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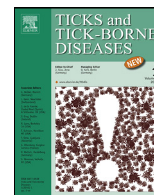

Original article

Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations

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ABSTRACT

In this study, we tested the survival of *Ixodes ricinus* under cold conditions in the laboratory. We investigated how the frequency of temperature variations (from −5 °C or −10 °C to 13 °C), and infection with *Borrelia burgdorferi* sensu lato (s.l.) influenced survival of questing nymphs collected in spring and autumn 2011. In experiment 1, survival of 1760 nymphs was tested at −10 °C over a short period of time to simulate very cold winter conditions. In experiment 2, survival of 1600 nymphs was tested under cold condition (−5 °C) over a long period of time to simulate common winter conditions. Ticks used for survival tests at −5 °C were screened for *Borrelia* by quantitative PCR, and genospecies identification was achieved by reverse line blotting. Tick age and frequency of temperature variations had a highly significant effect on *I. ricinus* survival while *Borrelia* infection was marginally significant. Hence, survival rate was higher in younger (autumn) than older (spring) nymphs and in nymphs exposed to low rather than high-frequency temperature variations. *Borrelia*-infected ticks tended to survive better than their uninfected counterparts. These findings suggest that in nature (i) frequent temperature changes in winter threaten tick survival more importantly than very low temperatures, (ii) older (spring) ticks are less resistant to cold than younger (autumn) individuals, and (iii) *Borrelia* infection plays a marginal role in *I. ricinus* survival during winter conditions.

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Introduction

The hard-bodied tick *Ixodes ricinus* (L.) (Acari: Ixodidae) is the vector of a variety of pathogens which have both medical and veterinary relevance, the most prevalent being *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of Lyme borreliosis. This tick, which is present in Europe, shows a seasonal activity. In spring, questing activity starts when the daily maximum temperature has reached 7 °C for around one week (MacLeod, 1936; Perret et al., 2000). In autumn, when temperatures fall below the temperature threshold of questing activity and day-lengths are shortened, ticks reduce their activity. Unfed ticks enter behavioural diapause (Belozerov, 1982) or more precisely winter quiescence (Dautel et al., 2008), which is a phase of inactivity that occurs when climatic conditions become harsh and stops as soon as favourable conditions are back. During this time, *I. ricinus* ticks remain in the leaf litter or in the upper layers of the soil (up to 5–7 cm deep) where temperatures are milder than on the vegetation (Dusbábek et al., 1971; Daniel et al., 1972; Gigon, 1985). Ticks belonging to the *I. ricinus* complex can resist temperatures falling far below 0 °C for short periods, as shown in nymphs of *I. persulcatus* and *I. nipponensis* (Fujimoto, 1994), *I. scapularis* (Burks et al., 1996; Vandyk et al., 1996; Neelakanta et al., 2010), and *I. ricinus* (MacLeod, 1935; Gigon, 1985; Dautel and Knülle, 1997). Increased winter temperatures due to global warming and climate change are affecting *I. ricinus* European populations, in particular, allowing ticks to colonize regions at higher latitudes (Lindgren et al., 2000; Jore et al., 2011; Jaenson et al., 2012), higher altitudes (Morán Cadenas et al., 2007; Jore et al., 2011), and allowing them to extend their winter activity (Dautel et al., 2008).

In this study, we evaluated in the laboratory how cold conditions and the frequency of exposure to cold temperatures may influence the survival of resident *I. ricinus* populations under winter conditions. In addition, it was recently reported that *Anaplasma phagocytophilum* increases *I. scapularis* nymph resistance to cold by inducing the expression of a gene coding for antifreeze proteins (Neelakanta et al., 2010). Consequently, we investigated whether *Borrelia* spirochaetes, known to be associated with a higher fat content in *I. ricinus* (Herrmann et al., 2013), may as well influence tick survival under cold conditions.

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Materials and methods

Tick collection and maintenance

The sampling site was a mixed (deciduous dominant) forest at 600 m above sea level in Neuchâtel on the Swiss Plateau, Switzerland (47°00′ N and 6°57′ E). Host-seeking *I. ricinus* nymphs were sampled by flagging low vegetation during several consecutive days in April 2011 (spring ticks) and September 2011 (autumn ticks). Spring ticks were maintained in tubes over water in a box with a tight-fitting lid (98% relative humidity; RH) in the dark within a cold chamber at 4 °C, as described in Crooks and Randolph (2006), for 5 months until autumn sampling was performed. Before the start of the survival tests, nymphs collected in spring (spring nymphs) and in autumn (autumn nymphs) were acclimated at 13 °C for a week, followed by another week at 4 °C and 98% RH.

Fat content quantification

Fat content was measured before the survival tests in a sample of 40 spring and 40 autumn nymphs to quantify the energy reserves as described by Herrmann et al. (2013). Briefly, ticks were dried at 70 °C for 24 h, weighed individually to the nearest 0.1 μg (UMT 5 Comparator, Mettler Toledo, Greifensee, Switzerland) (initial dry mass), washed in 3 changes of chloroform for 24 h each, redried at 70 °C for 24 h and reweighed (fat-free dry mass). Fat content was calculated according to Crooks and Randolph (2006), i.e. by subtracting fat-free dry mass (hereinafter referred to as body size, according to Randolph et al., 2002) from initial dry mass.

Survival tests

Survival tests were designed so that they recreated the winter conditions that ticks might experience in the field over short and long periods of time in Neuchâtel (located on the Swiss Plateau). Daily air temperature means of lowest and highest values of serial measures collected from 1864 to 2010 were obtained from MétéoSuisse. Data collected in Neuchâtel (47°00′ N and 6°57′ E, 485 m) from November to February were used to determine the temperature range. During the experiments, nymphs were kept in tubes (40 or 50 individuals per tube) at 98% RH when temperature was above 0 °C.

