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Ixodes ricinus ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves

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ABSTRACT

Ticks use their energy reserves to maintain their water balance, search for hosts and transmit tick-borne pathogens. However, the influence of tick-borne pathogens on the energy reserves of the tick vector has not been well studied. The relationship between *Borrelia burgdorferi* sensu lato (s.l.) infection status and fat content in questing *Ixodes ricinus* nymphs was examined. Nymphs were sampled from the field. Their body mass and fat content were measured, and their *Borrelia* genospecies infection status (using reverse line blot analysis), and spirochete load (using quantitative PCR) were analysed. Of the 900 nymphs tested, 21.2% were infected with a variety of *Borrelia* genospecies. *Borrelia*-infected nymphs had 12.1% higher fat content than uninfected ticks after correcting for body size. For the subset of *Borrelia*-infected nymphs, no relationship was found between spirochete load and fat content and bioenergetics calculations suggest that *Borrelia* spirochetes consume a negligible fraction of the tick energy reserves. While the mechanism that causes the association between *Borrelia* infection and higher fat content in *I. ricinus* nymphs remains unknown, the present study complements our previous findings that *Borrelia*-infected nymphs had higher survival times under desiccating conditions and walked less within a humidity gradient.

1. Introduction

Lyme borreliosis is the most common vector-borne disease in the northern hemisphere, accounting for approximately 85,000 new cases in Europe (Lindgren and Jaenson, 2006) and 20,000 in the United States each year (Bacon et al., 2008). The causative agents of this disease are spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex, which are transmitted to humans by hard-bodied ticks (Acari:Ixodidae) during blood feeding. In Europe, *Ixodes ricinus* is the most important tick species and to date at least 10 *B. burgdorferi* s.l. genospecies have been associated with this vector: *B. burgdorferi* sensu stricto (s.s.), *Borrelia afzelii*, *Borrelia garinii*, *Borrelia bissettii*, *Borrelia lusitaniae*, *Borrelia spielmanii*, *Borrelia valaisiana* (Rauter and Hartung, 2005; Gern et al., 2010), *Borrelia bavariensis* (Margos et al., 2009), *Borrelia carolinensis* (Cotté et al., 2010), *Borrelia finlandensis* (Casjens et al., 2011), as well as a genospecies related to relapsing fever, *Borrelia miyamotoi* (Fraenkel et al., 2002).

In haematophagous arthropods, fat content is a source of energy derived from the blood meal (Lehane, 1991). Although the blood meal primarily consists of proteins (up to 95%), the products obtained during digestion are largely converted to, and stored as, fat (lipids) (Lehane, 1991). In ticks, lipids are stored in epithelial cells of the midgut and in the fat body, which is a diffuse organ of highly dispersed strands of cells (primarily trophocytes) adhering to the branches of the tracheal system and other internal organs (Sonenshine, 1991). *Ixodes ricinus* ticks use their fat supplies to quest for hosts on vegetation and to maintain their water balance. Ticks are highly sensitive to desiccation (Lees, 1946) and have to move from their questing position to the underlying leaf litter where they take up atmospheric water (Lees and Milne, 1951; Knülle and Rudolph, 1982; Needham and Teel, 1991; Kahl and Alidousti, 1997). In *I. ricinus*, the rate of lipid consumption has been shown to increase under unfavourable conditions of temperature (Van Es et al., 1998) and humidity (Randolph and Storey, 1999). As ticks have no other energy sources, their body fat content declines between blood meals (Steele and Randolph, 1985). Thus tick fat content reflects both the history of the tick and the energy reserves remaining for future use (Randolph and Storey, 1999; Randolph et al., 2002).

Borrelia spirochetes influence both the survival and behaviour of their tick vector. Naumov (2003) reported that *I. ricinus* and *Ixodes persulcatus* ticks infected with *B. burgdorferi* lived slightly longer than uninfected individuals. More recently, we reported that field-collected *I. ricinus* ticks infected with *B. burgdorferi* s.l. survived better than uninfected individuals when exposed to unfavourable conditions of temperature and humidity (high saturation deficit) (Herrmann and Gern, 2010). With respect to tick

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behaviour, a number of studies have shown that *Borrelia*-infected ticks move less than uninfected individuals. Alekseev et al. (2000) noticed that the locomotor activity in *Borrelia*-infected *I. ricinus* and *I. persulcatus* ticks was reduced compared with uninfected individuals. Lefcort and Durden (1996) reported that *Borrelia*-infected adult *Ixodes scapularis* were less active than uninfected ticks. We observed that *I. ricinus* nymphs infected with *B. burgdorferi* s.l. moved less within a humidity gradient than uninfected individuals (Herrmann and Gern, 2012). These observations led us to hypothesise that *Borrelia* spirochetes are associated with high energy reserves (measured as fat content) in *I. ricinus*. The purpose of this experiment was to test our hypothesis.

