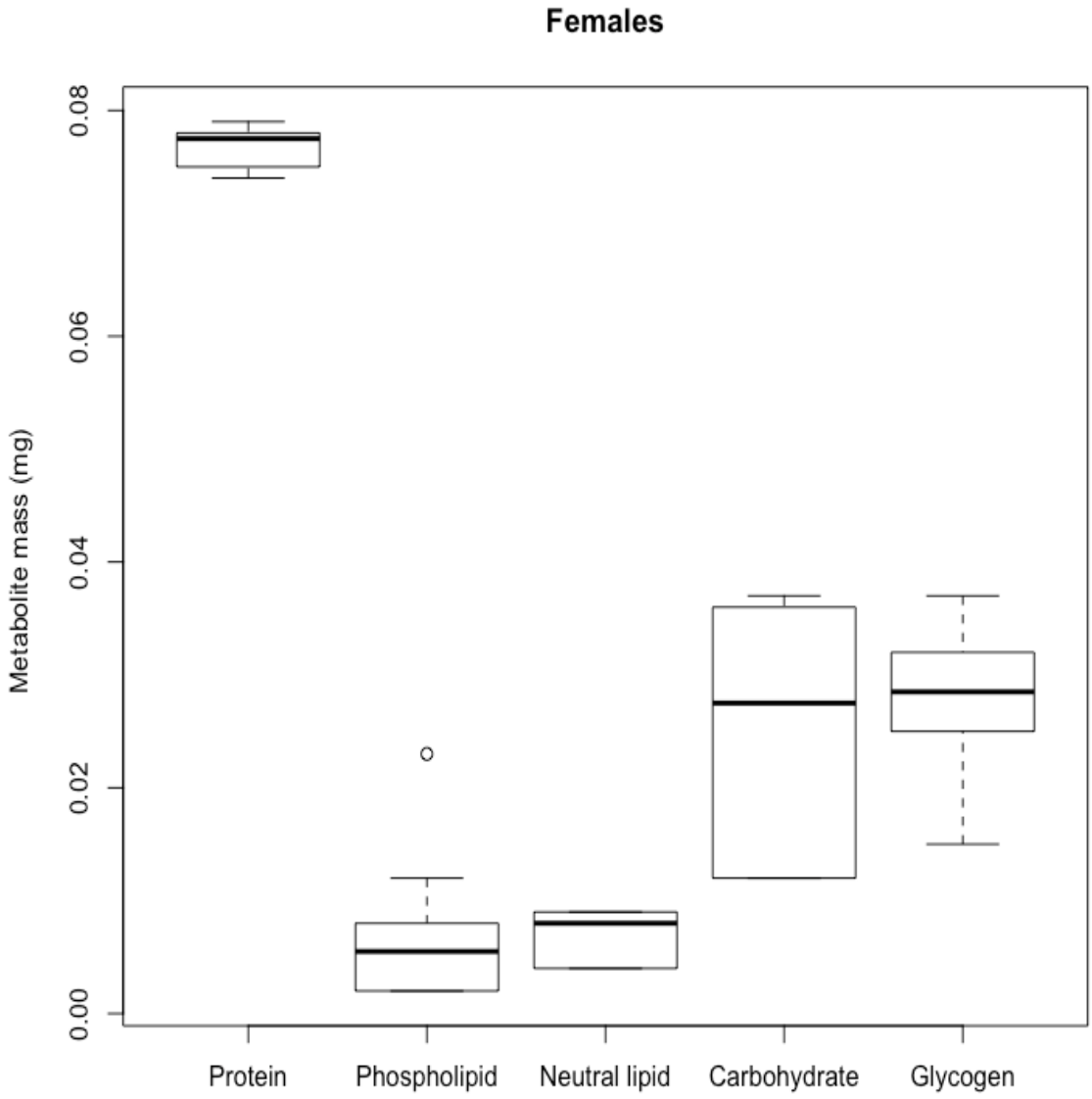

Figure 2. 6 The median mass of five metabolites (mg) in *Ixodes ricinus* females with 95% confidence interval (dashed lines) and lower and upper quartiles (box).

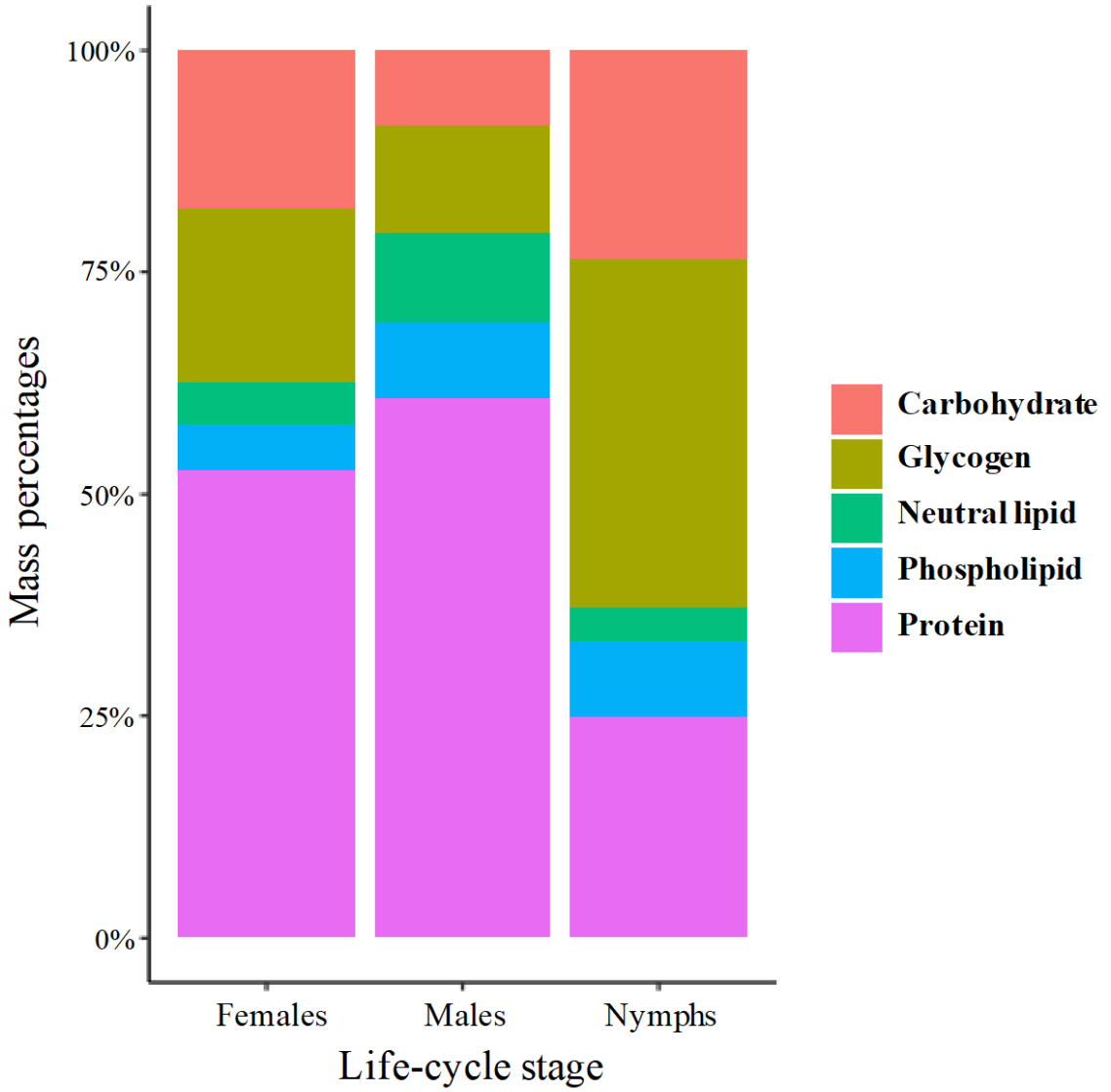

Figure 2. 7 The percentage mass for each metabolite category in individual *Ixodes ricinus* nymphs, males or females.

2.4 Discussion

Ticks need to be able to survive for extended periods between blood meals. During these off-host periods, they need to endure adverse conditions and maintain sufficient reserves to allow repeated episodes of host-seeking behaviour. Starvation of ticks for 18 or 36 weeks, in the absence of dehydration, was shown to result in the loss of 20-40% of protein and 60% of lipid reserves in the American dog tick, *Dermacentor variabilis* (Rosendale et al., 2017). The lipid reserves of field-collected *I. ricinus* ticks, collected in early summer, were estimated to be

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 that year, respectively (Abdullah et al., 2018).

The pattern of metabolic activity is not constant. A newly moulted tick is able to maintain relatively low levels of activity and minimise energy expenditure for several weeks; but once energy levels start to become depleted the level of activity and consequently energy expenditure may rise as questing activity becomes more prolonged and persistent. This may increase their susceptibility to environmental stresses such as dehydration (Rosendale et al., 2017). A significant increase in energy use has been associated with ovary development in female ticks (Xavier et al., 2019). A detailed understanding of the patterns of changing resource within ticks would therefore be expected to provide information in field-collected cohorts about their metabolic rate, feeding history and questing activity.

Early approaches to examining metabolic reserves used histological or anatomical examination of ixodid ticks to categorise them into feeding cohorts, but such approaches are relatively imprecise (Uspensky, 1995, Walker, 2001). Subsequently spectrophotometric methods have been used to examine total lipid (Abdullah et al., 2018), and the current paper extends these approaches to a consideration of the full metabolic profile in the tick *I. ricinus*. These methods have been used widely in insects, but have not previously been used comprehensively in ticks. The first analysis undertaken by the present study showed that there was no difference in the metabolite contents determined in either the ticks subjected to multiple analysis or ticks used for analysis of separate classes of metabolite. This was an essential initial step required to give confidence to the subsequent investigation, and to highlight the sensitivity and repeatability of the procedure. The ability to measure lipid, soluble carbohydrates/glycogen simultaneously in individual ticks highlights the suitability of these methods for use in ecological and epidemiological studies. Overall, the results indicate that the spectrophotometric approaches appear to allow accurate and reproducible quantification of the entire range of the energetic

reserves. This represents a considerable advance over the approaches used previously in ticks and the spectrophotometric approaches are able to deliver relatively rapid, inexpensive and reliable estimates of the total energetic budget.

The ticks used for the present study were collected by blanket dragging between March and May in southwest England when questing activity of nymphs and adults would have been expected to be at its peak. A range of stages of starvation would have been expected, with the population composed of cohorts that fed the previous year and either moulted the previous autumn or which overwintered or moulted in early spring prior to collection, depending on precisely when they had fed. The aim of the work in this initial data Chapter was to evaluate the applicability of the analytical methods to ticks, rather than attempt to explain the cause or meaning of the differences observed, which is dealt with more comprehensively in Chapter 4, nevertheless two key trends were apparent. In adults, protein values were high and relatively greater than in nymphs whereas in nymphs 39% of the metabolites were composed of glycogen. In contrast in females, glycogen composed only 15% and in males 10% of the mass of metabolites. The total water-soluble carbohydrates was also relatively more abundant in nymphs and females than males. The relatively high protein levels in adults may be associated with reproduction, whereas the relatively high glycogen levels in nymphs and very high variability in soluble carbohydrates values may indicate that the population of nymphs is divided into a cohort that moulted the previous autumn and a cohort that moulted in spring of the year they were collected. In contrast the adult population may be largely composed of individuals that moulted the previous autumn, so lipid and soluble carbohydrates values are relatively lower than in nymphs.

The data show that the proportion of neutral lipids (stored lipids) was about half that of structural phospholipids in nymphs, and considerably higher in adults. Hence the data suggest that since the levels of triglyceride can vary significantly between life-cycle stages, when

analysing lipid reserves, it is important to distinguish neutral lipids from structural phospholipids. In summary, the current study has demonstrated that a complete energy budget for an individual tick can be quantified using spectrophotometric approaches, although it is still necessary to quantify protein separately.

Chapter 3

Effects of temperature and starvation on metabolite contents in *Ixodes ricinus*

Summary
The work described in the current Chapter aimed to investigate the effects of temperature on the rate of depletion of energy reserves by nymphal and adult *Ixodes ricinus*. A cohort of nymphs, males and females were collected from the field, divided into groups and placed into incubators at temperatures of 5 °C, 10 °C, 15 °C, 20 °C, 25 °C and 30 °C. For nymphs the protein, the total water-soluble carbohydrates, total lipid, neutral lipid and glycogen levels were measured at days 0, 14, 28, 42, 56 and 70. For the adult males and females the measurements were performed only on days 0, 14, 28 and 42. In nymphs, the rate of soluble carbohydrates and glycogen utilisation was higher than in males or females and the concentrations of neutral lipids (a proxy for stored lipids) were significantly affected by higher temperatures (20-30 °C). In tick adults, the concentrations of protein and structural lipid (phospholipid) responded rapidly to changes in treatment time and the ambient temperature.

3.1 Introduction

Strong non-linear relationships between temperature and many aspects of tick life-history have been demonstrated, although this relationship is not a simple one because saturation deficit will affect desiccation (Needham Teel, 1991), and temperature in combination with daylength may affect various forms of behavioural or physiological diapause (Belozerov, 2009). In addition, patterns of metabolic activity are not uniform throughout life and increases are associated with feeding and moulting, followed by reduced levels of metabolic activity and energy utilisation for several weeks (Cuber *et al*., 2016). However, once energy levels have been depleted, the levels of activity and energy expenditure then increase as questing becomes more prolonged

and increasingly persistent and, during periods of questing, ticks are highly susceptible to environmental constraints such as temperature and dehydration (MacLeod, 1935; Needham Teel, 1991; Bowman *et al*., 1996). For example, for *I. scapularis*, Ogden *et al*. (2004) found that the power relationships observed between temperature and development rate in the laboratory could be used to predict dates for moulting, oviposition, and field-observed seasonal activity of questing larvae and nymphs. However, other factors such as seasonal activity of questing adult ticks were poorly predicted.

An understanding of the pattern of energy sources use and depletion, particularly in responses to factors such as temperature, is important, not only for helping to explain tick population dynamics and phenology but also because it may contribute to a better understanding of the likely impacts of climatic changes on ticks, which is likely to affect seasonal activity patterns and, consequently, may affect pathogen transmission (Burtis et al., 2016; Gray *et al.*, 2009).

The aim of the work described in this Chapter, therefore, was to investigate the effects of temperature on the rate of depletion of metabolic reserves over time in nymphs and adults of *I. ricinus* collected from the field.

3.2 Materials and Methods

3.2.1 Tick collection and environmental conditions

A total of 2,160 ticks were collected from Ashton Court Park near Bristol on a single day (day -14) in the middle of March 2019 by blanket dragging. The samples collected were temporarily stored at 4 °C for 6 h before being divided into groups based on life-cycle stage. Nymphs, adult males and adult females of *I. ricinus* were divided into six groups of about 100-150 individuals, which were transferred into 1.2-L plastic buckets with lids (12.5 x 18 cm). Each bucket

contained 3 kg of dampened Horticultural Silver Sand (Melcourt, Tetbury, UK) to raise the humidity experienced by the ticks to > 80%; humidity was not controlled further, but conditions were similar for all groups and humidity was sufficiently high to allow high rates of survival. All buckets were placed in incubators (Sanyo, MLR-351) set at temperatures of 5 °C, 10 °C, 15 °C, 20 °C, 25 °C or 30 °C, and kept under diel periods of L8:D16 h. A relatively short photoperiod was used to reflect the time of year when the ticks were collected, as a change from short to long daylight could induce physiological changes associated with diapause (Gray et al., 2016). Ticks were initially maintained under these conditions for two weeks before the experiment was considered to have started (day 0), to allow them to adapt to the laboratory conditions.

For nymphs, the concentrations of protein, soluble carbohydrates, total lipid, neutral lipid and glycogen were measured at days 0, 14, 28, 42, 56 and 70. For the adult males and females, the measurements of the same energy sources were performed on days 0, 14, 28 and 42 only. The spectrophotometric methods used were as described in Chapter 2. The experiments for males and females were completed earlier than those for the nymphs because of the smaller number of adults available in the field on the collection day. For each temperature condition on each of the sample days, extractions were performed on 12 nymphs, 12 adult males and 12 adult females.

3.2.2 Data analysis

All analyses were performed with the R-Studio statistical package (R v.3.5.3, 2019, R Foundation for Statistical Computing). A generalised linear model was used to investigate the effects of the relationship between the content of each energy sources and temperature and time, with an identity link that assumed a Gaussian distribution of the residuals. For the analysis of deviance of the generalised linear model fits an F test was used. Subsequently, to consider

the nature of the relationships between temperature and lipid concentration in more detail a simple exponential regression model was fitted to the metabolite contents over time and the slopes of these regressions were used to examine the change in the rate of depletion of metabolic components with temperature.

3.3 Results

3.3.1 Determinating the effect of temperature and time on metabolite contents

3.3.1.1 Nymphs

3.3.1.1.1 Protein content

There was a significant effect of temperature on protein content for nymphs, ($F_{5,426}$ = 48.60, P<0.001) and a significant effect of incubation time ($F_{5,421}$ = 219.46, P<0.001). The interaction between temperature and time showed a statistically significant effect ($F_{25,396}$ = 1.86, P<0.01). The relative changes in protein concentration showed that at 5 °C protein content fell 27% over 42 days whereas at 30 °C it fell 55%. At 70 days protein content had declined by 44% at 5 °C whereas at 30 °C it had declined by 80%. The protein content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.1).

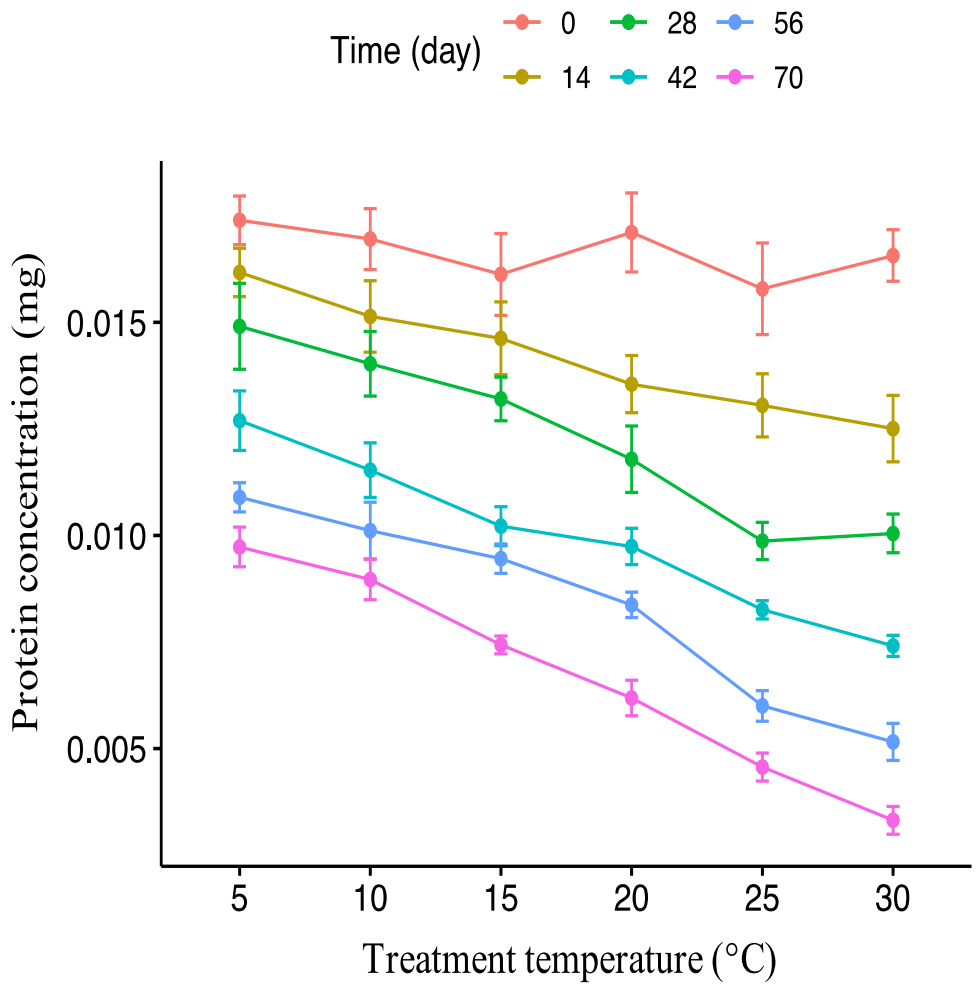

Figure 3. 1 The protein concentration (mean ±SE) (mg) of *Ixodes ricinus* nymphs that were maintained at a range of temperatures (°C) for up to 70 days. Points joined for clarity.

3.3.1.1.2 Phospholipid content

There was a significant effect of temperature on phospholipid concentration for nymphs ($F_{5,426}$ = 39.79, P<0.001) and a significant effect of incubation time ($F_{5,421}$ = 179.93, P<0.001). There was a significant interaction between temperature and incubation time ($F_{25,396}$ = 1.68, P<0.05). The relative changes in phospholipid concentration showed that at 5°C phospholipid content fell 25% over 42 days whereas at 30 °C it fell 58%. At 70 days phospholipid content had declined by 42% at 5°C whereas at 30 °C it had declined by 78%. The phospholipid content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.2).

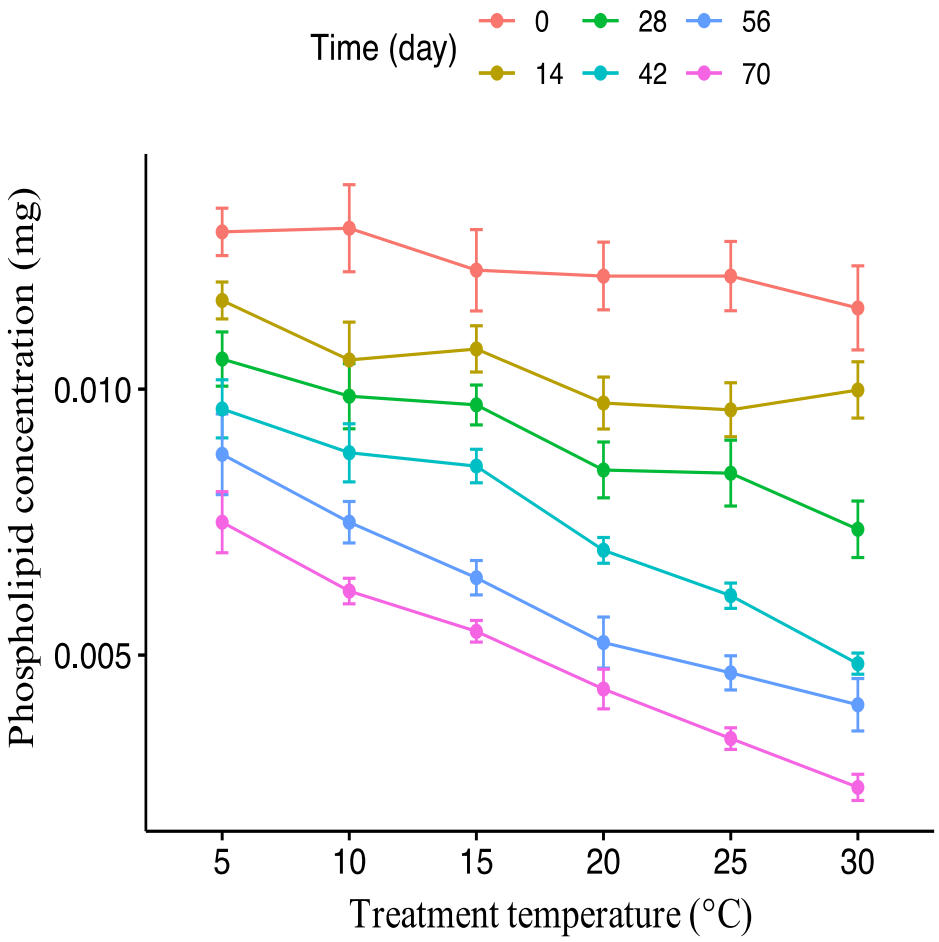

Figure 3. 2 The phospholipid concentrations (mean ±SE) (mg) of *Ixodes ricinus* nymphs that were maintained at a range of temperatures (°C) for up to 70 days. Points joined for clarity.

3.3.1.1.3 Neutral lipid content

There was a significant effect of temperature on neutral lipid content for nymphs ($F_{5,426} = 34.45$, $P<0.001$), and a significant effect of incubation time ($F_{5,421} = 242.12$, $P<0.001$). There was a significant interaction between temperature and time ($F_{25,396} = 2.39$, $P<0.001$). The relative changes in neutral lipid concentration showed that at 5 °C neutral lipid content fell 22% over 42 days whereas at 30 °C it fell 50%. At 70 days neutral lipid content had declined by 65% at 5°C whereas at 30 °C it had declined by 87%. The neutral lipid content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.3).

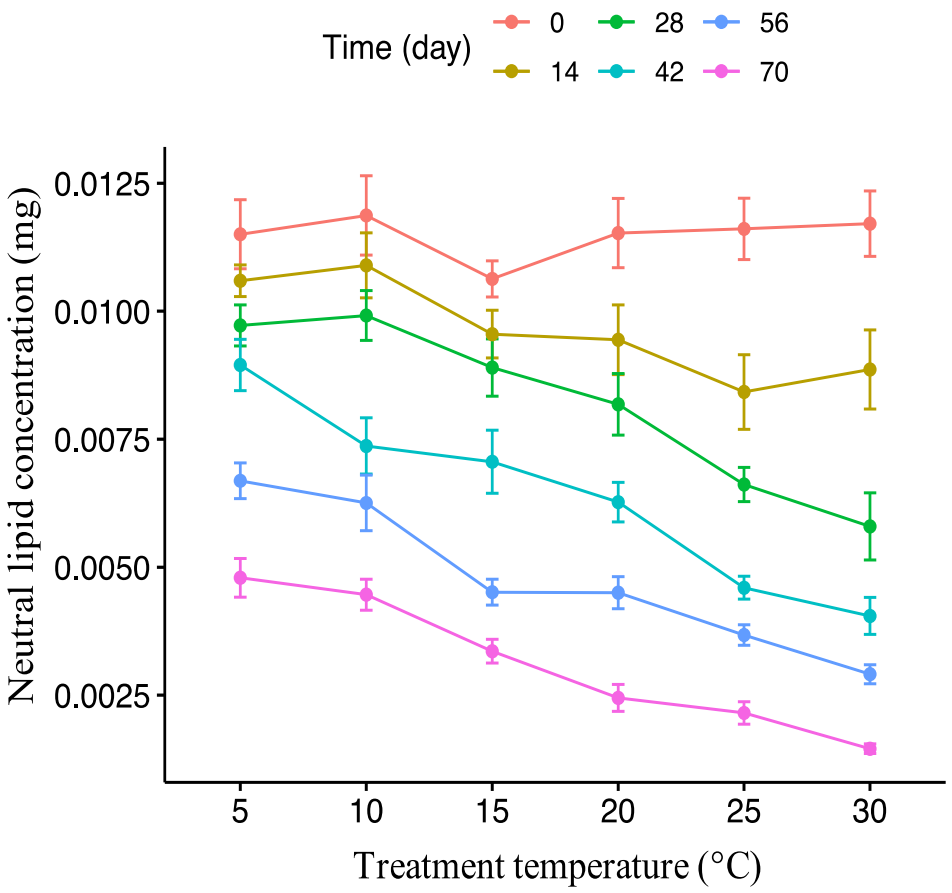

Figure. 3. 3 The neutral lipid concentrations (mean ±SE) (mg) of *Ixodes ricinus* nymphs that were maintained at a range of temperatures (°C) for up to 70 days. Points joined for clarity.

3.3.1.1.4 Total water-soluble carbohydrates content

There was a significant effect of temperature on soluble carbohydrates concentration for nymphs ($F_{5,426}$ = 21.99, P<0.001), and a significant effect of incubation time increase ($F_{5,421}$= 204.41, P<0.001). There was a significant interaction between temperature and time ($F_{25,396}$ = 2.36, P<0.001). The relative changes in phospholipid concentration showed that at 5°C soluble carbohydrates content fell 18% over 42 days whereas at 30°C it fell 48%. At 70 days soluble carbohydrates content had declined by 43% at 5°C whereas at 30 °C it had declined by 84%. The soluble carbohydrates content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.4).