Experiment 1: survival under very cold conditions

Based on MétéoSuisse data, −10 °C was chosen as a very cold temperature, with an exposure time of 4 days. To test whether the frequency of temperature variations had an effect on tick survival under very cold conditions ($n = 1760$), we maintained 440 spring and 440 autumn nymphs at −10 °C (in a freezer) for 4 days (low-frequency temperature variations, LF) while additional 440 spring and 440 autumn nymphs were exposed every day to temperature variations (18 h at −10 °C, 2 h at 4 °C (in a cold chamber), 2 h at 13 °C (in a fridge), and 2 h at 4 °C) for 4 days (high-frequency temperature variations, HF).

Experiment 2: survival under cold conditions

Based on MétéoSuisse data and on results obtained in experiment 1, −5 °C was chosen as a cold temperature. We tested 2 frequencies of temperature variations on tick survival. Low-frequency temperature variations (LF) involved maintaining nymphs at −5 °C (in a freezer) for 9 days followed by 24 h during which they experienced temperature variations (−5 °C for 18 h, at 4 °C for 2 h, at 13 °C for 2 h, and at 4 °C for 2 h). This 10-day cycle was repeated until the end of the experiment. High-frequency temperature variations (HF) involved exposing nymphs to the same temperature variations, but every day, rather than every 10 days. In this experiment, 400 spring and 400 autumn nymphs were exposed to HF and an additional 400 spring and 400 autumn nymphs were exposed to LF ($n = 1600$). For each treatment group, the time to 50% mortality was determined using a subset of 100 spring and 100 autumn nymphs for each temperature variation frequency. The proportion of ticks surviving was assessed every day for the HF groups and every 10 days for the LF groups. The point in the temperature cycle at which survival was assessed was the point after which nymphs had spent 2 h at 13 °C after having spent 2 h at 4 °C. Survival was assessed on the mobility of ticks. Any immobile ticks were exposed to human skin and breath at room temperature. If they did not move after a few minutes, they were considered dead. The time at which 50% of ticks within a treatment group had died was recorded, and the ticks from that group were harvested for laboratory analyses. This protocol is termed experiment 2a in the following. The proportions of ticks surviving in the subset of ticks subject to daily or 10-daily monitoring continued to be assessed until all the ticks had died, and in the following this protocol is termed experiment 2b. All ticks submitted to survival tests at −5 °C (experiments 2a and 2b) were frozen at −80 °C until they were analyzed for *Borrelia* infection.

Borrelia detection and quantification by real-time PCR

DNA was extracted from ticks using ammonium hydroxide as previously described (Herrmann and Gern, 2010, 2012; Herrmann et al., 2013). A real-time PCR amplifying a fragment of the flagellin gene (Schwaiger et al., 2001) was used to detect and quantify *Borrelia* DNA in nymphs. *B. afzelii* NE1817 was used as quantification standard. Spirochaete concentration in culture was evaluated using the Helber chamber. DNA was extracted by heating spirochaetes for 15 min at 100 °C (Postic et al., 1994). Serial dilutions were made from stored spirochaete DNA in order to obtain 5 standard solutions with concentrations of *Borrelia* DNA ranging from 10 to 10^5 copies per μl (Herrmann and Gern, 2012).

The 50-μl real-time PCR mixture (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), 20.75 μl of water and 10 μl of the extracted DNA. In each run, one extraction negative control (10 μl of extraction reagents without template DNA), one PCR negative control (10 μl of water), 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).

Borrelia genospecies identification by PCR and RLB

PCR followed by RLB was used to identify the *Borrelia* genospecies in ticks that were detected positive by real-time PCR as described in Herrmann and Gern (2010, 2012) and in Herrmann et al. (2013). 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) in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) by using a touchdown PCR program (Burri et al., 2007). Positive [*B. afzelii* (NE632), *B. lusitaniae* (PotiB1), *B. burgdorferi* s.s. (B31), *B. valaisiana* (VS116), or *B. garinii* (N11)] and negative (water) controls were included in each PCR.

RLB analysis was performed using 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) held in a Miniblotter 45 (Immunetic, Cambridge, MA). 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

Body size and fat content: we used an independent two-sample *t*-test to test whether there was a difference in body size between spring ($n = 40$) and autumn nymphs ($n = 40$). We used an ANCOVA to test 3 hypotheses: (1) whether there was a relationship between body size and fat content (a measure of tick energy reserves), (2) whether tick fat content for a given body size varied between spring and autumn nymphs, and (3) whether the slope of the body size-fat content relationship was the same between spring and autumn nymphs. We log-transformed the variables tick body size and fat content to control the variances and to linearize their relationship.

We analyzed the effect of *Borrelia* genospecies on spirochaete load in ticks that were infected with *B. afzelii*, *B. garinii*, or *B. valaisiana* ($n = 242$ nymphs). Infections by *B. burgdorferi* s.s., *B. bavariensis*, *B. miyamotoi*, and mixed infections were excluded from the statistical analyses due to their low frequency (Table 1). We used a two-way ANOVA to test whether sampling season and the presence of *B. afzelii*, *B. garinii*, or *B. valaisiana* in single infections influenced spirochaete load in nymphs. Since spirochaete load data were not normally distributed, we used Spearman's rank correlation to test whether spirochaete load among infected ticks varied over time during the survival tests.

Experiment 1, survival under very cold conditions ($-10\,^{\circ}$C): Simple chi-square tests were used to compare survival rate between spring and autumn nymphs and between high- and low-frequency temperature variations.