2. Material and methods

2.1. Pilot study

A pilot study was performed to determine whether it was possible to quantify fat content in field-collected *I. ricinus* ticks, followed by *Borrelia* detection. We wanted to make sure that the different steps used to remove fat from ticks (such as exposure to high temperature and 3-day chloroform immersion, described in Section 2.3) did not interfere with the efficiency of *Borrelia* detection and genospecies identification in tick carcasses (described in Sections 2.4 and 2.5). The pilot study was conducted on 80 nymphs collected in May ($n = 40$) and September ($n = 40$) 2010, and maintained as described in Section 2.2.

2.2. Tick collection and maintenance

The sampling site was a mixed forest (dominated by deciduous trees) situated 600 m above sea level on the south-facing slope of Chaumont Mountain, Neuchâtel, Switzerland (47°00′ N, 6°57′ E). Nine hundred questing *I. ricinus* nymphs were sampled by flagging low vegetation using a 1 m^2 cotton towelling flag on April 15th 2011. In the laboratory, ticks were kept over water in a box with a tight-fitting lid (98% relative humidity; RH) and placed within a cold chamber at 4 °C in the dark for 3 months as described in Crooks and Randolph (2006).

2.3. Fat content quantification

The fat content of each of 900 nymphs was measured as described in Randolph and Storey (1999). Ticks were incubated in an oven at 70 °C for 24 h to remove the water from their body. Following incubation, the dried ticks were immediately transferred to a desiccator until subsequent weighing (initial dry mass). Each nymph was individually weighed to the nearest 0.1 μg using an ultra-microbalance (UMT 5 Comparator, Mettler Toledo, Greifensee, Switzerland) kept in a room with controlled temperature, atmospheric pressure and light intensity. To remove the fat content, each nymph was immersed in chloroform for 24 h and this step was repeated twice. Nymphs were re-dried in an oven at 70 °C for 24 h, transferred to a desiccator and re-weighed to obtain the fat-free dry mass (hereafter referred to as "body size") and thereby the fat content (Crooks and Randolph, 2006). Tick carcasses were stored at −80 °C for *Borrelia* detection and genospecies identification.

Randolph et al. (2002) observed that fat content was positively correlated with tick body size. Fat content was therefore corrected for body size using the two different methods described by Randolph et al. (2002) and Crooks and Randolph (2006) to facilitate comparisons between our study and the published literature. The simpler correction is achieved by dividing fat content by body size (Crooks and Randolph, 2006), whereas using the square root of fat content as the numerator is necessary to achieve a more complete correction for body size (referred to as the 'fat index' by Randolph et al., 2002).

2.4. Borrelia detection and quantification using quantitative PCR

DNA was extracted from tick carcasses using ammonium hydroxide as previously described (Herrmann and Gern, 2010, 2012). Briefly, ticks (each one in its own Eppendorf tube) were boiled for 15 min at 100 °C in 100 μl of 0.7 M NH_4OH and then cooled for a few minutes. The ticks were boiled again for 15–20 min at 100 °C in the open tubes to evaporate the ammonia and cooled at room temperature. Negative controls were included during DNA extraction, which consisted of reagents without template DNA.

A quantitative PCR amplifying a fragment of the flagellin gene (Schwaiger et al., 2001) was used to detect and quantify *Borrelia* DNA in each field-collected tick that had been subjected to fat content analysis. The strain *B. afzelii* NE1817 was used as the quantification standard as follows. Spirochete concentration in culture was estimated using a Helber chamber. To extract spirochete DNA, the culture was washed twice with PBS/$MgCl_2$, the pellet was resuspended in 30 μl of distilled water and heated for 15 min at 100 °C (Postic et al., 1994). The *Borrelia* DNA stock was aliquoted at 10^5 spirochetes per μl and stored at −20 °C. Serial dilutions were made from stored spirochete DNA in order to obtain five standard solutions with concentrations of *Borrelia* DNA ranging from 10 to 10^5 copies per μl.

The 50-μl quantitative PCR mixture (Schwaiger et al., 2001; Herrmann and Gern, 2010, 2012) consisted of 10 μl of 5× buffer, 5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 1 μl of 20 μM FlaF1A forward primer, 1 μl of 20 μM FlaR1 reverse primer, 1 μl of 10 μM FlaProbe1 probe, 0.25 μl of HotStart Taq Polymerase (Kapa Biosystems, Woburn, MA, USA), 20.75 μl of water and 10 μl of the extracted DNA. In each run, one extraction negative control (10 μl, see above), one PCR negative control (10 μl of water instead of 10 μl of the extracted DNA) and three series of the five standards were included. Following an incubation step at 95 °C for 10 min, the samples were submitted to 45 repeated amplification cycles (95 °C for 15 s, 60 °C for 1 min) (Schwaiger et al., 2001) in an iCycler Optical Module (Bio-Rad, Reinach, Switzerland) using strip PCR tubes and flat caps (Scientific Specialties Inc, Lodi, CA, USA).