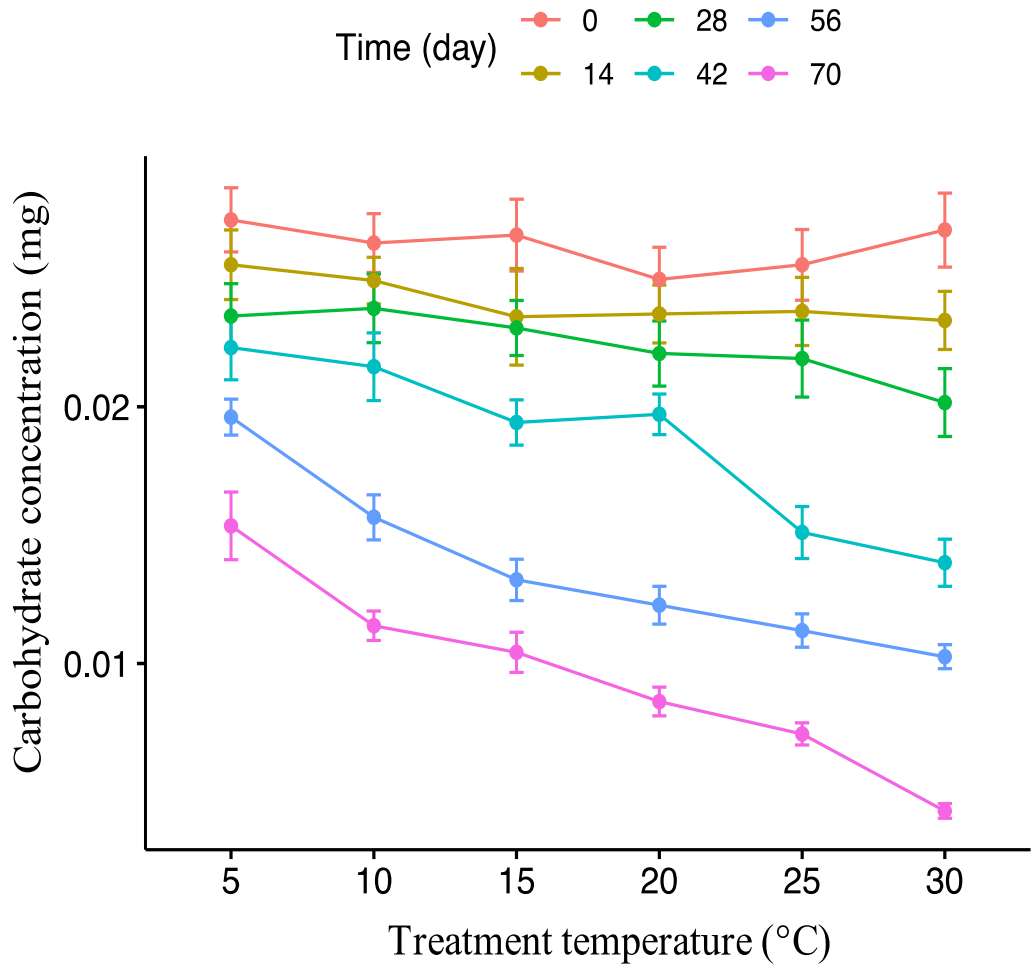

Figure 3. 4 The total water-soluble carbohydrates concentrations (mean ±SE) (mg) of *Ixodes ricinus* nymphs that were maintained at a range of temperatures (°C) for up to 70 days. Points joined for clarity.

3.3.1.1.5 Glycogen content

There was a significant effect of temperature on glycogen concentration for nymphs ($F_{5,426}$ = 24.51, P<0.001), and a significant effect of incubation time ($F_{5,421}$ = 281.61, P<0.001). There was a significant interaction between temperature and time ($F_{25,396}$ = 2.29, P<0.001). The relative changes in glycogen concentration showed that at 5°C glycogen content fell 32% over 42 days whereas at 30 °C it fell 62%. At 70 days glycogen content had declined by 48% at 5 °C whereas at 30 °C it had declined by 85%. The glycogen content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.5).

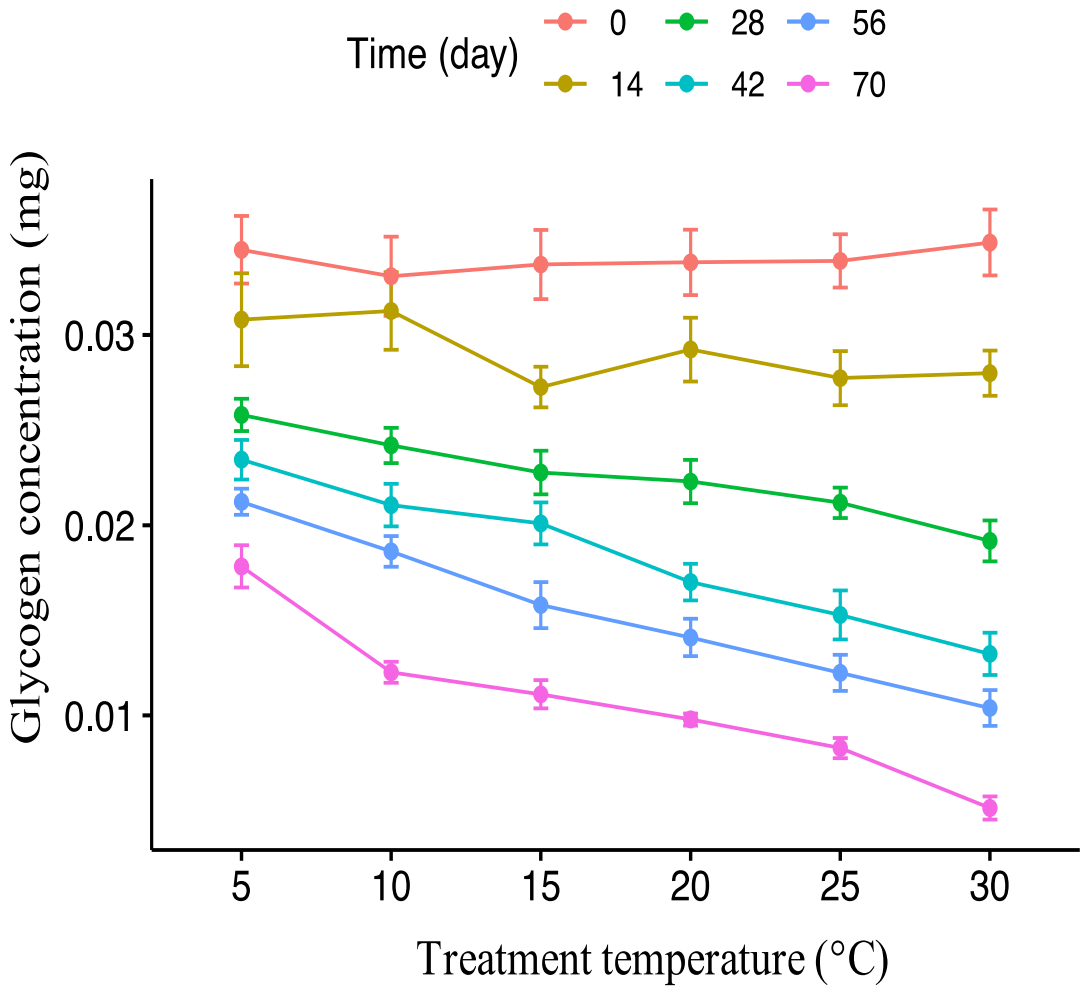

Figure 3. 5 The glycogen concentrations (mean ±SE) (mg) of *Ixodes ricinus* nymphs that were maintained at a range of temperatures (°C) for up to 70 days. Points joined for clarity.

3.3.1.2 Males

3.3.1.2.1 Protein content

There was a significant effect of temperature on protein content for males ($F_{5,282}$ = 90.78, P<0.001), and a significant effect of incubation time ($F_{3,279}$ =168.31, P<0.001). There was a significant interaction between temperature and time ($F_{15,264}$ = 5.36, P<0.001). The relative changes in protein concentration showed that at 5 °C protein content fell 3% over 14 days whereas at 30 °C it fell 44%. At 42 days protein content had declined by 21% at 5°C whereas at 30 °C it had declined by 70%. The protein content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.6).

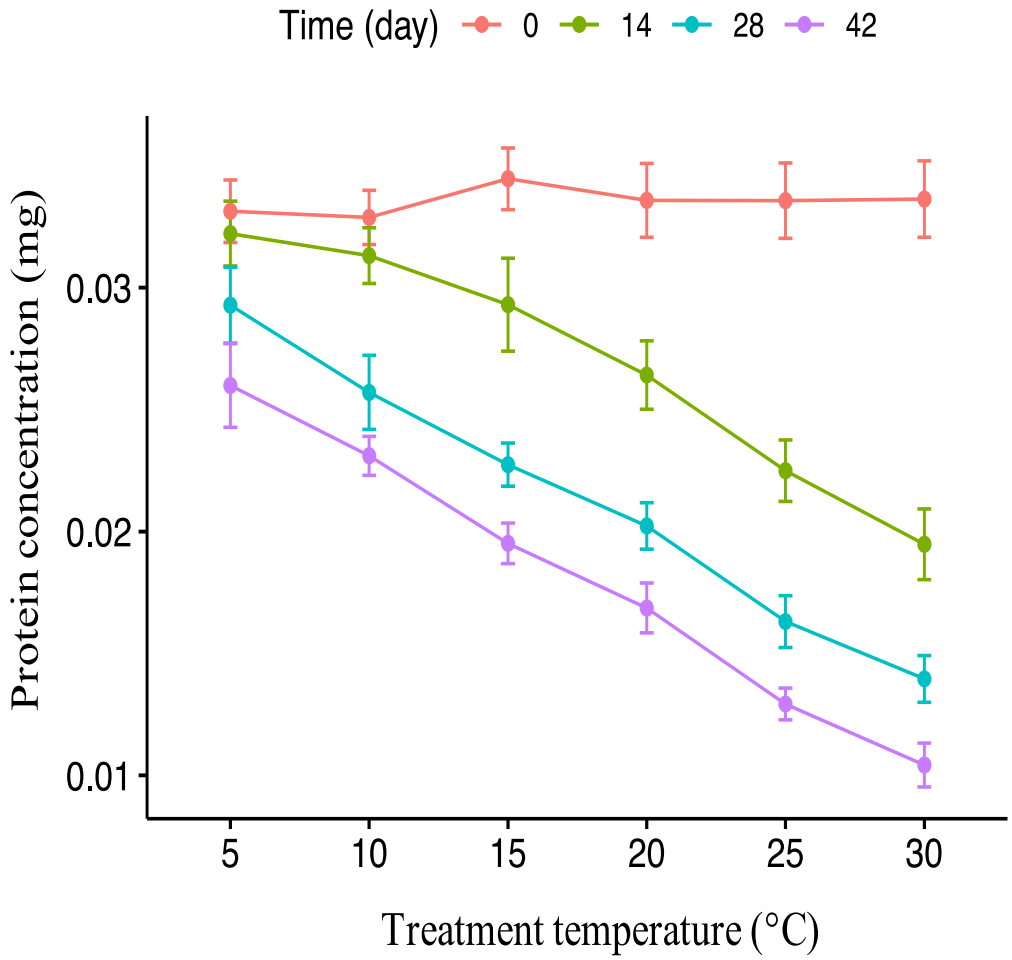

Figure 3. 6 The protein concentrations (mean ±SE) (mg) of *Ixodes ricinus* males that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.2.2 Phospholipid content

There was a significant effect of temperature on phospholipid concentration for males ($F_{5,282}$ = 59.16, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 221.76, P<0.001). There was a significant interaction between temperature and time ($F_{15,264}$ = 2.94, P<0.001). The relative changes in phospholipid concentration showed that at 5°C phospholipid content fell 6% over 14 days whereas at 30 °C it fell 45%. At 42 days phospholipid content had declined by 30% at 5°C whereas at 30 °C it had declined by 77%. The phospholipid content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.7).

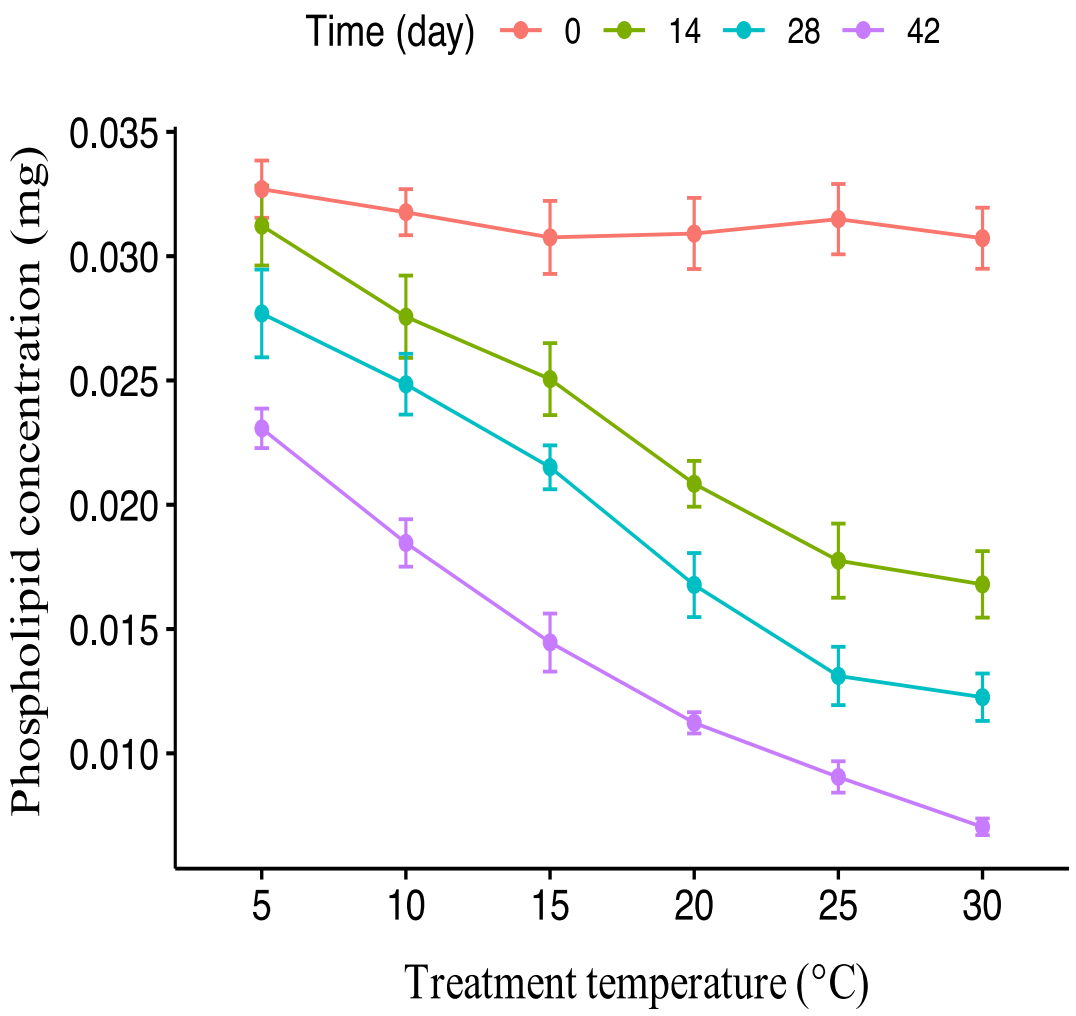

Figure 3. 7 The phospholipid concentrations (mean ±SE) (mg) of *Ixodes ricinus* males that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.2.3 Neutral lipid content

There was a significant effect of temperature on neutral lipid concentration for males ($F_{5,282}$ = 39.99, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 197.29, P<0.001). There was a significant interaction between temperature and time ($F_{15,264}$ = 4.92, P<0.001). The relative changes in neutral lipid concentration showed that at 5 °C neutral lipid content fell 4% over 14 days whereas at 30 °C it fell 30%. At 42 days neutral lipid content had declined by 52% at 5°C whereas at 30 °C it had declined by 78%. The neutral lipid content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.8).

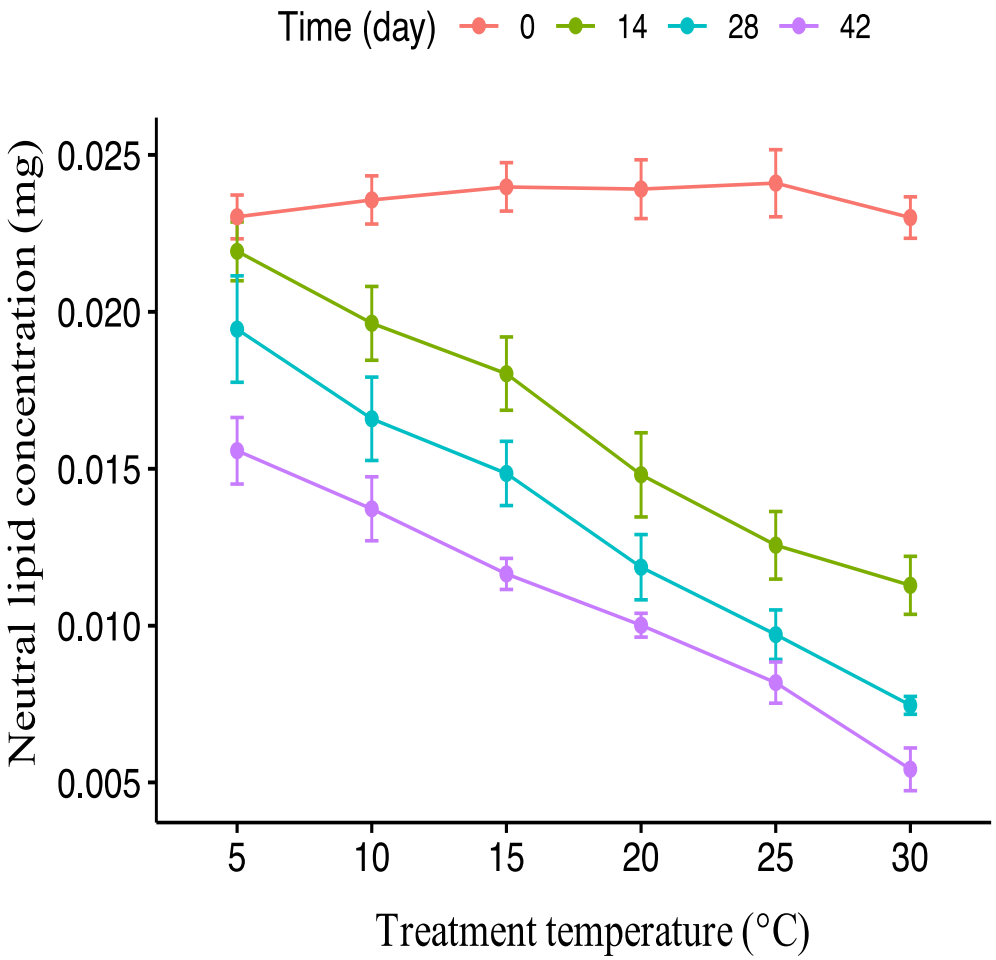

Figure 3. 8 The neutral lipid concentrations (mean ±SE) (mg) of *Ixodes ricinus* males that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.2.4 The total water-soluble carbohydrates content

There was a significant effect of temperature on soluble carbohydrates concentration for males ($F_{5,282}$ = 19.25, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 244.17, P<0.001). There was a significant interaction between temperature and time ($F_{15,264}$ = 2.61, P<0.01, Fig. 3.9). The relative changes in soluble carbohydrates concentration showed that at 5°C soluble carbohydrates content fell 13% over 14 days whereas at 30 °C it fell 40%. At 42 days soluble carbohydrates content had declined by 40% at 5°C whereas at 30 °C it had declined by 77%. The total water-soluble carbohydrates content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.9).

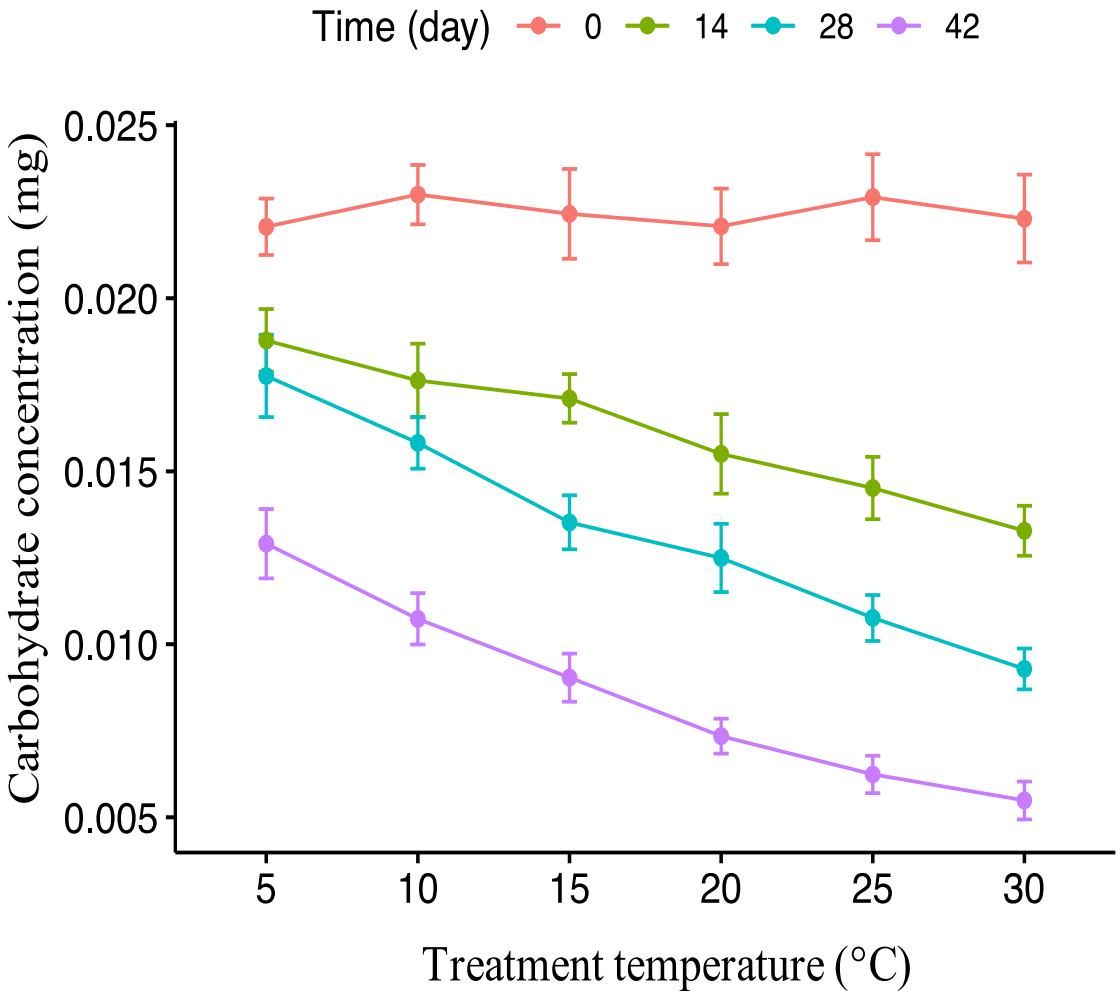

Figure 3. 9 The total water-soluble carbohydrates concentrations (mean ±SE) (mg) of *Ixodes ricinus* males that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.2.5 Glycogen content

There was a significant effect of temperature on glycogen concentration for males ($F_{5,282}$ = 16.23, P<0.001), and with a significant effect of increased incubation time ($F_{3,279}$ = 87.99, P<0.001), There was a significant interaction between temperature and time ($F_{15,264}$ = 2.20, P<0.01, Fig. 3.10). The relative changes in glycogen concentration showed that at 5 °C glycogen content fell 8% over 14 days whereas at 30 °C it fell 41%. At 42 days glycogen content had declined by 29% at 5°C whereas at 30 °C it had declined by 66%. The glycogen

content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.10).

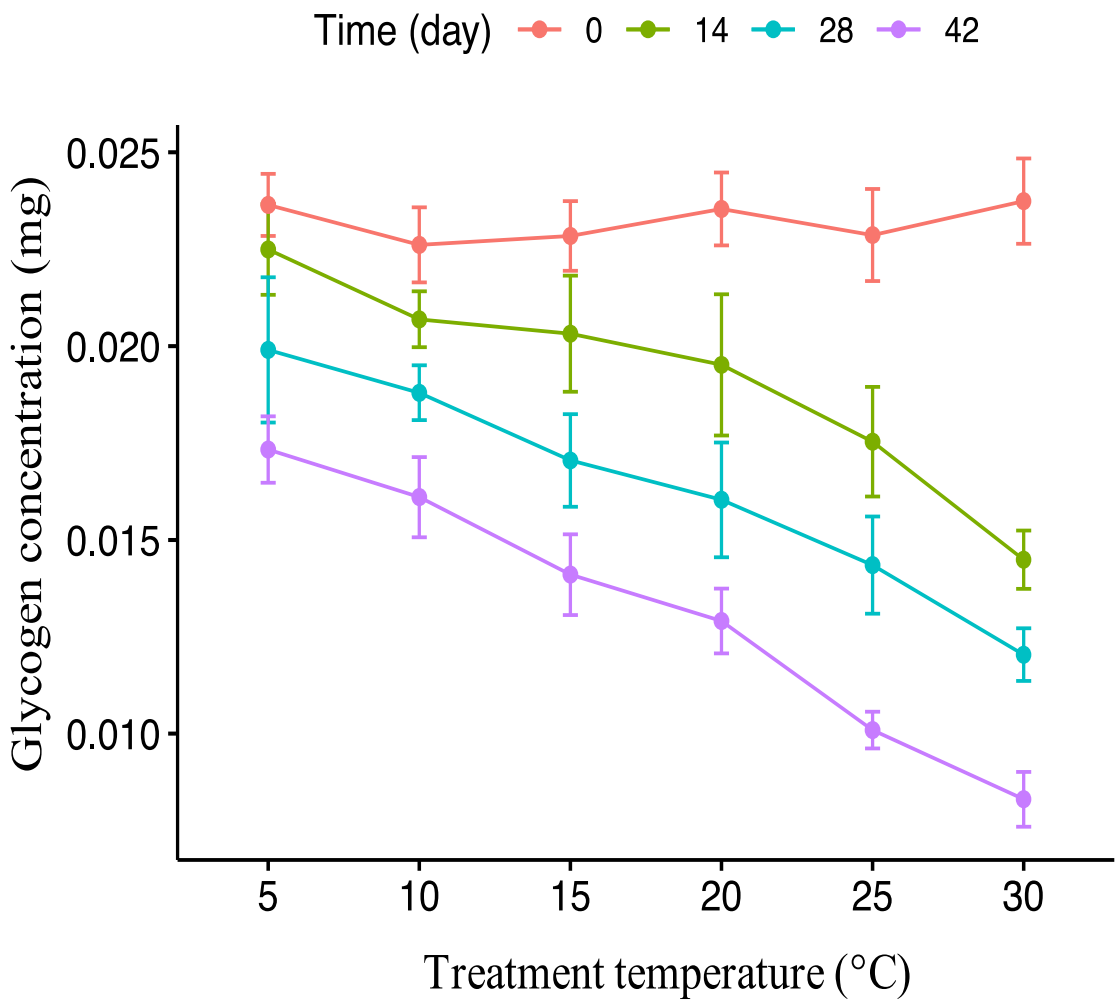

Figure 3. 10 The glycogen concentrations (mean ±SE) (mg) of *Ixodes ricinus* males that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.3 Females

3.3.1.3.1 Protein content

There was a significant effect of temperature on protein concentration for females ($F_{5,282}$ = 15.43, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 283.67, P<0.001). There was a significant interaction between temperature and time ($F_{15,264}$ = 3.36, P<0.001, Fig. 3.11). The relative changes in protein concentration showed that at 5 °C protein

content fell 8% over 14 days whereas at 30 °C it fell 17%. At 42 days protein content had declined by 37% at 5°C whereas at 30 °C it had declined by 77%. The protein content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.11).

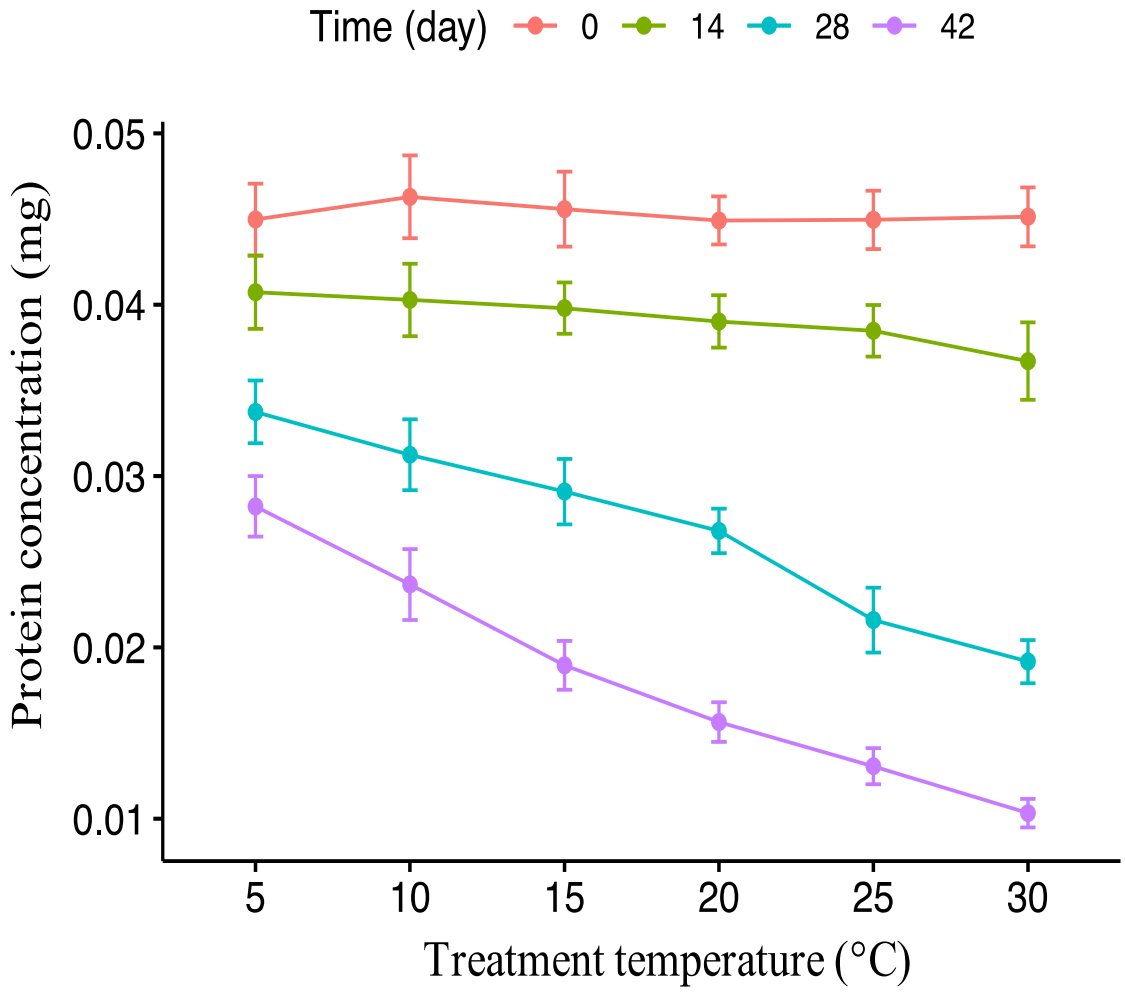

Figure 3. 11 The protein concentrations (mean ±SE) (mg) of *Ixodes ricinus* females that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.3.2 Phospholipid content

There was a significant effect of temperature on phospholipid concentration for females ($F_{5,282}$ = 20.21, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 236.47, P<0.001). There was a significant interaction between temperature and time ($F_{15,264}$ = 2.65, P<0.001). The relative changes in phospholipid concentration showed that at 5 °C phospholipid content fell 10% over 14 days whereas at 30 °C it fell 26%. At 42 days phospholipid content had declined by 39% at 5°C whereas at 30 °C it had declined by 77%. The phospholipid content

in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.12).

