Infection with *Borrelia afzelii* reduces moulting time of *Ixodes ricinus* ticks

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Abstract

Arthropod vectors carry vector-borne pathogens that cause infectious disease in vertebrate hosts, and arthropod-associated non-pathogenic microorganisms. Both types of micro-organisms can influence the fitness of their arthropod vectors, and hence the epidemiology of vector-borne diseases. The bacterium *Borrelia afzelii*, which causes Lyme disease in Europe, is transmitted among vertebrate reservoir hosts by *Ixodes ricinus* ticks, which also harbour a large diversity of non-pathogenic bacteria. The purpose of this study was to test experimentally whether *B. afzelii* and tick-associated non-pathogenic bacteria influence the fitness of *I. ricinus*. Eggs obtained from field-collected adult female ticks were washed with bleach, which reduced the abundance of non-pathogenic bacteria in the hatched *I. ricinus* larvae by 28-fold compared to larvae that hatched from eggs washed with water. The dysbiosed and control larvae were subsequently fed on *B. afzelii*-infected or uninfected control mice, and the engorged larvae were allowed to moult into nymphs under laboratory conditions. *I. ricinus* larvae that fed on *B. afzelii*-infected mice had a significantly faster larva-to-nymph moulting time compared to larvae that fed on uninfected control mice. In contrast, we found no evidence that *B. afzelii* infection or larval dysbiosis influenced the four other life history traits of the immature *I. ricinus* ticks, which included engorged larval weight, unfed nymphal weight, larva-to-nymph moulting success, and immature tick survival. A power analysis found that our sampling effort had sufficient power (>80%) to detect small effects (difference of 5% – 10%) of either *B. afzelii* infection or larval dysbiosis. Under the laboratory conditions of our study, *B. afzelii* appears to be a mostly passive passenger in *I. Ricinus* ticks.

Introduction

The epidemiology of vector-borne diseases is highly sensitive to the biology of the arthropod vector. Theoretical models that derive the reproduction number (R_0) of vector-borne diseases show that this quantity depends on a number of vector behavioural and life history traits, such as the biting rate, survival, and reproduction [1-3]. Infection by vector-borne pathogens can reduce the survival and reproduction of the arthropod vector [4-7], and thereby reduce the R_0 of the vector-borne disease. Conversely, vector-borne pathogens can manipulate their arthropod vectors (e.g., increase the biting rate, shift resources from reproduction to survival) to increase their own transmission, and thereby increase the R_0 of the vector-borne disease [8-11]. Measuring the effects of vector-borne pathogens on the fitness of their arthropod vectors is therefore important for understanding the epidemiology of vector-borne diseases.

Arthropod vectors also form intimate associations with microorganisms that do not cause diseases in vertebrate hosts [12]. These non-pathogenic bacteria can be inherited from the mother (i.e., vertical transmission) or acquired from the external environment [13]. Some are obligate symbionts that provide a critical function, for example providing a vitamin that is missing from a nutrient-poor hematophagous (blood-based) diet [14]. The general importance of arthropod-symbiont associations can be demonstrated by dysbiosis treatments (e.g., antibiotics) that decrease the abundance of the symbiont resulting in

dramatic reductions in the fitness of the arthropod vector [15-20]. These non-pathogenic bacteria can also influence vector competence, which is the ability of the arthropod vector to acquire, maintain, and transmit vector-borne pathogens over its lifecycle [21-25]. Thus, arthropod-associated bacteria can influence the R_0 of vector-borne diseases by their effects on both vector fitness and vector competence for vector-borne pathogens.

Hard ticks of the genus *Ixodes* transmit a diversity of tick-borne pathogens such as the spirochete bacteria belonging to the *Borrelia burgdorferi* sensu lato (sl) complex, which includes the causative agents of Lyme disease [3,26,27]. Some reviews have suggested that *B. burgdorferi* sl can adaptively manipulate its *Ixodes* tick vector to enhance its own transmission, and hence the R_0 of Lyme disease [11,28]. *Ixodes* ticks infected with *B. burgdorferi* sl differ from their uninfected counterparts in a number of tick phenotypes including questing behaviour, survival rates, body weight, and energy reserves [29-35]. However, as most of these studies are correlational in nature, there is a need for more controlled infection experiments to quantify the effects that *B. burgdorferi* sl pathogens have on the fitness of their tick vectors. *Ixodes* ticks also contain a diversity of non-pathogenic bacteria [36,37], but their effects on tick fitness are even less clear [38,39].