2.5. Borrelia genospecies identification

PCR and Reverse Line Blotting (RLB) were used to identify the *Borrelia* genospecies in the ticks that were positive for *Borrelia* DNA in the quantitative PCR as described in Herrmann and Gern (2010, 2012). The variable spacer region between two repeated copies of the 23S and 5S ribosomal genes was amplified (Alekseev et al., 2001).

The 25-μl touchdown PCR mixture (Herrmann and Gern, 2010, 2012) consisted of 2.5 μl of 10× buffer, 0.5 μl of 10 mM dNTPs, 0.5 μl of 10 μM 23S-Bor forward primer, 0.5 μl of 10 μM B5S-Bor reverse primer, 0.125 μl of Taq Polymerase (Qiagen, Basel, Switzerland), 15.875 μl of water and 5 μl of the extracted DNA. Positive and negative controls were included in each PCR run. Positive controls consisted of isolates of *B. afzelii* (NE632), *B. lusitaniae* (PotiB1), *B. burgdorferi* s.s. (B31) or *B. garinii* (NE11) and negative controls contained water. PCR amplifications were run in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) by using a touchdown PCR program (Burri et al., 2007). The amplification started with an initial denaturation at 94 °C for 3 min, followed by denaturation at 94 °C for 20 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. During the subsequent cycles, the annealing was lowered by 1 °C until it reached 52 °C. Another 40

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repeated amplification cycles (94 °C for 20 s, 52 °C for 30 s, 72 °C for 30 s) followed the touchdown program. The PCR ended with a final extension at 72 °C for 7 min.

For *Borrelia* identification by RLB, PCR products were hybridised to three generic oligonucleotide probes (SL1, SL2, SL3) and 14 specific oligonucleotide probes (SS, GA, GANE, AF, VSNE, LusiNE, RFLNE, SpiNE, SpiNE1, LusiNE1, LusiNE2, GANE1, BisNE, BisNE1) (Poupon et al., 2006; Gern et al., 2010) blotted in lines on an activated Biodyne C membrane (Pall Europe Ltd, Portsmouth, UK) using a Miniblotter 45 (Immunetic, Cambridge, MA, USA). Hybridization was visualised by incubating the membrane with enhanced chemiluminescence detection liquid (Amersham Biosciences Europe, Switzerland) and by exposing the membrane to X-ray film (Hyperfilm, GE Healthcare, UK).

2.6. Statistical analysis

2.6.1. Pilot study

2.6.1.1. Effect of season on the body size-fat content relationship. An independent two-sample *t*-test was used to test whether there was a difference in body size between spring and autumn nymphs. Analysis of co-variance (ANCOVA) was used to test the following three hypotheses: (i) whether there was a relationship between body size and fat content, (ii) whether fat content for a given body size changed with season, and (iii) whether the slope of the body size-fat content relationship was the same between spring and autumn nymphs. As tick body size and fat content are related to each other via a power relationship, where fat content = a (body size)^b (a and b being two constants), both variables were log-transformed to control the variances and to linearise the relationship, where log (fat content) = log(a) + b $*$ log(body size).

2.6.2. Main study

2.6.2.1. Effect of Borrelia genospecies on spirochete load in infected ticks. The effect of genospecies on spirochete load was analysed for the subset of ticks that were infected with at least one of the four most common *Borrelia* genospecies (n = 183 ticks). Infections by *B. bavariensis* (n = 5) and *B. miyamotoi* (n = 6) were excluded from the statistical analyses due to their low prevalence. Parasite burden variables such as spirochete load are almost never normally distributed and are best modelled using generalised linear models with a negative binomial distribution (Wilson et al., 1996; Wilson and Grenfell, 1997). The glm.nb() function in R was therefore used to test whether a particular genospecies alone or in combination with another genospecies (interaction) had a positive or negative effect on *Borrelia* spirochete number. The glm.nb() function was also used to test whether there was a relationship between spirochete load and body size or fat content.

2.6.2.2. Effect of Borrelia infection status on the body size-fat content relationship. An independent two-sample *t*-test was used to test whether there was a difference in body size between *Borrelia*-infected and uninfected nymphs. ANCOVA was used to test the following three hypotheses: (i) whether there was a linear relationship between body size and fat content (after log-transforming these two variables), (ii) whether *Borrelia* infection status influenced tick fat content for a given body size, and (iii) whether the slope of the body size-fat content relationship was the same between *Borrelia*-infected and uninfected ticks. For the subset of nymphs (n = 183) infected with the four most common *Borrelia* genospecies (i.e. *B. afzelii*, *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana*), ANCOVA was also used to test whether *Borrelia* genospecies influenced the slope or the intercept of the body size-fat content relationship.

