Effect of a *Borrelia burgdorferi* sensu lato infection on the life history and desiccation resistance of *Ixodes ricinus*

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Abstract

Lyme borreliosis is the most common tick-borne human disease in the northern hemisphere and can be transmitted by the sheep tick (*Ixodes ricinus*). It is well known that vector behaviour and physiology can be influenced by parasitic infections, but not much is known about the relation between *Borrelia burgdorferi* sensu lato, which causes Lyme borreliosis, and *I. ricinus*. In this study, two experiments were conducted to test the effect of a *B. burgdorferi* s.l. infection on the life history and desiccation resistance of *I. ricinus*. Experiment I tested the effect of a *B. burgdorferi* s.l. infection in wood mice (*Apodemus sylvaticus*) and *I. ricinus* on the scutal index, body weight, feeding time, and interstadial development time of wild and captive bred *I. ricinus* larvae. Infected wild engorged larvae were lighter (0.439 mg) and had a lower scutal index (4.03) compared to non-infected wild larvae (0.507 mg and 4.41, respectively). When moulted to a nymph, infected wild ticks were lighter (0.229 mg) than non-infected wild ticks (0.271 mg). Feeding time and interstadial development time varied between 1-10 and 35-63 days, respectively and were not influenced by a *B. burgdorferi* s.l. infection in ticks. These results corresponded to the effects of a *B. burgdorferi* s.l. infection in mice. A linear relation was found between the scutal index of larvae and the scutal index of nymphs, larval weight and nymphal weight, and between feeding time and interstadial development time. Experiment II tested the effect of a *B. burgdorferi* s.l. infection on the desiccation resistance of captured wild *I. ricinus* nymphs at different saturation deficits. Mean survival and survival curves were not influenced by a *B. burgdorferi* s.l. infection, but were influenced by saturation deficit. Mean survival of the total of ticks was 1.83, 1.97, and at least 6.86 days at 50, 70, and 95% relative humidity and 20 °C, respectively. A linear relation was found between body length and body weight of *I. ricinus* nymphs. It was hypothesized that smaller ticks and therefore perhaps males are more susceptible to a *B. burgdorferi* s.l. infection.

Content

Introduction

Lyme borreliosis is the most common tick-borne human disease in the northern hemisphere (Rauter and Hartung, 2005). The disease is a zoonosis and is caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex (hereafter named *B. burgdorferi* s.l.), which can be transmitted by at least seven different tick species (Sonenshine, 1991). One of these species is the sheep tick (*Ixodes ricinus*, Acari: Ixodidae), which is the most common tick species in Europe. *Ixodes ricinus* can parasitize a wide range of mammals and birds and some reptiles (Gern *et al*., 1998; Matuschka *et al*., 1991). It has a three host life cycle, where each life stage; larvae, nymph, and adult, feeds for several days on a host. To find a blood meal, *I. ricinus* climbs into vegetation and waits until a suitable host passes; this behaviour is defined as questing. During questing, water is lost from the tick through transpiration (Sonenshine, 1991). Therefore, ticks have to climb down the vegetation to the litter layer where the relative humidity is higher. Here they can reabsorb water from the subsaturated air by actively secreting and then reingesting hygroscopic fluid before they can continue questing (Kahl and Knulle, 1988). After a host is found and a blood meal is taken, the engorged larvae and nymphs drop of the host and seek a suitable microhabitat, where relative humidity exceeds 80%. Here they moult to the next developmental stage and engorged adult females mate and lay eggs. Moulting time is influenced by temperature and was 41 and 42 days at 25 ± 1 ºC, for *I. pacificus* and *I. scapularis* larvae, which are closely related to *I. ricinus* (Troughton and Levin, 2007).

It is well known that vector behaviour and physiology can be influenced by parasitic infections (reviewed by Hurd, 2003). Parasites can for example increase the amount of contact between vectors and hosts, like *Leishmania* in sand flies (Beach *et al.*, 1985), reduce vector reproductive output to increase nutrient availability, like *Plasmodium* does in the yellow fever mosquito (Freier and Friedman, 1976), and increase vector longevity to enhance transmission, like some malaria parasites in mosquito's (Reviewed by Ferguson *et al*., 2002). Gassner *et al*. (in prep.) showed that a *B. burgdorferi* s.l. infection increased *I. ricinus* activity and energy use. For the *B. burgdorferi* s.l. spirochetes, this would be positive if it increased questing success and therefore transmission from vectors to hosts. The ticks on the other hand used more of their energy reserves and are probably more likely to die if no host is found in time. Not much is known on the influences of *B. burgdorferi* s.l. on the biology of ticks. Because *Leishmania* reduces blood uptake speed and therefore blood meal size, which results in an increase in host contact in sand flies (Beach *et al.*, 1985), it is hypothesized that a *B.*

burgdorferi s.l. infection increases blood meal size or consumption speed of ticks to increase nutrient availability and therefore *B. burgdorferi* s.l. transmission.

Ticks can maintain a stable water balance as long as the relative humidity of their microclimate does not decrease below 80%. Spatial and temporal variation in tick abundance was closely related to air temperature, relative humidity, soil water content and vegetation type (Schwarz *et al.*, 2009). Randolph and Storey (1999) found that the number of unfed *I. ricinus* nymphs that quested in the upper layers of the vegetation, where they quest for larger host species, decreased when relative humidity decreased, whereas the rate of fat use increased. Alekseev and Dubinina (2000) found a change in questing activity between non-infected and infected *I. persulcatus* nymphs. They found an infection rate of 0.0% when soil surface temperature was 13.1 ± 0.8 °C and soil surface humidity was 80.8 ± 1.5 % and an infection rate of 20.13 ± 3.68 when soil surface temperature was 19.5 ± 0.45 °C and soil surface humidity was 59.9 ± 1.2 %. This indicates that temperature and relative humidity determine the saturation deficit of the air, which is correlated to tick questing activity (Perret *et al*., 2003; Randolph *et al*., 2002). Although *I. ricinus* is present, Lyme borreliosis is rarely reported from south and southeast Europe (Gray, 1998; Rauter and Hartung, 2005). Because 1) this area is relatively drier, 2) an increased activity might increase respiratory water loss, and 3) because an increased activity reduces available energy to reabsorb water from the subsaturated air, we hypothesized that a *B. burgdorferi* s.l. infection negatively influences desiccation resistance of ticks. If a *B. burgdorferi* s.l. infection affects desiccation resistance, the ratio of infected to non-infected nymphs questing on lower height and as a result feeding on rodents, could then be influenced. A difference in questing height was also seen between larvae and nymphs (Mejlon and Jaenson, 1997), which differ in desiccation resistance due to different body mass to surface ratios. A difference in desiccation resistance would also influence seasonal and geographical variation in Lyme borreliosis transmission as is also seen in seasonal activity differences between larvae and nymphs.

The aim of this study was to investigate the effect of a *B. burgdorferi* s.l. infection on the development and desiccation resistance of *I. ricinus*. Two experiments were conducted. Experiment I focused on the effect of a *B. burgdorferi* s.l. infection in mice or ticks on the interstadial development of wild and captive bred *I. ricinus* larvae. Experiment II focused on the effect of a *B. burgdorferi* s.l. infection on the desiccation resistance of wild unfed *I. ricinus* nymphs.

Materials and Methods

Experiment I

Ticks. Wild mice were caught with 106 Longworth life-traps on the estate of Oostereng in The Netherlands, 2 km north of the town of Renkum (52°00'36N, 5°45'36E), 40 m above sea level, as described in Gassner *et al*. (2008). An ear puncture was collected from each not lactating adult Wood mouse (*Apodemus sylvaticus*), which was kept in 70% alcohol and brought to the lab to determine *B. burgdorferi* s.l. infection. Four mice with and 6 without *B. burgdorferi* s.l. infection were then transported to the lab and housed individually in Makrolon III cages, which were placed in basins with 5 cm water for 9 days. Engorged ticks drooped from the mice, climbed out of the cage and dropped into the water basin, from where they were collected daily at 11:00 AM. As soon as all mice were tick free, the mice were sedated with an intraperitonial injection with 0.15 ml hypnorm (1:4 diluted) and 0.15 ml dormicum (1:4 diluted). All mice were then placed individually in clean cages. Twenty five *B. burgdorferi* s.l. free captive bred *I. ricinus* larvae of approximately 1 month of age (supplied by a tick rearing facility in Neuchâtel, Switzerland) were then placed between the ears using a small brush. During the next 9 days, the engorged captive bred larvae were collected from the water basins as described above. All experiments involving rodents were approved by the animal welfare committee (DEC) of Wageningen University and Research centre under nr 2009042.