Experiment 2a, survival under cold conditions ($-5\,^{\circ}$C): Mean daily survival rate was calculated for spring and autumn nymphs exposed to HF and LF. Daily survival rate was calculated in each tube according to the following formula:

$$D_{50\%}\sqrt{\frac{\text{liv}_{50\%}}{\text{tot}}}$$

where $D_{50\%}$ is the number of days until 50% mortality was reached, $\text{liv}_{50\%}$ is the number of living nymphs in the tube when 50% mortality was reached, and tot is the total number of nymphs in the tube. We used a paired *t*-test to test whether there was a difference in survival between *Borrelia*-infected ($n = 278$) and uninfected ticks ($n = 922$) without taking into account whether nymphs had been collected in spring or autumn, or exposed to HF or LF. The paired *t*-test compared the survival rate of infected nymphs to that of uninfected nymphs in the 24 tubes used in this experiment ($n = 24 \times 50$ nymphs = 1200). We then used an ANOVA to test whether survival ratio (= logarithm of the survival rate of infected nymphs divided by the survival rate of uninfected individuals) in the 24 tubes was different between spring and autumn nymphs and between nymphs exposed to high and low frequencies of temperature variation.

Experiment 2b, survival under cold conditions ($-5\,^{\circ}$C): We used GLMs with a Gamma error function to test whether sampling season, frequency of temperature variations, *Borrelia* infection, or the interactions between sampling season and *Borrelia* infection, and between frequency of temperature variations and *Borrelia* infection had an effect on the hazard rate, i.e. the daily probability of dying, of *I. ricinus* nymphs.

All statistics were calculated with R for Mac OS X (R Development Core Team, 2012).

Results

Body size and fat content in ticks collected in spring and autumn

Mean body size of questing *I. ricinus* was slightly higher in the autumn ($62.3 \pm 12.0\,\mu$g; $n = 40$) than in the spring nymphs ($62.0 \pm 12.0\,\mu$g; $n = 40$), but this difference was not significant ($t = -0.101$, df = 78, $p = 0.920$). The ANCOVA found no significant interaction between sampling season and tick body size ($F_{1,76} = 0.015$, $p = 0.903$). However, there was a significant relationship between body size and fat content ($F_{1,77} = 4.628$, $p = 0.035$). In addition, the mean fat content was 1.6 times higher in the autumn nymphs ($7.4\,\mu g \pm 3.5\,\mu g$) than in the spring nymphs ($4.6\,\mu g \pm 3.9\,\mu g$) ($F_{1,77} = 17.730$, $p < 0.001$).

Borrelia infection in ticks

B. burgdorferi s.l. was more frequently detected in spring (23.4%, 187/800) than in autumn ticks (20.0%, 160/800), but not significantly ($\chi^2 = 0.192$, df = 1, $p = 0.661$). Six *Borrelia* species were identified (Table 1). Both spring and autumn ticks were primarily infected by one *Borrelia* genospecies (87.9% and 83.0%, respectively). Infections by 2 *Borrelia* genospecies were less frequent (12.1% in spring and 14.3% in autumn ticks) while infections by 3 genospecies were only observed in autumn ticks (2.7%).

Spirochaete load among infected ticks

The mean spirochaete load in infected nymphs was 6584 and 5696 spirochaetes per nymph in spring ($n = 187$) and autumn samples ($n = 160$), respectively, while the median spirochaete load was 1060 and 536 spirochaetes per nymph, respectively. In spring nymphs, the mean spirochaete load of *B. afzelii* infections was higher than that of *B. garinii* and *B. valaisiana* infections (Table 1). In autumn nymphs, the mean spirochaete load of *B. garinii* infections was higher than that of *B. afzelii* and *B. valaisiana* infections. The ANOVA found no significant interaction between sampling season and the presence of *B. afzelii*, *B. garinii*, or *B. valaisiana* (df = 2, LR stat = 2.998, $p = 0.223$). Similarly, no difference in the spirochaete load was found between spring and autumn nymphs (df = 1, LR stat = 0.022, $p = 0.882$) or between ticks infected by these 3 *Borrelia* species (df = 2, LR stat = 3.921, $p = 0.141$).

Experiment 1: survival under very cold conditions

Nymphs submitted to low-frequency temperature variations survived better (spring ticks: 12.7%, 56/440; autumn ticks: 89.6%, 394/440) than those exposed to high-frequency temperature variations (spring ticks: 6.8%, 30/440; autumn ticks: 85.0%, 374/440) ($\chi^2 = 8.71$, df = 1, $p = 0.003$ spring ticks; $\chi^2 = 4.09$, df = 1, $p = 0.043$ autumn ticks) independently of the sampling season (Fig. 1). Moreover, autumn ticks survived significantly better at $-10\,^{\circ}$C (87.3% survival rate, 768/880) than spring individuals (9.8%, 86/880) ($\chi^2 = 1058.02$, df = 1, $p < 0.001$) independently of the frequency of temperature variations.

Experiment 2: survival under cold conditions

Experiment 2a: 50% survival in spring nymphs exposed to high-frequency temperature variations (HF) and low-frequency temperature variations (LF) was reached after 3 days (survival rate 57.3%, $n = 300$) and 5 days (survival rate 47.5%, $n = 300$), respectively. In autumn nymphs exposed to HF and LF, 50% survival

Table 1
Distribution of *Borrelia* genospecies and mean spirochaete load in questing *I. ricinus* nymphs collected in Neuchâtel, Switzerland, in spring and autumn 2011.