All statistics were calculated with R for Mac OS X (R Development Core Team, 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org). All means are reported with their standard error. The *P* value was considered significant when below 0.05.

3. Results

3.1. Pilot study

Tick body size of questing *I. ricinus* nymphs was slightly higher in the spring (72.9 ± 16.5 μg; n = 40) than the autumn sample (67.9 ± 14.7 μg; n = 40) but this difference was not significant (t = 1.509, df = 78, P = 0.135). The ANCOVA found no significant interaction between season and tick body size ($F_{1,76}$ = 2.052, P = 0.156) and there was no significant relationship between body size and fat content ($F_{1,77}$ = 1.618, P = 0.207). However, the mean fat content of autumn nymphs (13.2 ± 10.7 μg; range = 2–47 μg) was 1.7 times greater than that of the spring nymphs (7.8 ± 7.1 μg; range = 1–31 μg) and this difference was statistically significant ($F_{1,77}$ = 6.452, P = 0.013). The estimates of fat content divided by body size and the fat index following Randolph et al., 2002 are shown in Table 1.

With respect to *Borrelia* infection, 25.0% (10/40) of the spring nymphs and 27.5% (11/40) of the autumn nymphs were infected. The mean and median spirochete numbers were higher in spring than in autumn (Table 2). Identification of *Borrelia* genospecies was successful in nine of 10 spring nymphs and 10 of 11 autumn nymphs. Spring nymphs were infected with *B. afzelii* (n = 7), *B. valaisiana* (n = 1) and unidentified *B. burgdorferi* s.l. (n = 1). Autumn individuals were infected with *B. garinii* (n = 3), *B. afzelii* (n = 1), *B.*

Table 1
Fat content of *Ixodes ricinus* nymphs in the present study and in previously published studies conducted in Switzerland and the United Kingdom. For each study we report three fat content variables (i) absolute fat content (measured in μg), (ii) fat content corrected for size (following Crooks and Randolph, 2006), and (iii) the fat index (following Randolph et al., 2002). For each of the three fat content variables we report the mean, SD and median. *n* refers to the number of nymphs in the study. Cells with data missing are left blank.

Study	Season and year	*n*	Absolute fat content		Fat content corrected for size		Fat index	
			Mean (±SD)	Median	Mean (±SD)	Median	Mean (±SD)	Median
Pilot study	Spring 2010	40	7.8 (±7.1)	5.5	0.118 (±0.119)	0.077	0.037 (±0.021)	0.032
	Autumn 2010	40	13.2 (±10.7)	9.5	0.205 (±0.171)	0.162	0.052 (±0.028)	0.047
Main study	Spring 2011	900	7.8 (±4.3)	7.1	0.119 (±0.069)	0.106	0.041 (±0.013)	0.039
Herrmann and Gern (2010)	Spring 2009	40	7.1 (±6.8)	5	0.100 (±0.103)	0.071	0.034 (±0.018)	0.033
Herrmann and Gern (2012)	Spring 2010	40	9.3 (±6.6)	8	0.137 (±0.099)	0.115	0.043 (±0.018)	0.039
Steele and Randolph (1985)	Spring 1982		~6.0					
Randolph et al. (2002)	Spring 1998	36					~0.015	
	Autumn 1998	15					~0.032	
Crooks and Randolph (2006)	Spring 2004	40	4.7 (±2.7)					

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Table 2
Borrelia prevalence, mean and median spirochete number in questing *Ixodes ricinus* nymphs sampled during three different years in Neuchâtel, Switzerland.

Study	Season and year	Number of tested ticks	Prevalence of *Borrelia*-infected ticks	Spirochete number per tick	
				Mean	Median
Pilot study	Spring 2010	40	10 (25.0%)	41,475	2,410
	Autumn 2010	40	11 (27.5%)	14,752	1,390
Main study	Spring 2011	900	191 (21.2%)	15,556	3,410
Herrmann and Gern (2010)	Spring 2009	500	126 (25.5%)	18,638	2,760
Herrmann and Gern (2012)	Spring 2010	1500	448 (29.9%)	33,971	4,300

valaisiana ($n = 1$), and mixed infections of *B. garinii* and *B. valaisiana* ($n = 4$), and *B. afzelii* and *B. miyamotoi* ($n = 1$).