Feeding time and body measurements. Time between the moment the mice were placed in the Makrolon III cages and tick collection from the water basin was recorded to estimate feeding time of the wild larvae. Time between the placement of captive bred larvae on the sedated mice and tick collection from the water basin was recorded to determine feeding time of the captive bred larvae. After collection from the water basin, larvae were dried for 24 hours between filter papers, which were placed in Petri dishes (90 mm diameter). The Petri dishes were placed in a closed transparent plastic box (200*95*75 mm) with 1 cm of water to keep the relative humidity around 95%. Ticks were then weighed on a micro-balance to the nearest microgram. Freshly emerged nymphs were weighed again. When ticks were weighed as engorged larva and freshly emerged nymph, physiological status was determined with the scutal index, which was obtained by calculating the ratio of the maximum width of the scutum to the length of the idiosoma as was also done by Falco *et al*. (1996) and Gray *et al.* (2005). Ticks were photographed with a euromex CMEX-1 camera fitted to an Olympus SZX12

microscope at 50x magnification. Scutum width and idiosoma length were analysed from the photos using Image Focus 2.5 (Euromex microscopen B.V., Arnhem, The Netherlands).

Tick housing and interstadial development time. After the body measurements were completed, all engorged larvae were housed individually in small glass tubes with a piece of wet paper and perforated lids. These tubes were placed in plastic boxes to keep the relative humidity around 95% as described earlier and the boxes were placed in a climate chamber set at 20 °C. Lights were on between 06:00 AM and midnight. Time between collection of larvae from the water basin and emergence of the nymphs was recorded to estimate interstadial development time. Emergence of nymphs was recorded once per day at 11:00. Ticks were considered dead when they did not moult after 2 months.

Detection of *Borrelia burgdorferi* s.l. To extract DNA from mice and ticks, the ear puncture and tick were boiled in 100 µl 4 M ammonium hydroxide solution for 60 and 20 minutes, respectively. These solutions were then centrifuged at 14000 rpm for 20 seconds and heated at 90 °C for 20 min in opened vials to let the ammonia evaporate, as was done by Schouls *et al*. (1999). Samples were then stored at -20 °C until further analysis. DNA extracts were analyzed for *B. burgdorferi* s.l. DNA using a PCR targeting the HBB gene of *B. burgdorferi* s.l.. This method was adopted from Portnoï *et* al. (2006). For each DNA extract, 13.375 µl nuclease free H_2O, 5 µl loading buffer, 1.5 µl $MgCl_2$, 0.5 µl dNTP's, 1 µl forward primer, 1 µl reverse primer and 0.125 µl GoTaq polymerase was vortexed and pipetted in a 0.5 ml PCR vial. From the DNA extract 2.5 µl was added to the PCR mix and 2 negative controls (DNA extract and PCR H_2O) and 2 positive controls (*B. burgdorferi* sensu stricto and *B. afzelii*) were included in each PCR run. Step 1 of the PCR was set at 95 °C for 5 min, step 2 was set at 95 °C for 8 sec, step 3 was set at 50 °C for 10 sec, and step 4 was set at 72 °C for 10 sec. Step 2 till 4 were then repeated for 49 times. To prevent aspecific amplification of mouse DNA, the annealing temperature (step 2) was increased to 55 °C during mice sample PCR. Of each PCR product, 10 µl was evaluated using ethidium bromide stained gel electrophoresis.

Statistical analysis. Data analysis was done using SPSS 16.0. (SPSS Inc., Chicago, IL). Tick or mouse was used as the smallest experimental unit. Mean body weight, scutal index, feeding time, and interstadial development time were analyzed using a one way ANOVA with *B. burgdorferi* s.l. infection and tick origin as factor variable. Means were compared using Bonferroni adjustment for multiple comparisons. Larval and nymphal weight with *B. burgdorferi* s.l. infection in mice and tick origin as factor variable, were analyzed with an independent samples T-test. Model assumptions were checked by examining the skewness and kurtosis of the distributions of each variable. Scutal index, length, weight, feeding time, and interstadial development time were analyzed for linear relations between variables. Values are expressed as means ± 1 SEM. Treatment effect was analyzed for significance at $P \leq 0.05$.

Experiment II

Ticks. In August 2009, *I. ricinus* nymphs were collected on the estate of De Sysselt in The Netherlands, east of the town of Ede (52°01'44N, 5°41'58E), 40 m above sea level, by dragging a white cotton blanket (1*1 m) through the vegetation as was described by Daniels *et al*. (2000). Attached nymphs were collected with forceps and transported to the lab in 10 ml screw cap centrifuge tubes containing a piece of wet paper and stored at 4 °C until the start of the body measurements, which were determined prior to the experiment as in experiment I.

Capillary infection. After the weight and scutal index of nymphs were determined, 300 nymphs were placed on their back on a piece of double sided adhesive Tesa tape (Tesa AG, Hamburg, Germany). A glass capillary was heated, stretched and broken where the diameter was approximately 0.16 mm was pushed over the hypostome and nymphs were left in this position for 2 h. One hundred fifty nymphs were treated with capillaries filled with *Borrelia afzelii* strain DT1 (Provided by G.A. Oei, University of Amsterdam), which were cultured in BSK-II medium incubated at 33 °C and 150 nymphs with BSK-II medium only (incubated at 33 °C). Motility and cell length were observed under a dark field microscope at 400x magnification prior to the experiment. Spirochete concentration was $2.8*10^3$ per µl. Five ticks that were treated with medium with *B. burgdorferi* spirochetes and 5 ticks that were treated with medium only were weighed before and after treatment to estimate medium uptake. One hundred fifty nymphs were not weighed, measured and not treated with capillaries. Treatments were distributed at random to the ticks and performed randomly over a period of 9 days.

Tick housing. All nymphs that were alive at the start of the experiment were housed individually in 2 ml transparent eppendorf tubes. Lids were ventilated by making 5 small holes in it with a sharp needle. Tubes were divided over 3 transparent plastic boxes with air tight lids (50*35*25 cm, Xenos B.V., Waalwijk, The Netherlands). Two boxes had a layer of 5 cm of water at the bottom. Ventilation of these boxes was regulated to maintain the relative humidity in the eppendorf tubes at, 70 ± 5, or 95 ± 5%, which equal saturation deficits of 5.1 ± 0.9 and 0.9 ± 0.9 mmHg, respectively. One box contained no water to maintain the relative humidity in the eppendorf tubes at 50 ± 5%, which equals a saturation deficit of 8.6 ± 0.9 mmHg. Boxes were placed in a climate room set at 20 ± 1 °C. Lights were on between 06:00 AM and midnight. Climate conditions were constantly monitored using 2 MSR 145 dataloggers (MSR electronics, Henggart, Switzerland).

Mortality. Ticks were observed daily at 02:00 PM to determine mortality macroscopically. Ticks were considered dead when their legs and palps were shrunk back against the body and did not actively hold on to the tube wall. The location of ticks that were not clearly dead was marked on the tube. These ticks were considered to be dead when they did not move for 48h.

When all ticks in the 50 and 70% RH had died, the remaining ticks in the 95% RH group were killed and scored as if they died the next day. Dead ticks were stored at 4 °C before infection status with DNA extraction and PCR was determined as in experiment I.