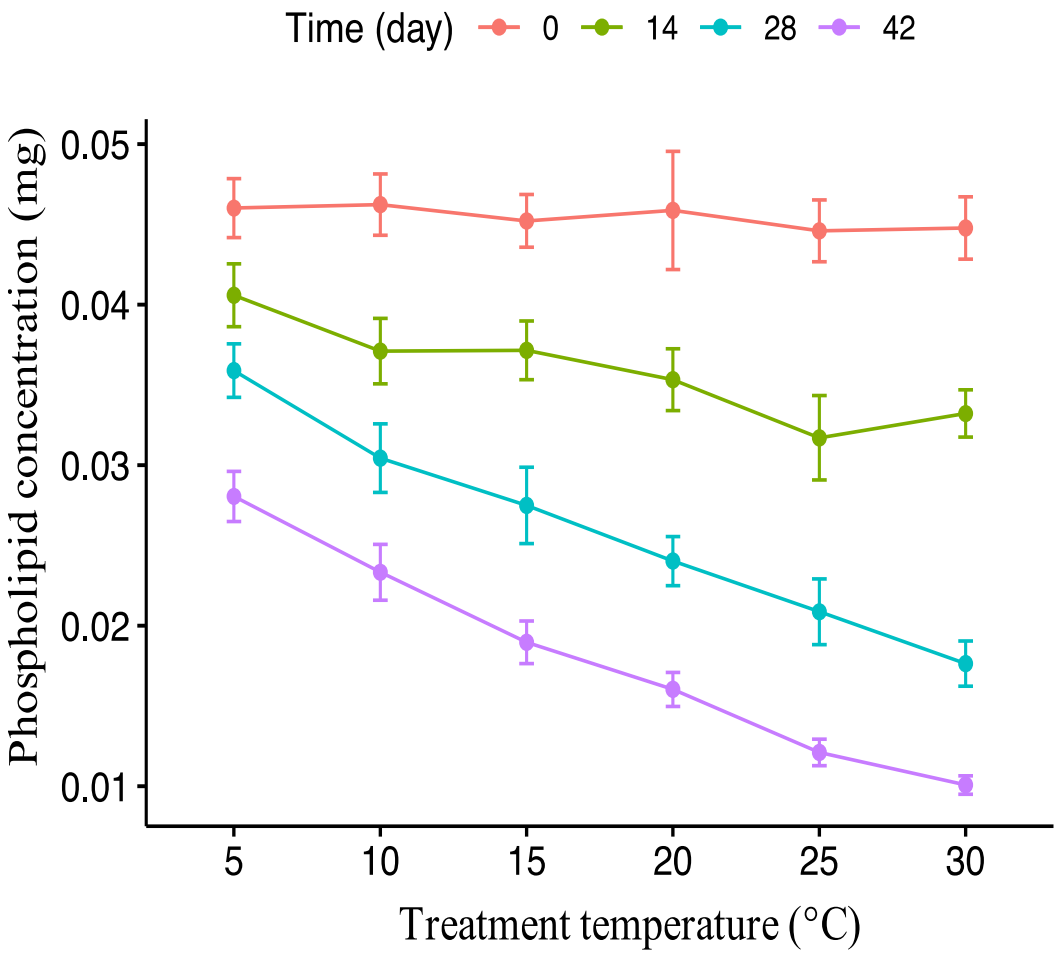

Figure 3. 12 The phospholipid concentrations (mean ±SE) (mg) of *Ixodes ricinus* females that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.3.3 Neutral lipid content

There was a significant effect of temperature on neutral lipid concentration for females ($F_{5,282}$ = 15.12, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 188.45, P<0.001). There was a significant interaction between temperature and time ($F_{15,264}$ = 2.16, P<0.01, Fig. 3.13). The relative changes in neutral lipid concentration showed that at 5°C neutral lipid content fell 15% over 14 days whereas at 30 °C it fell 45%. At 42 days neutral lipid content had declined by 45% at 5°C whereas at 30 °C it had declined by 85%. The neutral

lipid content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.13).

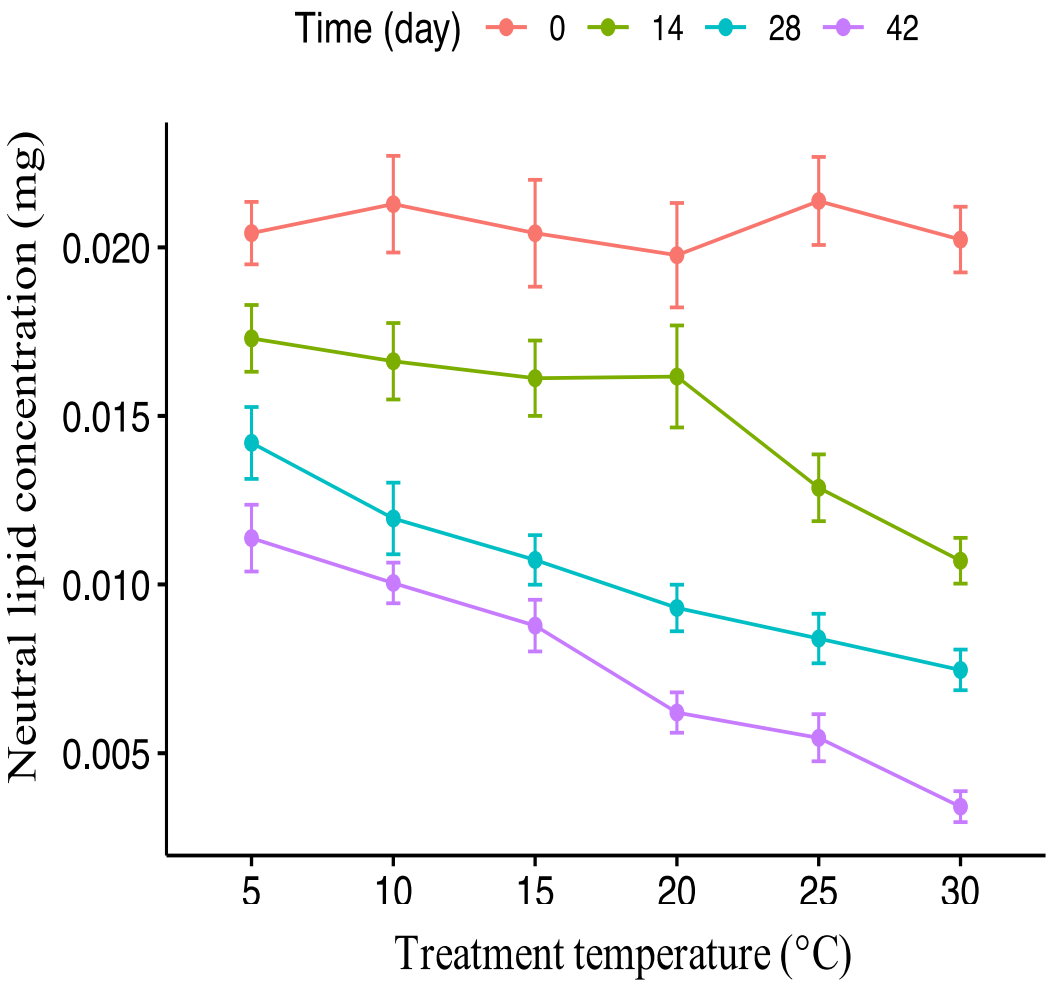

Figure 3. 13 The neutral lipid concentrations (mean ±SE) (mg) of *Ixodes ricinus* females that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.3.4 The total water-soluble carbohydrates content

There was a significant effect of temperature on soluble carbohydrates concentration for females ($F_{5,282}$ = 7.42, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 133.66, P<0.001), but, the interaction between temperature and time was not significant ($F_{15,264}$ = 1.30 P = 0.2). The relative changes in soluble carbohydrates concentration showed that at 5°C soluble carbohydrates content fell 14% over 14 days whereas at 30 °C it fell 29%. At 42

days soluble carbohydrates content had declined by 37% at 5°C whereas at 30 °C it had declined by 77%. The soluble carbohydrates content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.14).

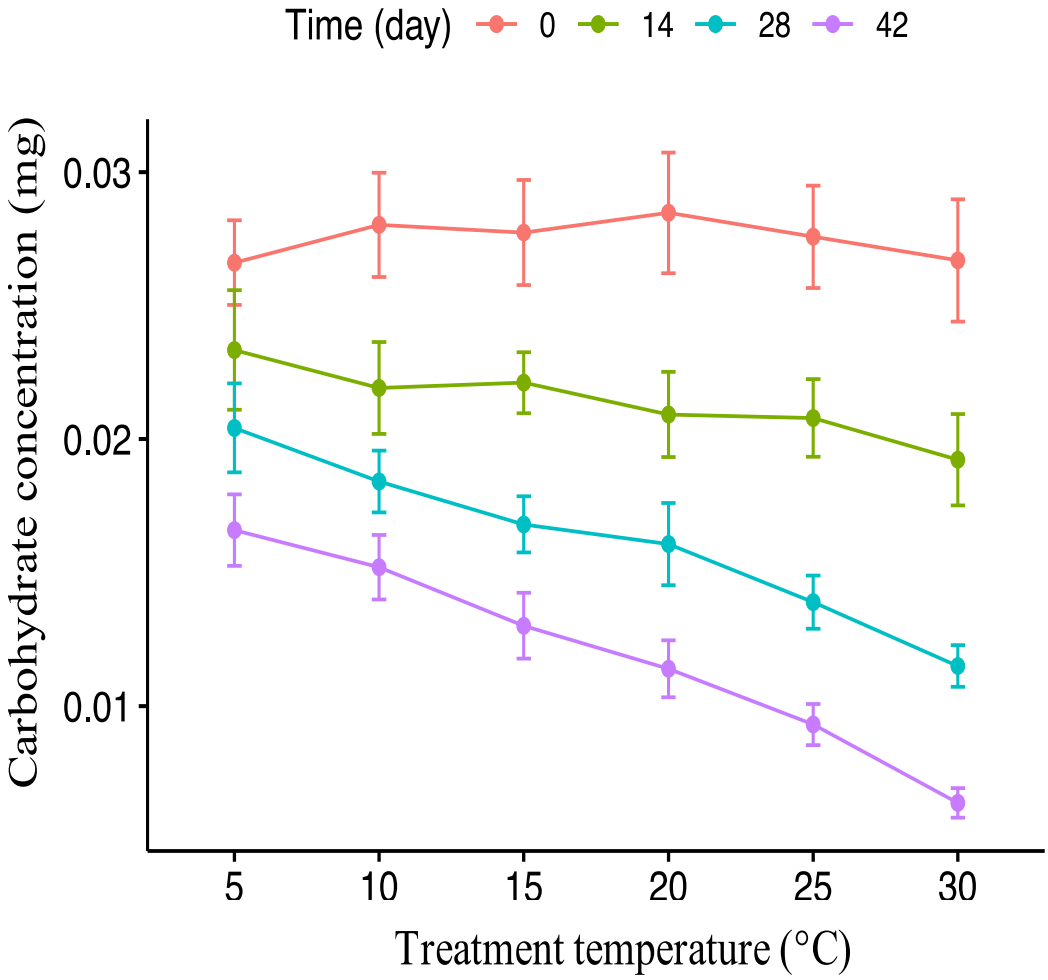

Figure 3. 14 The total water-soluble carbohydrates concentrations (mean ±SE) (mg) of *Ixodes ricinus* females that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.1.3.5 Glycogen content

There was a significant effect of temperature on glycogen concentration for females ($F_{5,282}$ = 22.23, P<0.001), and a significant effect of increased incubation time ($F_{3,279}$ = 151.08 P<0.001), but the interaction between temperature and time was not significant ($F_{15,264}$ = 1.62, P = 0.06, Fig. 3.15). The relative changes in glycogen concentration showed that at 5 °C glycogen

content fell 11% over 14 days whereas at 30 °C it fell 44%. At 42 days glycogen content had declined by 44% at 5°C whereas at 30 °C it had declined by 76%. The glycogen content in ticks sampled at intermediate times and temperatures declined between these values (Fig. 3.15).

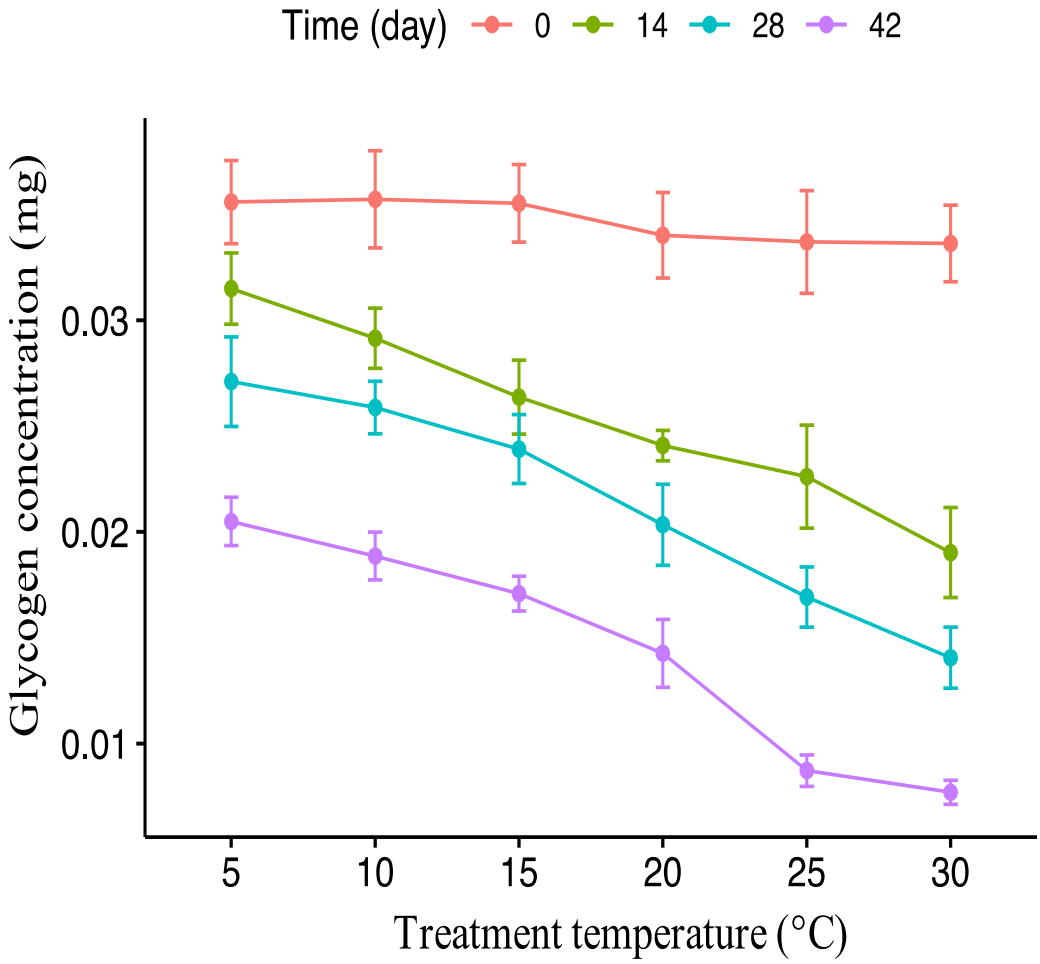

Figure 3. 15 The glycogen concentrations (mean ±SE) (mg) of *Ixodes ricinus* females that were maintained at a range of temperatures (°C) for up to 42 days. Points joined for clarity.

3.3.2 The relationships between time, temperature and starvation rates

To summarise the rate of energy sources, use with temperature, the relationships between metabolite contents and time were first described by fitting negative exponential lines of best fit for nymphs (Table 3.1), males (Table 3.2) and females (Table 3.3). Negative exponential relationships provided the best fit to the raw data, as determined by examination of the r^2

values. Statistically significant reductions in metabolite contents were observed at all temperatures and for all life-cycle stages (Figs. 3.16 – 3.33). The slopes of the regressions indicate that nymphs generally show lower rates of resource depletion at all temperatures than adults. The overall mean rate of depletion for nymphs was 0.016 mg/day (SD±0.0058), for males was 0.019 (SD± 0.0085) and for females was 0.023 (SD± 0.0088). Over a period of 42 days at 5 °C, nymphs lost an average of 25% of their initial metabolite mass. In contrast, over the same temperature and over the same time period adults showed losses of on average 35% for males and 40%. At 30 °C nymphs lost an average of 55% of their initial metabolite mass over 42 days, while adults lost an average of 73% and 78% for males and females respectively. Extrapolation from the results suggest that starvation would occur at between 50-70 days at 25-30 °C and 100-200 days at 5 °C.

Subsequently, the slopes of the exponential relationships between metabolite contents and time were plotted against temperature and linear regressions fitted to show the rate of metabolite loss (Figs. 3.4-3.6). The slopes of the relationships between metabolite contents and time describe the rate of loss of each class of compound (mg/day). A plot of the slope of the relationship between metabolite contents and time, against temperature therefore describes the relationship between the rate of loss of each compound class and temperature. Consistent patterns in the rate of use of particular energy sources was evident between different life-cycle stages. For nymphs. The rate of use of neutral lipid and glycogen were consistently high and the rate of loss of protein was low, particularly at low temperatures. In contrast for males, soluble carbohydrates concentrations declined most rapidly and glycogen the least. Finally, for females, there was much less variation in the rate of energy sources use, compared to males or nymphs, but a consistent pattern with lipid concentrations declining at a higher rate than other energy sources . The Q_{10} values, determined from a linear regression fitted to all the metabolite data (Figs. 3.4-3.6). gave values of 1.5 for nymphs, 1.71 for males and 1.63 for females.

Extrapolation of this linear regression indicates that the lower temperature threshold for metabolic activity in *I. ricinus* for is -8.8 °C for nymphs, -4.1 °C for males and -5.9 °C for females.

Table 3. 1 Regression statistics for the relationship between the concentration of each metabolic component and time (days) for *Ixodes ricinus* (nymphs) that were maintained at temperatures of between 5 to 30°C, with the equation of the best fit negative exponential line, R^2, F and significance of the regression (P).

Energetic components	Temperature	Exponential regression			
		Equation	R^2	F	P
Protein	5°C	$y = 0.0179e^{-0.008x}$	0.62	118	0.0001
	10°C	$y = 0.0171e^{-0.009x}$	0.59	101.3	0.0001
	15°C	$y = 0.0165e^{-0.010x}$	0.71	178.7	0.0001
	20°C	$y = 0.0168e^{-0.013x}$	0.74	208.6	0.0001
	25°C	$y = 0.0161e^{-0.017x}$	0.81	304.4	0.0001
	30°C	$y = 0.0176e^{-0.023x}$	0.83	348.4	0.0001
Phospholipid	5°C	$y = 0.013e^{-0.008x}$	0.47	63.3	0.0001
	10°C	$y = 0.0125e^{-0.009x}$	0.58	96.86	0.0001
	15°C	$y = 0.0126e^{-0.011x}$	0.74	205.7	0.0001
	20°C	$y = 0.0122e^{-0.015x}$	0.72	181.5	0.0001
	25°C	$y = 0.0125e^{-0.018x}$	0.82	326.1	0.0001
	30°C	$y = 0.0125e^{-0.022x}$	0.78	254.9	0.0001
Neutral lipid	5°C	$y = 0.0126e^{-0.012x}$	0.64	124.5	0.0001
	10°C	$y = 0.0128e^{-0.014x}$	0.66	140.2	0.0001
	15°C	$y = 0.0126e^{-0.017x}$	0.75	212.2	0.0001
	20°C	$y = 0.0128e^{-0.021x}$	0.74	208.8	0.0001
	25°C	$y = 0.0118e^{-0.023x}$	0.84	378.8	0.0001
	30°C	$y = 0.0123e^{-0.028x}$	0.85	399.3	0.0001
Soluble carbohydrates	5°C	$y = 0.0283e^{-0.007x}$	0.46	61.33	0.0001
	10°C	$y = 0.0294e^{-0.011x}$	0.65	131.3	0.0001
	15°C	$y = 0.0289e^{-0.013x}$	0.64	127.5	0.0001
	20°C	$y = 0.0291e^{-0.015x}$	0.70	169.2	0.0001
	25°C	$y = 0.0295e^{-0.018x}$	0.76	223.8	0.0001
	30°C	$y = 0.0327e^{-0.024x}$	0.80	286.9	0.0001
Glycogen	5°C	$y = 0.0336e^{-0.009x}$	0.58	100.6	0.0001
	10°C	$y = 0.035e^{-0.013x}$	0.73	193	0.0001
	15°C	$y = 0.0342e^{-0.015x}$	0.74	206.4	0.0001
	20°C	$y = 0.0352e^{-0.017x}$	0.83	349.7	0.0001
	25°C	$y = 0.0353e^{-0.02x}$	0.83	345.3	0.0001
	30°C	$y = 0.0386e^{-0.027x}$	0.83	360.7	0.0001

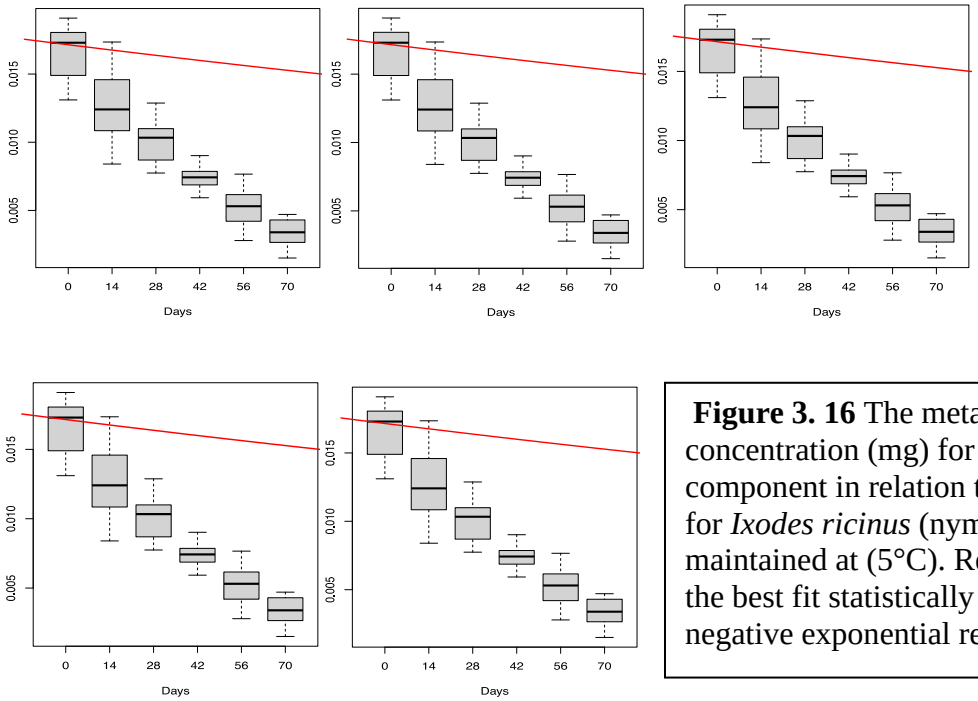

Figure 3. 16 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (nymphs) maintained at (5°C). Red line shows the best fit statistically significant negative exponential relationship.

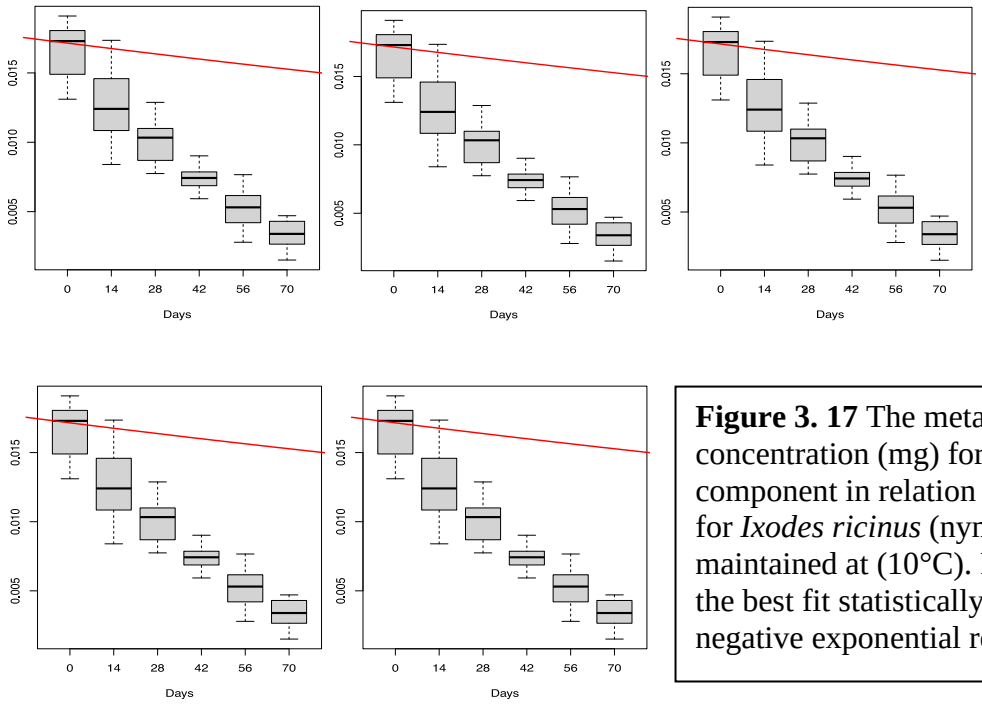

Figure 3. 17 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (nymphs) maintained at (10°C). Red line shows the best fit statistically significant negative exponential relationship.

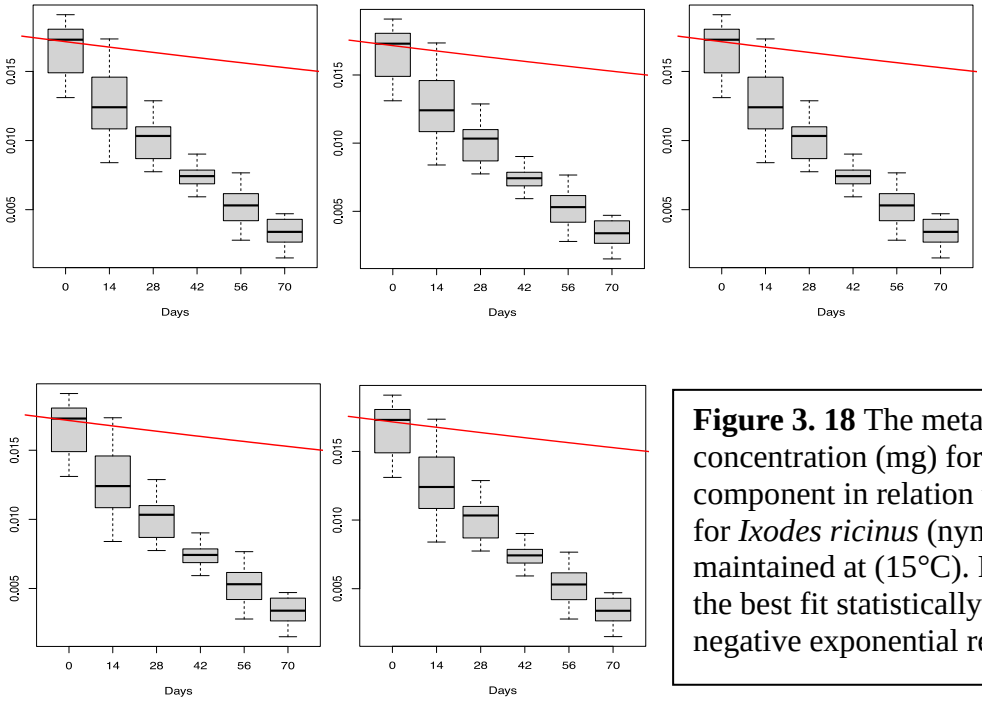

Figure 3. 18 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (nymphs) maintained at (15°C). Red line shows the best fit statistically significant negative exponential relationship.

