Do the level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks?

C. HERRMANN *and* L. GERN*

Institute of Biology, Laboratory of Eco-Epidemiology of Parasites, University of Neuchâtel, Emile Argand 11, Case Postale 158, 2000 Neuchâtel, Switzerland

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SUMMARY

Ixodes ricinus horizontal movement within a humidity gradient and the influence of infection by *Borrelia burgdorferi* sensu lato (s.l.) on tick walking were investigated. Nymphs were placed within an arena containing a humidity gradient ranging from 45 to 95% relative humidity (RH). After 1 h of acclimation at 70% RH ticks were released so that they could either stay, or walk towards either the wet or the dry end. Their position was recorded 2 h post-release. Fat content was quantified and *Borrelia* infection was detected using real-time PCR and PCR followed by Reverse Line Blotting. Among the 1500 ticks tested, 29·85% were infected. More low-fat nymphs walked inside the arena than high-fat individuals. When nymphs walked, more low-fat ticks walked towards wetter than drier air, whereas more high-fat individuals walked towards drier than wetter air. Among high-fat nymphs, a lower proportion of *Borrelia*-infected ticks walked inside the arena compared to uninfected individuals, as though spirochetes manipulated their arthropod vector to stay. However, *Borrelia* infection had no effect on walking direction towards the dry or the wet end. Hence, it appears that *I. ricinus* nymphs walk horizontally over short distances within a humidity gradient depending on both energy resources and *Borrelia* infection.

Key words: *Borrelia burgdorferi* s.l., *Ixodes ricinus*, energy resource, humidity, tick movement, vector behaviour.

INTRODUCTION

In Europe the hard-bodied tick *Ixodes ricinus* (L.) (Acari: Ixodidae) is the main vector of *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of the most commonly reported tick-borne disease of the northern hemisphere, Lyme borreliosis. Eleven *Borrelia* genospecies have been found associated with this tick species in Europe: *B. afzelii, B. bavariensis, B. bissettii, B. burgdorferi* sensu stricto (s.s.), *B. carolinensis, B. finlandensis, B. garinii, B. lusitaniae, B. spielmanii, B. valaisiana*, and a *Borrelia* genospecies related to relapsing fever spirochetes, *B. miyamotoi* (Rauter and Hartung, 2005; Margos *et al*. 2009; Gern *et al*. 2010; Cotté *et al*. 2010; Casjens *et al*. 2011).

The biggest survival challenge for ticks is to maintain water balance whilst living in a relatively dry environment. This is even more essential to *I. ricinus* since this tick is extremely sensitive to temperature and humidity compared with other tick species (MacLeod, 1935; Lees, 1946; Aeschlimann, 1972; Knülle and Rudolph, 1982; Sonenshine, 1991). Hence, saturation deficit, a measure of the drying power of the atmosphere depending on both temperature and relative humidity (Randolph and Storey, 1999), limits *I. ricinus* duration of questing (Perret *et al*. 2003, 2004) and survival in nature (Perret, 2002; Perret *et al*. 2000, 2004; Burri *et al*. 2007; Morán Cadenas *et al*. 2007) and in laboratory settings (Herrmann and Gern, 2010).

Whilst questing on vegetation for their vertebrate hosts, *I. ricinus* ticks increase water loss so that they must return periodically to moister surroundings, such as the litter layer. There, they can extract water vapour from the atmosphere above a certain humidity level (Knülle and Rudolph, 1982). Hence, ticks are considered to move primarily in the vertical rather than the horizontal plane in order to seek the two resources that are necessary to their survival, i.e. water vapour and/or hosts (Goddard, 1993). However, Perret *et al*. (2003) observed that nymphs walked great distances, up to 9·56 m in a laboratory setting. In these experiments, ticks were constrained within vertical channels and, although it was difficult to evaluate the part that would represent horizontal movements, Perret *et al*. (2003) assumed that some of the displacements could represent horizontal movements. Later, Crooks and Randolph (2006) showed that nymphs with high-energy resources walked horizontally but over short distances only. Furthermore, they also reported that ticks that were slightly dehydrated were more likely to walk towards fully saturated than drier air.