Statistical analysis. Data analysis was done using SPSS 16.0. (SPSS Inc., Chicago, IL). Tick was used as the smallest experimental unit. Mean survival time was analyzed using a one way ANOVA with *B. burgdorferi* s.l. infection and treatment or relative humidity as factor variable. Means were compared using Bonferroni adjustment for multiple comparisons. Model assumptions were checked by examining the distributions of each variable. The effect of *B. burgdorferi* s.l. infection, capillary treatment or relative humidity on the desiccation resistance of *I. ricinus* was also analyzed using Kaplan-Meier pairwise comparisons (Cox, 1972). Scutal index, length, weight, and survival were analyzed for linear relations between variables. Values are expressed as means ± 1 SEM. Treatment effect was analyzed for significance at $P \leq 0.05$.

Results

Experiment I

Tick collection and infection

See appendix I for an overview of the captured small mammals and their sex, age and lactation activity.

From the 10 mice in total, 114 wild *I. ricinus* larvae were collected (Table 1). One infected nymph was collected from mouse 4 but was excluded from the analysis. Forty-four of the 114 wild larvae moulted (39%) and 14 of these moulted larvae were infected (32%). From the 4 mice that were infected with *B. burgdorferi* s.l., 50 wild larvae were collected. Twenty-one of these 50 wild larvae moulted (42%) and 12 of these moulted larvae were infected (57%). From the 6 mice that were not infected with *B. burgdorferi* s.l., 64 wild larvae were collected. Twenty-three of these 64 wild larvae moulted (36%) and 2 of these moulted larvae were infected (9%). From the 250 captive bred larvae that were released on the 10 mice, 40 were collected (16%). Thirty of these 40 captive bred larvae moulted (75%) and 16 of these moulted larvae were infected (53%). From the 4 mice that were infected with *B. burgdorferi* s.l., 12 captive bred larvae were collected (12%). Nine of these captive bred larvae moulted (75%) and 6 of these moulted larvae were infected (67%). From the 6 mice that were not infected with *B. burgdorferi* s.l., 28 captive bred larvae were collected (19%). Twenty-one of these captive bred larvae moulted (75%) and 10 of these moulted larvae were infected (36%).

Table 1. Experiment I: *Borrelia burgdorferi* s.l. infection in mice and number of collected, moulted, and infected *I. ricinus* larvae per mouse

			Wild larvae			Captive bred larvae			
Mouse	Sex	Infected	Collected	Moulted	Moulted and infected	Released	Collected	Moulted	Moulted and infected
1	♀	-	3	2	1	25	4	4	2
2	♂	-	19	1	0	25	2	2	0
3	♂	-	4	0	0	25	9	5	4
4	♂	+	6	4	3	25	3	2	2
5	♂	+	16	3	0	25	2	2	1
6	♂	-	22	13	0	25	1	1	0
7	♂	-	14	7	1	25	6	4	0
8	♂	+	16	8	5	25	7	5	3
9	♂	-	2	0	0	25	6	5	4
10	♂	+	12	6	4	25	0	0	0
Total			114	44	14	250	40	30	16

Effect of a *B. burgdorferi* s.l. infection in ticks on scutal index, weight, feeding time, and interstadial development time of ticks

Scutal index of wild engorged larvae was lower in infected larvae (4.03) compared to non-infected larvae (4.41), whereas this difference was not seen between infected captive bred larvae (3.99) and non-infected captive bred larvae (4.01) (P=0.008) (Table 2). After moulting to a nymph, scutal index of infected wild ticks was higher (1.95) than of infected captive bred ticks (1.86) (P=0.013). Infected wild engorged larvae were lighter (0.439 mg) than non-infected wild larvae (0.507 mg) (P=0.009). When moulted to a nymph, infected wild ticks were lighter (0.229 mg) than non-infected wild ticks (0.271 mg) (P=0.014). Feeding time of wild infected larvae was shorter (3.64 d) than of captive bred infected larvae (5.25 d) (P<0.001). Interstadial development time did not differ between infected and non-infected ticks, and wild and captive bred ticks (P=0.092).

Effect of a *B. burgdorferi* s.l. infection in mice on scutal index, weight, feeding time, and interstadial development time of ticks feeding on them

Scutal index of wild larvae from infected mice was lower (3.99) compared to wild larvae from non-infected mice (4.21) (P=0.046) (Table 3). After moulting to a nymph, scutal index of captive bred ticks from infected mice was lower (1.87) than of wild ticks from infected mice (1.95) (P=0.019). Wild larvae from infected mice were lighter (0.433 mg) compared to wild larvae from non-infected mice (0.468 mg) (P=0.040). Wild freshly emerged nymphs from infected mice were lighter (0.225 mg) compared to wild nymphs from non-infected mice (0.261 mg) (P=0.030). Feeding time of wild larvae on infected mice was shorter (3.48 d) than of captive bred larvae from infected mice (5.21 d) (P<0.001). Interstadial development time did not differ between ticks from infected and non-infected mice, and wild and captive bred ticks (P=0.442).

Table 2. Experiment I: Effect of a *B. burgdorferi* s.l. infection in ticks on scutal Index, weight, feeding time and development time of wild and captive bred *I. ricinus*

		Scutal index		Weight (mg)		Feeding time (d) *		Interstadial development time (d)	
Ticks	*Borrelia* infection in ticks	Engorged larvae	Emerged nymphs	Engorged larvae	Emerged nymphs	Mean	Min. / Max.	Mean	Min. / Max.
Wild	+	4.03 ± 0.04 [a]	1.95 ± 0.02 [b]	0.439 ± 0.01 [a]	0.229 ± 0.01 [a]	3.64 ± 0.20 [a]	1 / 10	45.33 ± 1.20	35 / 61
	-	4.41 ± 0.08 [b]	1.96 ± 0.02 [b]	0.507 ± 0.02 [b]	0.271 ± 0.01 [b]	4.71 ± 0.47 [a b]	3 / 9	48.93 ± 1.30	40 / 59
Captive bred	+	3.99 ± 0.08 [a]	1.86 ± 0.03 [a]	0.478 ± 0.01 [a b]	0.251 ± 0.01 [a b]	5.25 ± 0.31 [b]	3 / 9	45.76 ± 1.35	40 / 63
	-	4.01 ± 0.10 [a]	1.91 ± 0.02 [a b]	0.475 ± 0.02 [a b]	0.264 ± 0.02 [a b]	5.38 ± 0.27 [b]	4 / 8	43.31 ± 1.35	36 / 54
N		150	73	152	74	152	152	74	74
P-value		0.008	0.013	0.009	0.014	<.0001		0.092	

[a] Means within a column lacking a common superscript differ ($P \leq 0.05$).
* Note that feeding time of wild *I. ricinus* was measured from capture moment and not from release moment.

Table 3. Experiment I: Effect of a *B. burgdorferi* s.l. infection in mice on scutal Index, weight, feeding time and development time of wild and captive bred *I. ricinus*

		Scutal index		Weight (mg)		Feeding time (d) *		Interstadial development time (d)	
Ticks	*Borrelia* infection in mice	Engorged larvae	Emerged nymphs	Engorged larvae	Emerged nymphs	Mean	Min. / Max.	Mean	Min. / Max.
Wild	+	3.99 ± 0.39 [a]	1.95 ± 0.02 [b]	0.433 ± 0.01 [a]	0.225 ± 0.04 [a]	3.48 ± 0.24 [a]	1 / 10	46.09 ± 1.41	35 / 61
	-	4.21 ± 0.06 [b]	1.96 ± 0.02 [b]	0.468 ± 0.01 [b]	0.261 ± 0.05 [b]	4.08 ± 0.29 [a b]	1 / 9	46.90 ± 1.26	36 / 59
Captive bred	+	4.01 ± 0.08 [b]	1.87 ± 0.02 [a]	0.478 ± 0.07 [b]	0.253 ± 0.01 [b]	5.21 ± 0.26 [b]	3 / 9	45.38 ± 1.24	38 / 63
	-	3.97 ± 0.09 [b]	1.91 ± 0.04 [a b]	0.475 ± 0.08 [a b]	0.264 ± 0.02 [b]	5.50 ± 0.38 [b]	4 / 9	43.11 ± 1.44	36 / 52
N		150	73	152	74	154	154	74	74
P-value		0.026	0.019	0.040	0.030	<0.001		0.442	

[a] Means within a column lacking a common superscript differ ($P \leq 0.05$).
* Note that feeding time of wild *I. ricinus* was measured from capture moment and not from release moment.