To investigate whether *B. burgdorferi* sl and tick-associated non-pathogenic bacteria can influence the population ecology of *Ixodes* ticks, it is important to consider the tick lifecycle [1,3]. *Ixodes* ticks have three motile stages, larva, nymph, and adult female, that must take a blood meal from a vertebrate host in order to graduate to the next step in the lifecycle. Larvae acquire *B. burgdorferi* sl pathogens after engorging on an infected host, and subsequently moult into infected nymphs. The size of the larval blood meal determines the body size and energy reserves of the resultant nymph, which influence nymphal survival and the ability to search for a host the following year [28,34]. The density of infected nymphs (DIN) largely determines the risk of infection for vertebrate hosts including humans [40]. Adult female ticks typically feed on non-competent vertebrate hosts, such as deer [41,42] and their main contribution to the R_0 of Lyme disease is via the production of larvae. In summary, the R_0 of *B. burgdorferi* sl is highly sensitive to the life history traits of immature *Ixodes* ticks, such as larva-to-nymph moulting success, survival of immature ticks, and body weight of immature ticks [1-3].

Our research group uses experimental infections with the pathogen *B. afzelii*, the tick *I. ricinus*, and rodent reservoir hosts to ask questions about the ecology of this common European Lyme disease system [43]. The purpose of the present study was to test whether *B. afzelii* infection and tick-associated non-pathogenic bacteria influence the life history traits of immature *Ixodes ricinus* ticks under standard laboratory conditions.

Materials And Methods

Background of the study

In a previous study, we set out to test whether manipulation of the larval microbiota would affect the probability that *I. ricinus* would acquire *B. afzelii* during the larval blood meal [44]. The larval microbiota was reduced 28-fold by washing eggs with bleach, whereas the control eggs were washed with water. These dysbiosed larvae and control larvae were fed on either *B. afzelii*-infected mice or uninfected control mice and the resultant engorged larvae were allowed to moult into nymphs under laboratory conditions [44]. In addition to the *B. afzelii* and microbiome dynamics presented in [44], we measured five life history traits including (i) engorged larval weight, (ii) unfed nymphal weight, (iii) larva-to-nymph moulting success, (iv) larva-to-nymph moulting time, and (v) immature tick survival. These life history data therefore represent an excellent opportunity to test whether *B. afzelii* infection and the larval microbiota influence the fitness of immature *I. ricinus* ticks under laboratory conditions.

Mice, *Borrelia afzelii*, and *Ixodes ricinus* ticks

We used *Mus musculus* BALB/c mice as the rodent host because we have a history of successful experimental infections with this mouse strain [45-47]. During the study, the mice were maintained in individual cages and were given food and water ad libitum. The mice were experimentally infected via tick bite with *B. afzelii* strain NE4049. This strain was originally isolated from an *I. ricinus* tick in Switzerland and has multilocus sequence type (MLST) 679 and strain identification number 1887 in the *Borrelia* MLST database, and *ospC* major group A10. We chose to work with strain NE4049 because it is highly infectious to both rodents and *I. ricinus* ticks [45,46,48]. The adult female *I. ricinus* ticks used to produce the eggs and larvae for this study were collected from wild roe deer captured in the Sylve d'Argenson forest near Chizé, France. The *I. ricinus* ticks used to experimentally infect the mice with *B. afzelii* strain NE4049 came from a laboratory colony established in 1978 at the University of Neuchâtel.

Approval for the use of experimental animals in the study

The authors complied with the ARRIVE guidelines. This study followed the Swiss legislation on animal experimentation. The commission that is part of the "Service de la Consommation et des Affaires Vétérinaires (SCAV)" of the canton of Vaud, Switzerland evaluated and approved the ethics of this study. The SCAV of the canton of Neuchâtel, Switzerland issued the animal experimentation permits for the study (NE04-2014) and for the maintenance of the *I. ricinus* tick colony on vertebrate hosts at the University of Neuchâtel (NE05-2014).

Egg washing treatment of ten field-collected families of *I. ricinus*

Ten engorged adult female *I. ricinus* ticks were collected from wild roe deer captured in the Sylve d'Argenson forest near Chizé, France. The ten female ticks were allowed to lay their eggs in the laboratory (Figure 1). Four weeks after deposition, each of the 10 clutches of eggs was split into two batches. One batch was rinsed with 10% bleach while the other batch was rinsed with distilled water. The purpose of the bleach rinsing treatment was to remove bacteria on the outer surface of the eggshell, whereas the water rinsing treatment functioned as a control. Eggs were placed in plastic petri dishes sealed with parafilm and allowed to hatch into larvae. Thus, for each of the 10 field-collected tick families, we

obtained batches of larvae that had hatched either from bleach-rinsed eggs or from water-rinsed eggs (20 batches in total). To determine whether the bleach treatment had reduced the microbiota in the larval ticks, a group of ~400 larvae was frozen for each of the 20 batches at six weeks after hatching.