3.2. Main study

3.2.1. Fat content quantification

Mean and median tick body size of questing *I. ricinus* nymphs sampled in spring 2011 were 66.4 ± 13.2 μg and 66 μg, respectively ($n = 900$; range = 23.6–128.4 μg). Mean fat content in these nymphs was 7.8 ± 4.3 μg while median fat content was 7.1 μg (range = 1–29 μg). The estimates of fat content corrected for body size and the fat index following Randolph et al. (2002) are shown in Table 1.

3.2.2. Borrelia infection in ticks

Of the 900 questing *I. ricinus* nymphs, 21.2% ($n = 191$) were infected with *B. burgdorferi* s.l. Identification of *Borrelia* genospecies was possible in 188 of 191 infected nymphs. Ticks were primarily infected by one *Borrelia* genospecies (89.9%, 169/188). Infections by two *Borrelia* genospecies were considerably less frequent (10.1%, 19/188) while no tick was infected by three or more genospecies. Among single infections, *B. afzelii* was the most common (64.5%, 109/169), followed by *B. valaisiana* (13.6%, 23/169), *B. garinii* (11.8%, 20/169), *B. burgdorferi* s.s. (7.1%, 12/169) and *B. bavariensis* (3.0%, 5/169). The 19 mixed infections consisted of three types: *B. afzelii* and *B. miyamotoi* (31.6%, 6/19), *B. afzelii* and *B. burgdorferi* s.s. (36.8%, 7/19), and *B. garinii* and *B. valaisiana* (31.6%, 6/19). The genospecies *B. bissettii*, *B. lusitaniae* and *B. spielmanii* were not detected.

3.2.3. Effect of Borrelia genospecies on spirochete load in infected ticks

The mean spirochete load in *Borrelia*-infected nymphs was 15,556 spirochetes per nymph ($n = 191$ nymphs) while the median spirochete load was 3,410 spirochetes (range = 1–496,000 spirochetes; Table 2). The mean spirochete load of *B. garinii* infections (22,442) was higher than *B. afzelii* (11,144), *B. burgdorferi* s.s. (9,886) and *B. valaisiana* (7,116) infections. However, the generalised linear model (GLM) analysis found no effect of any of the genospecies or their two-way interactions on the spirochete load inside the tick ($\chi^2 = 5.93$, df = 5, $P = 0.313$; Fig. 1). Similarly, the GLM found no effect of body size or fat content on the spirochete load inside the tick ($\chi^2 = 3.78$, df = 2, $P = 0.151$). For the GLM analyses, the residual deviances were similar to the residual degrees of freedom, suggesting that the negative binomial error function was a reasonable fit for the spirochete load data.

3.2.4. Effect of Borrelia infection on the body size – fat content relationship

Borrelia-infected nymphs had a larger body size (69.3 ± 12.3 μg; $n = 191$) than uninfected individuals (65.7 ± 13.3 μg; $n = 709$) and the difference was highly significant ($t = 3.639$, df = 898, $P < 0.001$). The ANCOVA found no significant interaction between *Borrelia* infection status and tick body size ($F_{1,896} = 0.303$, $P = 0.582$), indicating that the slopes of the fat content versus tick body size regressions were the same between infected and uninfected ticks (Fig. 2). Larger nymphs had more fat as there was a po-

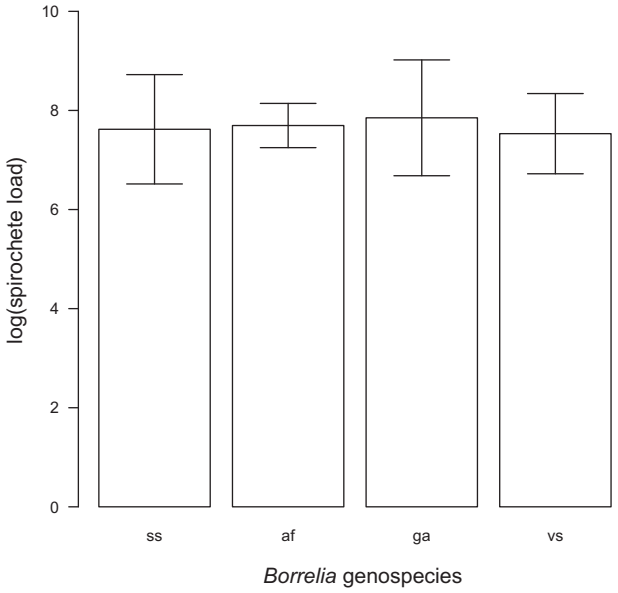

Fig. 1. Log-transformed spirochete loads for the four most common *Borrelia* genospecies: *Borrelia afzelii* (af), *Borrelia burgdorferi* sensu stricto (ss), *Borrelia garinii* (ga) and *Borrelia valaisiana* (vs) found in *Ixodes ricinus* nymphs sampled in Neuchâtel, Switzerland. Shown are the means and the 95% confidence intervals.