Borrelia genospecies[a]	Spring (n = 800 ticks)		Autumn (n = 800 ticks)	
	Infected ticks (n = 173)[b]	Mean spirochaete load[c]	Infected ticks (n = 147)[b]	Mean spirochaete load[c]
af	80 (46.2%)	8361	47 (32.0%)	5663
bav	8 (4.6%)	na	7 (4.8%)	na
ga	30 (17.3%)	5242	22 (15.0%)	9324
miy	0 (0.0%)	na	0 (0.0%)	na
ss	8 (4.6%)	na	9 (6.1%)	na
vs	26 (15.0%)	3156	37 (25.2%)	4719
Infection by one species	152 (87.9%)	7286	122 (83.0%)	6156
af & *bav*	1 (0.6%)	na	0 (0.0%)	na
af & *ga*	1 (0.6%)	na	1 (0.7%)	na
af & *miy*	2 (1.2%)	na	1 (0.7%)	na
af & *ss*	5 (2.9%)	na	4 (2.7%)	na
af & *vs*	1 (0.6%)	na	6 (4.1%)	na
bav & *vs*	1 (0.6%)	na	1 (0.7%)	na
ga & *ss*	2 (1.2%)	na	0 (0.0%)	na
ga & *vs*	8 (4.6%)	na	8 (5.4%)	na
Infection by 2 species	21 (12.1%)	7198	21 (14.3%)	3516
af & *ga* & *vs*	0 (0.0%)	na	3 (2.0%)	na
af & *miy* & *vs*	0 (0.0%)	na	1 (0.7%)	na
Infection by 3 species	0 (0.0%)	na	4 (2.7%)	na

na, not answered.
[a] af, *B. afzelii*; bav, *B. bavariensis*; ga, *B. garinii*; miy, *B. miyamotoi*; ss, *B. burgdorferi sensu stricto*; *vs*, *B. valaisiana*.
[b] *Borrelia* species identification by RLB in 173/187 infected spring and 147/160 infected autumn nymphs.
[c] Mean spirochaete number was not calculated when frequency was below 10.

was reached after 12 days (survival rate 48.8%, n = 300) and 24 days (survival rate 48.5%, n = 300), respectively. Mean daily survival rate was greater in autumn nymphs exposed to LF (0.96), followed by autumn nymphs exposed to HF (0.93), and spring nymphs exposed to LF (0.86). Mean daily survival rate was lowest in spring nymphs exposed to HF (0.84). No difference in survival was observed between nymphs infected by *Borrelia* (n = 302) and uninfected individuals (n = 998) (t = 0.680, df = 23, p = 0.504). Moreover, the ANOVA found no significant effect of sampling season (df = 1, F = 0.0101, p = 0.921) or frequency of temperature variations (df = 1, F = 0.0439, p = 0.836) on survival ratio of *Borrelia*-infected versus uninfected nymphs.

Experiment 2b: spring nymphs submitted to LF (n = 100) were all dead when their survival was assessed for the first time, i.e. 10 days after the start of the experiment and were therefore not used in the GLM. Mortality reached 100% after 7 days in spring nymphs (n = 100) exposed to HF and after 42 days and 80 days in autumn nymphs exposed to HF (n = 100) and LF (n = 100), respectively (Fig. 2). The GLM found that frequency of temperature variations and sampling season had both a significant effect on *I. ricinus* survival time (df = 1, deviance = 46.161, p < 0.001 and df = 1, deviance = 92.752, p < 0.001, respectively). In fact, the hazard rate, i.e. the daily probability of dying, of spring nymphs (0.293) was more than 4 times that of autumn nymphs (0.072), while the hazard rate of nymphs exposed to HF (0.072) was more than twice that of nymphs exposed to LF (0.028). In contrast, the interaction between frequency of temperature variations and *Borrelia* infection and the interaction between sampling season and *Borrelia* infection had no significant effect on survival time (df = 1, deviance = 0.0003, p = 0.972; df = 1, deviance = 0.0218, p = 0.759, respectively). The main effect of *Borrelia* infection was marginally significant on tick survival time (df = 1, deviance = 0.7111, p = 0.077) so that the

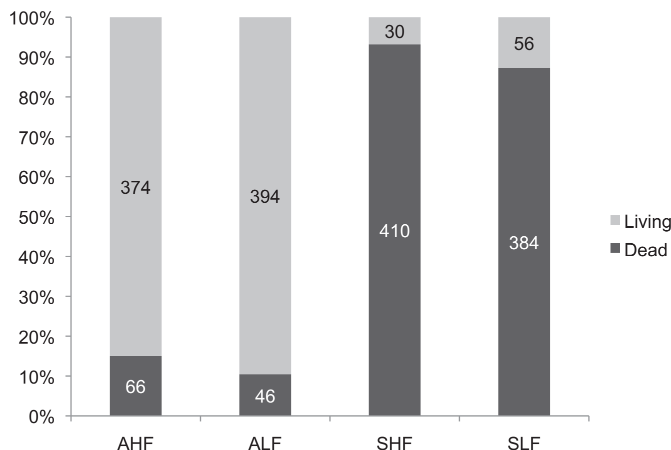

Fig. 1. Survival rate under very cold conditions (−10 °C) in autumn and spring nymphs exposed to high- and low-frequency temperature variations after 4 days. AHF and ALF: autumn nymphs exposed to high- and low-frequency temperature variations (n = 440 and n = 440), respectively. SHF and SLF: spring nymphs exposed to high- and low-frequency temperature variations (n = 440 and n = 440), respectively. Dead ticks are represented in dark grey while living ticks are represented in light grey.