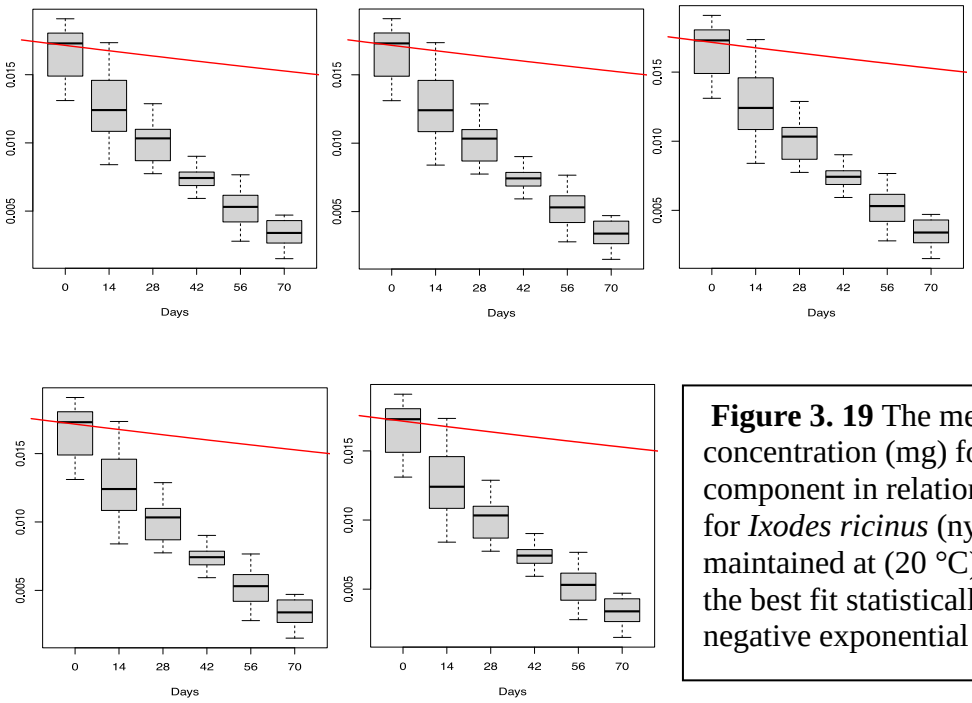

Figure 3. 19 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (nymphs) maintained at (20 °C). Red line shows the best fit statistically significant negative exponential relationship.

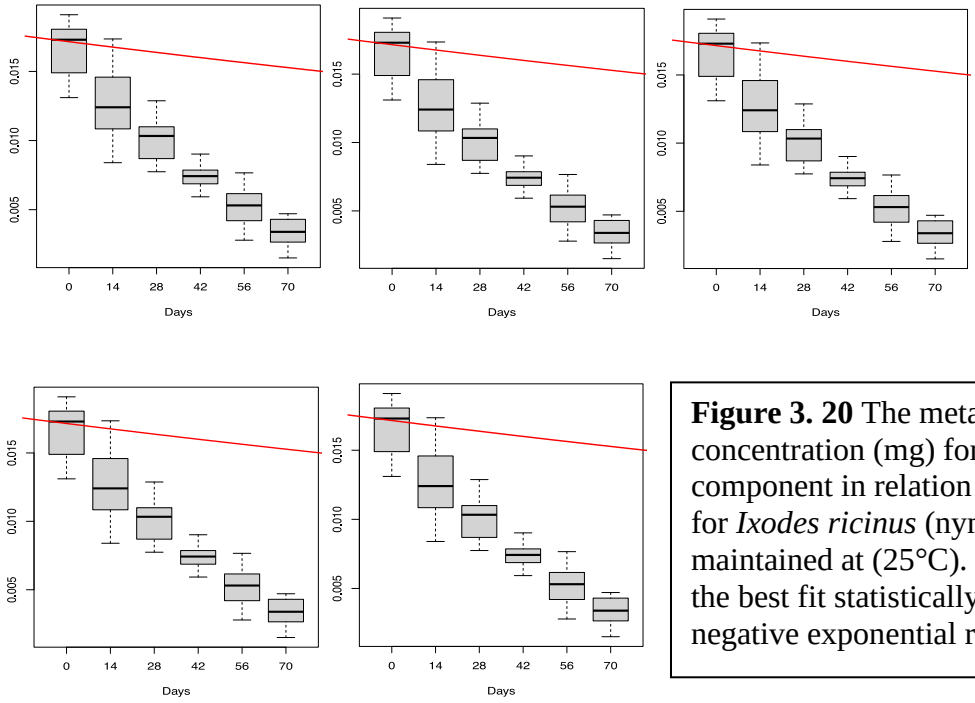

Figure 3. 20 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (nymphs) maintained at (25°C). Red line shows the best fit statistically significant negative exponential relationship.

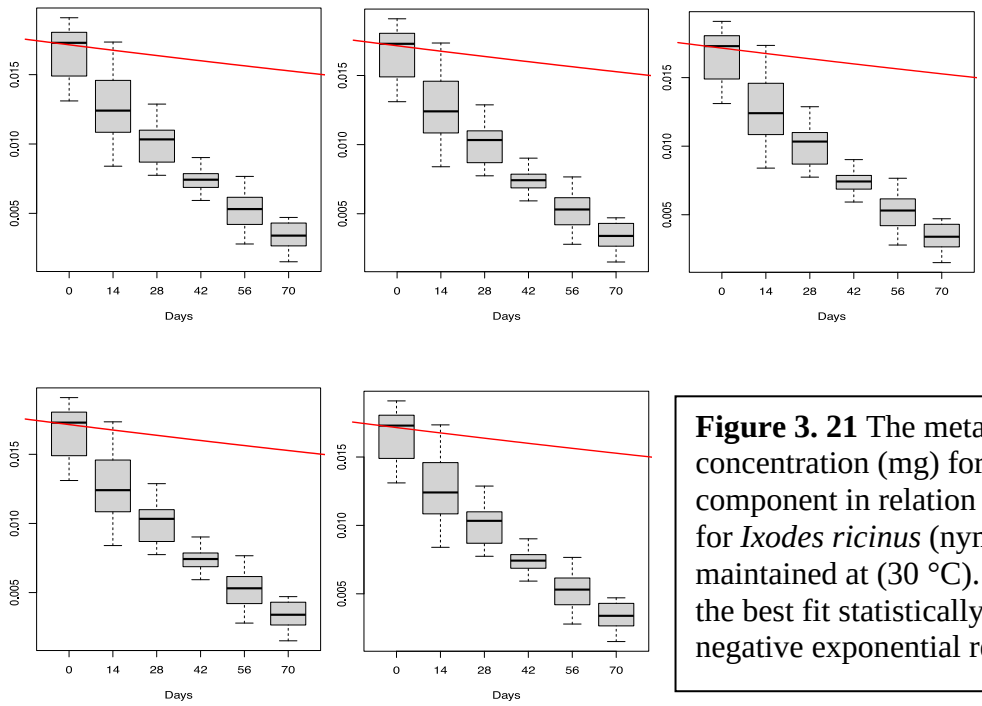

Figure 3. 21 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (nymphs) maintained at (30 °C). Red line shows the best fit statistically significant negative exponential relationship.

Table 3. 2 Regression statistics for the relationship between the concentration of each metabolic component and time (days) for *Ixodes ricinus* (males) that were maintained at temperatures of between 5 to 30°C, with the equation of the best fit negative exponential line, R^2, F and significance of the regression (P).

Energetic components	Temperature	Exponential regression			
		Equation	R^2	F	P
Protein	5°C	$y = 0.0337e^{-0.006x}$	0.24	14.81	0.0003
	10°C	$y = 0.0335e^{-0.009x}$	0.50	46.52	0.0001
	15°C	$y = 0.0342e^{-0.013x}$	0.65	87.07	0.0001
	20°C	$y = 0.0329e^{-0.016x}$	0.70	107	0.0001
	25°C	$y = 0.0317e^{-0.022x}$	0.78	167.1	0.0001
	30°C	$y = 0.0308e^{-0.028x}$	0.76	146.3	0.0001
Phospholipid	5°C	$y = 0.0334e^{-0.008x}$	0.37	28.02	0.0001
	10°C	$y = 0.0323e^{-0.012x}$	0.56	58.95	0.0001
	15°C	$y = 0.0315e^{-0.017x}$	0.64	82.44	0.0001
	20°C	$y = 0.03e^{-0.023x}$	0.80	190.6	0.0001
	25°C	$y = 0.0288e^{-0.029x}$	0.75	139.4	0.0001
	30°C	$y = 0.0289e^{-0.033x}$	0.84	249.8	0.0001
Neutral lipid	5°C	$y = 0.0238e^{-0.009x}$	0.33	23.09	0.0001
	10°C	$y = 0.0233e^{-0.013x}$	0.48	42.83	0.0001
	15°C	$y = 0.0231e^{-0.016x}$	0.66	89.92	0.0001
	20°C	$y = 0.0213e^{-0.02x}$	0.61	72.78	0.0001
	25°C	$y = 0.0206e^{-0.025x}$	0.64	84.72	0.0001
	30°C	$y = 0.0207e^{-0.035x}$	0.79	174.6	0.0001
Soluble carbohydrates	5°C	$y = 0.0224e^{-0.012x}$	0.48	42.81	0.0001
	10°C	$y = 0.023e^{-0.017x}$	0.65	85.65	0.0001
	15°C	$y = 0.0227e^{-0.021x}$	0.73	129.3	0.0001
	20°C	$y = 0.0221e^{-0.025x}$	0.73	126.5	0.0001
	25°C	$y = 0.0226e^{-0.030x}$	0.80	193.7	0.0001
	30°C	$y = 0.0218e^{-0.033x}$	0.80	194.1	0.0001
Glycogen	5°C	$y = 0.0239e^{-0.008x}$	0.24	15.21	0.0003
	10°C	$y = 0.0228e^{-0.008x}$	0.39	29.47	0.0001
	15°C	$y = 0.0229e^{-0.012x}$	0.40	31.32	0.0001
	20°C	$y = 0.0231e^{-0.014x}$	0.43	35.19	0.0001
	25°C	$y = 0.0225e^{-0.018x}$	0.61	75.03	0.0001
	30°C	$y = 0.0223e^{-0.024x}$	0.74	131.2	0.0001

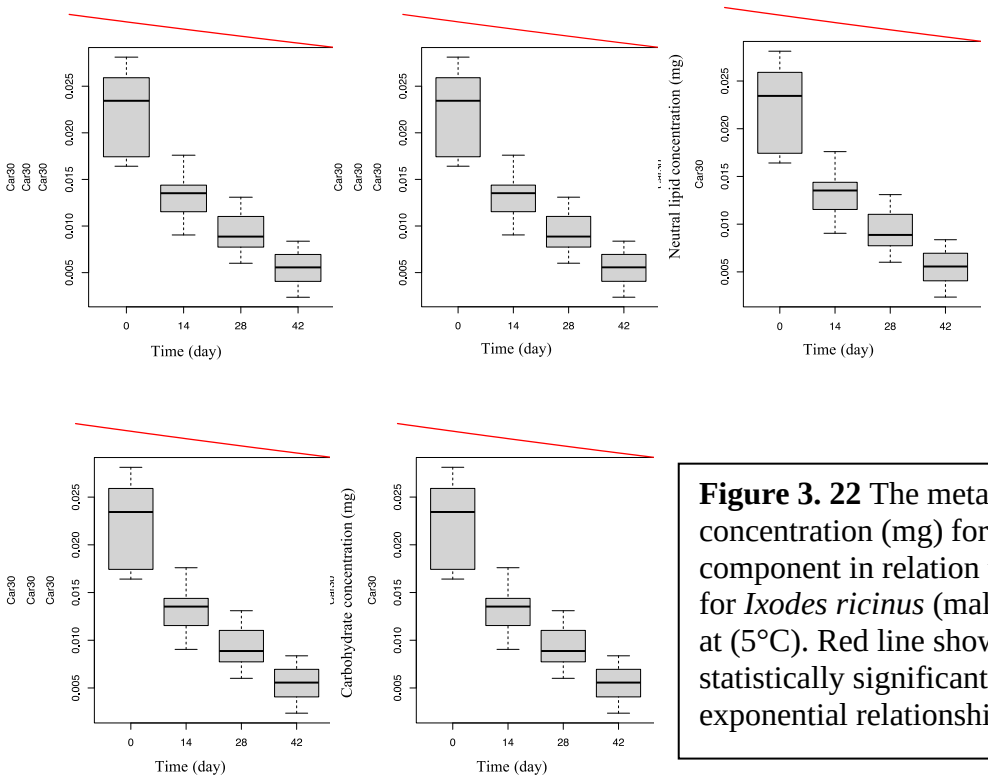

Figure 3. 22 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (males) maintained at (5°C). Red line shows the best fit statistically significant negative exponential relationship.

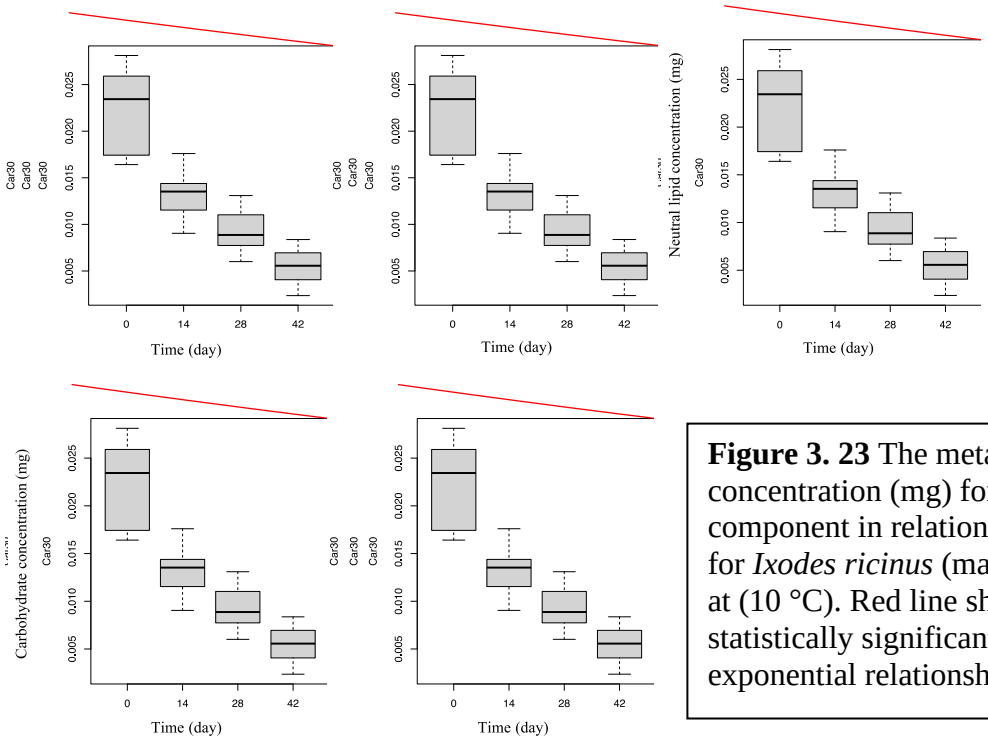

Figure 3. 23 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (males) maintained at (10 °C). Red line shows the best fit statistically significant negative exponential relationship.

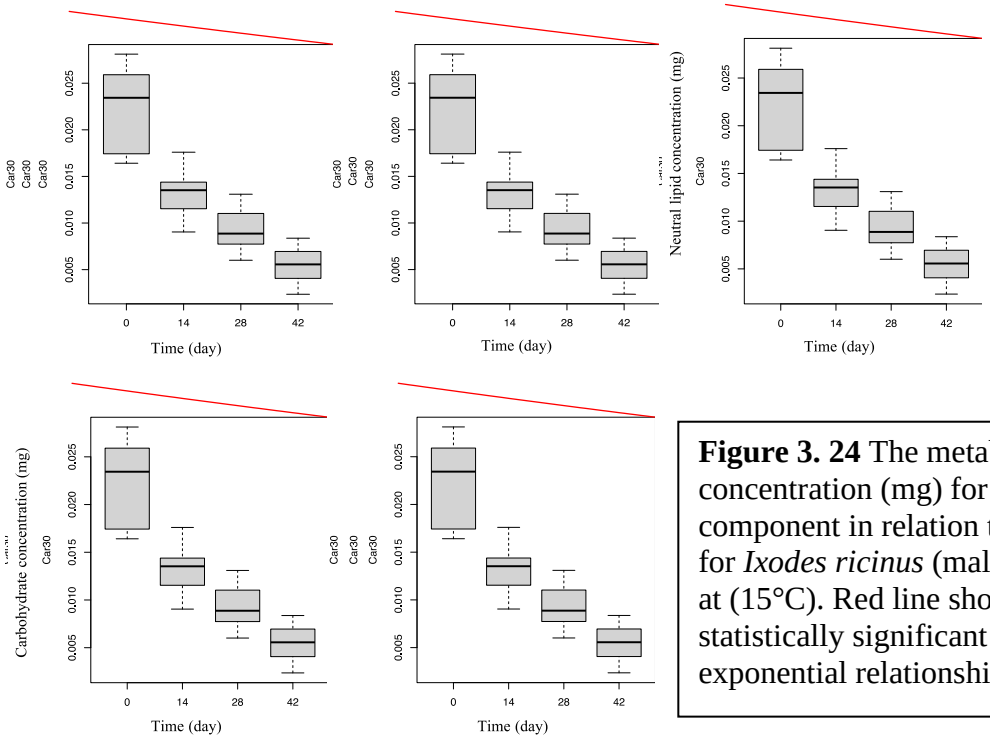

Figure 3. 24 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (males) maintained at (15°C). Red line shows the best fit statistically significant negative exponential relationship.

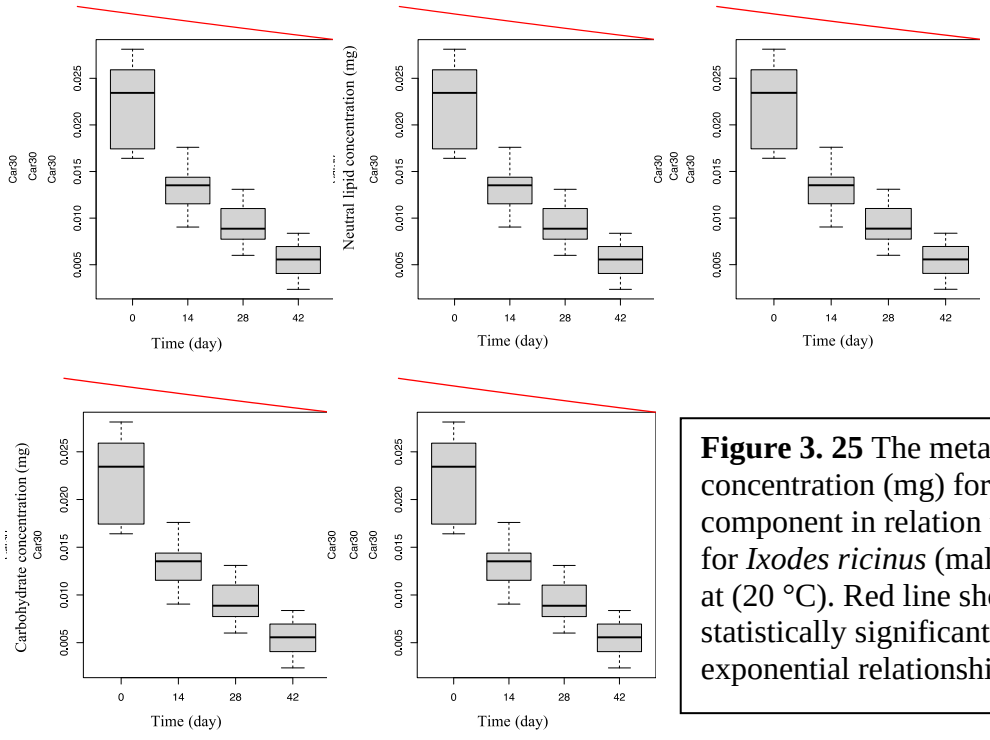

Figure 3. 25 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (males) maintained at (20 °C). Red line shows the best fit statistically significant negative exponential relationship.

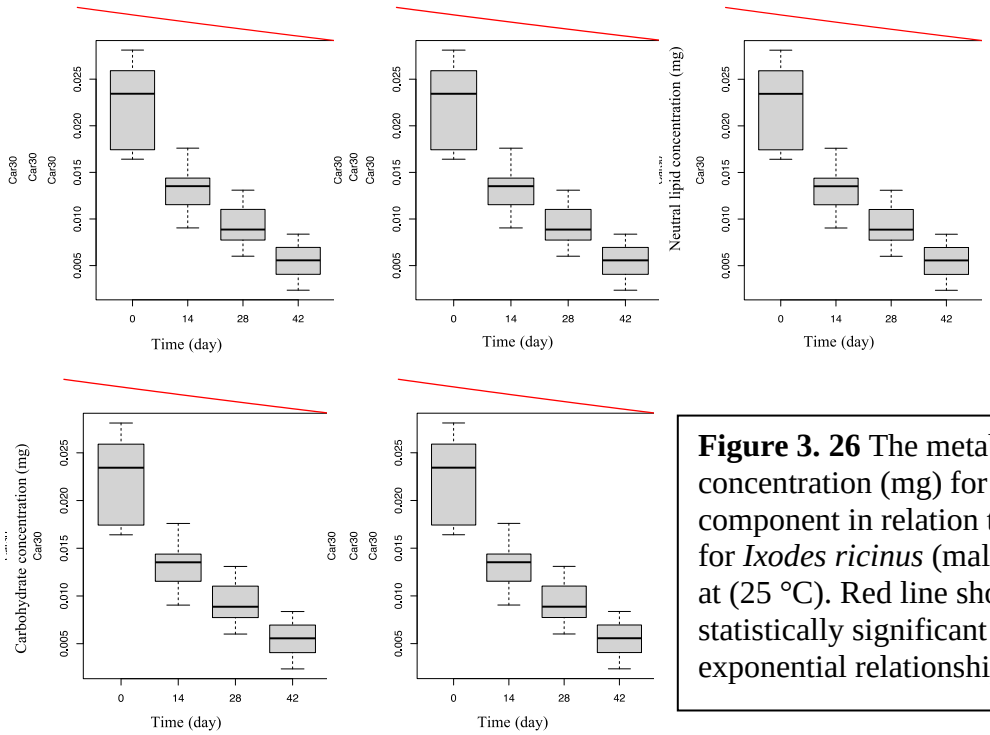

Figure 3. 26 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (males) maintained at (25 °C). Red line shows the best fit statistically significant negative exponential relationship.

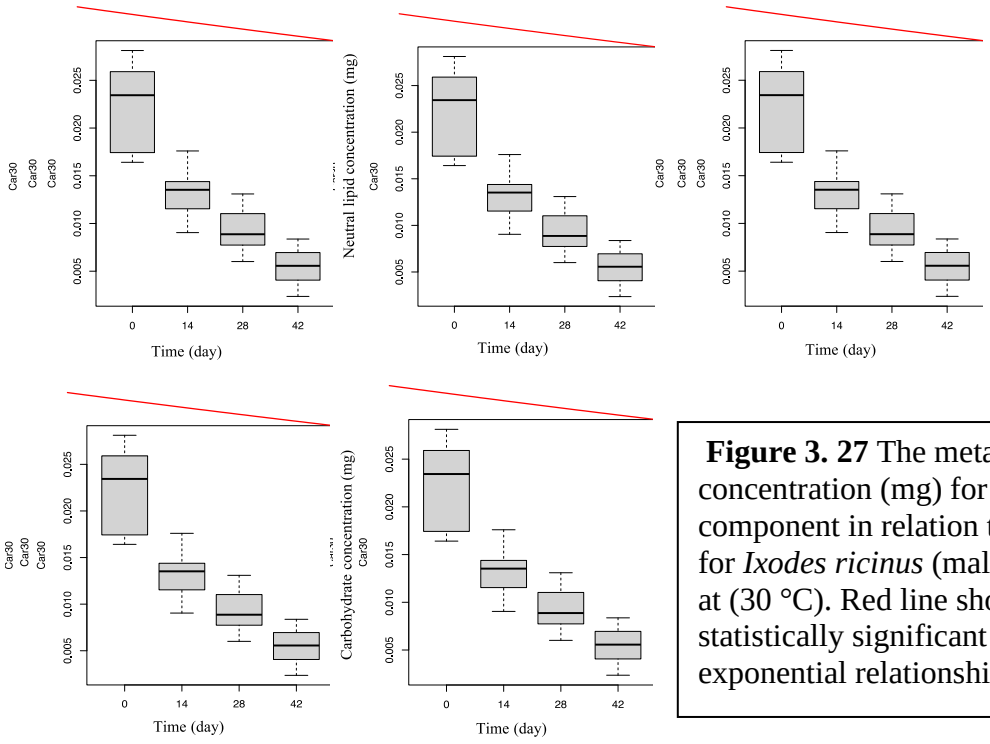

Figure 3. 27 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (males) maintained at (30 °C). Red line shows the best fit statistically significant negative exponential relationship.

Table 3. 3 Regression statistics for the relationship between the concentration of each metabolic component and time (days) for *Ixodes ricinus* (females) that were maintained at temperatures of between 5 to 30°C, with the equation of the best fit negative exponential line, R^2, F and significance of the regression (P).

Energetic components	Temperature	Exponential regression			
		Equation	R^2	F	P
Protein	5°C	$y = 0.0456e^{-0.011x}$	0.47	41.98	0.0001
	10°C	$y = 0.0476e^{-0.016x}$	0.56	59.39	0.0001
	15°C	$y = 0.0488e^{-0.021x}$	0.70	110.4	0.0001
	20°C	$y = 0.0497e^{-0.025x}$	0.80	187.1	0.0001
	25°C	$y = 0.05e^{-0.031x}$	0.79	181.7	0.0001
	30°C	$y = 0.0507e^{-0.036x}$	0.84	259.5	0.0001
Phospholipid	5°C	$y = 0.0467e^{-0.011x}$	0.56	59.16	0.0001
	10°C	$y = 0.0461e^{-0.016x}$	0.61	72.32	0.0001
	15°C	$y = 0.0469e^{-0.021x}$	0.69	105.1	0.0001
	20°C	$y = 0.0463e^{-0.024x}$	0.74	132.8	0.0001
	25°C	$y = 0.0458e^{-0.031x}$	0.78	170.6	0.0001
	30°C	$y = 0.0482e^{-0.036x}$	0.87	309.8	0.0001
Neutral lipid	5°C	$y = 0.0205e^{-0.014x}$	0.50	47.14	0.0001
	10°C	$y = 0.0205e^{-0.018x}$	0.56	60.57	0.0001
	15°C	$y = 0.0201e^{-0.021x}$	0.61	73.19	0.0001
	20°C	$y = 0.0206e^{-0.029x}$	0.68	100.8	0.0001
	25°C	$y = 0.0206e^{-0.033x}$	0.75	140.2	0.0001
	30°C	$y = 0.0202e^{-0.042x}$	0.80	191.7	0.0001
Soluble carbohydrates	5°C	$y = 0.0262e^{-0.011x}$	0.28	18.08	0.0001
	10°C	$y = 0.0267e^{-0.014x}$	0.45	38.41	0.0001
	15°C	$y = 0.0275e^{-0.018x}$	0.59	66.3	0.0001
	20°C	$y = 0.0276e^{-0.021x}$	0.56	60.73	0.0001
	25°C	$y = 0.0279e^{-0.026x}$	0.72	119.5	0.0001
	30°C	$y = 0.0275e^{-0.034x}$	0.76	146.7	0.0001
Glycogen	5°C	$y = 0.0362e^{-0.013x}$	0.49	44.39	0.0001
	10°C	$y = 0.0356e^{-0.014x}$	0.57	62.8	0.0001
	15°C	$y = 0.0344e^{-0.016x}$	0.60	70.12	0.0001
	20°C	$y = 0.0333e^{-0.021x}$	0.58	64.28	0.0001
	25°C	$y = 0.0337e^{-0.031x}$	0.69	105.8	0.0001
	30°C	$y = 0.0317e^{-0.033x}$	0.74	135	0.0001

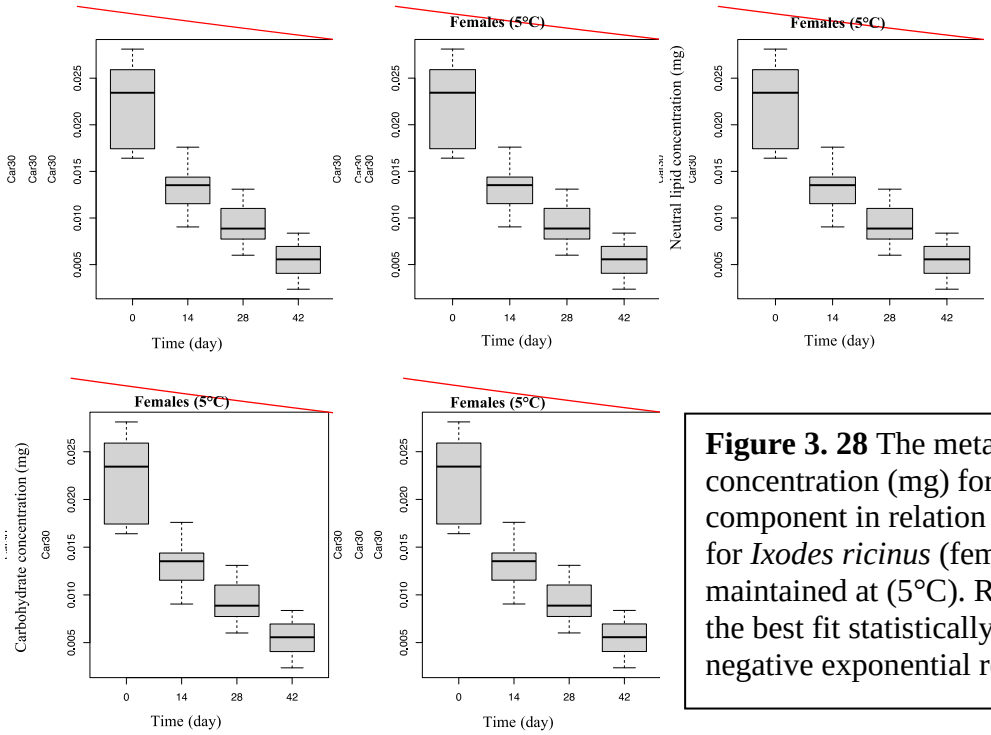

Figure 3. 28 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (females) maintained at (5°C). Red line shows the best fit statistically significant negative exponential relationship.