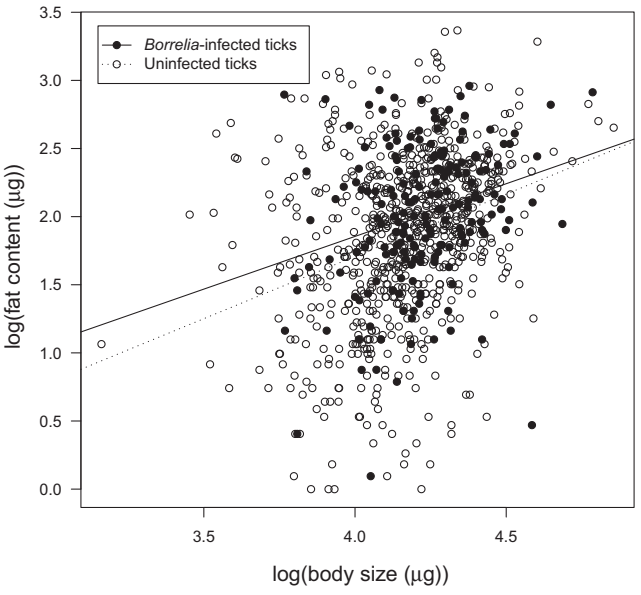

Fig. 2. The relationship between fat content and tick body size for uninfected and *Borrelia*-infected *Ixodes ricinus* nymphs.

sitive and statistically significant relationship between body size and fat content ($F_{1,897} = 89.979$, $P < 0.001$). The main effect of infection status was also statistically significant ($F_{1,897} = 6.265$, $P = 0.012$), indicating that infected ticks had a higher fat content than uninfected ticks after controlling for body size. For an average body size of 66.4 μg, the mean fat content of a *Borrelia*-infected tick (7.40 μg) was 12.1% higher than that of an uninfected tick (6.60 μg). The lines of best fit are as follows: uninfected ticks: log (fat content) = $-1.854 + 0.891 * \log$ (body size); infected ticks: log (fat content) = $-1.738 + 0.891 * \log$ (body size).

There was no effect of *Borrelia* genospecies on the slope ($F_{3,156} = 1.176$, $P = 0.321$) or intercept ($F_{3,159} = 0.978$, $P = 0.405$) of the body size-fat content relationship in the subset of ticks infected with the four most common *Borrelia* genospecies ($n = 183$). Thus for a given body size, infected ticks had a similar fat content regardless of the *Borrelia* genospecies they harboured. As before, there was a significant and positive relationship between body size and fat content ($F_{1,159} = 11.062$, $P = 0.001$).

4. Discussion

Parasites of arthropods can affect the resource levels of their hosts. For example, in a triatomine-trypanosomatid system, *Rhodnius prolixus* bugs infected by the parasite *Trypanosoma rangeli* show a twofold increase in their amount of lipids compared with uninfected individuals (Ferreira et al., 2010). In this study, we found that for a given body size, *Borrelia*-infected *I. ricinus* nymphs had a higher fat content than uninfected ticks. Similarly, Gassner (2010) showed that infected nymphs had a higher fat content than uninfected nymphs, but that study was restricted to *I. ricinus* nymphs infected by *B. afzelii*. Here, ticks were infected by various *Borrelia* genospecies (*B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. bavariensis* and by *B. miyamotoi*, a relapsing fever like spirochete), but for a given body size, infected ticks had a similar fat content regardless of the *Borrelia* genospecies they harboured.

The higher fat content in *Borrelia*-infected nymphs may be caused by a variety of processes operating on tick-host interactions (blood feeding), on tick physiology (digestion, and moulting), and/or tick behaviour. The presence of *Borrelia* spirochetes in the vertebrate host might modify the quantity or quality of the larval blood meal, resulting in higher fat content in the nymph. Previous work on *Babesia microti* found that this tick-borne parasite increased the blood meal size and thus the engorged weight in immature *Ixodes trianguliceps* (Randolph, 1991) and *I. scapularis* ticks (Hu et al., 1997). One potential explanation for this phenomenon is that the *B. microti* parasites suppress the acquired immune response of rodents against ticks. Previous studies have shown that rodents can develop an acquired immunity to ticks, which reduces the quality of the tick blood meal (Oberem, 1984; Dizij and Kurtenbach, 1995) and that *B. microti* suppresses the rodent immune system (Purvis, 1977; Gray and Phillips, 1983). Thus, ticks feeding on *Babesia*-infected, immunosuppressed mice may obtain larger blood meals (resulting in higher fat content) than ticks feeding on uninfected mice. Although it is not known whether a similar mechanism operates at the interface between *Borrelia*, *I. ricinus* and the reservoir host, there is evidence that *Borrelia* suppresses the immune response in vertebrate hosts (Giambartolomei et al., 1998; Rupprecht et al., 2008), which presumably would also help blood feeding of ticks. Additionally, *Borrelia* might modify the quality of the blood meal in the vertebrate host (e.g. by increasing the glucose concentration) thereby increasing tick fat content. However, this phenomenon has never been reported in the literature.