Relations between variables

A significant linear regression was found between the scutal index of larvae and the scutal index of nymphs (R^2 = 0.111, *P*=0.002), between weight of larvae and weight of nymphs (R^2 = 0.819, *P*<0.001), and between feeding time and interstadial development time (R^2 = 0.144, *P*=0.008) (Table 4 and figures 1, 2, and 3 in appendix II for the regressions per tick group).

Table 4. Experiment I: *P*-values and R-squares for each linear regression between variables. *P*-values are expressed above the "X" and R-squares are expressed below the "X"

	Scutal index larvae	Scutal index nymphs	Weight larvae	Weight nymphs	Feeding time	Development Time
Scutal index larvae	X	0.002	0.128	0.162	0.542*	0.990
Scutal index nymphs	0.111	X	0.339	0.935	0.672*	0.727
Weight larvae	0.322	0.004	X	>0.001	0.155*	0.243
Weight nymphs	0.369	0.007	0.819	X	0.257*	0.384
Feeding time	0.016*	0.001*	0.001*	>0.001*	X	0.008*
Development time	0.001	0.003	0.024	0.005	0.144*	X

* Note that feeding time of wild *I. ricinus* was measured from capture moment and not from release moment and that this overall regression should therefore be observed with caution.

Experiment II

Effect of capillary treatment on *B. burgdorferi* s.l. infection

Medium intake of 5 ticks that were treated with *B. burgdorferi* s.l. spirochetes in medium varied between 0.000 and 0.101 mg (Average: 0.021 mg, $5.9*10^4$ spirochetes) and intake of 5 ticks that were treated with medium only varied between 0.000 and 0.048 mg (Average: 0.053 mg). From the 3 groups of 150 nymphs that were treated with capillaries filled with *B. burgdorferi* s.l. spirochetes in BSK-II medium, BSK-II medium only or not treated, 99, 101, and 144 were alive at the beginning of the experiment, respectively. Of these nymphs, 59 (60%), 17 (17%), and 31 (22%) were infected with *B. burgdorferi* s.l., respectively.

Effect of capillary treatment and *B. burgdorferi* s.l. infection on desiccation resistance

Mean survival in days at 50, 70, and 95% RH was not influenced by a *B. burgdorferi* s.l. infection (Table 5). However, in the 50% RH group, nymphs that were capillary treated died earlier than the nymphs that were not treated, irrespective of a *B. burgdorferi* s.l. infection (*P*<0.001). In the 70% RH group, nymphs that were capillary treated but not infected died earlier than nymphs that were not treated (*P*<0.001). In the 95% RH group, nymphs that were capillary treated but not infected died earlier (6.04 d) than nymphs that were not treated and not infected (7.72 d) (*P*=0.003). From the total of nymphs, nymphs that were capillary treated

but not infected died earlier than nymphs that were not treated ($P<0.001$). The survival curves of the nymphs were not influenced by a *B. burgdorferi* s.l. infection (Figure 1 and table 6). In the 50 and 95% RH groups, the survival curve of nymphs that were capillary treated differed from the nymphs that were not treated ($P<0.05$). In the 70% RH group, the survival curve of nymphs that were capillary treated but not infected differed from the nymphs that were not treated and not infected ($P<0.05$).

Table 5. Experiment II: Effect of capillary treatment and *B. burgdorferi* s.l. infection on mean survival of *I. ricinus* nymphs at different relative humidities

	Mean survival (d)			
Ticks	50% RH	70% RH	95% RH *	Total
Capillary treated and infected	1.31 [a]	2.12 [a b]	7.00 [a b]	3.43 [a b]
Capillary treated but not infected	1.40 [a]	1.44 [a]	6.04 [a]	2.90 [a]
Not treated but infected	3.25 [b]	2.42 [b]	7.46 [a b]	4.79 [b]
Not treated and not infected	2.40 [b]	2.50 [b]	7.72 [b]	4.10 [b]
N	120	111	113	344
P-value	<0.001	<0.001	0.003	<0.001

[a] Means within a column lacking a common superscript differ ($P\leq0.05$).
* Mean survival at 95% RH is low, because the experiment was terminated when all ticks that were housed at 50 and 70% RH had died.

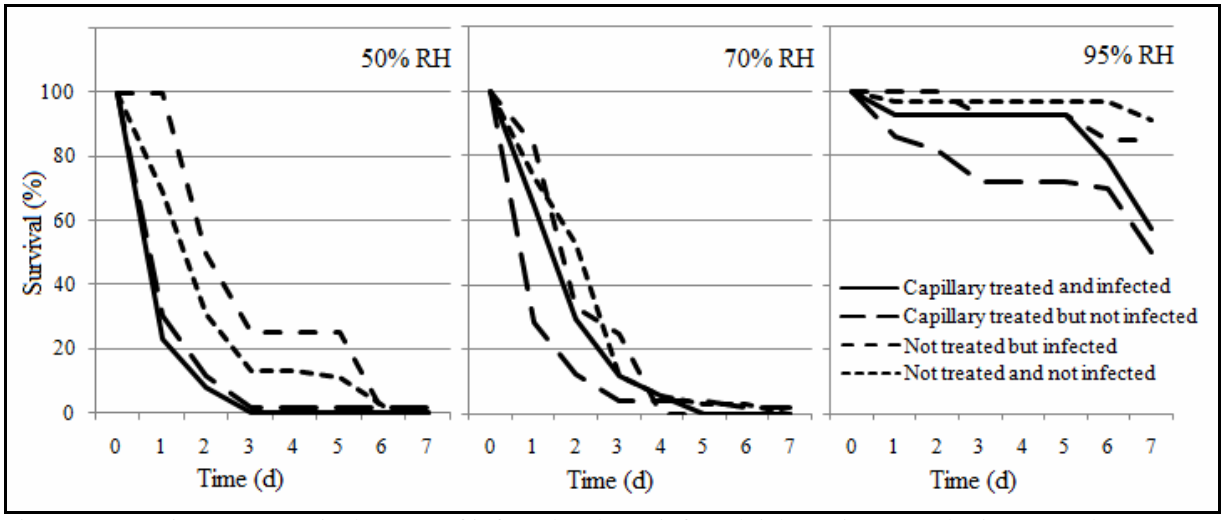

Figure 1. Experiment II: Survival curves of infected and non-infected ticks at 50, 70, and 95% RH and 20 ºC.

Table 6. Experiment II: Effect of capillary treatment and *B. burgdorferi* s.l. infection on survival curves of *I. ricinus* nymphs at different relative humidities

	50% RH		70% RH		95% RH	
Survival curve	N	Curve[a]	N	Curve[a]	N	Curve[a]
Capillary treated and infected	22	a	27	a b	27	a
Capillary treated but not infected	77	a	72	a	73	a
Not treated but infected	4	b	13	b c	13	b
Not treated and not infected	46	b	36	c	37	b

[a] Survival curves within a column lacking a common letter differ ($P\leq0.05$).

Effect of humidity on desiccation resistance

Nymphs died earlier at 50 and 70% RH compared to 95% RH and no difference in mean survival was found between 50 and 70% RH, irrespective treatment and infection status (P<0.001) (Table 7). The survival curves of the nymphs were influenced by the relative humidity (Figure 2 and table 8). In the capillary treated and infected nymphs, the curves of the 50, 70, and 95% RH groups differed from each other (P<0.05). In the capillary treated but not infected, not treated but infected, and the not treated and not infected nymphs, the curve of the 95% RH group differed from the 50 and 70% RH groups (P<0.05).