* Corresponding author: Institute of Biology, Laboratory of Eco-Epidemiology of Parasites, University of Neuchâtel, Emile Argand 11, Case Postale 158, 2000 Neuchâtel, Switzerland. Tel: +41 32 718 30 52. Fax: +41 32 718 30 01. E-mail: lise.gern@unine.ch

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Recently, we observed that field-collected *I. ricinus* ticks infected by *B. burgdorferi* s.l. survived better under unfavourable conditions of temperature and humidity (saturation deficit), suggesting that infected ticks might be affected differently by surrounding humidity (Herrmann and Gern, 2010). In that context, it was interesting to determine whether field-collected *I. ricinus* nymphs of varying hydration status and level of energy reserves displayed a different walking activity and direction within a humidity gradient in the horizontal plane when infected by *B. burgdorferi* s.l. spirochetes.

MATERIALS AND METHODS

Tick collection and maintenance

The sampling site was a mixed forest (deciduous dominant) situated at 600 m above sea level (47°00′N and 6°57′E) on the south-facing slope of Chaumont Mountain (Neuchâtel, Switzerland) (Herrmann and Gern, 2010). Host-seeking *I. ricinus* nymphs were sampled by flagging the low vegetation using a 1-m^2 terry flag on 3 consecutive days in May 2010. In the laboratory, collected nymphs were divided into 3 groups exposed to different conditions. To obtain high-fat and fully hydrated ticks (H&F, Group 1), ticks were held over water in a box with a tight-fitting lid (98% relative humidity; RH) in the dark within a cold chamber at 4 °C for at least 4 weeks as described by Crooks and Randolph (2006). Group 2 contained low-fat and moderately hydrated (L&M) ticks. These ticks were maintained over water in a transparent plastic box with small holes allowing airflow (87% RH) at room temperature (~23 °C); while group 3 ticks, low-fat and fully hydrated (L&F), were held over water in a transparent plastic box with a tight-fitting lid (98% RH) at room temperature (~23 °C). L&M and L&F ticks were exposed to the natural daylight conditions in May–June for 4 weeks before being returned to the cold chamber until use (Crooks and Randolph, 2006).

Fat content analysis

Fat content was quantified in order to verify whether fat content of high-fat and low-fat groups of ticks differed. Samples of 40 nymphs from each group (H&F, L&F, and L&M) were analysed for their fat content as described by Randolph and Storey (1999) and as calculated by Crooks and Randolph (2006). Briefly, ticks were dried at 70 °C for 24 h, weighed individually to the nearest 1 μg, washed in 3 changes of chloroform for 24 h each, re-dried at 70 °C for 24 h and re-weighed using a MX5 microbalance (Mettler Toledo, Greifensee, Switzerland).

Attraction tests

Choice arenas were slightly modified from those described by Crooks and Randolph (2006). They were made of 2 transparent polystyrene boxes (19 cm × 9 cm × 9 cm) with tight-fitting lids, joined by a transparent polystyrene tunnel (3 cm diameter, 28 cm long) sealed into holes cut in one side of each box. Humidity was monitored using an EE07-PFT5 hygrometer (E+E Elektronik, Engerwitzdorf, Austria) with a probe within different parts of the arena. A small dish of water was placed in one box whereas the other box remained empty, producing a humidity gradient of 90–45% RH within 30 min. The humidity gradient did not evolve much after 3 h (end time of test, see details below), only reaching 95–45% RH. In the experimental room at ambient temperature (~23 °C), this corresponds to saturation deficits (SD) ranging from 1·10 mmHg to 10·98 mmHg (1 mmHg = 133·3 Pa), as calculated by Randolph and Storey (1999).

Each replicate consisted of a set of 20 nymphs placed into a 1 cm × 5 cm plastic culture tube (Milian, Meyrin, Switzerland) that was closed at both ends by pieces of cotton wool. Each piece of cotton wool was tied to a length of cotton thread. Experimental runs were conducted as described by Crooks and Randolph (2006). Briefly, 1 tick-laden tube was positioned in the centre of the arena tunnel with the cotton threads passing out through small holes cut into the box side that were closed by laboratory film (Parafilm M, Pechiney Plastic Packaging, Menasha, WI, USA). The arena was left undisturbed for 1 h with the lights on. Then the threads were gently pulled to remove the cotton wool from each end of the tube. The cotton wool pieces were left very close to the tube. Lights were left on for 1 h and switched off for 1 additional hour since, according to Perret *et al.* (2003), darkness induces *I. ricinus* ticks to move. Ticks were collected 2 h post-release and their position was recorded. Testing time was determined so that half of the ticks would walk out of the tube, based on the protocol of Crooks and Randolph (2006). Twenty-five replicates were performed for each tick group (H&F, L&F, and L&M). Humidity side and tick group were changed for every new experimental run.