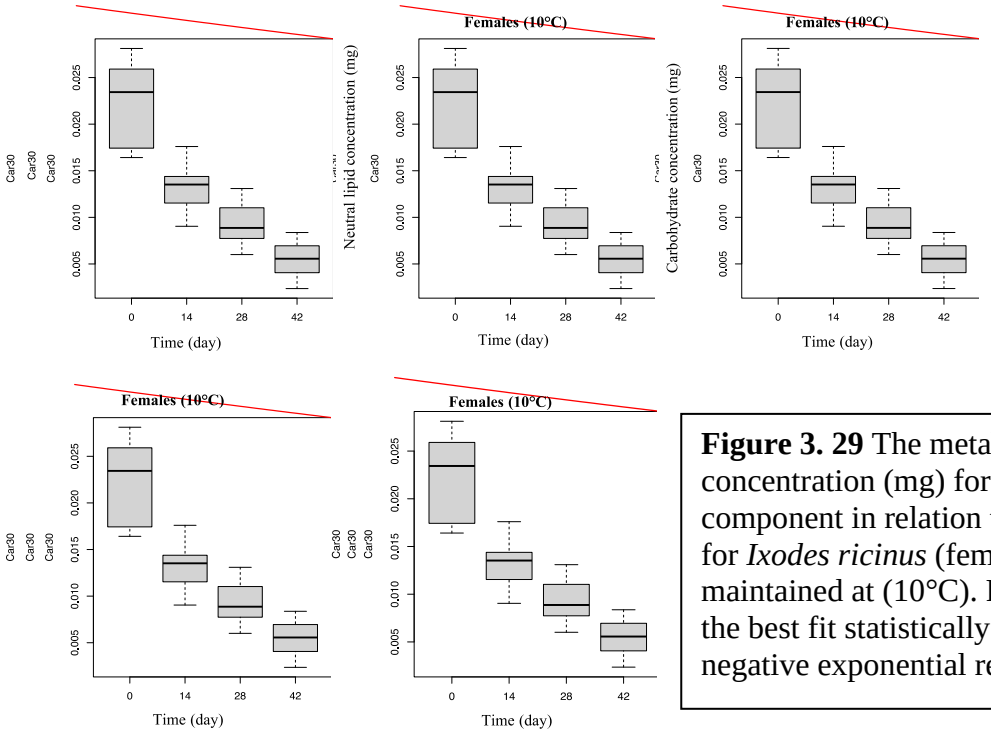

Figure 3. 29 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (females) maintained at (10°C). Red line shows the best fit statistically significant negative exponential relationship.

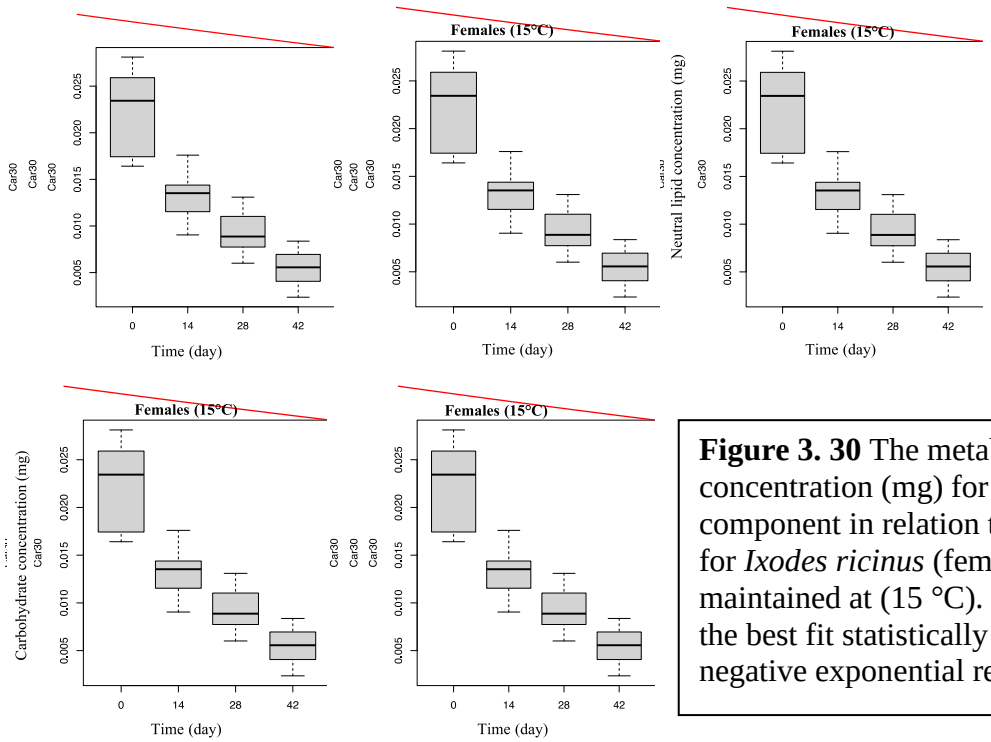

Figure 3. 30 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (females) maintained at (15 °C). Red line shows the best fit statistically significant negative exponential relationship.

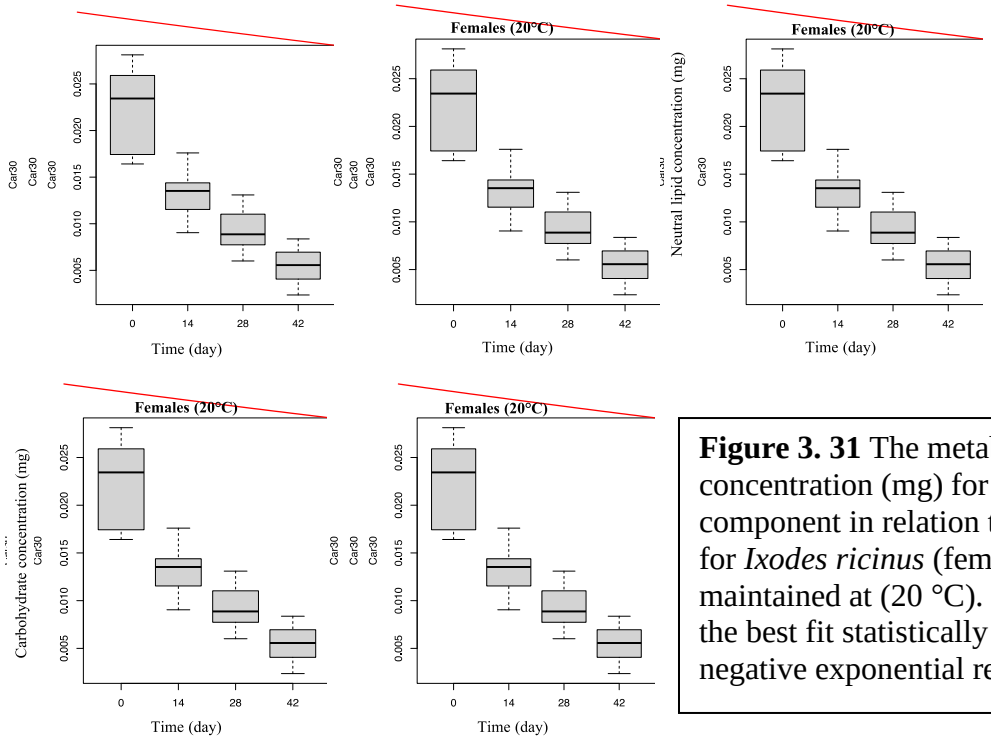

Figure 3. 31 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (females) maintained at (20 °C). Red line shows the best fit statistically significant negative exponential relationship.

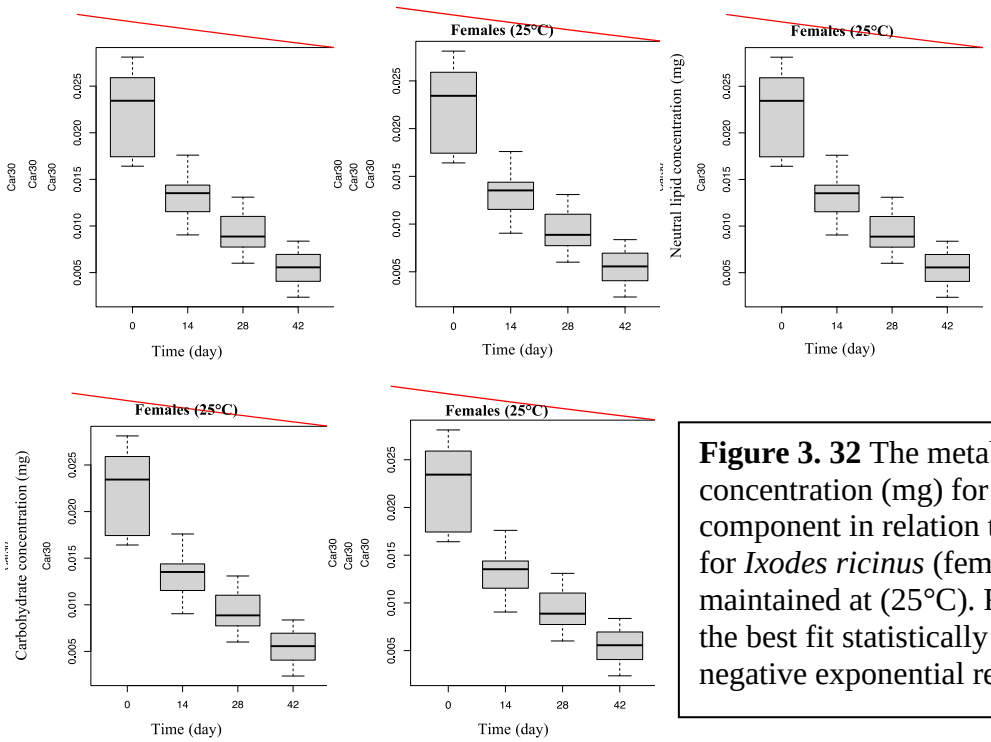

Figure 3. 32 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (females) maintained at (25°C). Red line shows the best fit statistically significant negative exponential relationship.

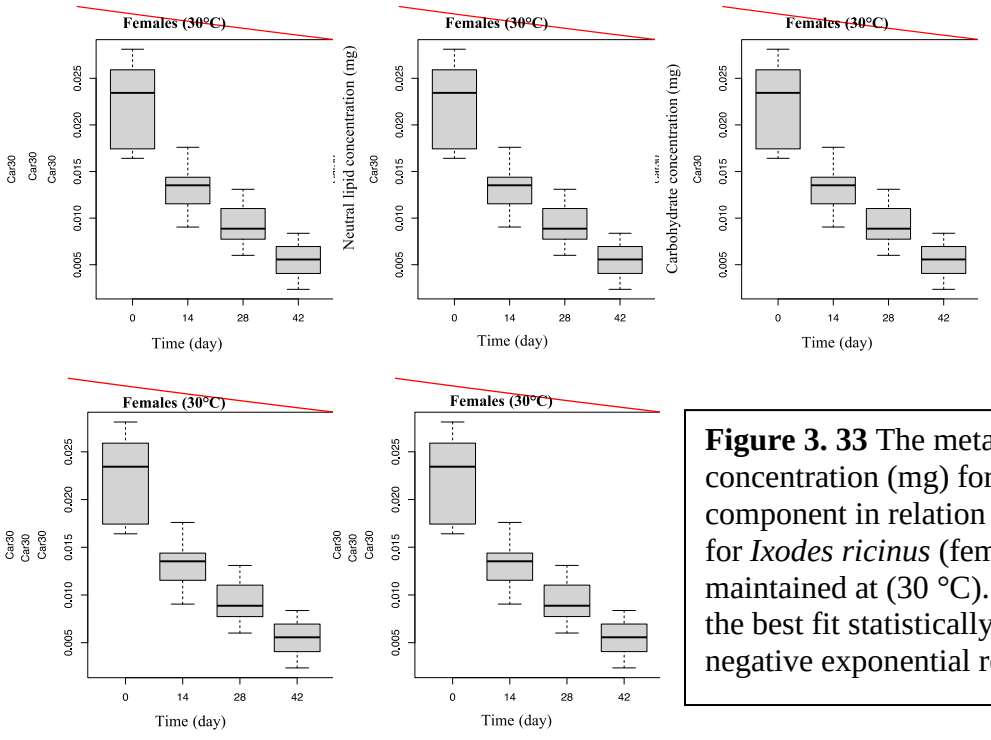

Figure 3. 33 The metabolite concentration (mg) for each energetic component in relation to time (days) for *Ixodes ricinus* (females) maintained at (30 °C). Red line shows the best fit statistically significant negative exponential relationship.

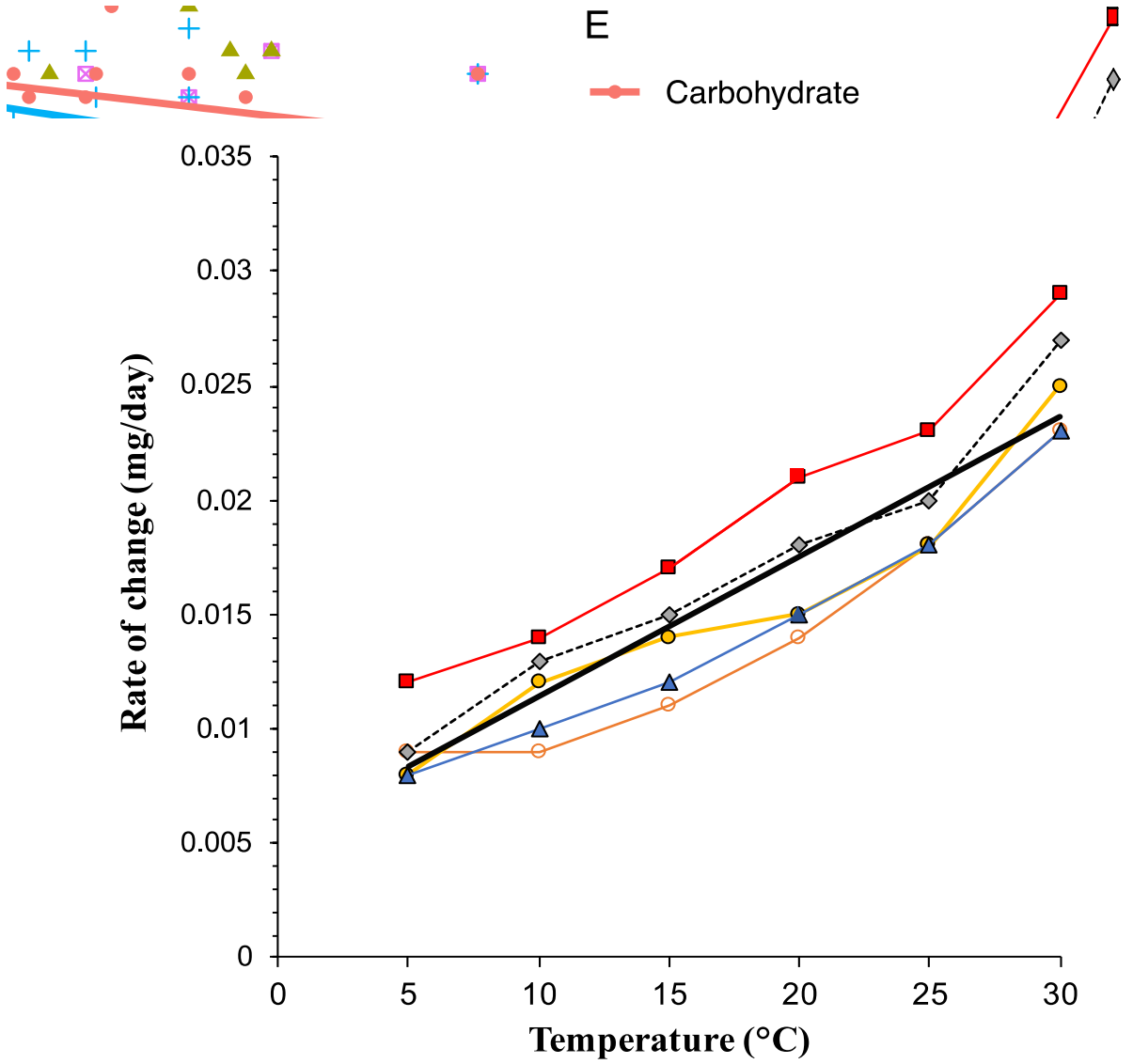

Figure 3. 34 Rate of change of various metabolites over time in *Ixodes ricinus* nymphs at 5-30 °C. Points are joined for clarity. The solid black lines indicate the best fit linear regression fitted to all data ($Y = 0.0006.X + 0.0053$, $R^2 = 0.834$, $P<0.001$).

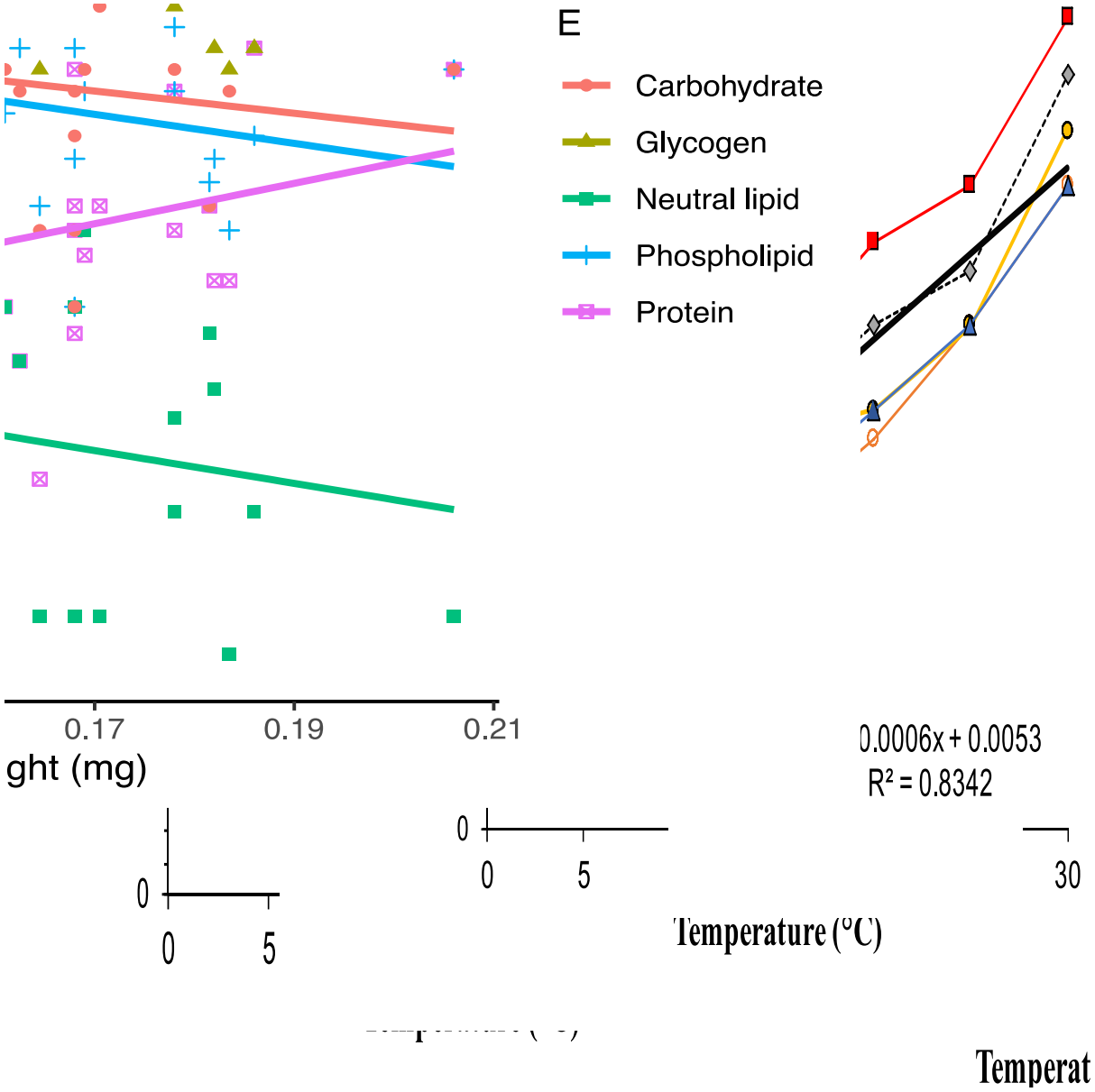

Figure 3. 35 Rate of change of various metabolites over time in *Ixodes ricinus* males at 5-30 °C. Points are joined for clarity. The solid black lines indicate the best fit linear regression fitted to all data ($Y = 0.0009.X + 0.0033$, $R^2 = 0.804$, $P<0.001$).

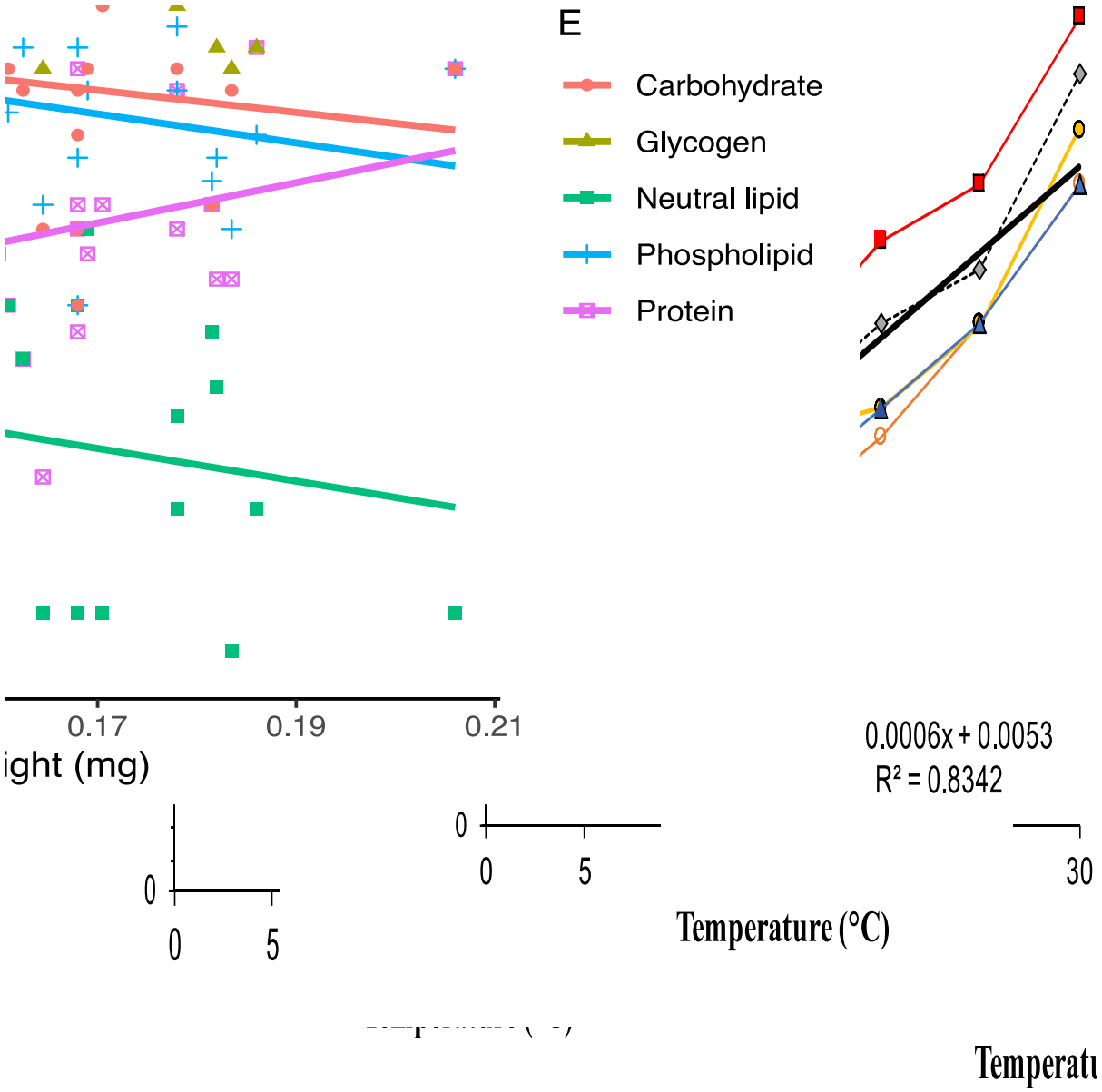

Figure 3. 36 Rate of change of various metabolites over time in *Ixodes ricinus* females at 5-30 °C. Points are joined for clarity. The solid black lines indicate the best fit linear regression fitted to all data ($Y = 0.001.X + 0.0059$, $R^2 = 0.916$, $P<0.001$).

3.4 Discussion

The ticks used for the present study were collected by blanket dragging in March 2019. At this time of year, questing activity of nymphs and adults in southwest England would be expected to be at its peak. The populations of nymphs and adults collected would be expected to be largely composed of cohorts which fed the previous year and either moulted the previous autumn or which overwintered or moulted in early spring prior to collection, depending on precisely when they had fed.

Amongst the ticks collected, at experimental day 0, clear differences in energy source contents between life cycle stages were apparent: these were largely associated with the differences in the relative amounts of protein compared to glycogen. In nymphs, glycogen represented 34% of the total mass of metabolites measures, followed by soluble carbohydrates at 26%, whereas protein represented only 16% of the mass of metabolites present in nymphs. In contrast, glycogen composed only 12% of the mass of metabolites in females and 18% in males, whereas protein represented 26% of the mass in females and 25% in males. These differences are broadly consistent with those recorded previously (Chapter 2). The relatively low glycogen and soluble carbohydrates levels in adults may be associated with the fact that the adult population is composed predominantly of individuals that fed as nymphs early the previous year and moulted the previous autumn and so by March are relatively resource depleted (Randolph et al., 2002; Abdullah et al., 2018). In contrast, the cohort of nymphs may be derived from larvae that fed later in the previous year and which may have moulted in late autumn or early spring and so have relatively higher energy reserves and are particularly richer in glycogen and soluble carbohydrates. This hypothesis could be tested in the future by examining the full energy sources profile of adults collected at different times of year, particularly in late autumn. If this is correct, over time, because nymphs were relatively better resourced with higher initial short-term energy resources, their lipid and glycogen rates of depletion would be higher than those of other energy sources and their rate of protein loss would be low. In contrast to nymphs, for

adults, males were depleting soluble carbohydrates and lipid at the highest rates, whereas in females the highest rates of depletion were seen in lipid and protein concentrations; in adults, the rates of glycogen depletion were relatively low because their initial glycogen concentrations available at day predicted in nymphs as they depleted their glycogen and soluble carbohydrates reserves. The rate of depletion of each energy sources during the trial was relatively constant in response to temperature and was best described by an exponential model. No major changes in the rank order of the rates of metabolite depletion over time in response to changes in temperature could be detected, although a greater sample size might have been needed to detect this.

In a study that considered only stored lipids, the lipid reserves of field collected *I. ricinus* nymphs, collected in early summer, were estimated to be sufficient to allow survival without feeding for up to 100 to 250 days at 15 °C, depending on whether they had fed as larvae the previous autumn or that year, respectively (Abdullah et al., 2018). Here, the results suggest that, amongst the cohort collected, complete resource depletion would occur at 45–70 days at 25–30 °C and 200 days at 5 °C, assuming humidity was sufficiently high not to result in desiccation. This fits with previous estimates from observations in the field (Steele and Randolph, 1985) and arenas (Randolph and Storey, 1999), which indicated a maximum questing period of about 120 days for questing nymphs.

The thermal sensitivity of metabolic rate, often described by an organism's Q_{10}, is the magnitude of change in metabolic rate for a 10 °C change in temperature. In insects, Q_{10} values range from 1.5 to 3, with a mode of 2.5 (Woods et al., 2003). Here, Q_{10} values were 1.5 for nymphs, 1.71 for males and 1.63 for females. It has previously been suggested that ixodid ticks have a metabolic rate which is typically 13% below that of most arthropods (Lighton and Fielden, 1995).Why the metabolic rate of nymphs was slightly lower than those of adults is unclear. A maximum daily air temperature of 7–8°C is generally proposed as necessary to

initiate questing in *I. ricinus* (MacLeod, 1935). In constant-temperature laboratory trials, the minimum threshold temperature for metabolic activity was estimated to be 5.7°C for populations derived from Scotland, 7.9°C for Wales, 7.0°C for England, 9.3°C for France at low altitude, and 6.9°C for France at high altitude (Tomkins et al., 2014). Furthermore, (Perret et al. (2000) found that questing ticks could be collected consistently when the air temperature reached or exceeded 5.2 °C. Environmental conditions with a relative humidity of higher than 45% RH and an ambient temperature of greater than 2.5 °C were considered to be required for questing by *I. ricinus* (Hubálek et al., 2003). Some element of genetic variation reflecting local adaptation is likely (Gilbert et al., 2014). Here, however, assuming that a linear extrapolation from the observed temperature range is appropriate, the data presented suggest that the lower threshold for metabolic activity is between 10 and 5 °C, which is likely to be close to the lower lethal temperature. An understanding that metabolic activity is likely to be occurring even at relatively low temperatures may have important impacts on attempts to model tick phenology using climate simulations, since measurable levels of resource depletion will occur at temperatures well below those considered to be appropriate for questing. Further work explicitly examining tick metabolism at low temperatures would be of value.

Chapter 4

Climatic and environmental constraints on the behaviour and energy storage in *I. ricinus*

Summary

Nymphal and adult *I. ricinus* were sampled each month from February 2018 to January 2019 and subjected to spectrophotometric analysis of energy sources . In general, for nymphs, the changing patterns of metabolite contents over the year present a clear and consistent picture. energy source content declined to a seasonal minimum in July before rising once again. These data suggest that there exists a well-defined cohort of relatively well-fed nymphal ticks in the early spring, most probably derived from larvae that fed the previous summer and moulted the previous autumn. They then start to quest as temperatures rise sufficiently in spring to permit activity. Those that are unable to find a blood-meal continue questing but gradually exhaust their reserves. By mid-summer the only nymphs left questing are close to starvation. In the second half of summer, nymphs that fed as larvae relatively early in the year and have now completed digestion, moulted, quest again as nymphs with high levels of metabolic resource which they carry with them into winter. For females, the pattern of changing energy source contents was more complex than seen in nymphs. The population at the start of the year is composed of a cohort that started questing at early in the year that were relatively hungry; they may have been derived from nymphs that fed relatively early the previous year, moulted to become adults that were unable to feed, so overwintered and started questing early the following year in an already resource depleted state. By April, the cohort of questing females has now been joined by individuals that moulted to become adults late the previous year or early in the year and are relatively well resourced. However, those that do not feed gradually start to exhaust their resources. In the second half of the year, females with high levels of metabolic resource appear – presumably from nymphs that fed early in the year of the study, digested their blood meals, moulted to become adults and are now re-joining the questing population. In males the patterns of changing energy sources were much less distinct than seen in nymphs or females.