Table 7. Experiment II: Effect of relative humidity on mean survival of *I. ricinus* nymphs with or without a *B. burgdorferi* s.l. infection after different treatments

	Mean survival (d)				
	Capillary treated		Not treated		Total
Humidity	Infected	Not infected	Infected	Not infected	
50%	1.31 [a]	1.40 [a]	3.25 [a]	2.40 [a]	1.83 [a]
70%	2.12 [a]	1.44 [a]	2.42 [a]	2.50 [a]	1.97 [a]
95% *	7.00 [b]	6.04 [b]	7.46 [b]	7.72 [b]	6.86 [b]
N	44	156	29	115	344
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

* Mean survival at 95% RH is low, because the experiment was terminated when all ticks that were housed at 50 and 70% RH had died.

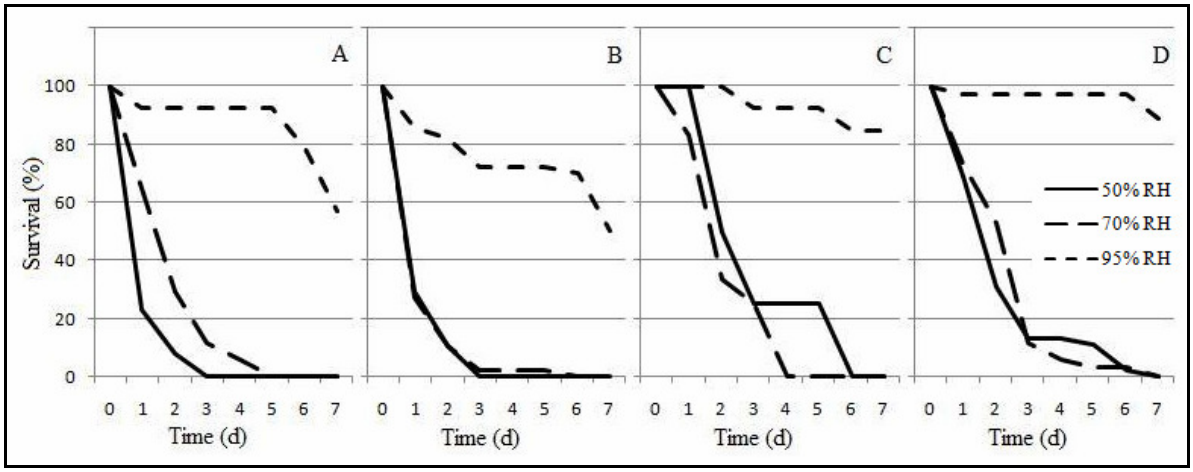

Figure 2. Experiment II: Survival curves of capillary treated and infected ticks (A), capillary treated but not infected ticks (B), not treated but infected ticks (C), and not treated and not infected ticks (D) at 50, 70, and 95% RH and 20 ºC.

Table 8. Experiment II: Effect of relative humidity on survival curves of *I. ricinus* nymphs with or without a *B. burgdorferi* s.l. infection after different treatments

	Capillary treated				Not treated			
	Infected		Not infected		Infected		Not infected	
Survival curve	N	Curve[a]	N	Curve[a]	N	Curve[a]	N	Curve[a]
50% RH	22	a	77	a	4	a	46	a
70% RH	27	b	72	a	13	a	36	a
95% RH	27	c	73	b	13	b	37	b

[a]Survival curves within a column lacking a common letter differ (P≤0.05).

Relations between variables

A linear regression was found between body length and body weight (R^2 = 0.015, *P*=0.033) (Table 9 and figure 4 in appendix II for the regression per tick group).

Table 9. Experiment II: *P*-values and R-squares for each linear regression between variables. *P*-values are expressed above the "X" and R-squares are expressed below the "X"

	Scutal index	Length	Weight	Survival
Scutal index	X	0.058	0.538	0.628
Length	0.011	X	0.031	0.929
Weight	<0.001	0.015	X	0.167
Survival	<0.001	<0.001	0.007	X

Discussion

Experiment I

The infection rates of *B. burgdorferi* s.l. from infected mice to wild and captive bred larvae were 57 and 67%, respectively. From the moment of spirochete transmission from the host to the tick till the moment the DNA was extracted from the ticks, the suitability of the tick depended on the extent to which the spirochetes could colonize the tick, reproduced, and survived in the midgut during the moult (Tsao, 2009). Because the infection rate of larvae that were collected from infected mice was not 100%, one of these was not optimal, but which one is not known. The infection rate of the larvae was determined after they moulted to a nymph. From the 154 larvae that were collected from the 10 mice in total, only 74 succeeded to moult. Most of the 80 larvae that did not complete their moult were overgrown by fungus, which made detection of *B. burgdorferi* s.l. impossible. Of the collected wild and captive bred larvae, 61% and 25% did not moult to nymphs, respectively. This difference between the wild and captive bred ticks was probably caused by a higher humidity in the tubes of the wild ticks. The pieces of paper that were added to the tubes of the wild larvae were soaked in water, whereas only 10 µl of water was pipetted on the pieces of paper that were added to the tubes of the captive bred larvae. It is not known whether the ticks that did not moult and were overgrown by fungus had the same infection rate as the ticks that moulted because the *B. burgdorferi* s.l. spirochetes could have had an effect on this. The *B. burgdorferi* s.l. transmission from host to tick could therefore be higher than was measured. Burkot *et al.* (1994) placed uninfected *I. scapularis* larvae on *B. burgdorferi* s.l. infected mice and collected them after they dropped from the mice. They found that *B. burgdorferi* s.l. infection rate of larvae that just dropped from the mice was higher than infection rate of larvae that moulted to a nymph (98 and 85% respectively). This was perhaps caused by a decrease in the number of spirochetes during moulting (Piesman *et al.*, 1990), which indicates that the colonization of *B. burgdorferi* s.l. spirochetes from mice to larvae could have been high, whereas an unsuccessful reproduction or survival in the midgut during the moult could have reduced spirochete numbers, which resulted in a lower infection rate in nymphs. The infection rates of 9 and 36% in nymphs that moulted from engorged wild and engorged captive bred larvae that were collected from non-infected mice were higher than expected. The *I. ricinus* larvae that were used did not take a blood meal other than from the mice in the experiment. Furthermore, unfed *I. ricinus* larvae are only rarely infected with *B. burgdorferi* s.l., which indicates that transovarial infection of *B. burgdorferi* s.l. is rare (Rauter and Hartung, 2005). It

is therefore likely that the *B. burgdorferi* s.l. spirochetes were transmitted from the mice that were used in the experiment. It is therefore expected that the non-infected mice from which infected nymphs were collected could actually have been infected with *B. burgdorferi* s.l., but that spirochete numbers in the ear punctures that were collected were too low to detect. To make sure that the non-infected mice were indeed non-infected, organ autopsy has to be done on each mouse to determine *B. burgdorferi* s.l. infection in these organs.

Infected wild engorged larvae had a lower scutal index than non-infected wild engorged larvae. This could suggest that a *B. burgdorferi* s.l. transmission from the host to the tick has a negative effect on blood meal size. Because no difference in feeding time was found between infected and non-infected ticks, it is likely that the *B. burgdorferi* s.l. transmission negatively influenced blood uptake rate. A reduced blood meal size after a lower blood consumption speed was also found in sandflies infected with *Leishmania* (Beach *et al.*, 1985). However, in this case it was advantageous for the *Leishmania* transmission because it resulted in an increase in number of blood meals in sandflies and therefore also host contact. *Ixodes ricinus* on the other hand is not known to quest for a second host after a relatively small blood meal is consumed. A smaller blood meal is therefore not necessarily advantageous for the transmission of the *B. burgdorferi* s.l. spirochetes. The difference in scutal index between infected and non-infected *I. ricinus* larvae could also indicate that the ticks that took a smaller blood meal were already less resistant to a *B. burgdorferi* s.l. infection compared to ticks that took a larger blood meal. In this case, the ticks that consumed blood at a lower rate were then more susceptible for a *B. burgdorferi* s.l. infection.