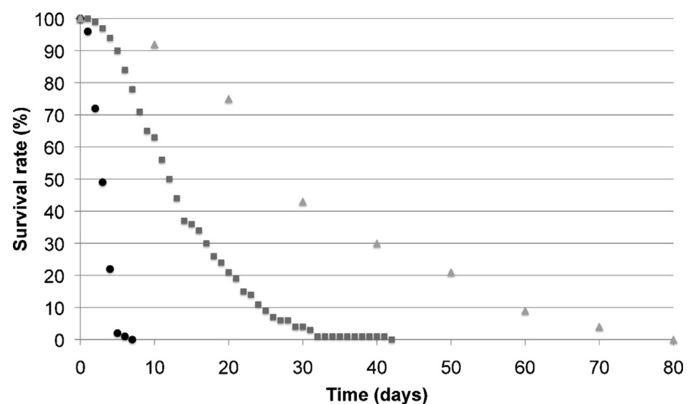

Fig. 2. Survival rate under cold conditions (−5 °C) in spring nymphs exposed to high-frequency temperature variations (n = 100, black circles), autumn nymphs exposed to high-frequency temperature variations (n = 100, dark grey squares), and autumn nymphs exposed to low-frequency temperature variations (n = 100, light grey triangles) over time (experiment 2b).

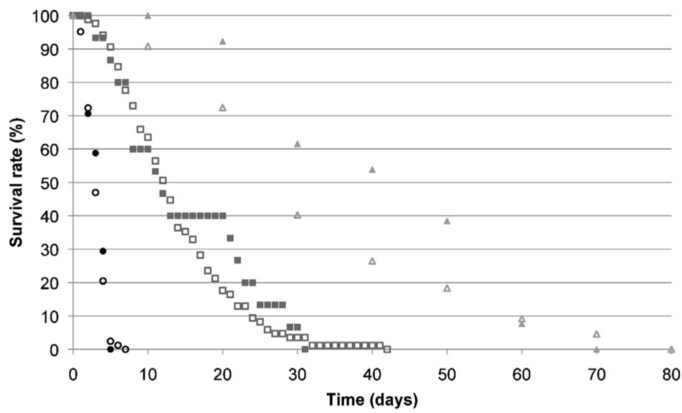

Fig. 3. Survival rate under cold conditions (−5 °C) in *Borrelia*-infected individuals (filled markers) versus uninfected individuals (empty markers) over time (experiment 2b). Spring nymphs exposed to high-frequency temperature variations (black circles), autumn nymphs exposed to high-frequency temperature variations (dark grey squares), and autumn nymphs exposed to low-frequency temperature variations (light grey triangles) are treated separately.

hazard rate of *Borrelia*-infected nymphs (0.066) was 1.1 times lower than that of uninfected nymphs (0.072) (Fig. 3). Fig. 3 shows that infected spring nymphs exposed to HF displayed a 10% higher survival rate on days 3 and 4, and autumn nymphs exposed to HF 22% on days 14–20. It also shows that infected autumn nymphs exposed to LF displayed a higher survival rate until day 50. At other points in time, *Borrelia*-infected and uninfected nymphs had similar survival rates. When the last infected nymph died, few uninfected ticks were still alive (2 spring nymphs exposed to HF, 3 and 4 autumn nymphs exposed to HF and LF, respectively). Among infected ticks (n = 45) spirochaete load was negatively correlated to the number of days that ticks survived (S = 22248.27, rho = −0.466, p = 0.001), i.e. the longer nymphs survived, the lower the spirochaete load they harboured (Fig. 4).

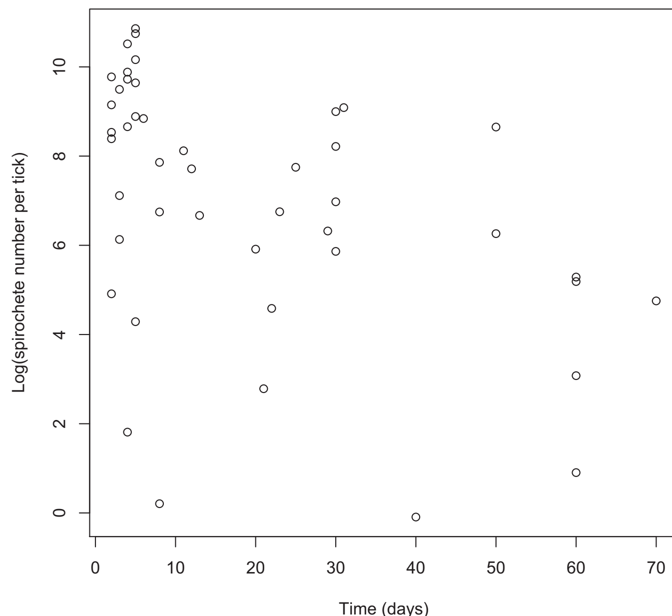

Fig. 4. Log-transformed spirochaete load depending on time (measured in days) that infected (spring and autumn) nymphs (n = 45) survived under cold conditions (−5 °C).

Discussion

Fat content in ticks collected in spring and autumn

Autumn nymphs possessed close to twice the fat content of spring individuals (representing tick energy reserves according to Randolph et al., 2002). It is likely that the lower fat content in spring nymphs was due to the fact that these ticks were older and belonged to a different cohort. Nymphs collected in spring had emerged the previous autumn and survived cold winter conditions, while nymphs collected in autumn had newly emerged. Moreover, a sampling bias might have occurred in spring. In April, we might have collected smaller ticks, because larger ticks had already been picked up by hosts, since it is known that larger ticks start questing earlier in the spring (Randolph et al., 2002).

The spring (4.6 μg) and autumn (7.4 μg) nymphs sampled in 2011 which were used in this study possessed lower mean fat content than nymphs collected during the same spring season (7.8 μg) and nymphs collected in autumn 2010 (13.2 μg) (Herrmann et al., 2013). One explanation for the latter differences is that fat content is known to decrease over time (Steele and Randolph, 1985; Herrmann and Gern, 2012). Since we maintained spring nymphs at 4 °C for 6 months versus 3 months in the study by Herrmann et al. (2013), the difference between spring ticks was possibly related to the difference in maintenance time. In addition, fat content is consumed faster when climate conditions are unfavourable, i.e. warmer and drier (Van Es et al., 1998; Randolph and Storey, 1999). Hence, nymphs collected in autumn 2011 probably consumed more fat to maintain their water balance than nymphs collected in autumn 2010 (Herrmann et al., 2013) since 2011 was warmer and drier than 2010 (MétéoSuisse).