Borrelia *infection in ticks*

Borrelia infection in ticks was detected using real-time PCR. Before DNA isolation, ticks were soaked in 70% ethanol and air-dried. Extraction of DNA from ticks was achieved using ammonium hydroxide as previously described (Guy and Stanek, 1991; Rijpkema *et al.* 1996; Herrmann and Gern, 2010). Negative controls were included during DNA isolation, which consisted of reagents without template DNA.

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A real-time PCR amplifying a fragment of the flagellin gene (Schwaiger *et al*. 2001; Herrmann and Gern, 2010) was used to detect and quantify *Borrelia* DNA in all field-collected ticks that were subjected to the humidity gradient tests. The strain *B. afzelii* NE1817 was used as quantification standard. Spirochete concentration in culture was evaluated using the Helber chamber. To extract DNA, the culture was washed twice with phosphate-buffered saline/$MgCl_2$, and the pellet was resuspended in 30 μl of 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 5 standard solutions with concentrations of *Borrelia* DNA ranging from 10^2 to 10^5 copies per μl.

The 50 μl real-time PCR mixture (Schwaiger *et al*. 2001) 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, 1 extraction negative control (10 μl, see above), 1 PCR negative control (10 μl of water instead of 10 μl of the extracted DNA) and 3 series of the 5 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).

PCR and RLB were used to identify the *Borrelia* species in the field-collected ticks that were detected positive by real-time PCR as described in Herrmann and Gern (2010). The variable spacer region between 2 repeated copies of the 23S and 5S ribosomal genes was amplified with primers 23S-Bor and B-5S-Bor (Alekseev *et al*. 2001). PCR amplifications were run in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) by using a touchdown PCR program (Burri *et al*. 2007; Herrmann and Gern, 2010). Positive and negative controls were included in each PCR. In positive controls, isolates of *B. valaisiana* (VS116), *B. lusitaniae* (PotiB1), *B. burgdorferi* s.s. (B31) or *B. garinii* (NE11) replaced DNA samples, whereas water substituted them in negative controls.

For *Borrelia* identification by RLB, PCR products were hybridized to 15 oligonucleotide probes (Rijpkema *et al*. 1995; 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 visualized 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).

Statistical analysis

The Mann-Whitney test was performed in order to determine whether there was a difference in fat content between tick groups, and whether spirochete load differed among *Borrelia* genospecies. Simple χ^2 tests were used to compare walking activity, direction of movement between tick groups. All statistics were calculated with R for Mac OS X (R Development Core Team, 2011).

RESULTS

Fat content analysis

The fat content of the low-fat moderately hydrated (L&M) ticks (4·15 ± 3·98 μg) was not statistically different from that of the low-fat fully hydrated (L&F) ticks (4·28 ± 3·38 μg) (Mann-Whitney test; $P = 0{\cdot}51$). In contrast, the fat content of high-fat fully hydrated (H&F) ticks (9·25 ± 6·64 μg) was significantly higher than that of the L&M and L&F ticks (Mann-Whitney test; H&F-L&M $P = 0$, H&F-L&F $P = 0{\cdot}0001$). According to Crooks and Randolph (2006), fat content is positively correlated with tick size, measured by tick fat-free (reduced) dry mass. Therefore a correction for size was calculated by dividing fat content by tick reduced dry mass (Crooks and Randolph, 2006). After correction for size, fat content of the H&F ticks (0·137 ± 0·099) remained significantly higher than that of the L&M (0·073 ± 0·076) and L&F (0·074 ± 0·059) ticks (Mann-Whitney test; H&F-L&M $P = 0{\cdot}0003$, H&F-L&F $P = 0{\cdot}001$) and it did not differ between the L&M and L&F ticks (Mann-Whitney test; $P = 0{\cdot}78$).

Borrelia *infection in ticks*

Among the 1500 questing nymphs that were tested for *B. burgdorferi* s.l. by real-time PCR, 29·85% ($n = 448$) were infected. Globally, infection load in questing nymphs ranged from 2 to 896 000 spirochetes per tick. Mean spirochete number was 33 971 spirochetes per nymph while median spirochete number was 4300.