Infected wild engorged larvae were lighter than non-infected wild larvae and a linear relation was found between the weight of engorged larvae and the weight of emerged nymphs. Furthermore, infected wild emerged nymphs were lighter than non-infected wild emerged nymphs. This indicates that a *B. burgdorferi* s.l. infection in engorged larvae reduces the weight of the emerged nymphs too. The difference in scutal index and weight between infected and non-infected wild *I. ricinus* larvae and nymphs was not found in captive bred *I. ricinus* larvae. Caution should therefore be taken if one wants to extrapolate results from experiments with captive bred ticks to wild ticks. The captive bred larvae originated from Switzerland and came from the same cohort while the wild larvae originated from the Netherlands and came from different cohorts. It is speculated that larvae from different cohorts differ in their susceptibility to a *B. burgdorferi* s.l. infection, which could have been caused by a different fitness, which resulted in a different blood consumption rate. If this is the case, it can be speculated that smaller ticks are more susceptible to a *B. burgdorferi* s.l. infection and that it would be advantageous for ticks to be larger. Gassner *et al.* (in prep.) showed that infected ticks were more active and burned more energy, which would result in a negative selection against ticks with a *B. burgdorferi* s.l. infection. A higher infection rate in

smaller ticks is then perhaps not a positive mechanism from the *B. burgdorferi* s.l. spirochetes point of view, but maybe the only option because the immune system of larger ticks could be too strong. Dusbábek (1996) found that *I. ricinus* nymphs with smaller body dimensions moulted predominantly to a male. Therefore, infection in males could be a higher than in females. This could be the reason why infection rate in *I. ricinus* does not reach 100% in the wild. Further research is needed to test this hypothesis.

A *B. burgdorferi* s.l. infection in the host resulted in the same trend in differences in scutal index of larvae, scutal index of nymphs, weight of larvae, weight of nymphs, feeding time, and interstadial development time between the infected and non-infected ticks compared to a *B. burgdorferi* s.l. infection in the ticks. This was most likely caused by a higher percentage of infected ticks from infected mice compared to infected ticks from non-infected mice.

Feeding time of wild larvae varied between 1 and 10 days. Because feeding time of wild larvae was recorded from the moment the mice were placed in their cages till tick collection from the water basin, feeding time of wild larvae could have been longer. Feeding time of captive bred larvae varied between 3 and 9 days and was recorded from moment of release till tick collection from the water basin. This is in contradiction to the feeding time of 3-5 days found in literature (Needham and Teel, 1991; Sonenshine, 1991). This difference could have been caused by a difference in collection method. In the present study ticks first had to climb out the Makrolon cages before they fell in the water basin and could be collected. Other authors could have used other methods, like cages with grid floors from which the ticks fell in the water basins immediately (De Boer *et al*., 1993). Although no ticks were observed to hide in the soil of the cages, there could have been some time between the moment ticks dropped off their hosts and the moment they fell in the water basin. Because only 40 of the 250 released captive bred larvae were collected and none were found in the soil at the end of the experiment it can be speculated that a part of the other 210 larvae took a blood meal and died or were killed by the mice before they could climb out of the cage. It can be expected that this also happened with a part of the wild larvae that were on the mice when they were captured and that the numbers of wild ticks that were on the mice were actually higher than the numbers that were collected.

From experiment I was concluded that a *B. burgdorferi* s.l. infection in *I. ricinus* had no effect on feeding time and interstadial development time between *I. ricinus* larvae and nymphs, but that it reduced blood meal size of wild larvae and weight of both wild larvae and nymphs. Further research is needed to test whether body size of *I. ricinus* affects *B. burgdorferi* s.l. susceptibility and whether an infection is disadvantageous for the survival of *I. ricinus*.

Experiment II

Only 60% of the nymphs that were treated with capillaries filled with *B. burgdorferi* s.l. spirochetes in BSK-II medium was infected. This was probably below 100% because not all nymphs took up the medium and therefore no spirochetes. Although a *B. burgdorferi* s.l. infection had a negative effect on energy stock (Gassner *et al.,* in prep.) and reabsorbtion of water from the subsaturated air costs energy (Kahl and Knulle, 1988), no effect of a *B. burgdorferi* s.l. infection was found on the desiccation resistance of *I. ricinus* nymphs. Therefore, no support was found for our hypothesis that a *B. burgdorferi* s.l. infection negatively influences desiccation resistance of ticks. However, Alekseev and Dubinina (2000) found that infection of *B. burgdorferi* s.l. in *I. persulcatus* nymphs was 20.13% at a saturation deficit of the soil surface of 6.7 mmHg (T = 19.5 °C and RH = 59.9%), whereas 0% was infected at a saturation deficit of 2.1 mmHg (T = 13.1 °C and RH = 80.8%). It is therefore suggested that ticks that are infected with *B. burgdorferi* s.l. are not more resistant to high saturation deficits, but that the higher questing activity found by Alekseev and Dubinina (2000) was a result of an increased activity of *I. persulcatus*, caused by the *B. burgdorferi* s.l. spirochetes, as was also found in *I. ricinus* by Gassner *et al.* (in prep.). An increased activity could increase host contact and therefore the transmission of the *B. burgdorferi* s.l. spirochetes from the ticks to the host.

Ticks have to survive between blood meals for more than 90% their life and water maintenance is an important process that influences off-host survival (Needham and Teel, 1991) and is influenced by the saturation deficit of the air. The relative humidities of 50, 70, and 95% at 20 °C that were used in the experiment equal saturation deficits of 8.6, 5.1, and 0.9 mmHg, respectively. At all these saturation deficits, mean survival was higher for nymphs that were not capillary treated, indicating that the capillary treatment had a negative effect on the *I. ricinus* nymphs. Because of this and the low infection success, capillary infection is perhaps not an optimal method to infect *I. ricinus* nymphs. Even though the live ticks of the 95% RH group were killed and scored as if they died the day after all ticks in the 50 and 70% RH had died, mean survival time was longer at 95% RH compared to 50 and 70% RH for all groups of nymphs. Mean survival between the 50% and 70% RH groups did not differ. Meyer-König *et al.* (2001) found that survival time of unfed adult *Dermacentor marginatus* and *D. reticulates* prolonged with decreasing saturation deficits. These authors housed adult males and females at 33, 55, 76, or 95% RH at 20 °C, which equal saturation deficits of 11.5, 7.7, 4.1, and 0.9 mmHg, respectively and determined the 50% mortality period and maximum survival time. Although no significance was determined the results indicate a longer 50% mortality period at a saturation deficit of 4.1 mmHg, compared to a saturation deficit of 7.7

mmHg (on average 152 and 81 days, respectively) and a longer maximum survival time (on average 235 and 128 days, respectively). Rodgers *et al*. (2007) housed *Ixodes scapularis* nymphs for 96 h at 40, 60, 75, or 82% RH and 23 °C, which equal saturation deficits of 12.4, 8.3, 5.2, and 3.7 mmHg, respectively and found that approximately 70% of the ticks survived at a saturation deficit of 3.7 mmHg whereas only approximately 8% of the ticks survived at a saturation deficit of 5.2 mmHg. Stafford (1994) housed *I. ricinus* nymphs at 65, 75, 85, 93, and 100% RH at 27 °C, which equal saturation deficits of 9.3, 6.6, 4.0, 1.9, and 0.0 mmHg, respectively and found a large decrease in survival at saturation deficits higher than 4.0 mmHg. These results are in line with our results. It is therefore supported that a saturation deficit of approximately 4 mmHg is the threshold for *I. ricinus* (See Perret *et al*., 2000, 2003) and perhaps also for *I. scapularis*, *D. marginatus* and *D. reticulates*. In that case, these species can not quest for passing blood meals for long periods if the saturation deficit of their micro-habitat is below this threshold. Schulze and Jordan (2003) found peak numbers of questing *I. scapularis* and *Amblyomma americanum* nymphs at different times of the day, which suggests that different tick species or genera require different environmental conditions. The relation between saturation deficit, *B. burgdorferi* s.l. infection and survival of *I. ricinus* nymphs, increases our knowledge of the inability of this species to withstand high saturation deficits. Why *B. burgdorferi* s.l. is absent from southern Europe needs more research. Because *B. burgdorferi* s.l. infection might be higher in small ticks (See experiment I) and smaller ticks have a disadvantageous body volume to surface ratio, they could have to quest at lower heights where relative humidity is higher, which might influence tick bite occurrence in humans. To investigate this, the *B. burgdorferi* s.l. infection percentage and body size of *I. ricinus* in southern Europe should be compared to northern Europe.