Survival under cold and very cold conditions

Milder climate (especially during winter) due to climate change and global warming has allowed tick species such as *I. persulcatus*, *I. ricinus*, and *I. scapularis* to colonize regions at higher latitudes (Dennis et al., 1998; Lindgren et al., 2000; Yasyukevich et al., 2009; Jore et al., 2011; Jaenson et al., 2012; Leighton et al., 2012) and higher altitudes (Morán Cadenas et al., 2007; Jore et al., 2011) and has allowed *I. ricinus* to quest during winter months (Dautel et al., 2008). In such context, we focused on the influence of cold conditions on the survival of resident *I. ricinus* populations during winter. Brunner et al. (2012) reported that cold-related overwintering mortality did not impact greatly on *I. scapularis* populations. Similarly, we observed that *I. ricinus* ticks resisted constant cold conditions rather well. However, experiencing frequent temperature variations was more detrimental to *I. ricinus* survival than being maintained under constant cold temperature. Nymphs survived better when they stayed at −5 °C or −10 °C for a few days without temperature changes than when they experienced frequent temperature variations. These results are consistent with Gigon (1985), although he used more extreme conditions (temperature variations from −4.5 °C to 21 °C, −10.5 °C to 21 °C, or −36 °C to 21 °C) and smaller samples (n = 100). Lower survival rates in ticks exposed to frequent temperature variations (from quiescence to questing activity temperatures, i.e. temperatures below and over 7 °C) were likely due to the adaptations (costing energy) these variations generated. Moreover, when the temperature reaches an activity threshold (i.e. above 7 °C), tick metabolism increases. Energy reserves were probably depleted faster in ticks exposed to high-frequency temperature variations, resulting in shorter survival time. The fact that variations of temperature are more detrimental to ticks than constant temperature conditions is interesting in the context of climate change. During the past decades, a decrease in snowfall frequency and quantity was reported on the

Swiss Plateau (main distribution area of *I. ricinus* and tick collection site) (North et al., 2007). This may have a negative impact on tick population, since overwintering ticks in this area are most likely no longer protected by snow cover and may be more exposed to severe variation in temperatures. However, it was shown that day-to-day temperature variability decreased during the last century in Neuchâtel (tick collection site), mainly due to a loss of extremely low temperatures, particularly in winter (Rebetez, 2001). Hence, according to our results, we expect overwintering *I. ricinus* ticks to benefit from the observed reduced snow cover associated with a reduction of temperature variability, resulting in lower tick mortality during winters with these conditions. Such a trend may even increase in the future due to global warming. However, if reduced snow cover was associated with important temperature fluctuations during a particular winter, we would expect *I. ricinus* populations to be more severely impaired, resulting in higher tick mortality during that winter.

I. ricinus nymphs sampled in autumn survived better at −5 °C and −10 °C than individuals sampled in the spring of the same year. Autumn nymphs possessed close to twice more fat content than spring individuals. The lower fat content in spring nymphs was likely due to the fact that these ticks were older and belonged to a different cohort, as mentioned above. The combination of these differences likely explains why younger autumn nymphs resisted longer under cold conditions than older spring nymphs.

In experiment 2a, no effect of *Borrelia* infection on nymph survival was observed. In contrast, in experiment 2b, nymphs infected with *Borrelia* spirochaetes tended to survive slightly better (marginally significant) than uninfected individuals. As it has recently been reported that nymphs infected with *B. burgdorferi* s.l. have a higher fat content (12% more after correction for body size) than uninfected ticks (Herrmann et al., 2013), we expected infected ticks to survive better. When the survival rate was closely examined in experiment 2b (Fig. 3), it appeared, in fact, that at some time points, *Borrelia*-infected nymphs survived better. However, the last surviving nymphs were uninfected independently of sampling season (i.e. tick age) or frequency of temperature variations. The latter probably occurred because uninfected ticks were more numerous than infected nymphs, resulting in higher numbers of uninfected ticks with a high fat content (due to a normal distribution of fat content, unpublished data). Such ticks would therefore survive longer than infected nymphs. The fact that no difference could be observed in experiment 2a might be because the point in time at which higher survival in infected nymphs occurred had not been reached or had already been passed when survival was assessed. However, further investigations are needed to confirm such a phenomenon.

Borrelia infection in ticks

Prevalence of *Borrelia* infection in spring (23.4%) and autumn nymphs (20.0%) was consistent with previous reports from Neuchâtel varying from 17.4% to 29.8% between 1999 and 2011 (Jouda et al., 2004; Morán Cadenas et al., 2007; Gern et al., 2010; Herrmann and Gern, 2010, 2012; Herrmann et al., 2013). *Borrelia* genospecies distribution was similar to that previously reported in the same area, *B. afzelii*, *B. garinii*, and *B. valaisiana* being the most common genospecies (Herrmann and Gern, 2010, 2012; Herrmann et al., 2013).