Alternatively, *Borrelia* spirochetes might alter tick gene expression in such a way that fat storage is increased. A recent study found that entomopathogenic fungal parasites changed the lipid profiles in cattle ticks (Angelo et al., 2010) suggesting that parasites can change the expression of genes related to fat metabolism and fat levels in ticks. Similar phenomena might take place via mechanisms related to the digestion and moulting process in *Borrelia*-infected ticks, resulting in increased fat content in nymphs harbouring spirochetes. The *4E-BP* gene involved in lipid storage (Kume et al., 2012) or the *ATG* genes involved in autophagy (Umemiya-Shirafuji et al., 2010) in ticks might be potential targets for spirochete manipulation.

Finally, *Borrelia* spirochetes might alter behaviour of ticks in a way that conserves fat reserves. Fat reserves can be metabolised to provide energy as well as water since the latter is a by-product of fat metabolism. *Ixodes ricinus* uses this energy for questing activity and maintenance of water balance under desiccating environmental conditions (Van Es et al., 1998; Randolph and Storey, 1999). A number of studies have shown that *Borrelia*-infected nymphs move less than uninfected individuals (Lefcort and Durden, 1996; Alekseev et al., 2000; Herrmann and Gern, 2012). Therefore, we expect *Borrelia*-infected, low-activity ticks to have higher fat levels than their uninfected, high-activity counterparts. In conformity with this hypothesis, *Borrelia* induces low activity levels in the tick, thereby conserving the tick fat reserves. In addition, energy is better conserved by infected ticks since their larger body size enhances water retention. Indeed, since surface to volume ratio is smaller in larger individuals, larger ticks lose proportionally less water than smaller ticks. Our study thus suggests that *Borrelia*-infected ticks can spend more time questing, thereby increasing their chances of finding a host compared with uninfected ticks, which have to return more frequently to the leaf litter to rehydrate. There is the potential for positive feedback mechanisms where larger, *Borrelia*-infected ticks do not suffer the costs of rehydration as often as smaller, uninfected ticks, which allows them to further conserve their fat supplies.

The present study also suggests that *Borrelia*-infected ticks should be more tolerant to desiccation than uninfected ticks, which is consistent with the results from our previous studies: (i) that *Borrelia*-infected ticks survived better under desiccating conditions (Herrmann and Gern, 2010) and (ii) that *Borrelia*-infected ticks were less likely to move to an environment that was favourable for maintaining water balance (Herrmann and Gern, 2012). Our results may also explain observations by Naumov (2003) who reported longer survival in *I. ricinus* and *I. persulcatus* ticks infected with *B. burgdorferi* compared with uninfected individuals. Ticks with a higher fat content relative to body size have larger energy reserves and therefore a higher likelihood of survival.

Parasites consume host energy resources. In *Borrelia*-infected larval ticks, the spirochete load increases dramatically following the blood meal (Gern et al., 1990; Piesman et al., 1990; De Silva and Fikrig, 1995) presumably by consuming the tick energy reserves. However, no significant relationship between tick energy reserves and spirochete load was found, suggesting that the spirochete load represents a negligible energetic cost for the tick. To check this hypothesis, we calculated how much energy it takes to grow one spirochete using tick glucose under anaerobic conditions. *Borrelia burgdorferi* uses glycolysis to meet its energetic demands for maintenance and growth (Von Lackum and Stevenson, 2005) as the genome lacks enzymes for both the citric acid cycle and oxidative phosphorylation (Fraser et al., 1997). A large cylindrically shaped spirochete (length: 30 μm, diameter: 0.3 μm) has a volume of 2.121 μm^3 and a dry mass of $5.089 * 10^{-13}$ g (cells have a density of 1.2 g/cm^3 of which 80% is water). Under anaerobic conditions, it takes $5.089 * 10^{-12}$ g of glucose to grow a single spirochete, corresponding to $8.143 * 10^{-11}$ kJ of energy. The average tick has a fat content of 7.8 μg, which corresponds to $2.886 * 10^{-4}$ kJ of energy. Thus the energy requirements to grow the median population of 3,410 spirochetes using glucose under

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anaerobic conditions corresponds to 0.10% of the total fat reserves of the nymphal tick. These bioenergetics calculations suggest that the median spirochete population consumes a very small portion of the tick energy budget and provide a potential explanation for the lack of a negative relationship between tick energy reserves and spirochete load.