From experiment II was concluded that a *B. burgdorferi* s.l. infection in *I. ricinus* had no effect on the desiccation resistance of wild unfed *I. ricinus* nymphs, but that capillary infection had a negative effect. Further research is needed to test whether a *B. burgdorferi* s.l. infection in *I. ricinus* has a positive effect on questing success and whether infection percentage and body size of *I. ricinus* in southern Europe differs from northern Europe.

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Appendix I

Experiment I: Overview of the captured small mammals and their sex, age, and lactation activity.

	Species	Sex	Age / stage		Species	Sex	Age / stage
1	*Apodemus sylvaticus*	f	Lactating	29	*Clethrionomys glareolus*	m	Adult
2	*Clethrionomys glareolus*		Juvenile	30	*Clethrionomys glareolus*	m	Adult
3	*Clethrionomys glareolus*	m	Adult	31	*Apodemus sylvaticus*	f	Adult
4	*Apodemus sylvaticus*	m	Adult	32	*Clethrionomys glareolus*		Juvenile
5	*Apodemus sylvaticus*	m	Adult	33	*Clethrionomys glareolus*	f	Adult
6	*Apodemus sylvaticus*		Juvenile	34	*Clethrionomys glareolus*	f	Lactating
7	*Apodemus sylvaticus*	f	Lactating	35	*Clethrionomys glareolus*	f	Adult
8	*Apodemus sylvaticus*		Juvenile	36	*Apodemus sylvaticus*	m	Adult
9	*Clethrionomys glareolus*		Juvenile	37	*Apodemus sylvaticus*	f	Lactating
10	*Apodemus sylvaticus*		Juvenile	38	*Clethrionomys glareolus*		Juvenile
11	*Sorex* sp.			39	*Clethrionomys glareolus*	m	Adult
12	*Sorex* sp.			40	*Clethrionomys glareolus*		Juvenile
13	*Apodemus sylvaticus*	m	Adult	41	*Clethrionomys glareolus*	f	Lactating
14	*Apodemus sylvaticus*	m	Adult	42	*Apodemus sylvaticus*	f	Lactating
15	*Clethrionomys glareolus*		Juvenile	43	*Apodemus sylvaticus*		Juvenile
16	*Apodemus sylvaticus*		Juvenile	44	*Apodemus sylvaticus*	m	Adult
17	*Clethrionomys glareolus*		Juvenile	45	*Apodemus sylvaticus*	m	Adult
18	*Apodemus sylvaticus*		Juvenile	46	*Sorex* sp.		
19	*Clethrionomys glareolus*	m	Adult	47	*Clethrionomys glareolus*		Juvenile
20	*Clethrionomys glareolus*	m	Adult	48	*Clethrionomys glareolus*		Juvenile
21	*Clethrionomys glareolus*	m	Adult	49	*Clethrionomys glareolus*		Juvenile
22	*Clethrionomys glareolus*	f	Adult	50	*Clethrionomys glareolus*	f	Adult
23	*Apodemus sylvaticus*	f	Lactating	51	*Clethrionomys glareolus*		Juvenile
24	*Apodemus sylvaticus*	f	Lactating	52	*Clethrionomys glareolus*		Juvenile
25	*Apodemus sylvaticus*		Juvenile	53	*Clethrionomys glareolus*		Juvenile
26	*Clethrionomys glareolus*	f	Adult	54	*Clethrionomys glareolus*		Juvenile
27	*Clethrionomys glareolus*		Juvenile	55	*Clethrionomys glareolus*	m	Adult
28	*Clethrionomys glareolus*	f	Lactating	56	*Clethrionomys glareolus*	m	Adult

Appendix II

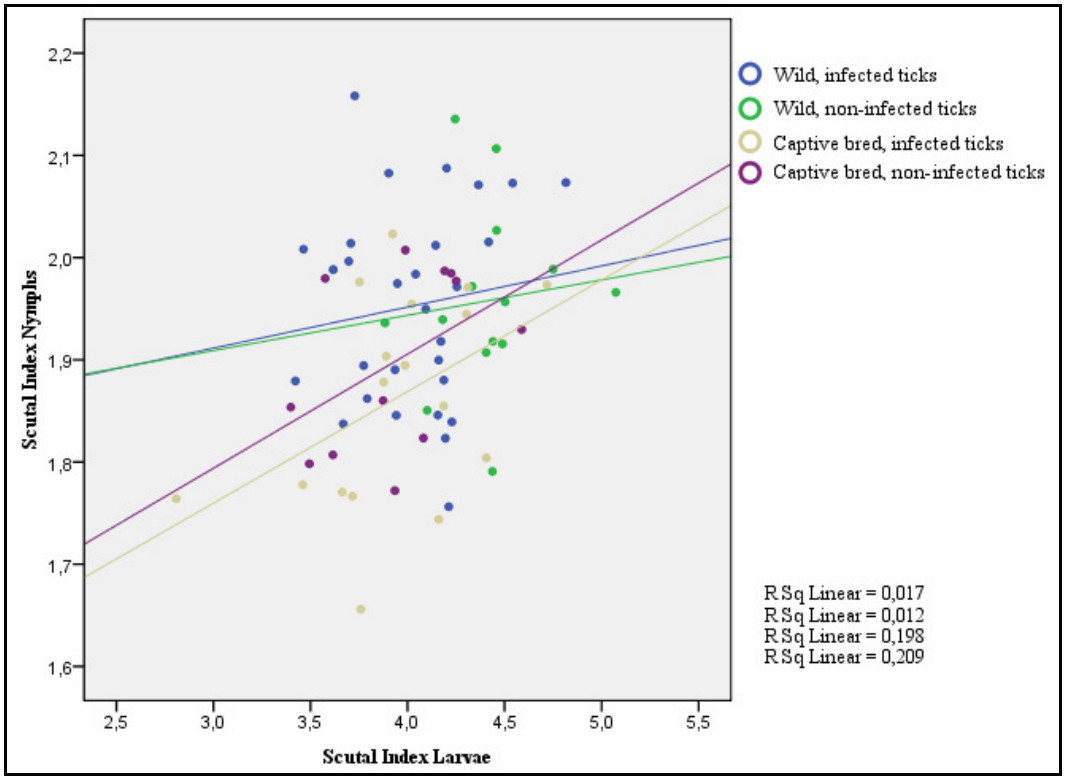

Figure 1, Experiment I: Relation between scutal index of larvae and scutal index of nymphs per group.