The load of *Borrelia* spirochaetes per nymph (spring mean = 6584; autumn mean = 6604) was lower than previously reported in the area (mean = 18,638, Herrmann and Gern, 2010; mean = 33,971, Herrmann and Gern, 2012; mean = 15,556, Herrmann et al., 2013). Although spirochaete numbers might fluctuate in questing *I. ricinus* ticks over time, spirochaete numbers observed in the present study were particularly low. We observed that spirochaete load and the number of days that nymphs survived were negatively correlated, suggesting that spirochaete number was reduced in nymphs during maintenance under cold conditions. Interestingly, a significant decrease of spirochaete numbers was also observed in *I. scapularis* adults that overwintered in nature (Sharon et al., 1992). Explanations may be that important resources for spirochaete survival were depleted in ticks when the temperature was reduced, resulting in reduced numbers of spirochaetes, and/or that spirochaetes, which are primarily located in tick midgut (Lebet and Gern, 1994), were gradually digested by cold-exposed nymphs and used as additional energy resources.

Conclusion

We observed that spring *I. ricinus* nymphs possessing less fat content than their autumn counterparts were less resistant to cold, most likely due to their lower energy reserves. Moreover, the presence of *Borrelia* spirochaetes in nymphs was associated with a slightly better survival, presumably because infected nymphs have higher fat contents than their uninfected counterparts (Herrmann et al., 2013). Last but not least, the frequency of temperature variations between temperatures below and above activity thresholds was the most important factor impairing *I. ricinus* nymphal survival under winter conditions. We might expect low survival of *I. ricinus* populations during winters with high temperature fluctuations and those associated with reduced snow cover (protecting ticks from extreme cold temperature variations) on the Swiss Plateau (North et al., 2007). However, since a reduction in the day-to-day temperature variability was observed during the past century in Neuchâtel, mainly due to less extreme cold temperatures in winter (Rebetez, 2001), ticks are likely to be less frequently exposed to temperature variations in the future. Under such conditions, we might expect a better survival of *I. ricinus* populations during winter in the Neuchâtel area.

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References

Alekseev, A.N., Dubinina, H.V., Van de Pol, I., Schouls, L.M., 2001. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J. Clin. Microbiol. 39, 2237–2242.

Belozerov, V.N., 1982. Diapause and biological rhythms in ticks. In: Obenchain, F.D., Galun, R. (Eds.), Physiology of Ticks. Pergamon Press, Oxford, pp. 469–500.

Brunner, J.L., Killilea, M., Ostfeld, R.S., 2012. Overwintering survival of nymphal *Ixodes scapularis* (Acari: Ixodidae) under natural conditions. J. Med. Entomol. 49, 981–987.

Burks, C.S., Stewart Jr., R.L., Needham, G.R., Lee Jr., R.E., 1996. The role of direct chilling injury and inoculative freezing in cold tolerance of *Amblyomma americanum*, *Dermacentor variabilis* and *Ixodes scapularis*. Physiol. Entomol. 21, 44–50.

Burri, C., Morán Cadenas, F., Douet, V., Moret, J., Gern, L., 2007. *Ixodes ricinus* density and infection prevalence with *Borrelia burgdorferi* sensu lato along a north-facing altitudinal gradient in the Rhône Valley (Switzerland). Vector Borne Zoonotic Dis. 7, 50–58.

Crooks, E., Randolph, S.E., 2006. Walking by *Ixodes ricinus* ticks: intrinsic and extrinsic factors determine the attraction of moisture or host odour. J. Exp. Biol. 209, 2138–2142.

Daniel, M., Černý, V., Dusbábek, F., 1972. Stratification of overwintering engorged larvae and nymphs of *Ixodes ricinus* (L.). Wiad. Parazytol. 18, 727–730.

Dautel, H., Dippel, C., Kämmer, D., Werkhausen, A., Kahl, O., 2008. Winter activity of *Ixodes ricinus* in a Berlin forest. Int. J. Med. Microbiol. 298 (Suppl. 1), 50–54.

Dautel, H., Knülle, W., 1997. Cold hardiness, supercooling ability and causes of low-temperature mortality in the soft tick, *Argas reflexus*, and the hard tick, *Ixodes ricinus* (Acari: Ixodidae) from Central Europe. J. Insect Physiol. 43, 843–854.

Dennis, D.T., Nekomoto, T.S., Victor, J.C., Paul, W.S., Piesman, J., 1998. Reported distribution of *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae) in the United States. J. Med. Entomol. 35, 629–638.

Dusbábek, F., Daniel, M., Černý, V., 1971. Stratification of engorged *Ixodes ricinus* larvae overwintering in soil. Folia Parasitol. 18, 261–266.

Fujimoto, K., 1994. Comparison of the cold hardiness of *Ixodes nipponensis* and *I. persulcatus* (Acari: Ixodidae) in relation to the distribution patterns of both species in the Chichibu mountains. Jpn. J. Sanit. Zool. 45, 333–339.

Gern, L., Douet, V., Lopez, Z., Rais, O., Morán Cadenas, F., 2010. Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting. Ticks Tick Borne Dis. 1, 23–29.

Gigon, F., 1985. Biologie d'*Ixodes ricinus* L. sur le Plateau Suisse – une contribution à l'écologie de ce vecteur. Université de Neuchâtel, Neuchâtel, Switzerland (Ph.D. Thesis).

Herrmann, C., Gern, L., 2010. Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection. J. Med. Entomol. 47, 1196–1204.

Herrmann, C., Gern, L., 2012. Do level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks? Parasitology 139, 330–337.

Herrmann, C., Voordouw, M.J., Gern, L., 2013. *Ixodes ricinus* ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves. Int. J. Parasitol. 43, 477–483.

Jaenson, T.G.T., Jaenson, D.G.E., Eisen, L., Petersson, E., Lindgren, E., 2012. Changes in the geographical distribution and abundance of the tick *Ixodes ricinus* during the past 30 years in Sweden. Parasite Vector 5, 8.

Jore, S., Viljugrein, H., Hofshagen, M., Brun-Hansen, H., Kristoffersen, A.B., Nygard, K., Brun, E., Ottesen, P., Saevik, B.K., Ytrehus, B., 2011. Multi-source analysis reveals latitudinal and altitudinal shifts in range of *Ixodes ricinus* at its northern distribution limit. Parasite Vector 4, 84.