Identification of *Borrelia* genospecies by RLB was possible in 413/448 nymphs. Nymphs were mainly infected by 1 *Borrelia* genospecies (86·4%) (Table 1). Six *Borrelia* genospecies were identified: *B. afzelii*, *B. bavariensis*, *B. burgdorferi* s.s, *B. garinii*, *B. miyamotoi*, and *B. valaisiana* (Table 1). *B. bissettii*, *B. lusitaniae*, and *B. spielmanii* were not observed.

Spirochete load varied between *Borrelia* genospecies (Table 1). Infections by *B. bavariensis*

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Table 1. Distribution of *Borrelia* genospecies and mean spirochete number in questing *Ixodes ricinus* nymphs in Neuchâtel, Switzerland

Borrelia genospecies[a]	Infected ticks[b]	Mean spirochete number[c]
af	165 (40·0%)	15 531
bav	24 (5·8%)	89 419
ga	86 (20·8%)	69 660
miy	2 (0·5%)	na
ss	18 (4·4%)	8985
vs	62 (15·0%)	12 981
Infection by 1 species	357 (86·4%)	32 678
af & *ga*	4 (1·0%)	na
af & *miy*	3 (0·7%)	na
af & *ss*	2 (0·5%)	na
af & *vs*	2 (0·5%)	na
bav & *vs*	4 (1·0%)	na
ga & *miy*	2 (0·5%)	na
ga & *vs*	32 (7·7%)	87 706
Infection by 2 species	52 (12·6%)	64 345
af & *ga* & *vs*	1 (0·2%)	na
ga & *miy* & *vs*	3 (0·7%)	na
Infection by 3 species	4 (1·0%)	na

[a] af, *B. afzelii*; bav, *B. bavariensis*; ga, *B. garinii*; miy, *B. miyamotoi*; ss, *B. burgdorferi* sensu stricto; *vs*, *B. valaisiana*.
[b] n = 413.
[c] Mean spirochete number was not calculated when frequency was below 10.

and by *B. garinii* consisted of significantly more spirochetes per tick (89 419 and 69 660, respectively) than infections by *B. afzelii* (15 531) (Mann-Whitney test; $P=0$ and $P=0{\cdot}008$, respectively), by *B. valaisiana* (12 981) (Mann-Whitney test; $P=0$ and $P=0{\cdot}005$, respectively), and *B. burgdorferi* s.s. (8985) (Mann-Whitney test; $P=0{\cdot}001$ and $P=0$, respectively). Infections by *B. miyamotoi* ($n=2$) were excluded from the statistical analyses due to their low frequency.

Walking activity

Within 2 h, a significantly higher number of low-fat ticks walked out of the tube than stayed inside (χ^2 test; L&M: $P=0$; L&F: $P=0$). Hence, 65% (323/500) L&M and 60% (298/500) L&F nymphs crawled out of the tube. In contrast, high-fat ticks had a higher tendency to stay inside the introduction tube (54%, 270/500) than walk out (χ^2 test; $P=0{\cdot}07$). Thus, more low-fat nymphs (L&M: 323/500 and L&F: 298/500) walked out of the tube than high-fat (H&F: 230/500) individuals (Fig. 1). The difference was highly significant for moderately hydrated ticks (χ^2 test; $P=0$) and for fully hydrated ticks (χ^2 test; $P=0$). Among low-fat individuals, there was no difference in walking activity between moderately (323/500 out of tube) and fully hydrated ticks (298/500 out of tube) (χ^2 test; $P=0{\cdot}12$).

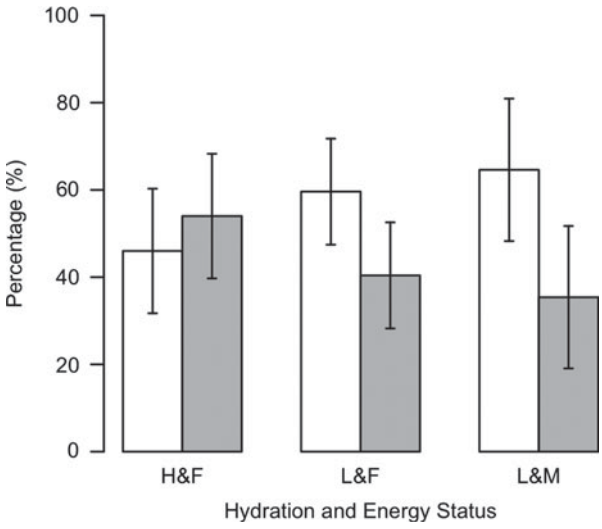

Fig. 1. The percentage of high-fat fully hydrated (H&F), low-fat fully hydrated (L&F), and low-fat moderately hydrated (L&M) *Ixodes ricinus* ticks that stayed inside (light grey bars) or walked (white bars) out of the introduction tube after 2 h within a humidity gradient.