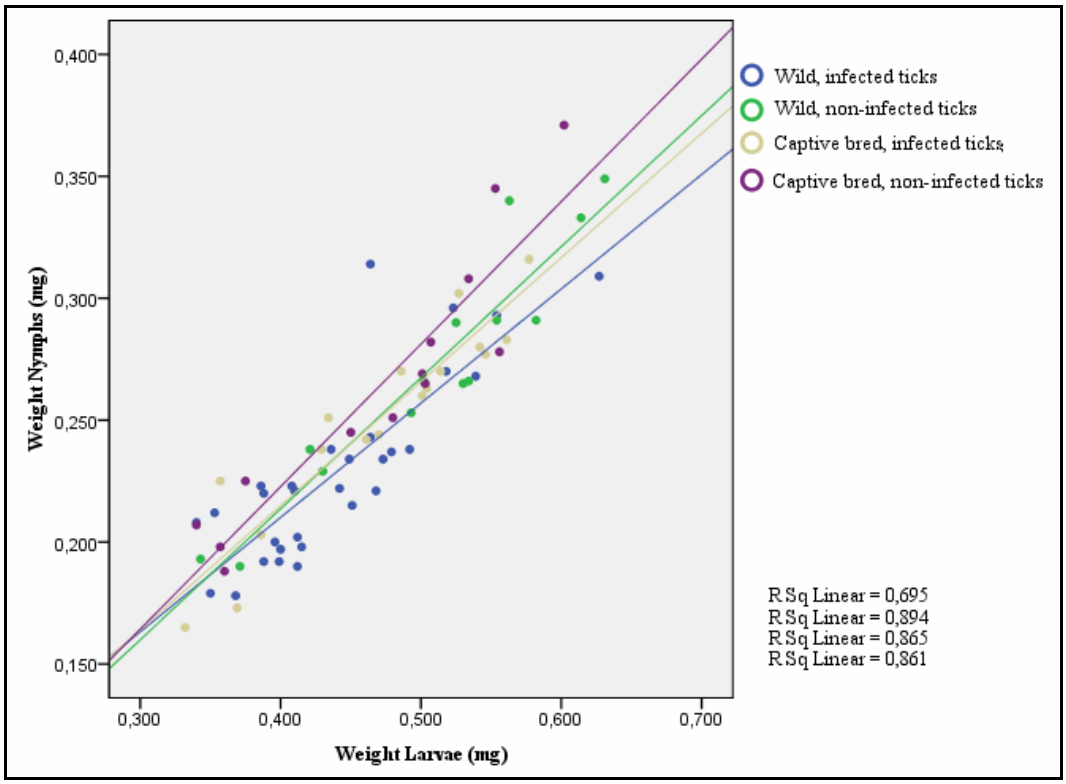

Figure 2, Experiment I: Relation between weight of larvae and weight of nymphs per group.

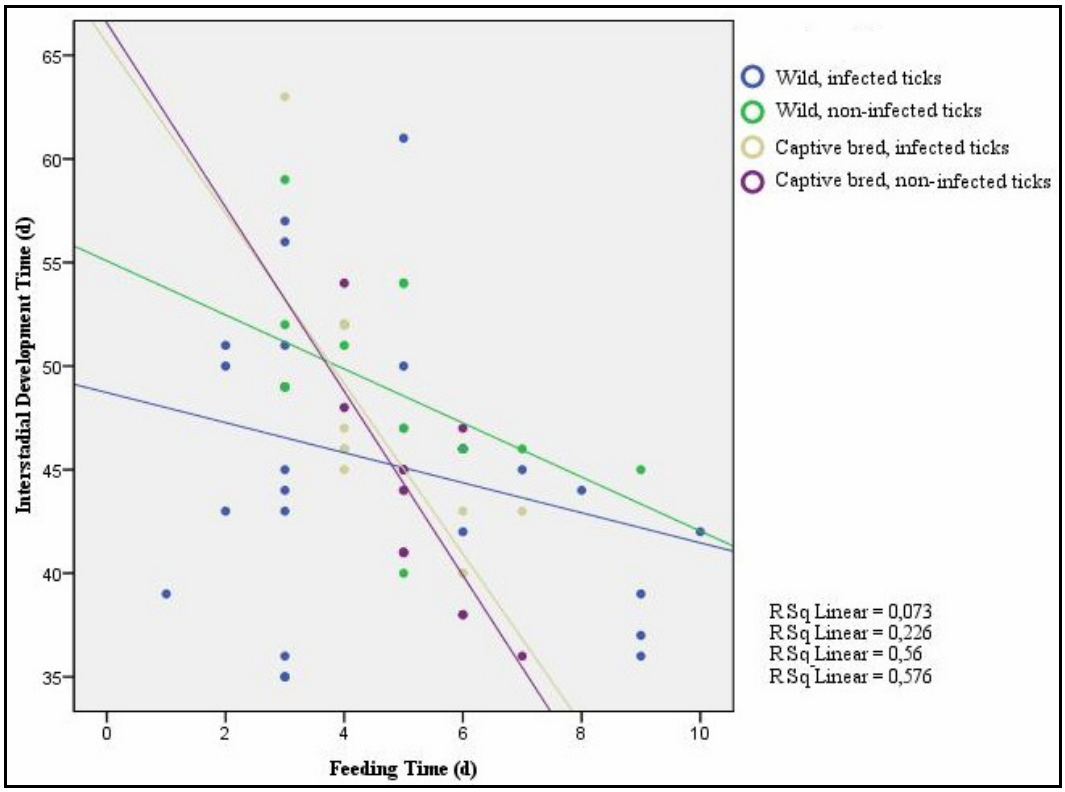

Figure 3, Experiment I: Relation between feeding time and interstadial development time per group.

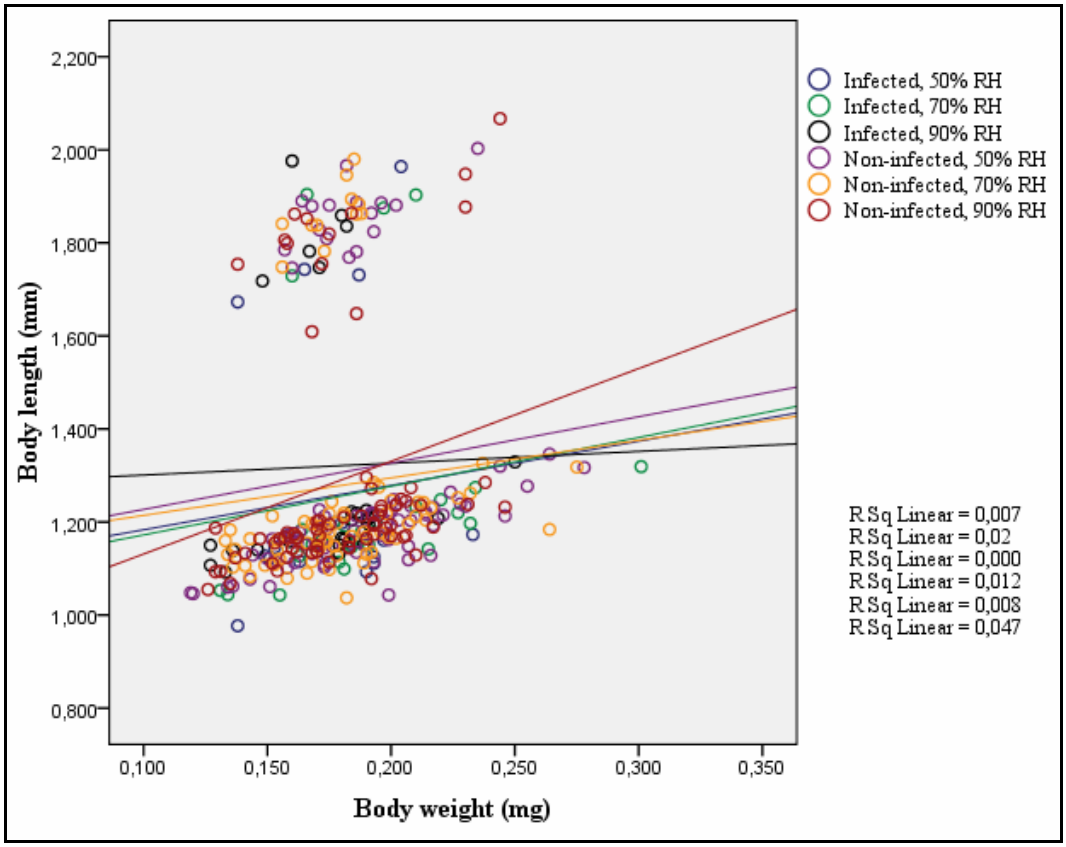

Figure 4, Experiment II: Relation between body weight and body length of capillary treated nymphs per group. Untreated nymphs were not weighed and measured and therefore not included in the figure.