4.1 Introduction

Each species of parasitic arthropod has its own optimal niche within an environmental envelope. How parasites manage fluctuations in conditions, within or beyond this niche, either behaviourally or physiologically, is a critically important element of their life-history strategy, particularly for species with an extensive off-host component in their life cycle (Poulin et al., 2011). This is especially important where the effects of wide environmental extremes of

temperature or humidity need to be mitigated over summer or winter (Cabezas-Cruz et al., 2017, Herrmann et al., 2013).

Behaviours among blood-feeding arthropods that allow them to survive periods where they are unable to feed, that may last a few day (fleas, kissing bugs, and bed bugs) to several months (ticks), are of particular interest. Sufficient resource must have been accumulated at the start of the non-feeding period to allow the organism to maintain physiological homeostasis, undergo development and then find a new host at the end of the period of adverse conditions. Tick densities are highly dependent on preceding winter temperatures, with higher tick densities strongly associated with mild weather conditions (Lauterbach et al., 2013). The complex relationship between temperature and humidity requirements, host-seeking behaviours, and seasonal conditions has been widely investigated and its understanding is essential to allow patterns of tick phenology to be explained (Belozerov, 2009, Gray, 2008, Gray et al., 2016, Randolph, 2004). Its understanding also helps to explain the geographical distribution of different tick species, along with the resulting spread of the pathogens which they transmit (Bouchard et al., 2019).

A range of adaptations are seen to allow ticks to mitigate the effects of unfavourable conditions. Even within the same species, local adaptation has been shown, for example populations of *I. ricinus* in Scotland may quest at cooler temperatures than populations from Wales or England, with higher temperatures still required by populations from France (Gilbert et al., 2014). These adaptations may have important impacts on population dynamics and affect their interactions with hosts (Ogden et al., 2014). For instance, fluctuation in temperature overwinter may stimulate eggs to have a shorter incubation time or allow ticks to lay eggs earlier in the year and this may have impacts on activity peaks, for example bringing peaks of larval activity in advance of peaks of nymphal activity or extending the inactive stage of the life cycle from September to June (Ogden et al., 2004; Gray et al., 2016; Furness and Furness, 2018).

Furthermore, it has been suggested that changes in activity patterns such as host seeking, feeding, moulting, or oviposition in response to seasonal temperatures can be more clearly observed in the nymphal and adult stages of the life cycle, whereas responses in egg and larval stages are less easily observed due to the greater importance of humidity and ground moisture (Gray, 1991, Randolph, 2008). Changes in phenology may also affect interactions with reservoir hosts and pathogen transmission. Reduced host numbers at a time when most ticks are questing, for example, may increase the proportion of the population that is likely to be infected (Krawczyk et al., 2020). While several studies have considered the behavioural responses of ticks to environmental conditions in the field, few have extended this to a consideration of physiological responses.

The aim of the work described in this chapter, therefore, was to consider the changes in resource availability of *I. ricinus* ticks collected from the field over the course of a year to more deeply understand their responses to seasonal constraints and evaluate the potential use of the full metabolic profile for explaining these responses.

4.2 Materials and Methods

4.2.1 Tick physiological age index (PAI) and field collection

Monthly sampling by blanket dragging was undertaken between February 2018 to January 2019 from Ashton Court Park near Bristol (as described in Chapter 2). The sampling aimed to collect sixty-eight specimens of *I. ricinus* for each lifecycle stage (nymphs, males, and females). However, this sampling was dependent on seasonal availability; relatively small numbers of nymphs were collected in November and December 2018, and no adults could be collected in July, November or December 2018. Ticks were brought back to the laboratory and subjected to a range of body and scutal measurements.

The ratio between the alloscutal (body – scutum) and scutal areas was calculated for each tick, as follows:

$$\text{PAI} = \frac{(\text{body length} * \text{body width}) - (\text{scutal length} * \text{scutal width})}{(\text{scutal length} * \text{scutal width})}$$

This ratio was termed the Physiological Age Index (PAI), and used as an index of physiological age within a life-cycle stage, following the method described by (Uspensky et al., 2006). This physiological age index depends on the fact that the alloscutal dimensions gradually diminish during tick starvation, while the scutal dimensions remain constant. All ticks collected were subjected to a full metabolic analysis using spectrophotometric methods as described in Chapter 2.

4.2.2 Data analysis

Two way ANOVA was used to compare differences in body weights for each life-cycle stage of the ticks used for protein analysis or for analysis of other energy sources , with analysis type and month, as factors, with month specified as a moderator variable. To consider whether differences in metabolite contents varied bodyweight between life cycle stage, the mass of each metabolite was first divided by the total bodyweight to correct for differences in size between life-cycle stages, and life-cycle stage and month, were included in a two-way ANOVA, with month specified as a moderator variable; Bonferroni adjustments were used to correct for multiple comparisons within groups. All analysis used the R-Studio statistical package (v.3.5.3, 2019, R Foundation for Statistical Computing),

4.3 Results

4.3.1 Body weight and PAI

To ensure that the ticks used for protein or other energy sources analysis were comparable, body weights were first examined. There was no significant difference in body weight between

the ticks used for protein analysis or for analysis of other energy sources , in each month throughout year: nymphs ($F_{11,764} = 0.190$, $P = 0.66$), males ($F_{8,594} = 1.28$, $P = 0.26$), females ($F_{8,594} = 0.47$, $P = 0.88$). In the month of maximum body weight (Table 4.1), the mean body weight of questing *I. ricinus* nymphs sampled in September 2018 was 0.234 mg (± 0.033 mg), adult males sampled in January 2019 were 0.929 mg (± 0.014 mg), and adult females sampled in April 2018 were 2.166 mg (± 0.055 mg).

The Physiological Age Index (Table 4.1), for nymphs, gradually decreased in value over the course of summer suggesting that the nymphs show a gradual increase in starvation. This then reached a minimum in August before increasing to a seasonal peak between September to October 2018. In adult females, however, there was a gradual increase in mean PAI value, which reached a peak between in April/May 2018. Lastly, in adult male**s**, the mean value of PAI showed little change (the highest mean value was seen in January 2019).

Table 4. 1 The mean body weight (mg) and physiological age index (PAI) of *Ixodes ricinus* for each month (±SD). A total 788 nymphs, 612 males, and 612 females were used in the analyses; cells with data missing are left blank.

Month	Nymph		Male		Female	
	Body weight (mg) (mean, ±SD)	PAI (mean, ±SD)	Body weight (mg) (mean, ±SD)	PAI (mean, ±SD)	Body weight (mg) (mean, ±SD)	PAI (mean, ±SD)
Feb-18	0.171 (±0.018)	1.692 (±0.461)	0.856 (±0.014)	0.358 (±0.009)	1.059 (±0.019)	1.086 (±0.034)
Mar-18	0.164 (±0.021)	1.603 (±0.237)	0.818 (±0.013)	0.241 (±0.006)	1.130 (±0.025)	1.196 (±0.020)
Apr-18	0.157 (±0.021)	1.495 (±0.156)	0.786 (±0.017)	0.205 (±0.016)	2.166 (±0.055)	2.436 (±0.097)
May-18	0.149 (±0.024)	1.524 (±0.123)	0.704 (±0.014)	0.192 (±0.014)	1.840 (±0.044)	1.747 (±0.072)
Jun-18	0.131 (±0.014)	1.371 (±0.204)	0.662 (±0.011)	0.156 (±0.006)	1.458 (±0.033)	1.416 (±0.054)
Jul-18	0.116 (±0.017)	1.180 (±0.305)				
Aug-18	0.148 (±0.027)	1.488 (±0.244)	0.792 (±0.014)	0.321 (±0.006)	0.913 (±0.15)	1.180 (±0.017)
Sep-18	0.243 (±0.033)	2.140 (±0.561)	0.741 (±0.010)	0.309 (±0.008)	1.028 (±0.24)	1.216 (±0.031)
Oct-18	0.179 (±0.030)	1.912 (±0.481)	0.789 (±0.18)	0.294 (±0.010)	0.785 (±0.16)	0.979 (±0.028)
Nov-18	0.137 (±0.025)	1.368 (±0.266)				
Dec-18	0.120 (±0.021)	1.263 (±0.286)				
Jan-19	0.146 (±0.026)	1.522 (±0.260)	0.929 (±0.014)	0.451 (±0.035)	1.133 (±0.40)	1.282 (±0.018)

4.3.2 Seasonal patterns in metabolite contents

The nymphs showed marked seasonal change in metabolite contents over time (Fig. 4.1). The overall pattern was similar for all metabolites, although the absolute values showed consistent differences. At the start of the year there was a decrease in the mean metabolite contents over the spring and these then reached the lowest levels in mid-summer indicating a loss of between 11 to 35%. The absolute values then increased to peak in autumn in October 2018 before falling once again to reach a second low in January to February 2019. The absolute values then increased to peak in autumn in October 2018, before falling once again. The most abundant metabolite, glycogen, declined from 0.052 mg in February to 0.014 mg in July (Fig. 4.1). In contrast, the mean mass of phospholipid and soluble carbohydrates were very similar and

fluctuated less than that of glycogen. Neutral lipid concentrations were lower than those of all other metabolites and fluctuated the least, but did follow the same broad seasonal pattern (Fig. 4.1). The metabolite contents then reached the second low point in December 2018 before a slight recovery in both glycogen and soluble carbohydrates, which increased to reach approximately 0.035 mg in January 2019, whereas, the mean phospholipid concentration decreased continuously to reach 0.015 mg (Fig. 4.1).

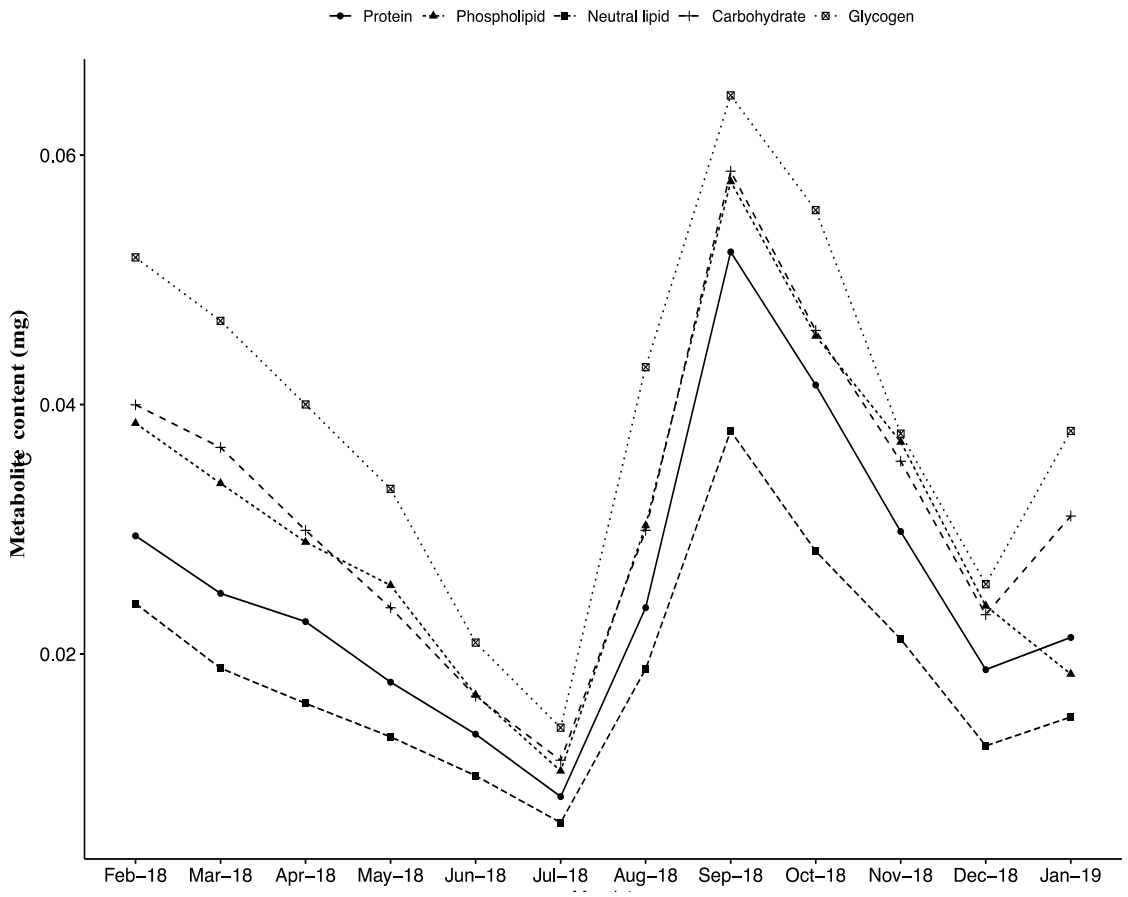

Figure 4. 1 The mean absolute metabolite contents (mg) of nymphal *Ixodes ricinus*, over a year. N = 68 each month, exccept in November and December where N = 54. Standard errors around the means not plotted for clarity.

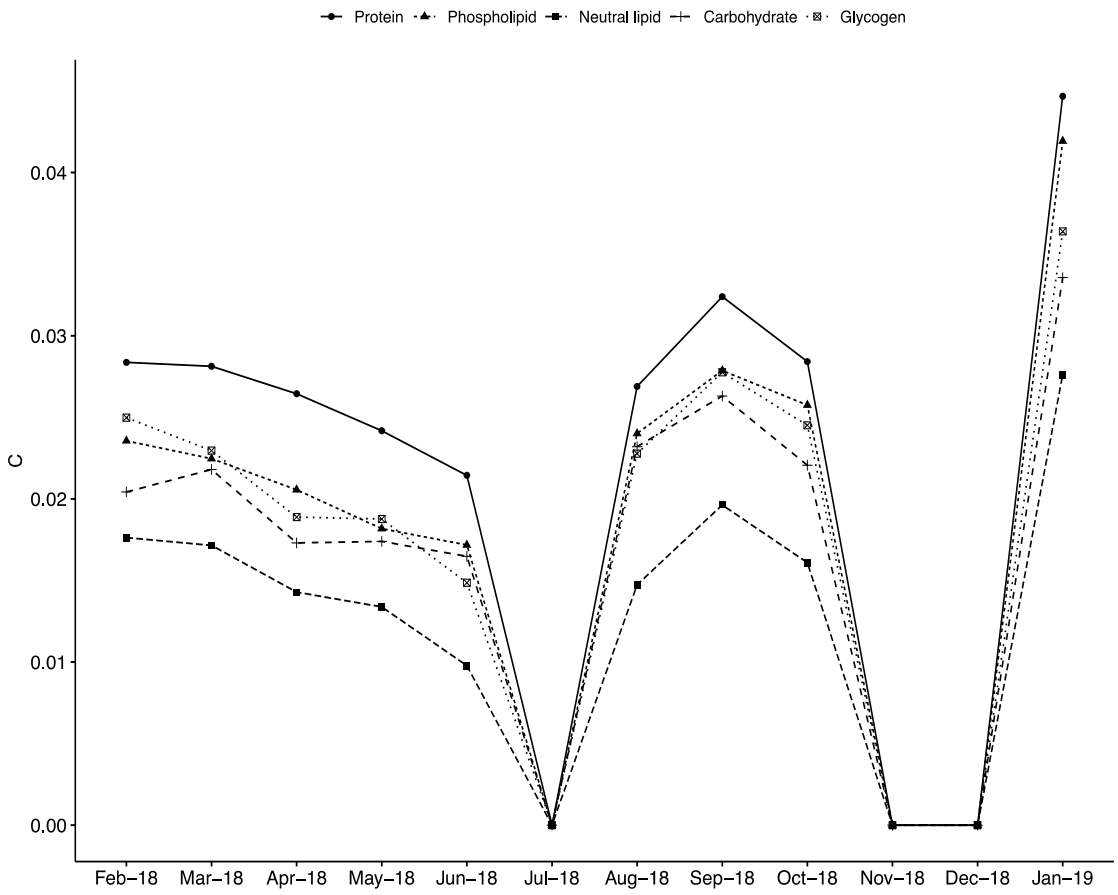

Figure 4. 2 The mean absolute metabolite contents (mg) of male *Ixodes ricinus*, over a year. N = 68 each month, exccept in November and December where N = 54. Standard errors around the means not plotted for clarity.

The pattern of changing mean absolute metabolite contents observed in adult males was much less pronounced than that seen in nymphs (Fig. 4.2). Males sampled between February to June showed a relatively small but steady decrease in the mean metabolic contents, indicated by a decline in the total metabolite mass of between -5 to -20%. This was followed by a progressive and continuous increase over the autumn and winter seasons. There was a positive gain in the total relative metabolite mass of 69% between August and September 2018, of 12% between September to October 2018, and 87% between January and February 2019. The mean protein mass was greater than that of all other energy sources throughout the year and neutral lipid the

lowest, declining from 0.024 mg in February to 0.014 mg in June and increasing to reach an average of 0.041mg by the end of the year (Fig. 4.2).

The seasonal patten of changing in mean absolute metabolite contents seen in females, was broadly similar to that seen in nymphs although the main seasonal peak in metabolite availability occurred much earlier in the year (Fig 4.3). energy source contents increased from February to peak in April, showing a gain in the total relative metabolite mass of up to 66%. This was followed by decrease between May to August to reach the seasonal minimum in October 2018. Phospholipid and protein concentrations were considerably greater than those of other energy sources in during the peak availability, Feb to June 2018, but fell to a similar level as other energy sources in autumn. Neutral lipid was the least abundant energy sources in all but two samples.

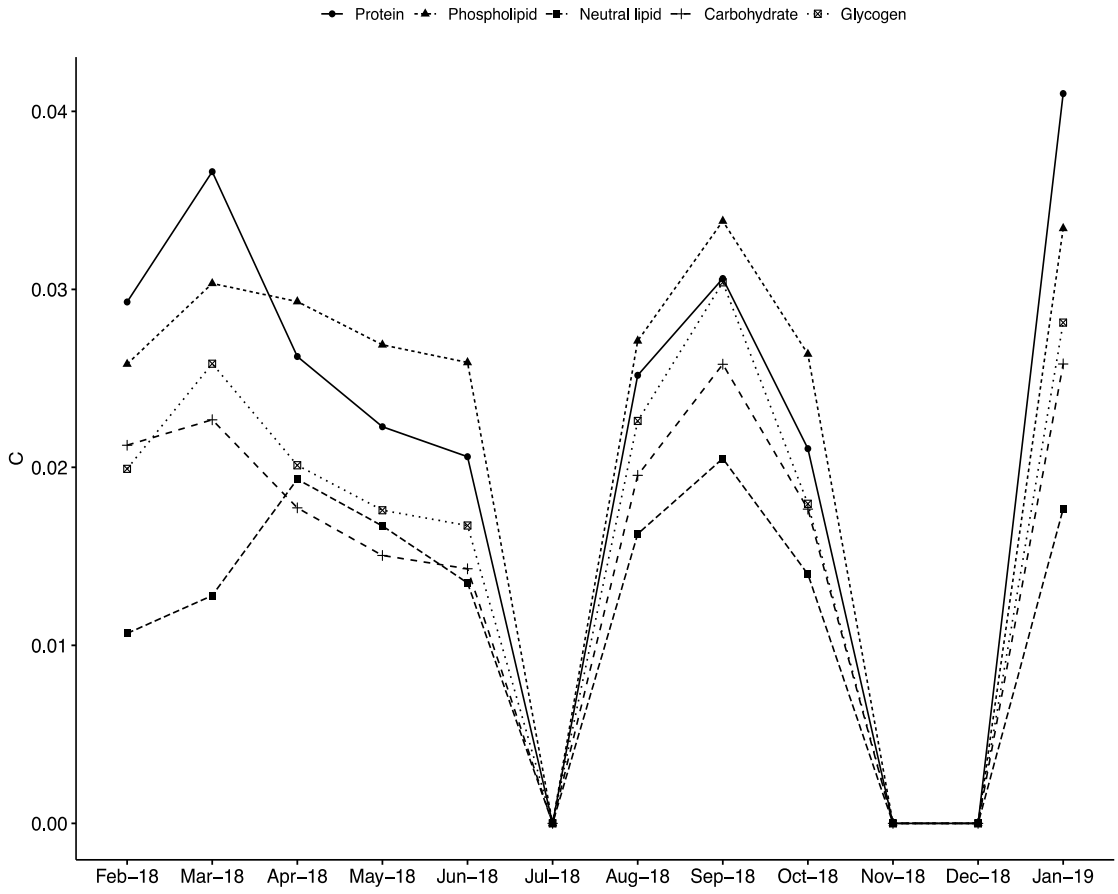

Figure 4. 3 The mean absolute metabolite contents (mg) of female *Ixodes ricinus*, over a year. N = 68 each month, exccept in November and December where N = 54. Standard errors around the means not plotted for clarity.

4.3.3 Metabolic differences between life cycle stages

Direct comparison in the energy source contents corrected for body weight, between the three life-cycle stages shows a pattern that broadly agrees with the data presented in Figs. 4.1-4.3. Overall, the proportionate contribution of each energy sources were relatively stable in males throughout the year compared to females or nymphs. The two-way ANOVA indicated that for protein, there was a statistically significant interaction between life-cycle stage and month ($F_{16, 891}$ = 96.3, P<0.0001, Fig. 4.4). Protein concentrations were elevated in nymphs in September and October but were highest in females during March to May. Protein in nymphs declined to reach its lowest seasonal contents in June (Fig. 4.4). There was a significant interaction between life-cycle stage and month for phospholipid ($F_{16,891}$ = 121.6, P<0.0001 Fig. 4.5). The

phospholipid concentrations were highest in females in summer (April to June) and highest in nymphs in later summer (August to October). A statistically significant interaction between life-cycle stage and month was also seen for neutral lipid ($F_{16, 891}$ = 66.7, P<0.0001 Fig. 4.6), with nymphs showing their highest contents in late summer (August to October) and females showing their highest concentrations between April and June. Similarly, both soluble carbohydrates and glycogen showed statistically significant interactions between life-cycle stage and month (soluble carbohydrates concentrations, $F_{16,891}$ = 66.7, P<0.0001; glycogen concentrations, $F_{16,891}$ = 71, P<0.0001). In nymphs, soluble carbohydrates (Fig. 4.7) and glycogen (Fig 4.8) were generally more abundant than in adults. Soluble carbohydrates concentrations were particularly high in nymphs in later summer. The glycogen concentration showed a relatively low concentration over summer in nymphal stages.

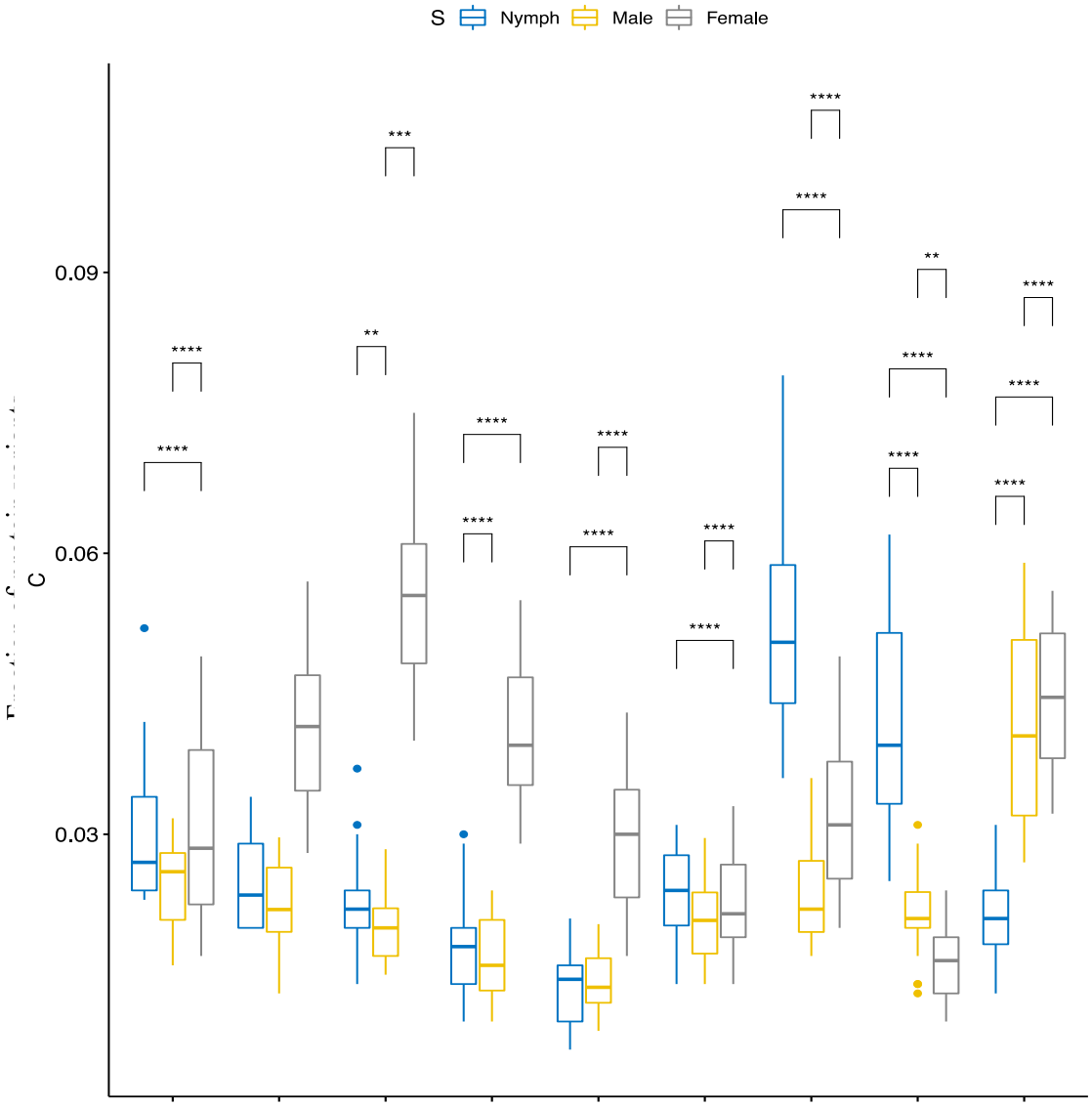

Figure 4. 4 Pairwise comparisons of protein concentrations of different metabolites expressed as a proportion of total bodyweight over a year, found in nymphal and adults *Ixodes ricinus.* Bar = mean, box = standard deviation and whiskers = range. The data in months of July, November and December were excluded from the analysis due to the lack of availability in adult samples. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.

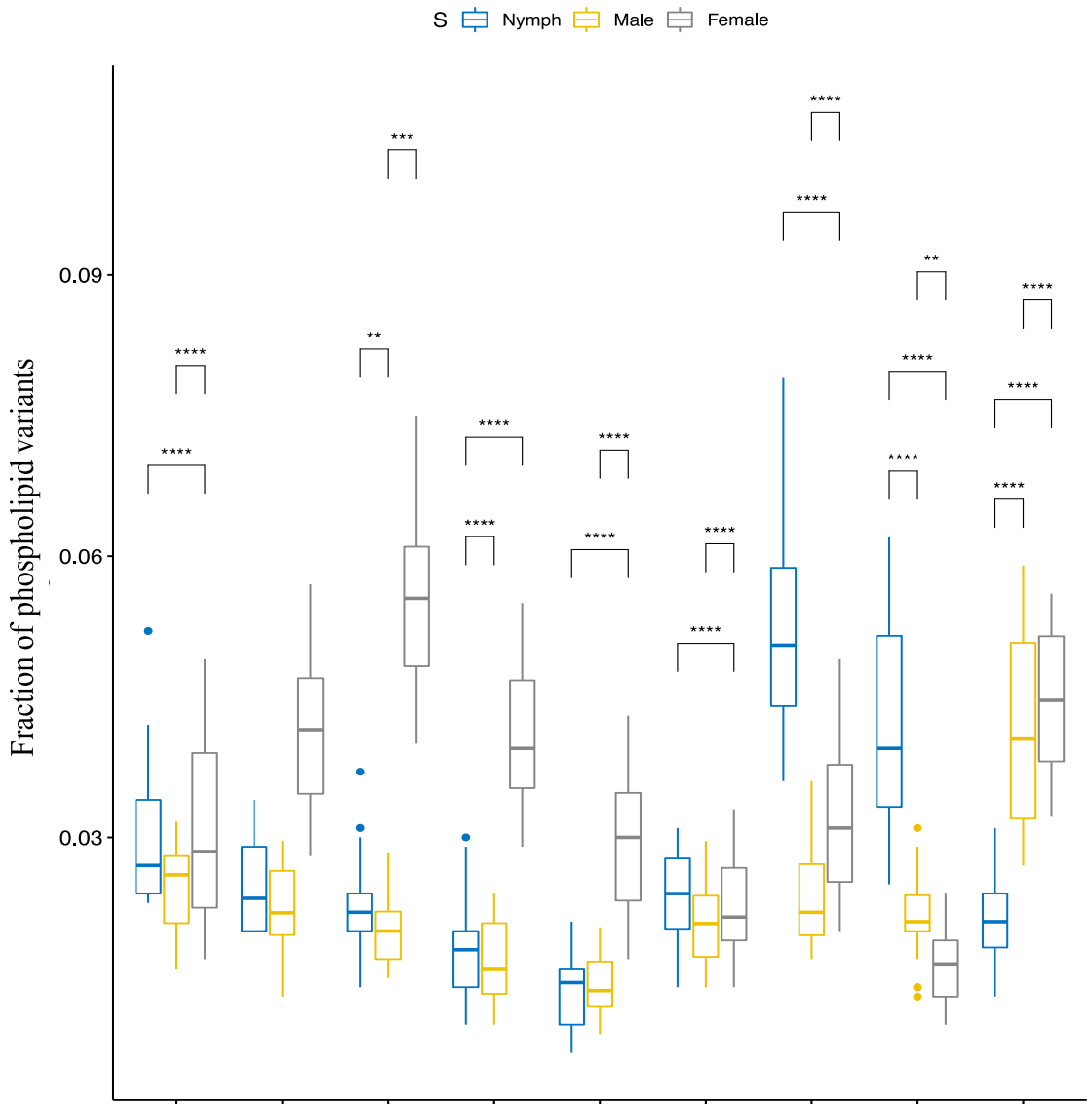

Figure 4. 5 Pairwise comparisons of phospholipid concentrations of different metabolites expressed as a proportion of total bodyweight over a year, found in nymphal and adults *Ixodes ricinus.* Bar = mean, box = standard deviation and whiskers = range. The data in months of July, November and December were excluded from the analysis due to the lack of availability in adult samples. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.