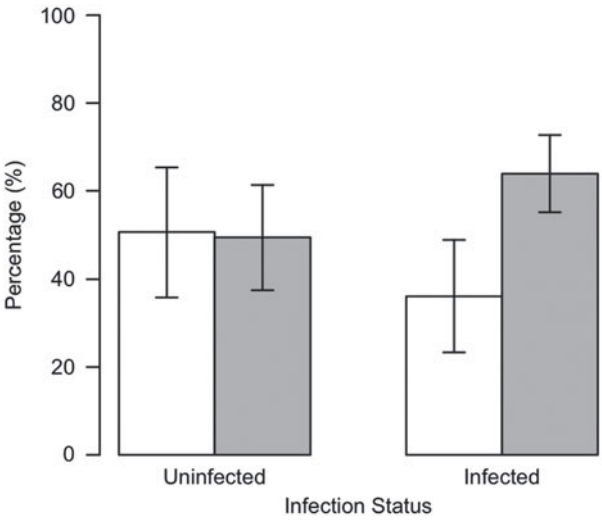

Fig. 2. The effect of *Borrelia* infection on walking activity of high-fat fully hydrated *Ixodes ricinus* nymphs after 2 h within a humidity gradient. Light grey bars represent ticks that stayed within the introduction tube while white bars represent those that walked out.

Among low-fat (L&M and L&F) ticks, walking activity was not different between uninfected and *Borrelia*-infected individuals. In fact, among L&M ticks 64% (222/347) uninfected and 66% (101/153) infected individuals crawled out of the tube (χ^2 test; $P=0{\cdot}74$), and among L&F nymphs 61% (221/363) uninfected and 56% (77/137) infected ticks walked out of the tube (χ^2 test; $P=0{\cdot}4$). In contrast, among H&F individuals, significantly more uninfected ticks (51%, 173/342) walked out of the tube than infected ticks (36%, 57/158) (χ^2 test; $P=0{\cdot}003$) (Fig. 2). Hence, H&F infected ticks rather stayed inside the

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introduction tube (64%, 101/158) than walked out of it (36%, 57/158).

Direction of movement (dry versus wet end of the arena)

To evaluate direction of movement, 3 categories of ticks were considered: (1) those that reached the empty box, i.e. moved to the dry end, (2) those that reached the box containing the dish of water, i.e. moved to the wet end, and (3) those that stayed in the tunnel, i.e. did not move. Although some ticks might have moved 1–2 cm inside the tunnel and have been categorized as 'did not move', such cases were rare (less than 1%). There was a clear distinction between ticks that moved to the boxes or stayed close to the plastic culture tube. A higher proportion of low-fat individuals moved towards the wet than the dry end of the arena (χ^2 test; L&M: $P=0$; L&F: $P=0$), whereas a lower proportion of high-fat nymphs (H&F) walked towards the wet than the dry end of the arena (χ^2 test; $P=0{\cdot}01$) (Fig. 3). Hence, 42% (210/500) L&M and 38% (191/500) L&F ticks moved towards the wet end, and 23% (113/500) L&M and 21% (107/500) L&F nymphs walked to the dry end of the arena. In contrast, 19% (96/500) H&F ticks walked towards the wet end and 27% (134/500) H&F nymphs moved towards the dry end of the arena. The difference in walking direction between groups of ticks with different fat contents was statistically significant (χ^2 test; L&M-H&F: $P=0$, L&F-H&F: $P=0$). However, tick hydration status (only tested among low-fat ticks) (moderate, L&M: 87% RH, or fully, L&F: 98% RH) had no effect on the direction of movement (χ^2 test; $P=0{\cdot}88$). Similarly, *Borrelia*-infection had no significant effect on walking direction towards the dry or the wet end of the arena (χ^2 test; H&F: $P=0{\cdot}09$, L&F: $P=0{\cdot}16$, L&M: $P=0{\cdot}29$).