Experimental infection of mice with *B. afzelii* via tick bite

For the main experiment, 40 female, 5-week-old, specific pathogen-free BALB/c mice were randomly assigned to either the control group or the infection group (Figure 1). Each mouse in the control group (n = 20) was infested with 5 uninfected *I. ricinus* nymphs, whereas each mouse in the infected group (n = 20) was infested with 5 nymphs infected with *B. afzelii* strain NE4049. In this way, each of the 40 mice had a similar immune experience with *I. ricinus* nymphs. These nymphs came from the University of Neuchâtel laboratory colony and the creation of these nymphs is explained in section 1 of the electronic supplementary material (ESM). Five weeks after the nymphal challenge, an ear tissue biopsy and a blood sample were taken from each of the 40 mice to determine their infection status. The ear tissue biopsy was tested for the presence of *B. afzelii* using qPCR. The blood sample was tested for the presence of *B. afzelii*-specific antibodies using a commercial ELISA. These tests confirmed that the 20 mice in the infected group were infected with *B. afzelii*, whereas the 20 mice in the control group remained uninfected.

Feeding *I. ricinus* larvae on *B. afzelii*-infected mice and uninfected control mice

The larvae for each of the 20 batches were split into two groups of ~100 larvae. One group was fed on an uninfected control mouse whereas the other group was fed on a *B. afzelii*-infected mouse (Figure 1). For each of the 40 mice, we collected a maximum of 60 engorged larvae; each engorged larva was placed in an individual Eppendorf tube and was allowed to moult into a nymph. Four weeks after the larva-to-nymph moult, 10 nymphs were randomly selected from each mouse and were frozen at –80°C. In summary, we froze ~400 nymphs (10 families*2 egg washing treatments* 2 mouse infection statuses*10 nymphs/mouse) for future molecular analyses of *B. afzelii* infection.

Life history traits of immature *I. ricinus* ticks

For each mouse, we randomly selected a maximum of 20 engorged larval ticks and weighed them within 2 days of their collection (n = 742 engorged larvae). Four weeks after the larva-to-nymph moult, we weighed the same group of nymphs that had been weighed as engorged larval ticks (n = 742 – 89 = 653 unfed nymphs, as 89 engorged larvae did not moult into nymphs). To increase the sample size, we weighed an additional 10 unfed nymphs for each of the 40 mice (n = 414 unfed nymphs). The engorged larvae and the unfed nymphs were weighed to a precision of 0.1 mg using an Ultra Microbalance (UMT 5 Comparator, Mettler Toledo, Greifensee, Switzerland).

To determine the larva-to-nymph moulting success, larva-to-nymph moulting time, and immature tick survival, the moulting status and alive status of the engorged larvae (n = 1739) were checked every 2 or 3 days over the larva-to-nymph moulting period (a period of 16 days from 8 August to 24 August 2016).

The moulting status and alive status of all the engorged larvae were checked one last time (13 September 2016), which was 74 days after the larvae were fed on the mice (1 July 2016).

In summary, we collected 5 life history traits for these immature *I. ricinus* ticks: (i) engorged larval weight within 2 days of drop-off (n = 742), (ii) unfed nymphal weight at 4 weeks after the larva-to-nymph moult (n = 1067), (iii) larva-to-nymph moulting success over 74 days following the start of the larval blood meal (n = 1739), (iv) larva-to-nymph moulting time over 55 days following the start of the larval blood meal (n = 1529), and (v) immature tick survival over 74 days following the start of the larval blood meal (n = 1739). The numbers of engorged larval ticks that were recovered from each of the 40 mice were also recorded.

Molecular methods for *I. ricinus* larval ticks

The larval ticks that had been frozen for molecular analysis were split into 40 sub-groups with ~200 larval ticks per sub-group (10 families x 2 egg washing treatments x 2 replicates). DNA extraction of these 40 sub-groups of larvae was done using a DNeasy Blood & Tissue spin-column kit (QIAGEN) and following the manufacturer's instructions. The DNA of each sub-group was eluted into 100 µl of distilled water, and the DNA concentration was measured using a Nanodrop. For qPCR, the DNA concentration of each sub-group was adjusted to 5 ng/µl. Two qPCR assays were performed independently for each DNA extraction: tick *calreticulin* and bacterial *16S rRNA*. Each qPCR assay contained 3 µl of template for a total of 15 ng of DNA. The molecular methods are described in detail in section 2 of the ESM.