The present study assumes that *Borrelia* infection is causal in influencing variation in tick body size and fat content. However, the alternative hypothesis that tick body size could influence the probability that a tick becomes infected cannot be excluded. For example, larger ticks might take larger blood meals, which are more likely to contain spirochetes. However, this explanation is unlikely as previous studies have shown that most of the variation in host-to-tick transmission rates is caused by differences among host individuals (Gern et al., 1994) and, in particular, by differences in spirochete load inside the host tissue (Raberg, 2012). In addition, the possibility that natural selection in the field might have caused differences in relative fat content between *Borrelia*-infected and uninfected ticks prior to sampling cannot be excluded. For example, if *Borrelia* spirochetes exert some sort of survival cost on ticks, only those individuals with a high fat content (relative to body mass) survived to be included in the sample. This explanation seems unlikely since our bioenergetic calculations suggest that the median spirochete population consumes a negligible portion of the tick energy budget. Sampling bias could also produce the observed results if flagging is more likely to capture *Borrelia*-infected ticks with high fat content than low fat content but no such fat content-related sampling bias exists for uninfected ticks. Future studies should include manipulative experiments to test whether ticks feeding on *Borrelia*-infected hosts are larger and have higher fat content than ticks feeding on control hosts.

The pilot study showed that the protocol for fat content quantification did not interfere with the subsequent protocol for *Borrelia* detection and genospecies identification. The mean and median spirochete loads in individuals collected in spring and autumn (Table 2) were similar to the levels observed in an earlier study (unpublished data; spring: mean = 45,850, median = 3,390 spirochetes per tick; autumn: mean = 16,231, median = 2,275 spirochetes per tick) where nymphs were not tested for fat content prior to the *Borrelia* infection analysis. *Borrelia* prevalences in the pilot study (spring: 25%; autumn: 17.5%) were similar to the unpublished study (spring: 26.4%, 595/2250; autumn: 26.7%, 120/450) and the prevalences of the *Borrelia* genospecies were similar to two previously published studies in the same area (Herrmann and Gern, 2010, 2012). Finally, the fat content of the spring nymphs was similar to that previously described in Switzerland (Table 1). The similarity between the pilot study and the literature confirmed our belief that the *Borrelia* quantification and identification results of the main study were not biassed by the upstream fat content protocol.

To date, studies quantifying fat content in *I. ricinus* have been conducted in Wales (Steele and Randolph, 1985), England (Randolph and Storey, 1999; Randolph et al., 2002; Crooks and Randolph, 2006), and Switzerland (Herrmann and Gern, 2010, 2012). In Switzerland, the fat content of spring nymphs was 1.5 to 2.0 times higher than in the United Kingdom (UK) (Table 1). The *Borrelia* infection rate of nymphs in Switzerland (>21.2%) was much higher than that in the UK (<12.4% according to Vollmer et al., 2011). Thus one potential explanation is that Swiss larvae are more likely to feed on *Borrelia*-infected hosts and thus moult into *Borrelia*-infected nymphs with higher fat content. Ticks in Switzerland and the UK also may have evolved different body sizes and/or fat content levels in response to the very different climatic conditions in these two countries with UK ticks experiencing much higher rainfall (Gray, 1998). Randolph and Storey (1999) observed that ticks under dry conditions consume their fat resources twice as fast as those under wet conditions, which might explain why Swiss ticks have evolved to have more fat than their UK counterparts.

In the pilot study, tick body size was slightly (although not significantly) higher in the spring than the autumn sample. This result is consistent with a previous study showing that larger ticks become active earlier in the spring while ticks questing in autumn reflect the full size range (Randolph et al., 2002). Fat content in autumn nymphs was 1.4 to 1.9 times higher than spring nymphs. This phenomenon has been reported previously in southern England (Randolph et al., 2002). The autumn nymphs have higher fat content because they obtained their larval meal that same summer whereas the spring nymphs obtained their larval blood meal the previous summer and consumed their fat stores during the winter months.

We found a strong positive association between *Borrelia* infection status and body size corrected for fat content. We suggest that *Borrelia* infection increases tick fat content through mechanisms operating in the host (e.g. *Borrelia*-induced immune suppression of anti-tick immunity) or the tick (e.g. *Borrelia*-induced manipulation of tick fat metabolism). Higher fat content allows *Borrelia*-infected ticks to better maintain their water balance under desiccating conditions. This explanation accounts for our previous observations that *Borrelia*-infected ticks are more likely to survive desiccating conditions than uninfected ticks and that the former will quest for a longer period without having to move to an environment that is favourable for maintaining water balance (Herrmann and Gern, 2010, 2012). Additional studies are needed and expected on this topic so as to understand the multiple ways in which *Borrelia* and *Ixodes* have evolved to benefit from their close and long-lasting association.

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