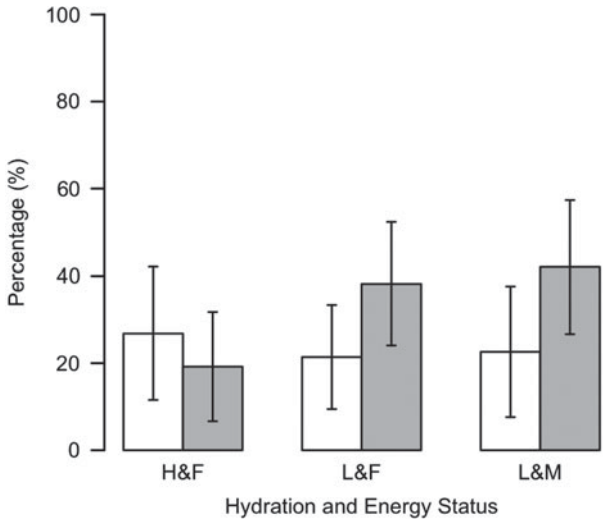

Fig. 3. The percentage of high-fat fully hydrated (H&F), low-fat fully hydrated (L&F), and low-fat moderately hydrated (L&M) *Ixodes ricinus* ticks that walked towards the wet (light grey bars) or the dry end (white bars) of the arena after 2 h.

DISCUSSION

Fat content analysis

Fat content is a source of energy derived from each bloodmeal (Uspensky, 1995). Here it was quantified in order to determine available energy reserves in *I. ricinus* nymphs among the different groups (high-fat and low-fat). Nymphs treated to conserve their fat did indeed contain higher lipid content (9·3 μg) than those treated to lose some of their fat (4·2–4·3 μg). However, it appeared that low-fat nymphs from the present study had a fat content that was in between that of high-fat and low-fat nymphs (4·7 μg and 3·9 μg, respectively) from the work by Crooks and Randolph (2006). In fact, nymphs collected in the spring in Neuchâtel did not only have a higher mean fat content (9·3 μg, this study; 7·12 μg; Herrmann and Gern, 2010) but also a higher fat range (1–25 μg, this study; 1–29 μg; Herrmann and Gern, 2010) than those sampled in southern England all over the year (0·2–15 μg; Randolph *et al.* 2002; 1·6–18·2 μg; Randolph and Storey, 1999). Moreover, when fat content of spring ticks from Neuchâtel was corrected for size and converted into fat index (Randolph *et al.* 2002) (mean = 0·035), it was strikingly higher than that of spring ticks from southern England (approximately 0·015), and even slightly higher than that of fall ticks from southern England (approximately 0·032). This suggests that *I. ricinus* ticks from central Europe and southern England are more distinct than previously thought. Hence, they seem to distinguish themselves not only by different host-seeking behaviour and activity as suggested by Kurtenbach *et al.* (2006), i.e. in southern England, ticks might quest higher in the vegetation and host-seeking activity is present in spring-summer while in central Europe questing activity is present in spring and occasionally in autumn, but also by overall fat content.

Borrelia infection in ticks

Prevalence of infection by *Borrelia* in *I. ricinus* nymphs (29·85%) was similar to that reported by Casati *et al.* (2004) (29%) and slightly higher than that observed by Herrmann and Gern (2010) (25·5%). All these studies were conducted in the same suburban forest of Neuchâtel, Switzerland. Such slight fluctuations of *B. burgdorferi* s.l. prevalence over the years have already been observed in a nearby area on Chaumont Mountain (Jouda *et al.* 2004; Morán Cadenas *et al.* 2007).

Borrelia genospecies frequency was in line with what was previously observed in this area: the most abundant species was *B. afzelii* (42·9%), followed by *B. garinii* (31%), *B. valaisiana* (25·4%), *B. burgdorferi* s.s. (5·6%), while *B. miyamotoi* (2·9%) was the least

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common one (Casati *et al*. 2004; Jouda *et al*. 2004; Morán Cadenas *et al*. 2007; Gern *et al*. 2010; Herrmann and Gern, 2010). *B. bavariensis* (6·8%), formerly *B. garinii* OspA serotype 4, a newly described genospecies (Margos *et al*. 2009), is reported for the first time in the area, although it was already described in Switzerland (Staatswald) (Hu *et al*. 2001; Huegli *et al*. 2002; Pérez *et al*. *manuscript submitted*).