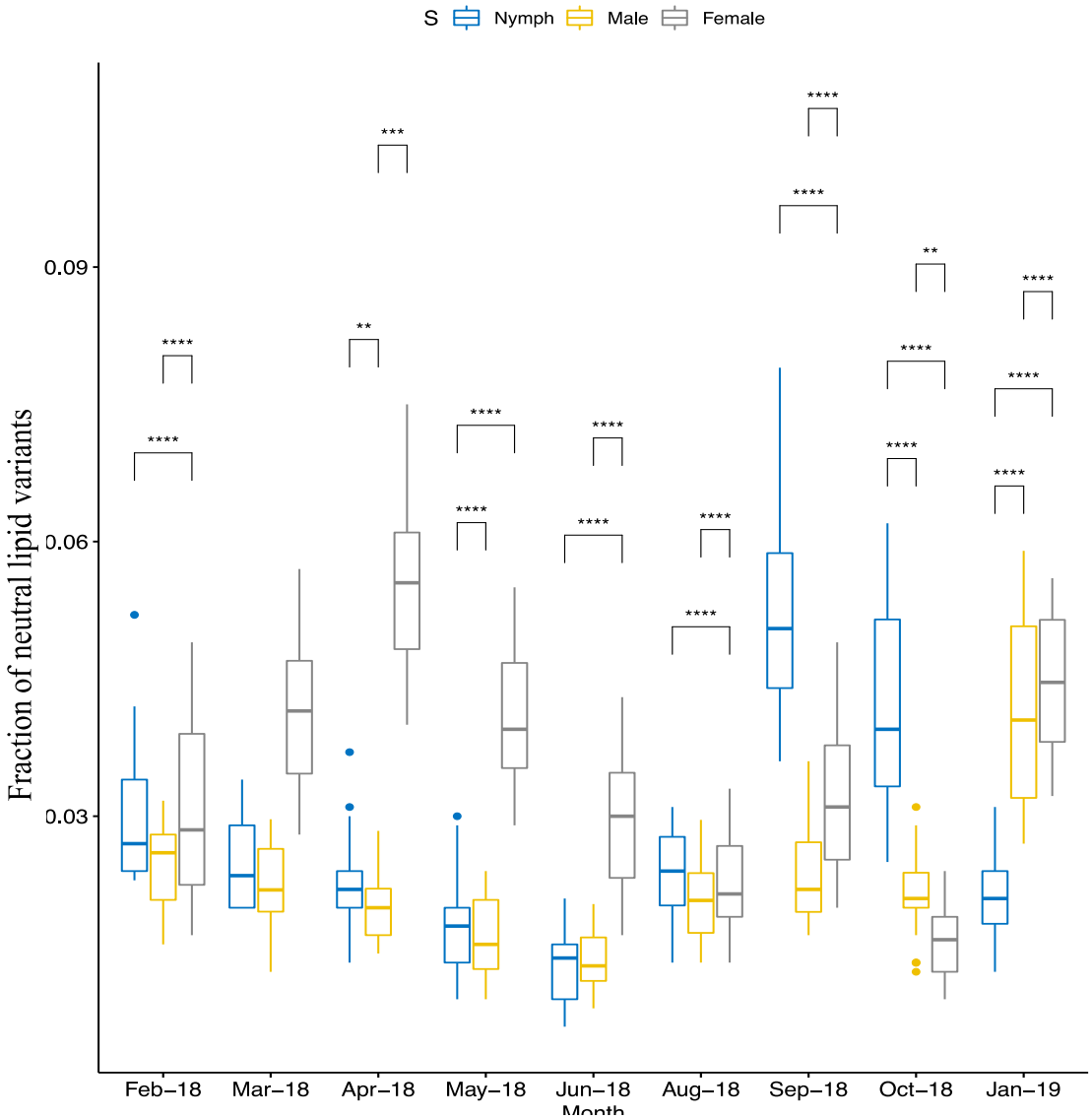

Figure 4. 6 Pairwise comparisons of neutral lipid concentrations of different metabolites expressed as a proportion of total bodyweight over a year, found in nymphal and adults *Ixodes ricinus.* Bar = mean, box = standard deviation and whiskers = range. The data in months of July, November and December were excluded from the analysis due to the lack of availability in adult samples. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.

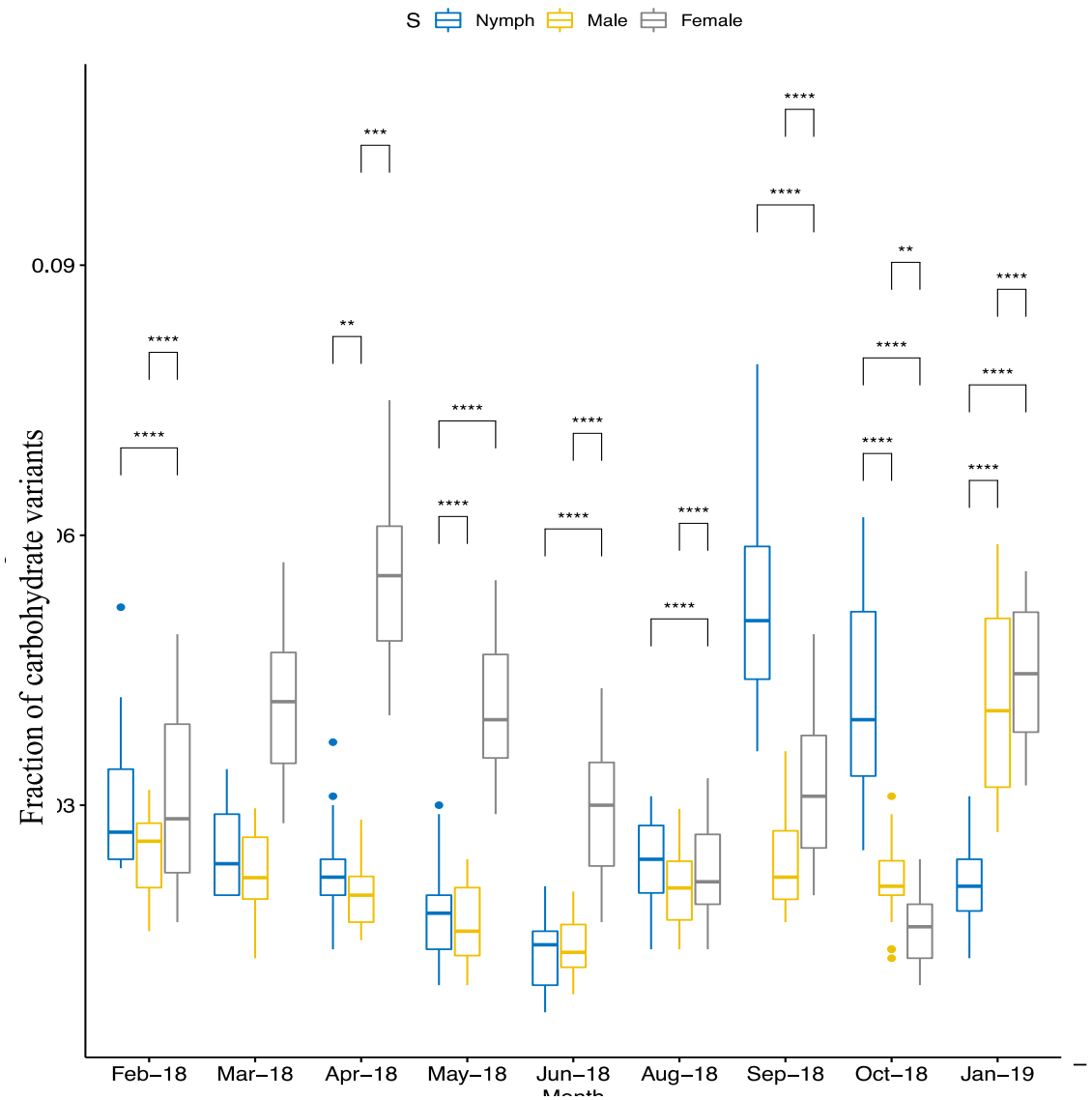

Figure 4. 7 Pairwise comparisons of soluble carbohydrates concentrations of different metabolites expressed as a proportion of total bodyweight over a year, found in nymphal and adults *Ixodes ricinus*. Bar = mean, box = standard deviation and whiskers = range. The data in months of July, November and December were excluded from the analysis due to the lack of availability in adult samples. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.

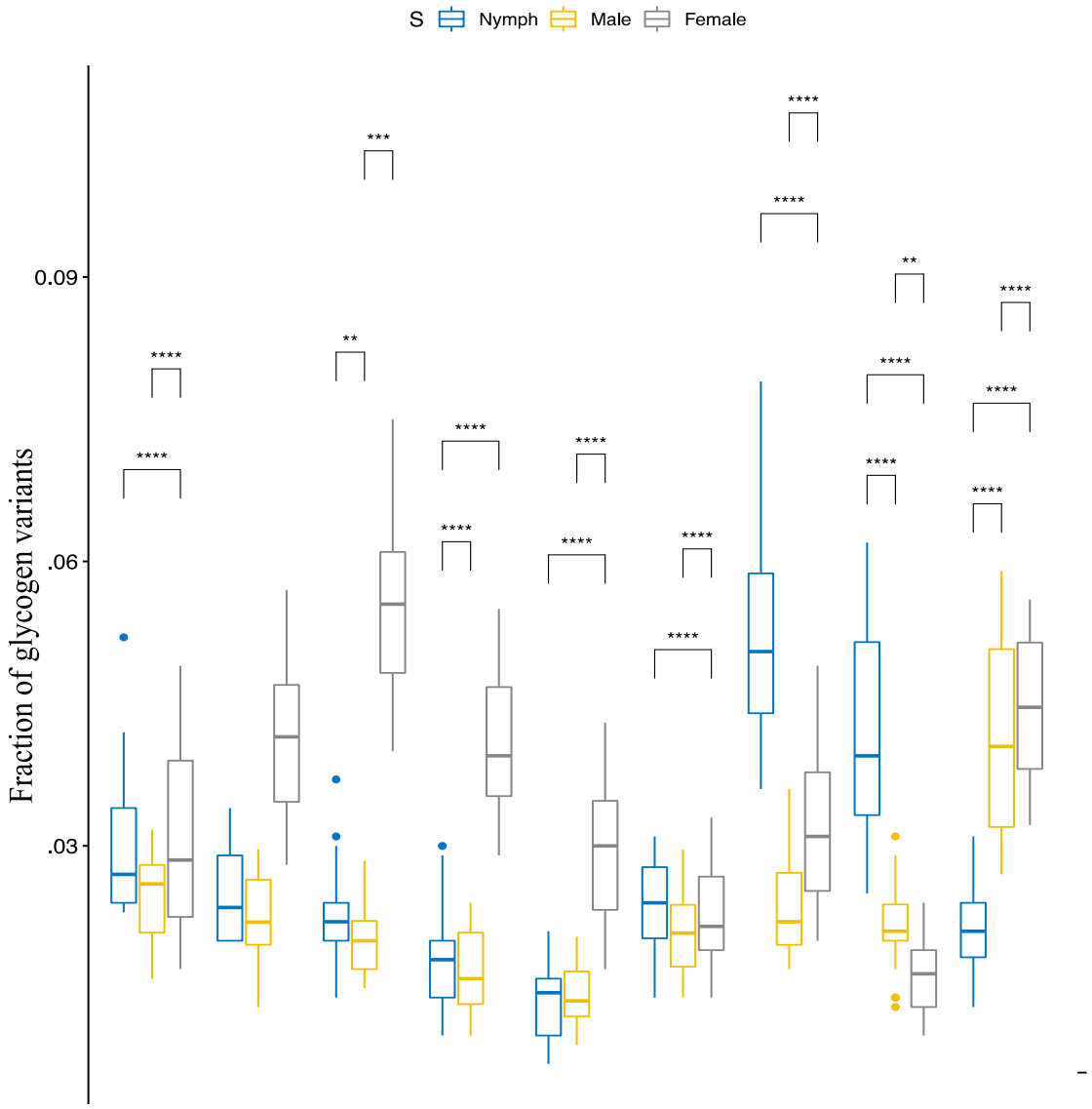

Figure 4. 8 Pairwise comparisons of glycogen concentrations of different metabolites expressed as a proportion of total bodyweight over a year, found in nymphal and adults *Ixodes ricinus.* Bar = mean, box = standard deviation and whiskers = range. The data in months of July, November and December were excluded from the analysis due to the lack of availability in adult samples. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.

4.4 Discussion

This chapter has examined the change in energy source contents in questing ticks with the aim of using these data to explain the feeding behaviour of tick populations in the field, based on

the assumption that the changes in contents of these metabolites reflect resource depletion following the bloodmeal. Resource depletion will also be affected by factors such as environmental temperature (Chapter 3) and activity such as questing and reproduction. Ticks have a limited ability to defend their energic contents during long periods between bloodmeals, other than switching between two dichotomous states, questing and not questing, with environmental variations such as weather conditions influencing how they switch between the two states (Spielman, 1994, Spielman et al., 1985, Wood and Lafferty, 2013).

The Physiological Age Index (PAI), as described by (Uspensky et al., 2006), depends on the fact that the alloscutal dimensions gradually diminish while the scutal dimensions remain constant, and effectively gives an independent measure of starvation over a life cycle stage and a useful confirmation of the feeding pattern derived from the energy sources measurement. In general, for nymphs, the PAI and the changing patterns of metabolite contents over the year presented a clear and consistent picture. The mean PAI value declined between February and July, but then jumped to its seasonal peak in September and October only to start to decline again. Similarly, in terms of metabolite contents, these followed a consistent pattern, declining to a seasonal minimum in July before rising once again. These data therefore show a clear pattern and suggest that a cohort of relatively well-fed nymphal ticks is present in the early spring, most probably derived from larvae that fed the previous summer and moulted the previous autumn. They then start to quest as the temperatures rise sufficiently in spring to permit activity. Those that are unable to find a blood-meal continue questing but gradually exhaust their reserves. By mid-summer the only larvae left questing are close to starvation. In the second half of summer, nymphs that fed as larvae relatively early in the year have now competed digestion, moulted to become nymphs and are questing again as nymphs with high levels of metabolic resource, which they carry with them into winter. The data suggest that nymphs may have a greater ability to maintain their activity levels during starvation compared

with adults (Williams et al., 1986; Meyer-König et al., 2001; Rosendale et al., 2017; (Meyer-König et al., 2001, Rosendale et al., 2017, Williams et al., 1986, Zhang et al., 2019).

For females, The PAI was highest in April/May and the pattern of changing energy source contents was more complex than seen in nymphs. The fluctuations were less marked than in nymphs, but it must be noted that this may reflect the fact that there is simply greater variation within the female population over time. In future studies, larger samples sizes taken over time would allow analysis of the frequency distribution in energy source contents, but such analysis was not considered statistically robust with the sample sizes collected here.

The female population at the start of the year has a relatively low PAI and low energy source contents, which quickly increased. Hence there would appear to be a cohort of females that started questing at early in the year that were relatively hungry; they may have been derived from nymphs that fed relatively early the previous year moulted to become adults that were unable to feed, so overwintered and started questing early the following year in an already resource depleted state. By April, the cohort of questing females had now been joined by individuals that moulted to become adults late the previous year or early in 2018 and were relatively well resourced with a high PAI. However, those that were unable to feed gradually started to exhaust their resources. However, in the second half of the year, females with high levels of metabolic resource appear – presumably derived from nymphs that fed early in the year of the study, digested their blood meals, moulted to become adults and were re-joining the questing population. Interestingly, this late summer/autumn cohort of well-resourced females is not seen in the PAI data.

For males the changing patterns of energy source contents are the least distinct of any life-cycle stage, suggesting that there is considerable variation in questing activity within the male population. This might be anticipated since questing behaviour in males is driven by both mate-

seeking as well as feeding requirements. Interestingly, it has been noted that larvae and nymphs that feed in the presence of males achieve a higher mean weight than those fed without males (Rechav and Nuttall, 2000). This study showed the mean protein concentration of male ticks was greater than all other energy sources throughout the year, which may in part reflect the fact that the salivary glands and haemolymph in males produce an important group of proteins, immunoglobulin-binding IGBPs), which facilitate the feeding performance of co-feeding females (Gong et al., 2014, Wang and Nuttall, 1994).

A study by Tomkins et al. (2014) argued that physiological temperature thresholds have the ability to constrain the distribution of tick populations. However, physiological thresholds are not uniform and recent evidence has suggested that geographically separate populations of *I. ricinus* show variations in questing behaviour in response to temperature and adaptation to local environmental conditions can influence activity and development in different parts of a tick's geographical range (Corbet et al., 1991; Mallorie and Flowerdew, 1994; Craine et al., 1995; Hoodless et al., 1998; Bale and Hayward, 2010). No attempt has been made here to relate the energy sources pattern seen to weather, because with only one year of field samples available, meaningful statistical analysis is difficult. Future studies, with longer sets of field-derived data will be need for such analysis. Weather might be expected to alter the precise timing of the fluctuations seen, though in most years not the overall pattern. For example, cool autumn, weather may limit questing activity of larvae and result in earlier diapause. Warmer autumn temperatures have been shown to increase survival/developmental rates, which subsequently increases nymphal densities the following summer.

Of particular interest in the present study, was that the energy source contents seen in different life cycle stages in the field broadly matched the differences seen in the laboratory (Chapter 2), with protein making a considerably higher contribution to resource in adults than seen in

nymphs. This role of protein may be linked to reproduction, though notably because females collected here had not yet taken their adult bloodmeal, vitellogenesis and embryogenesis would not have been expected to have initiated, but the synthesis of metabolic precursors may have been underway. It may also be that the long-term physiological responses of adults rely on protein and total lipid stores to maintain resistance levels for long durations of starvation, as has been suggested previously (Jaworski et al., 1984, Williams et al., 1986). In contrast, although carbohydrates are not a major contributing factor in the storage of energy, the critical roles of carbohydrate metabolism are high likely during reproduction, development and starvation progresses, which mainly shifting to break down into lipolysis and proteolysis, (McCue et al., 2016, Moraes et al., 2007, Rosendale et al., 2019).

Sharp changes in climate serve to synchronise insect populations in the field, and highly distinct seasons particularly in temperate habitats, serve to in limit the available feeding periods. Climate change, in the form of warmer wetter conditions might be expected to increase variation, making populations more heterogenous, removing distinct peaks in the tick season, increasing the proportion of tick population questing at any one time, but increasing the rates of starvation and the risks of parasitisation and pathogen infection (Gilbert et al., 2014). It might have been expected that the cohort of ticks collected would form two distinct groups,; those that fed the previous sutumn and those that fed in spring. However, no such bimodal distribution could be detected, and it is considered likely that the sample size used was not large enough to allow such resolution. Hence the sample was represented by a single mean value. Further work, to explicitly consider this point within in tick cohorts would be a useful step in future.

Chapter 5

General Discussion

Energy source ingestion from the bloodmeal, accumulation, and subsequent metabolism, affect almost every aspect of the life history of a tick (Randolph and Storey, 1999) and may also help determine their resilience to infection by pathogens and subsequent infectiousness (Angelo et al., 2013). Metabolites derived from the bloodmeal not only provide energy, but they also provide the starting materials for the onward synthesis of other important biomolecules. For example, sugar (glucose)-derived alcohols are required for adaptation to adverse temperature conditions and the formation of chitin, a key structural biomolecule. Lipids such as fatty acids (e.g. triglyceride) are key precursors for the synthesis of hormones (e.g. eicosanoids) and pheromones used for sensing and signalling between male-female adult ticks and detection of suitable host species by all tick stages (Stanley-Samuelson and Nelson, 1993). Fat content in ticks is primarily in the form of lipids with triglyceride being the dominant molecule (present in anhydrous form). Triglyceride provides more energy (has a higher calorific value per unit weight) than glucose and can be oxidised to form water more efficiently than glycogen-glucose. Triglyceride is synthesised from carbohydrates, smaller fatty acids and proteins present in ingested blood (Arrese and Soulages, 2010). Understanding the accumulation and use of resources by ticks is therefore of fundamental importance to understanding the pattern of development, behaviour and the vectoral importance of ticks.

5.1 Approaches to metabolism measurement

Studies of metabolism and energy balance have been carried out on a range of arthropods at different lifecycle stages and/or activity levels, a range of approaches (Foray et al., 2012, Olson et al., 2000, Phillips et al., 2018, Schilman, 2017), but primarily using histological or respiratory rate-based analysis. For example, (Lighton and Fielden, 1995) measured metabolic rates of adult ticks in standardised flow chambers using gas analysers to assess CO_2 respiratory quotients. However, whilst flow-through respirometry analysis remains a valuable indirect tool to assess insect metabolism, direct methods, such as calorimetry, have also been used to compare insect vector metabolism where characteristics such as direct heat produced (kilojoules-1) or comparing blood oxygen, carbon dioxide and water levels (Schilman, 2017). Schilman, (2017) analysed *Rhodonium proxilus* a vector of the *Trypanosoma cruzi* (Chagas disease) parasite that infects through via blood feeding like ticks or transmission through direct blood contact with insect faeces and identified higher metabolic rates than ticks but lower than other non-hematophagous insects.

A spectrophotometric-based method was initially developed for assessing lipid levels in mosquitoes by Van Handel (1985a,b), and more latterly was extended for the measurement of a range of different-sized arthropods and energy source in insects (Lee, 2019) and this allowed the direct measurement of lipids, free sugars, glycogen and proteins and the correlation of energy storage against activity, longevity and behavioural responses. Here, detailed laboratory investigations were used to modify these techniques for use in ticks and determine whether they could be applied on individual specimens and whether multiple biochemical assays could be undertaken on a single sample, simultaneously. The results presented in in Chapter 2 were able to demonstrate that spectrophotometric approaches do deliver relatively rapid and reliable estimates of the total energetic budget in *I. ricinus* and indicated that they could be used to quantify the metabolic profiles of individual ticks, demonstrating their suitability for use in

ecological and epidemiological studies. The technique is relatively rapid and inexpensive, though does require some relatively precise chemistry and high precision balances, which may not be available in some research laboratories in parts of the developing world. The use of bulk samples could provide cohort data and the additional support provided by the physiological age index (Uspensky et al., 2006), as discussed in Chapter 4, gives some valuable support. The fact that variance estimates around the mean metabolite values obtained from field-derived samples were generally small, indicates that cohort data could be useful in the examination of seasonal patterns, though clearly not as valuable as the analysis of individual ticks.

5.2 Metabolism, temperature and climate

Studies show that tick activity patterns are strongly correlated with temperature and humidity (MacLeod, 1935). Physiological temperature and humidity thresholds (with daylength probably also playing some role) constrain the phenology and distribution of tick populations (Tomkins et al., 2014) resulting in relatively well-defined cohorts of ticks in field populations at equivalent stages of nutrition, as was demonstrated here (Chapter 4). The data showed that there was a cohort of relatively well-fed nymphal ticks in the early spring, most probably derived from larvae that fed the previous summer and moulted the previous autumn; in contrast for females, the pattern of changing energy source contents was more complex, possibly reflecting greater variation in the population by this life-cycle stage. The importance of climatic factors in determining tick distribution means that climate change are likely to result in significant impacts; recent data suggests that their range is increasing northwards with ticks such as *I. ricinus* now being recorded more commonly in northern Scandinavian countries such as Sweden as well as Russia (Dantas-Torres, 2015). Research by Dantas-Torres, (2015) also highlights the non-linear increase in tick abundance and a potential habitat expansion of 3.8%

in Europe. The changing distribution of hosts, such as birds and mammals, as a result of climate change, may also indirectly affect tick populations (Dantas-Torres, 2015).

Threshold temperatures for larval activities of around 7–8°C are generally considered to be necessary to initiate questing in *I. ricinus* (MacLeod, 1935). Some element of genetic variation in threshold temperatures, reflecting local adaptation is likely (Gilbert et al., 2014) and in laboratory trials, minimum temperature thresholds for activity were estimated to be 5.7°C for populations derived from Scotland, 7.9°C for Wales, 7.0°C for England, 9.3°C for France at low altitude, and 6.9°C for France at high altitude (Tomkins et al., 2014). Humidity was not explicitly considered here, and the laboratory studies used humidity levels that were considered to be above what might be limiting (Crooks and Randolph, 2006). The effects of temperature on the rate of depletion of energy reserves by nymphal and adult *I. ricinus* was undertaken using the spectrophotometric approached developed and explained in Chapter 2. In nymphs, the rate of soluble carbohydrates and glycogen utilisation was higher than in males or females and the concentrations of neutral lipids (a proxy for stored lipids) were significantly affected by higher temperatures. In adults, the concentrations of protein and structural lipid (phospholipid) responded rapidly to changes temperature over time. Q_{10} values recorded were 1.5 for nymphs, 1.71 for males and 1.63 for females. These values are relatively low, and this reflects the low metabolic rate that helps to increase survival during the extended inter-feed intervals. Ticks have relatively low metabolic rates in comparison with other arthropods, similar to those of scorpions, and this is considered to be an adaptation to the sit-and-wait feeding strategies of both groups. Lighton and Fielden, (1995) compared standard metabolic rates in ticks with other insects and identified that at temperatures of 25 °C and 85% humidity ticks with a body mass of 11 mg were predicted to have a standard metabolic rate of 2.8 uW. A metabolic rate of 2.8 uW (20% body mass) corresponds with the catabolism of 2.3 mg lipids annually, which is significantly lower than species that do not employ a sit-and-wait strategy

(Lighton and Fielden, 1995). Of particular interest in the present data, however, was that a linear extrapolation from the observed temperature range suggested that the lower threshold for metabolic activity is between -10 and -5 °C. This may be an anomaly if the assumed linear extrapolation is incorrect and in fact some concave non-linear change in metabolic activity occurs at lower temperature, but this would be physiologically unusual. The data suggest that at temperatures well below 0 °C, some metabolic activity persists, and this is likely to be the case down to between -5 and -10°C which is probably close to the lower lethal temperature. An understanding that metabolic activity is likely to be occurring even at relatively low temperatures may have important impacts on attempts to model tick phenology using climate simulations, since measurable levels of resource depletion will occur at temperatures well below those considered to be appropriate for questing.

5.3 Future directions: metabolism and pathogen transmission

Given, that the work in this thesis has demonstrated that spectrophotometric techniques can measure the range of energy source in ticks (Chapter 2), that the rate of depletion is dependent of time and temperature (Chapter 3) and that these patterns can be detected in field populations (Chapter 4), the next step is to consider what further studies should follow in the application of the techniques developed here. The relationship between metabolism and the acquisition of and resilience to pathogens by ticks and their subsequent infectiousness, would appear to be particularly relevant and appropriate for further investigation. Because the feeding cycle is so long, and the accompanying changes in resource availability are so marked, ticks face very different metabolic challenges in relation to pathogen infection compared to haematophagous insects, such as mosquitoes or fleas, that feed frequently at short intervals throughout their adult life. Previous studies suggest that in ticks, the relationship between lipid metabolism and pathogen infection and transmission is complex and can vary between life stage (Parola and Raoult, 2001; Arrese and Soulages, 2010; Herrmann et al., 2013,). Ticks infected with

pathogens such as *Borrelia burgdorferi* were found to have over 12% higher fat content than uninfected ticks (Herrmann et al., 2013) . Angelo et al. (2015) showed that after infection by a pathogen, glycogen levels in *R. microplus* decreased significantly in the first 24-48 h and that glycogen was absorbed directly into the host haemolymphatic circulatory system supporting pathogen development and multiplication through homeostatic regulation. This suggests that the pathogen may be manipulating energy release by the vector to support its survival and reproduction. Studies have also demonstrated changes to lipid metabolism, protein synthesis and immune response in engorged ticks after infection (Angelo et al., 2013). Tick borne viruses have been found to infect tick cells, but few will grow in mosquito cells and this is potentially due to cellular transcription differences of the pathogen (Bell-Sakyi et al., 2007). However, the role of proteins and genetic signalling in the regulation of metabolic processes, such as iron absorption from blood, following ingestion can have similar significant metabolic effects (Hajdusek et al., 2009). This underlines the importance of assessing tick metabolism and energy balance alongside pathogen transmission risk. However, a holistic approach is essential as research has highlighted the importance of water, as well as energy balance, in determining feeding and famine tolerances (Kaufman and Sauer, 1982), and this should also inform future work.

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Appendix

Publications of Chapter 2: Determining the total energy budget of the tick *Ixodes Ricinus*.