Jouda, F., Perret, J.-L., Gern, L., 2004. *Ixodes ricinus* density, and distribution and prevalence of *Borrelia burgdorferi* sensu lato infection along an altitudinal gradient. J. Med. Entomol. 41, 162–169.

Lebet, N., Gern, L., 1994. Histological examination of *Borrelia burgdorferi* infections in unfed *Ixodes ricinus* nymphs. Exp. Appl. Acarol. 18, 177–183.

Leighton, P.A., Koffi, J.K., Pelcat, Y., Lindsay, L.R., Ogden, N.H., 2012. Predicting the speed of tick invasion: an empirical model of range expansion for the Lyme disease vector *Ixodes scapularis* in Canada. J. Appl. Ecol. 49, 457–464.

Lindgren, E., Tälleklint, L., Polfeldt, T., 2000. Impact of climatic change on the northern latitude limit and population density of the disease-transmitting European tick *Ixodes ricinus*. Environ. Health Persp. 108, 119–123.

MacLeod, J., 1935. *Ixodes ricinus* in relation to its physical environment. II. The factors governing survival and activity. Parasitology 27, 123–144.

MacLeod, J., 1936. *Ixodes ricinus* in relation to its physical environment. IV. An analysis of the ecological complexes controlling distribution and activities. Parasitology 28, 295–319.

MétéoSuisse, 2012. Evolution climatique pour des stations du réseau Suisse de mesures climatiques. http://www.meteosuisse.admin.ch/web/fr/climat/climat_aujourdhui/evolution_climatique_nbcn/Neuchatel.html

Morán Cadenas, F., Rais, O., Jouda, F., Douet, V., Humair, P.-F., Moret, J., Gern, L., 2007. Phenology of *Ixodes ricinus* and infection with *Borrelia burgdorferi* sensu lato along a North- and South-facing altitudinal gradient on Chaumont Mountain, Switzerland. J. Med. Entomol. 44, 683–693.

Neelakanta, G., Sultana, H., Fish, D., Anderson, J.F., Fikrig, E., 2010. *Anaplasma phagocytophilum* induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. J. Clin. Invest. 120, 3179–3190.

North, N., Kljun, N., Kasser, N., Heldstab, J., Maibach, M., Reutimann, J., Guyer, M., 2007. Changements climatiques en Suisse – indicateurs des causes, des effets et mesures. Etat de l'environnement No. 0728. Office Fédéral de l'Environnement, Bern, pp. 77.

Perret, J.-L., Guigoz, E., Rais, O., Gern, L., 2000. Influence of saturation deficit and temperature on *Ixodes ricinus* tick questing activity in a Lyme borreliosis-endemic area (Switzerland). Parasitol. Res. 86, 554–557.

Postic, D., Assous, M.V., Grimont, P.A.D., Baranton, G., 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rrf*(5S)-*rrl*(23S) intergenic spacer amplicons. Int. J. Syst. Bacteriol. 44, 743–752.

Poupon, M.-A., Lommano, E., Humair, P.-F., Douet, V., Rais, O., Schaad, M., Jenni, L., Gern, L., 2006. Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland. Appl. Environ. Microb. 72, 976–979.

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

Randolph, S.E., Green, R.M., Hoodless, A.N., Peacey, M.F., 2002. An empirical quantitative framework for the seasonal population dynamics of the tick *Ixodes ricinus*. Int. J. Parasitol. 32, 979–989.

Randolph, S.E., Storey, K., 1999. Impact of microclimate on immature tick–rodent interactions (Acari: Ixodidae): implications for parasite transmission. J. Med. Entomol. 36, 741–748.

Rebetez, M., 2001. Changes in daily and nightly day-to-day temperature variability during the twentieth century for two stations in Switzerland. Theor. Appl. Climatol. 69, 13–21.

Rijpkema, S.G.T., Molkenboer, M.J.C.H., Schouls, L.M., Jongejan, F., Schellekens, J.F.P., 1995. Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. J. Clin. Microb. 33, 3091–3095.

Schwaiger, M., Péter, O., Cassinotti, P., 2001. Routine diagnosis of *Borrelia burgdorferi* (sensu lato) infections using real-time PCR assay. Clin. Microbiol. Infect. 7, 461–469.

Sharon, M., Rowley, W.A., Novak, M.G., Platt, K.B., 1992. Rates of *Borrelia burgdorferi* infection in *Ixodes dammini* (Acari: Ixodidae) in southwestern Wisconsin. J. Med. Entomol. 29, 314–317.

Steele, G.M., Randolph, S.E., 1985. An experimental evaluation of conventional control measures against the sheep tick, *Ixodes ricinus* (L.) (Acari: Ixodidae). I. A unimodal seasonal activity pattern. Bull. Entomol. Res. 75, 489–499.

Van Es, R.P., Hillerton, J.E., Gettinby, G., 1998. Lipid consumption in *Ixodes ricinus* (Acari: Ixodidae): temperature and potential longevity. Bull. Entomol. Res. 88, 567–573.

Vandyk, J.K., Bartholomew, D.M., Rowley, W.A., Platt, K.B., 1996. Survival of *Ixodes scapularis* (Acari: Ixodidae) exposed to cold. J. Med. Entomol. 33, 6–10.

Yasyukevich, V.V., Kazakova, E.V., Popov, I.O., Semenov, S.M., 2009. Distribution of *Ixodes ricinus* L., 1758 and *Ixodes persulcatus* Schulze, 1930 (Parasitiformes, Ixodidae) in Russia and adjacent countries in view of observable climate changes. Dokl. Earth Sci. 427, 1030–1034.