The spirochete load in questing ticks observed in the present study reached a mean of 33 971 and a median of 4300 spirochetes per tick, showing a higher load of spirochetes than that reported in a previous study conducted in the same area (mean = 18 640; median = 2760 spirochetes per tick) (Herrmann and Gern, 2010). Since *Borrelia* genospecies influence infection load (Herrmann and Gern, 2010), these differences in spirochete load may be due to different genospecies distribution in the two studies. For example, infections by *B. garinii*, which consisted of high spirochete numbers (78 000 per tick), represented 23·4% of infections in the study by Herrmann and Gern (2010), while infection by *B. garinii* and by *B. bavariensis* (previously included in infections by *B. garinii*) (69 660 and 89 419, respectively) accounted for 37·8% of infections in this study.

Influence of Borrelia *infection on walking activity*

To date, numerous vector-borne parasites have been shown to alter phenotypic traits of their arthropod vectors, most likely in a way that enhances their probability of transmission (Hurd, 2003; Lefèvre and Thomas, 2008). For example, *Trypanosoma cruzi* increases biting rate in bug *Mepraia spinolai* (Botto-Mahan *et al*. 2006), *Plasmodium mexicanum* alters temperature preference in sand fly *Lutzomyia vexator* (Fiahlo and Schall, 1995), *P. falciparum* alters immune response in mosquito *Anopheles gambiae* (Lambrechts *et al*. 2007), *Babesia microti* increases lifespan in the tick *I. trianguliceps* (Randolph, 1991), or *B. burgdorferi* increases survival in *I. ricinus* under hot and dry conditions (Herrmann and Gern, 2010). Another example of behavioural/physiological alteration in the arthropod vector due to parasite presence is brought to light in the present study. Although walking activity was similar between low-fat uninfected and *Borrelia*-infected nymphs, it seems that among high-fat ticks, *Borrelia* infection influenced *I. ricinus* walking activity. Thus, more high-fat uninfected nymphs walked out of the introduction tube than high-fat *Borrelia*-infected individuals, which rather stayed inside the tube. Higher locomotion activity in uninfected ticks than in individuals infected by *B. burgdorferi* s.l. has been previously reported. In fact, Alekseev *et al*. (2000) observed that infection by *B. burgdorferi* s.l. in *I. persulcatus* and *I. ricinus* ticks suppressed walking activity of both adult and immature individuals compared to uninfected individuals. Hence, *B. burgdorferi* s.l. spirochetes seem to reduce tick motor activity in high-fat individuals (ticks used by Alekseev *et al*. in 2000 were field-collected and stored, similarly to high-fat individuals in this study), apparently manipulating ticks to behave in a beneficial way to them. It may be hypothesized that *Borrelia* spirochetes only affect the behaviour of ticks with high-energy reserves because they may have access to their host vector energy resources without threatening to make the tick run out of energy and die, which would be a disaster for the spirochetes. Thus, energy-using borreliae may manipulate the tick to reduce its motor activity when the arthropod is placed in favourable surroundings for the spirochete, for example when the tick is on the vegetation, increasing the tick chances to find a passing host, in turn allowing the spread of *Borrelia* spirochetes to a new host. In contrast, spirochetes in ticks with reduced energy reserves may not have access to their vector energy resources, forcing them to reduce their metabolism and preventing the spirochetes from manipulating tick behaviour in any way.

Influence of energy reserves and hydration status on walking activity

A 2-h period was chosen as testing time based on Crooks and Randolph (2006) so that half of the ticks would walk out of the tube. The estimate was quite correct since 46% of the high-fat and 62% of the low-fat ticks moved out of the tube during that period.

Interestingly, high-fat nymphs walked less than low-fat ones in the present study while the opposite phenomenon (about 70% and 40%, respectively) was observed by Crooks and Randolph (2006). Differing levels of energy reserves between the nymphs in the two studies seem to be one of the factors explaining this differential walking behaviour. The proportion of low-fat nymphs that walked (62%), which proved not to be affected by *Borrelia* infection, was in between that of high-fat (70%) and low-fat (40%) individuals that walked in the study by Crooks and Randolph (2006). Similarly, fat content in low-fat ticks in our study (4·2–4·3 μg) was in between that of high-fat (4·7 μg) and low-fat (3·9 μg) ticks, in Crooks and Randolph (2006). Thus, low-fat nymphs in our study did not correspond to either low-fat or high-fat individuals in the work by Crooks and Randolph (2006). They had more energy reserves than the first and less than the second, and behaved as such, i.e. walked more than low-fat ticks and less than high-fat ticks.