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Determining the total energy budget of the tick *Ixodes ricinus*

Saeed Alasmari[1] · **Richard Wall**[1]

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Abstract
Precise and accessible techniques for measuring metabolic responses to environmental stress are essential to allow the likely impacts of climate and climate change on tick distribution, abundance and phenology to be predicted. A more detailed understanding of the metabolic profile of ticks may also help the complex responses to pathogen infection and effects on transmission to be evaluated. Here, a series of biochemical protocols employing spectrophotometric methods are used to determine the entire energy budget of ticks. Protein, carbohydrate, total lipid, neutral lipid and glycogen were measured in individual *Ixodes ricinus* nymphs and adults. Two key trends were identified: in adults, protein was relatively more abundant than in nymphs, whereas in nymphs, glycogen and carbohydrate were more abundant than in adults, with glycogen alone composing 39% of the mass of metabolites in nymphs compared to 15 and 10% in females and males, respectively. The methods used were able to successfully separate neutral lipids from the polar phospholipids and the importance of distinguishing stored from structural lipid in estimates of lipid reserves is emphasised. The results demonstrate that the spectrophotometric approaches deliver relatively rapid and reliable estimates of the total energetic budget and can be used to quantify the metabolic profiles of individual ticks, demonstrating their suitability for use in ecological and epidemiological studies.

Keywords Carbohydrate · Glycogen · Lipid · Metabolism · Protein · Resource acquisition

Introduction

For multi-host ixodid ticks, the blood meal obtained by each lifecycle stage must provide the resource required for development and/or reproduction (Sonenshine and Roe 2014). Between feeding events, ticks spend the majority of their lives off the host where they need to survive adverse environmental conditions (Needham and Teel 1991). To facilitate survival during these extended inter-feed intervals and then ensure that sufficient stored resources are available to allow repeated questing until a host is located, ixodid ticks have a metabolic rate which is typically 13% below that of most arthropods (Lighton and Fielden 1995). Given this intermittent 'high-risk' sit-and-wait feeding strategy, understanding how

✉ Saeed Alasmari
sa16081@bristol.ac.uk

[1] School of Biological Sciences, University of Bristol, 24 Tyndall Avenue, Bristol BS8 1TQ, UK

ticks partition resources between maintenance, development, reproduction and storage for future requirements is of intrinsic physiological interest but their measurement can also allow insight into the feeding history and ecology of cohorts of field-derived ticks (Randolph et al. 2002; Abdullah et al. 2018) and can allow predictions about the likely impact of changes in rate determining environmental factors such as temperature and humidity (Randolph et al. 2002; Rosendale et al. 2017).

The main immediate source of energy for ticks is glucose, which is stored in a hydrated polymeric form as glycogen. Glycogen serves as an energy source to support the post-feeding stage, but is rapidly depleted (Moraes et al. 2007). Metabolism of glycogen involves its breakdown to glucose with resultant energy release. Carbohydrate can be used to replenish glycogen reserves so that energy is available for activity. Lipids also play key roles in tick metabolism, both as an energy source and structurally in cell membranes, in hormones and for egg development (Kluck et al. 2018). Lipid is stored primarily the form of triglycerides in adipocytes the main fat body cell and additionally as cytoplasmic lipid droplets. Storage is mostly regulated by hormones in response to physiological demands; in insects marked decreases in lipid content have been associated with key growth phases (Arrese and Soulages 2010). Lipids measured as a total concentration (stored lipids such as triglyceride plus structural lipids present in cell membranes) are commonly used as proxy for stored lipids to give an indication of energy reserves (Abdullah et al. 2018). However, only the stored lipids are functionally valuable as an energy reserve and consideration of total lipid alone may be misleading because the dominant triglyceride component fluctuates considerably with developmental stage, sex and even season and may vary independently of other lipid classes, such as diglyceride, monoglyceride, sterols, sterol esters, free fatty acids and pholipids (Angelo et al. 2013). Hence, accurate distinction between structural polar lipids and total lipids may be important. Finally, proteins are important and may act as a long-term energy reserve (Williams et al. 1986) and are structurally essential for muscle, cuticle synthesis, hormones, enzymes, carrier proteins (Kluck et al. 2018), in females for the synthesis of egg yolk (Tatchell 1971; Xavier et al. 2019) and in males for sperm and gonadal proteins that trigger engorgement in females (Weiss and Kaufman 2004). Glycogen resynthesis by protein degradation through the gluconeogenesis pathway has been demonstrated in ticks, notably at the end of the embryonic period (Martins et al. 2018).

The measurement of energy budgets and the factors affecting metabolic rate have been well studied in insects (Chown and Nicolson 2004) and approaches using spectrophotometric techniques, as developed by Van Handel (1985a, b), have been used widely for such measurements (Raubenheimer et al. 2009), for example allowing insect nutrient levels to be related to egg load or longevity (Otronen 1995), or to allow explanations of insect population dynamics and behavioural ecology in terms of resource availability (Pelosse et al. 2007). Such studies have looked in particular at the measurement of lipids, but the analytical approaches have also been extended to allow the estimation of glycogen and free sugars. These analytical methods are used increasingly because they are relatively inexpensive, rapid and precise. However, although applied extensively in insects, these techniques have rarely been used in ticks (Abdullah et al. 2018).

The aim of the work described here, therefore, was to evaluate the use of the range of spectrophotometric techniques for quantifying protein, carbohydrate, lipid and glycogen resources in ticks in the nymphal and adult life-cycle stages. A further aim was to explore the separate quantification of neutral lipids and structural polar phospholipids to allow more exact quantification of the contribution of lipids to energy reserves. In particular, the work aimed to determine whether several metabolites could be determined for an individual tick. To explore this, two analytical approaches were compared. The first applied

multiple biochemical measurements to individual ticks, whereas the second, used separate ticks for the measurement of complimentary groups of metabolites.

Material and methods

Sample collection

Ixodes ricinus ticks (nymphs, males and females) were collected every 2 weeks from the field between March to May in 2017 by blanket-dragging. The field site was a semi-urban park (51° 26′ 49.2″ N, 2° 38′ 01.9″ W) to the west of the city of Bristol in southwest England, containing managed herds of red and fallow deer and a population of wild roe deer and where ticks are abundant (Jennett et al. 2013). The ticks collected were identified to species, sex and lifecycle stage using standard keys (Arthur 1963; Hillyard 1996; Walker et al. 2014). They were weighed using an ultrasensitive microbalance (Sartorius-ME5, Goettingen, Germany) to the nearest microgram and then stored at − 20 °C for a maximum of 8 weeks prior to analysis.

The ticks collected were divided at random into two groups based on the analytical approach to be used. In the first group, referred to as the 'individual-analysis' group, a total of 60 ticks were used, 30 for protein analysis and 30 for the measurement of all other metabolites in each specimen. In the second, referred to as the 'multi-analysis' group, in total 90 ticks were used, 30 for protein analysis, 30 for both free sugars and glycogen and 30 for total lipids and neutral lipids. In all cases, each group of 30 ticks consisted of 10 nymphs, 10 males and 10 females.

Individual analysis ticks

Protein was measured following the method described by Bradford (1976). Ticks were first placed individually into a clean borosilicate tube (12 ml) and crushed using a clean glass rod. This was followed by the addition of 1500 µl of phosphate buffer solution to extract the protein (100 mM of monopotassium phosphate, 1 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of dithiothreitol (DTT), pH 7.4). Thereafter, the homogenate was placed on ice prior to processing (about 1 min). For nymphs, 1000 µl was transferred to a cuvette and mixed with 1000 µl of Bradford reagent (Sigma, Dorset, UK). For males and females, 50 µl was transferred to a cuvette and mixed with 1500 µl of Bradford reagent. Individual cuvettes were incubated at room temperature for 5 min and then the absorbance value was immediately measured at a wavelength of 595 nm using a Biochrome spectrophotometer (Biowave II, Cambridge, UK). The procedure is based on the formation of a protein–dye complex; coomassie dye in the Bradford reagent binds with proteins and results in a change in colour from brown to blue leading to a shift in the absorption maximum from 465 to 595 nm.

For analysis of other metabolites, a single individual tick was placed into a clean borosilicate tube and crushed, as above. To dissolve all water-soluble carbohydrates and the total fats, 200 µl of 2% sodium sulphate solution (VWR International, Leicestershire, UK), and 1500 µl of a chloroform/methanol mixture (1:2 vol/vol) were added to each tube. This mixture was then transferred into a 2 ml Eppendorf tube and centrifuged (Centrifuge 5418R; Eppendorf Lutterworth, UK) for 15 min at 180×*g* and 4 °C. For total lipid, neutral

lipid and carbohydrate analysis, the supernatant was removed into a new tube; and the pellets containing glycogen were retained for further analysis.

Beginning with carbohydrate determination, the method of Van Handel (1965) was used. 200 µl of the supernatant from individual samples were moved into a borosilicate tube and placed in a water bath at 90 °C for 40 s to evaporate the solvent to about 20 µl. Thereafter, 1 ml of freshly prepared anthrone reagent (Sigma) (1.42 g/l in 70% sulphuric acid; VWR International) was added to each sample and incubated for 15 min at 25 °C. Subsequently, the tubes were heated for 15 min at 90 °C and then cooled at room temperature for 15 min, after which the samples were placed in microcuvette and finally read in a spectrophotometer set at 625 nm, to determine the total water-soluble carbohydrates. Blue green coloration indicates the binding of anthrone to sugar.

For glycogen determination, pellets were washed on two occasions using 400 µl of 80% methanol to remove sodium sulphate. Vigorous vortexing was followed by centrifugation, for 5 min at 180×*g* at 4 °C. Once the supernatant was eliminated, 1 ml of fresh anthrone reagent was added and the mixture was incubated at 90 °C for 15 min. Each sample was cooled on ice to end the reaction and filtered on low-protein binding membranes of diameter 0.45 µm (Fisher Scientific, Leicestershire, UK). Finally, the absorbance was read in a spectrophotometer at 625 nm to quantify the presence of water-soluble carbohydrates.

The total lipids were quantified using a vanillin assay. For this, 200 µl of the supernatant was added into a new borosilicate tube and placed into a heating block at 90 °C until total evaporation was achieved. 40 µl of 95% sulphuric acid (VWR International), was added to the mixture and heated at 90 °C for 2 min and then cooled in ice. 960 µl of freshly prepared 1.2 g/l vanillin reagent (Fisher Scientific) in 68% phosphoric acid (Sigma) was added and followed by incubation at room temperature for 15 min. The absorbance was read in a spectrophotometer at 525 nm (Van Handel 1985b). Finally, the neutral lipid content was measured by placing 500 µl of the supernatant in a new tube, which was heated at 90 °C to evaporate off the solvents. One 1 ml of chloroform was added into each tube to re-solubilize the fats. 200 mg of dry silicic acid (Sigma) was added to each sample. Thereafter, all samples were mixed and then centrifuged at 180×*g* and 4 °C for 10 min to remove polar lipids in the silicic acid. From the final supernatant 200 µl was pipetted off and removed into new tube. Again, the absorbance was read in a spectrophotometer at 525 nm (Van Handel 1985b).

Multi-analysis ticks

For multi-analysis, 30 ticks were used for protein analysis, as described above. Thirty ticks were used for the analysis of carbohydrate and glycogen only and a further 30 were used only for the analysis of lipids, following the procedures described above.

Standard curves

Standard curves of absorbance against known metabolite concentration were created to allow spectrophotometric values to be related to metabolite concentrations. Care was taken to ensure linearity within the various concentrations and that they started below the lowest sample value and ended beyond the highest. For protein concentration a standard curve was generated with a dilution series of bovine serum albumin (BSA) (1 mg/ml) (Sigma) treated as described above. For carbohydrate and glycogen, a standard curve was generated using glucose (1 mg/ml) (Sigma) at a range of dilutions. For lipids a standard curve was

generated using glycerol trioleate (1 mg/ml) (Sigma) at various dilutions. Five independent repeats of different serial dilutions were conducted to produce each standard curve.

Statistical analysis

Generalized linear models (with a gamma distribution and inverse link) with Tukey multiple comparisons post-hoc tests were used to compare the mass of different metabolites within each lifecycle stage and the masses of metabolites measured using the individual-analysis or multiple-analysis approaches and the weights of ticks included in the two analysis-groups. One-way ANOVA was used to compare tick body weights between analysis-groups. All analyses were performed with the R-Studio statistical package (R v.3.5.3, 2019, R Foundation for Statistical Computing, Vienna, Austria). For graphical presentation of the metabolite data, medians are plotted with 95% confidence intervals.

Results

All the ticks collected from the field were *I. ricinus*. The mean (±SE) body mass of nymphs, males and females used in the individual analysis was 0.138 (±0.006), 0.884 (±0.034) and 1.649 (±0.069) mg, whereas the means of the weights of these lifecycle stages in the multi-analysis groups were 0.130 (±0.004), 0.843 (±0.022) and 1.634 (±0.006) mg, respectively. Within each lifecycle stage these weights were not significantly different from each other (nymphs: $F_{1,149}=1.44$, $P=0.23$; males: $F_{1,149}=1.14$, $P=0.29$; females: $F_{1,149}=0.03$, $P=0.86$). Statistical analysis showed that there were no significant differences between the individual- or multi-analysis methods in any of the metabolite concentrations recorded in nymphs, males or females (Table 1) and data were therefore pooled for subsequent analysis.

In nymphs, the metabolite contents show a significant difference between the amounts of different categories present ($F_{4,45}=12.31$, $P<0.001$). Glycogen was the most abundant metabolite with a median of 0.014 mg and was significantly more abundant than any other metabolite. Protein was the second most abundant metabolite, with a high degree of

Table 1 Statistical comparison of the mass of phospholipid, neutral lipid, carbohydrate and glycogen values recorded in nymphs, males and females of *Ixodes ricinus* (n = 30 in each case), where all were measured either in a single individual or where lipids and glycogen/carbohydrate were measured separately in different groups

Metabolite category	Lifecycle stages	F	P
Phospholipid	Nymphs	1.90	0.28
	Males	1.03	0.35
	Females	1.73	0.24
Neutral lipids	Nymphs	0.76	0.41
	Males	1.24	0.31
	Females	0.21	0.66
Carbohydrate	Nymphs	0.69	0.43
	Males	7.79	0.15
	Females	0.34	0.56
Glycogen	Nymphs	6.53	0.22
	Males	4.81	0.43
	Females	0.30	0.60

F and P values are based on a generalised linear model (GLM)

consistency between individuals. Total lipid and neutral lipid concentrations were low as was the median concentration of carbohydrate, but the latter was notable for its very high degree of variation between individuals (Fig. 1).

In adults, there were significant differences in the amounts of the different categories of nutrient present, in males ($F_{4,45}=172$, $P<0.001$) and females ($F_{4,45}=196.8$, $P<0.001$). Males had high median values for protein, at 0.056 mg, which was significantly greater than other metabolites, which had concentrations that were not significantly different from each other. The degree of variation around the median values was consistently relatively small (Fig. 2). Females also had concentrations of protein which were relatively high and similar to those of males (Fig. 3). They had low concentrations of lipid, both total lipid and neutral lipid, but they had concentrations of carbohydrate and glycogen at around 0.3 mg, which were considerably greater than those seen in males (Fig. 3).

Consideration of the amounts of each metabolite present as a percentage, to allow for comparison between lifecycle stages, shows that the greatest differences in metabolite concentrations between nymphal and adult stages were in protein and glycogen (Fig. 4). In nymphs, glycogen accounted for 39% of the metabolites measured, whereas protein accounted for 25%. However, in males and females the protein values were at 60 and 53%, respectively, whereas the glycogen contents were lower at 12 and 19%, respectively. Other

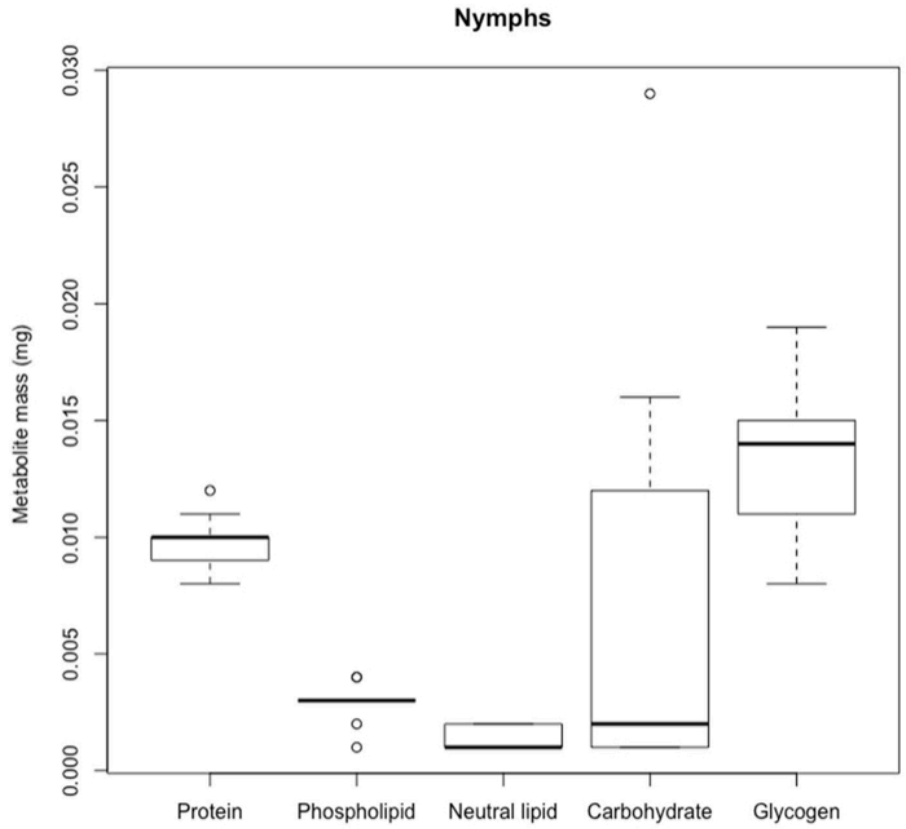

Fig. 1 The median mass of five metabolites (mg) in *Ixodes ricinus* nymphs with 95% confidence interval (dashed lines) and lower and upper quartiles (box)

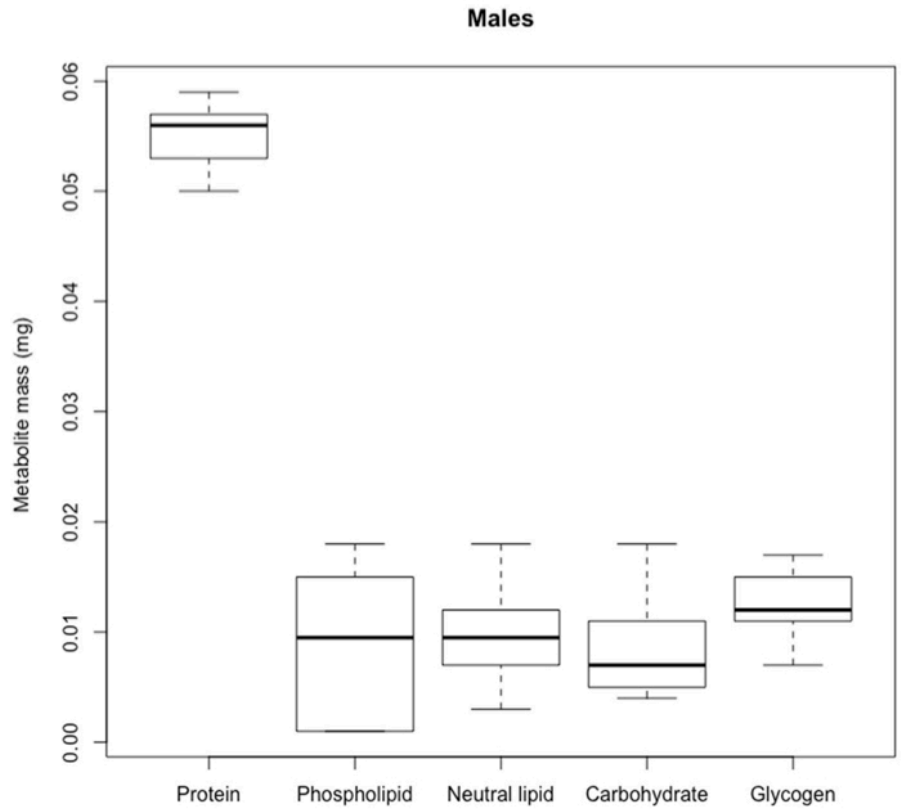

Fig. 2 The median mass of five metabolites (mg) in *Ixodes ricinus* males with 95% confidence interval (dashed lines) and lower and upper quartiles (box)

metabolites were relatively similar between lifecycle stages. Notably lipid was consistently the least abundant metabolite.

The methods used were able successfully to separate neutral lipids from the polar lipids; about 94.6% of polar lipid was extracted from the standardized phospholipid solution. In nymphs, 8.4% of the mass was composed of lipids of which neutral lipids represented 45.2%. In adults, total lipid composed about 10 and 5% of the total body mass in males and females, respectively, whereas neutral lipids composed about 85% of the lipid fraction in males and about 96% in females (Fig. 4).

Discussion

Ticks need to be able to survive for extended periods between blood meals. During these off-host periods, they need to endure adverse conditions and maintain sufficient reserves to allow repeated episodes of host-seeking behaviour. Starvation of ticks for 18 or 36 weeks, in the absence of dehydration, was shown to result in the loss of 20–40% of protein and 60% of lipid reserves in the American dog tick, *Dermacentor variabilis* (Rosendale et al. 2017). The lipid reserves of field-collected *I. ricinus* ticks, collected in early summer,

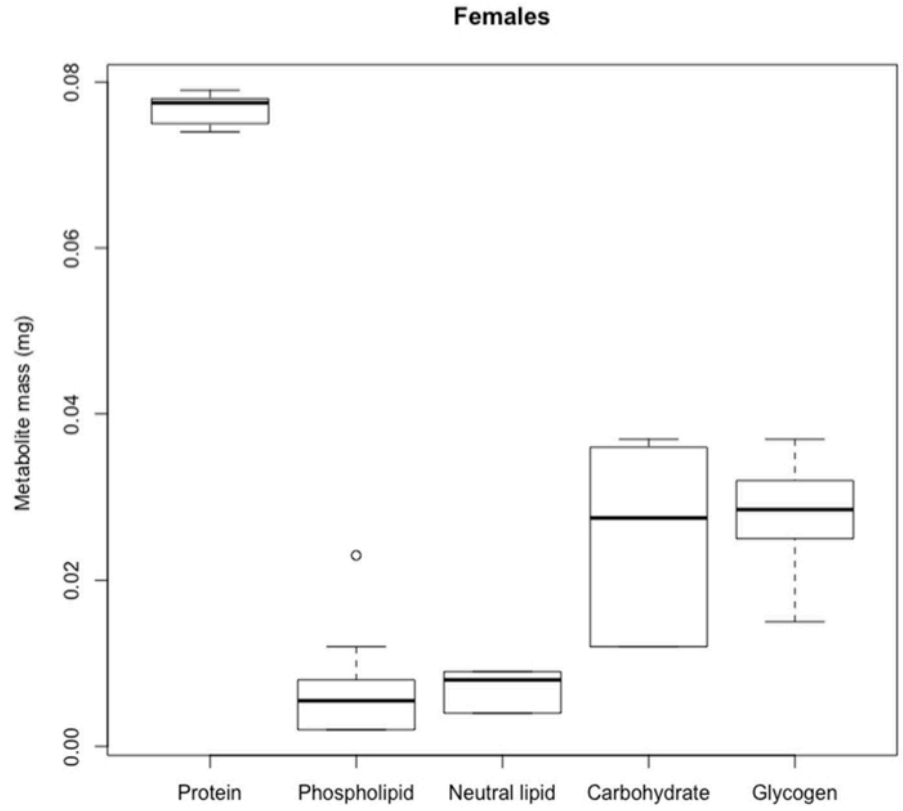

Fig. 3 The median mass of five metabolites (mg) in *Ixodes ricinus* females with 95% confidence interval (dashed lines) and lower and upper quartiles (box)

were estimated to be sufficient to allow survival without feeding for up to 100–250 days at 15 °C, depending on whether they had fed the previous autumn or that year, respectively (Abdullah et al. 2018).

The pattern of metabolic activity is not constant. A newly moulted tick is able to maintain relatively low levels of activity and minimise energy expenditure for several weeks; but once energy levels start to become depleted the level of activity and consequently energy expenditure may rise as questing activity becomes more prolonged and persistent. This may increase their susceptibility to environmental stresses such as dehydration (Rosendale et al. 2017). A significant increase in energy use has been associated with ovary development in female ticks (Xavier 2019). A detailed understanding of the patterns of changing resource within ticks would therefore be expected to provide information in field-collected cohorts about their metabolic rate, feeding history and questing activity.

Early approaches to examining metabolic reserves used histological or anatomical examination of ixodid ticks to categorise them into feeding cohorts, but such approaches are relatively imprecise (Uspensky 1995; Walker 2001). Subsequently spectrophotometric methods have been used to examine total lipid (Abdullah et al. 2018), and the current paper extends these approaches to a consideration of the full metabolic profile in the tick *I. ricinus*. These methods have been used widely in insects, but have not previously been used

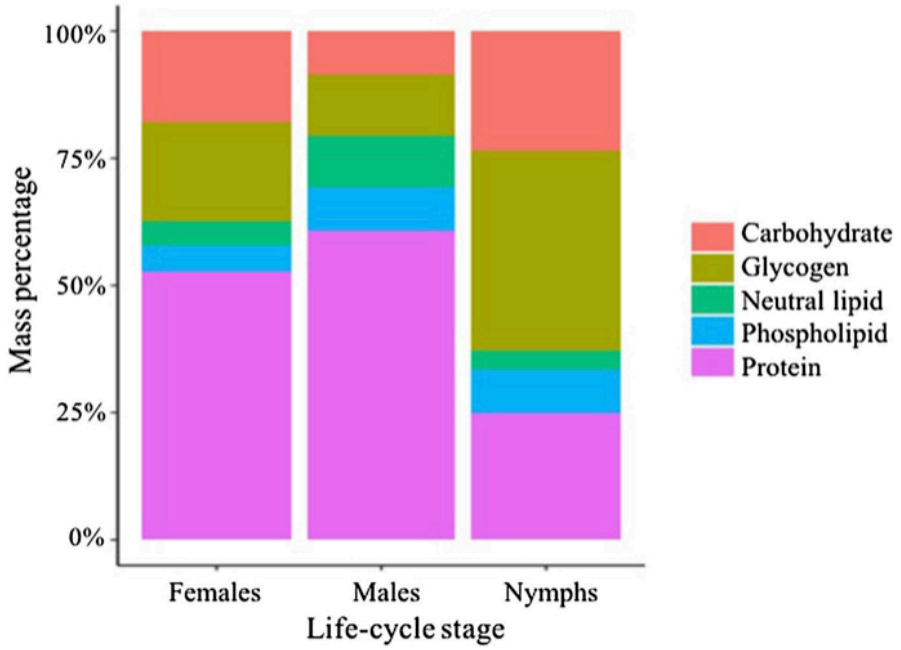

Fig. 4 The percentage mass for each metabolite category in individual *Ixodes ricinus* nymphs, males or females

comprehensively in ticks. The first analysis undertaken by the present study showed that there was no difference in the metabolite concentrations determined in either the ticks subjected to multiple analysis or ticks used for analysis of separate classes of metabolite. This was an essential initial step required to give confidence to the subsequent investigation, and to highlight the sensitivity and repeatability of the procedure. The ability to measure lipid, carbohydrate/glycogen simultaneously in individual ticks highlights the suitability of these methods for use in ecological and epidemiological studies. Overall, the results indicate that the spectrophotometric approaches appear to allow accurate and reproducible quantification of the entire range of the energetic reserves. This represents a considerable advance over the approaches used previously in ticks and the spectrophotometric approaches are able to deliver relatively rapid, inexpensive and reliable estimates of the total energetic budget.

The ticks used for the present study were collected by blanket dragging between March and May in southwest England when questing activity of nymphs and adults would have been expected to be at its peak. A range of stages of starvation would have been expected, with the population composed of cohorts that fed the previous year and either moulted the previous autumn or which overwintered or moulted in early spring prior to collection, depending on precisely when they had fed. The aim of this work was to evaluate the applicability of the analytical methods to ticks, rather than attempt to explain the cause or meaning of the differences observed, nevertheless two key trends were apparent. In adults, protein values were high and relatively greater than in nymphs whereas in nymphs 39% of the metabolites were composed of glycogen. In contrast in females, glycogen composed only 15% and in males 10% of the mass of metabolites. Carbohydrate was also relatively

more abundant in nymphs and females than males. The relatively high protein levels in adults may be associated with reproduction, whereas the relatively high glycogen levels in nymphs and very high variability in carbohydrate values may indicate that the population of nymphs is divided into a cohort that moulted the previous autumn and a cohort that moulted in spring of the year they were collected. In contrast the adult population is largely composed of individuals that moulted the previous autumn, so lipid and carbohydrate values are relatively lower than in nymphs. At this stage these suggestions remain preliminary and, now that the spectrometric techniques have been validated, further long-term seasonal studies of metabolic profiles will allow changing metabolite patterns to be mapped more clearly to observed activity. The data show that the proportion of neutral lipids (stored lipids) was about half that of structural phospholipids in nymphs, and considerably higher in adults. Hence the data suggest that since the levels of triglyceride can vary significantly between life-cycle stages, when analysing lipid reserves, it is important to distinguish neutral lipids from structural phospholipids.

In summary, the current study has demonstrated that a complete energy budget for an individual tick can be quantified using spectrophotometric approaches, although it is still necessary to quantify protein separately. The expectation, therefore, is that the use of these techniques will facilitate further research using studies of tick metabolism to elucidate the seasonal pattern of feeding activity and physiological drivers leading to, for example, diapause (Raubenheimer et al. 2009). A more detailed understanding of the metabolic profile of ticks may also help in understanding the complex interaction between metabolism, pathogen infection, and transmission (Angelo et al. 2013, 2015; Herrmann et al. 2013).

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