Another factor that may explain the difference between the two studies is *Borrelia* infection. Although Crooks and Randolph (2006) did not

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provide information about *Borrelia* infection, it may be considered that infection prevalence was much lower among the nymphs they tested. In fact, tested ticks were collected in southern England where infection prevalence is below 12·4% (Rauter and Hartung, 2005; Vollmer *et al*. 2011) whereas infection prevalence reached 30% in the present study. Hence, this difference in infection prevalence may explain the lower proportion of high-fat nymphs walking outside the introduction tube (since *Borrelia* reduced walking activity of high-fat individuals) in the present study (36% of infected ticks moved versus 51% of uninfected ticks).

Hydration status (only tested among low-fat nymphs) had no significant effect on tick walking activity. However, it appeared that moderately hydrated individuals had a higher tendency to walk than fully hydrated ones. This is in line with what Lees (1948) described. He reported that dehydrated ticks were intensely active in dry air (about 70% RH in this study) while hydrated individuals were not. This seems to indicate that ticks with higher deficiencies in water reserves are more prone to walk, probably in order to seek for what they lack.

Direction of movement (dry versus wet end of the arena)

Hydration status, which only differed among low-fat individuals, appeared not to influence direction of movement. This may probably be explained by the fact that their hydration status was very similar (they were maintained at 98% and 87% RH, respectively). Moderately hydrated ticks would probably have needed to be maintained under drier conditions in order to be differentiated from fully hydrated individuals.

The level of energy reserves played a role in the direction of movement of *I. ricinus* nymphs within a humidity gradient. Low-fat individuals (fully and moderately hydrated) tended to walk up the humidity gradient, i.e. towards the area saturated in water vapour, whereas high-fat individuals tended to move down the humidity gradient, i.e. towards an area containing less water vapour than where they were released. Walking of high-fat fully hydrated ticks towards the dry end of the arena is in line with what has been described by Lees (1948). He reported that nymphal and female *I. ricinus* ticks that had been previously exposed to saturated air avoided the wet side (95% RH) of the arena, preferring to walk towards the dry side (34% RH). On the contrary, ticks that were kept under unsaturated air conditions moved towards the opposite direction. This behaviour was observed on the horizontal and the vertical plane (Lees, 1948). It appears in the present study that when *I. ricinus* nymphs are treated so that they become low-fat, they behave similarly to ticks exposed to unsaturated air in the study of Lees (1948), suggesting that not only state of hydration but also the level of energy reserves plays a role in triggering the locomotion response.

These results seem to indicate that *I. ricinus* nymphs that are not in immediate need for energy or water move towards unfavourable dry surroundings, supposedly representing in nature the walk up the vegetation to quest for a host where humidity decreases. In contrast, ticks with lower energy reserves, despite high water reserves, tend to walk towards a wet comfortable zone where they remain fully hydrated, such as the litter layer in the field. Thus, it is as though nymphs with sufficient energy reserves may take the risk to wander randomly outside their comfort zone in order to find a potential host, while leaner counterparts need to be cautious about their energy-costly movements and move towards favourable (wet) conditions before waiting until a positive stimulus (such as host odour) trigger them to walk again.

Nymphs infected by *Borrelia* spirochetes did not walk preferentially towards the wet or the dry end within a humidity gradient. This seems to indicate that borreliae do not affect the need of *I. ricinus* nymphs for moist conditions as might have been expected because of higher survival of ticks infected by *B. burgdorferi* s.l. under hot and dry conditions (Herrmann and Gern, 2010).

In conclusion, the results described here point out that *I. ricinus* nymphs with lower energy reserves are more likely to walk horizontally than their counterparts with higher energy reserves. When *I. ricinus* nymphs walk, those with lower energy reserves are more likely to move towards fully saturated air than drier air, while those with higher energy reserves are more likely to move to the opposite direction. Moreover, it seems that *I. ricinus* immature ticks that are infected by *Borrelia* spirochetes are less likely to move on the horizontal plane than uninfected specimens, suggesting a manipulation by spirochetes in ticks with sufficient available energy reserves. Thus, it appears that *I. ricinus* nymphs will crawl horizontally over short distances within a humidity gradient depending both on their energy reserves and on the presence of *B. burgdorferi* spirochetes.

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