Molecular methods for *I. ricinus* nymphal ticks

For each of the 40 mice, a sample of 7 – 8 unfed nymphs (that had fed as larvae on the mice) were randomly selected and tested for *B. afzelii* infection. Nymphs were crushed using the TissueLyser II by shaking them with a stainless-steel bead (1.4 mm in diameter) at a frequency of 30 Hz for 1 min. Total DNA was extracted for each tick using the DNeasy Blood & Tissue 96-well extraction kit (QIAGEN) and following the manufacturer's instructions. The DNA of each tick was eluted into 65 µl of distilled water, and the DNA concentration was measured for each of the ~300 nymphs using a Nanodrop. For qPCR, the DNA concentration of each tick was adjusted to 5 ng/µl. Three qPCR assays were performed independently for each DNA extraction: tick *calreticulin*, bacterial *16S rRNA*, and *Borreliaflagellin*. Each qPCR assay contained 3 µl of template for a total of 15 ng of DNA. The molecular methods are described in detail in section 2 of the ESM.

ELISA to test whether mice developed IgG antibodies against *B. afzelii*

We used the SERION® ELISA classic *Borrelia burgdorferi* IgG/IgM immunoassay to detect the presence of IgG antibodies against *B. afzelii*. Mouse sera was diluted 1:100 in blocking solution that was composed of 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The ELISA plate was incubated with the mouse sera for 45 min at room temperature. The plate was washed three times with a solution of

0.1% TWEEN in PBS for 5 min. The plate was incubated with a goat anti-mouse IgG horseradish peroxidase conjugate diluted 1:5000 in the blocking solution for 45 min at room temperature. The plate was washed three times with PBS-TWEEN as described above. The final step was to add 100 µl of tetramethylbenzidine (TMB) to each well. The absorbance at a wavelength of 652 nm was measured every two minutes for one hour to determine the strength of the IgG antibody response against *B. afzelii*. We used the area under the curve function, *auc()*, in R to calculate the total absorbance over the 60 min of the reaction.

Statistical Methods

Statistical analyses were performed using RStudio Version 1.2.5042. The *lm()* and *glm()* functions in the base package were used to create the Linear Models (LMs) and the Generalized Linear Models (GLMs), respectively. The *lmer()* and *glmer()* functions in the lme4 package were used to create the Linear Mixed effects Models (LMMs), and the Generalized Linear Mixed effects Models (GLMMs), respectively. The *corrplot()* function in the corrplot package was used to graph the correlation matrix of the life history traits of the immature *I. ricinus* ticks. Effects with p-values < 0.050 were considered statistically significant. After Bonferroni correction for 5 life history traits, p-values < 0.010 were considered statistically significant.

Effect of egg bleaching on the microbiota of *I. ricinus* larvae

For each of the 40 sub-groups of larvae, we divided the *16S rRNA* gene copy number of the batches of *I. ricinus* larvae by their *calreticulin* gene copy number. These *16S rRNA* to *calreticulin* ratios were then log10-transformed to improve the normality of the data. To test whether the egg bleaching treatment reduced the microbiota of the resultant larvae, the log10-transformed *16S rRNA* to *calreticulin* ratios were analysed using linear mixed effects models (LMMs). Egg washing treatment (2 levels: water and bleach) and tick family were modelled as fixed and random factors, respectively.

Effect of *B. afzelii* infection and egg washing treatment on the life history traits of immature *I. ricinus* ticks

The response variables were the five life history traits of the immature *I. ricinus* ticks: (i) engorged larval weight, (ii) unfed nymphal weight, (ii) larva-to-nymph moulting success, (iv) larva-to-nymph moulting time, and (v) immature tick survival. We used mixed effects models to analyze these response variables to account for the fact that ticks that fed on the same mouse are not statistically independent. The log10-transformed engorged larval weight, log10-transformed unfed nymphal weight, and larva-to-nymph moulting time were treated as normally distributed response variables that were analyzed using LMMs. The larva-to-nymph moulting success and immature tick survival are binomial response variables that were analyzed using GLMMs. Egg washing treatment (2 levels: bleach and water) and mouse infection status (2 levels: infected and control) were modelled as fixed effects. Mouse ID and tick family were

modelled as random effects, with mouse ID nested in tick family. If the interaction between egg washing treatment and mouse infection status was not significant, the model was re-run without the interaction.

Effect of tick family and mouse ID on the life history traits of immature *I. ricinus* ticks

To test whether there were significant differences among the 10 tick families, the 5 life history traits were modelled using LMMs with normal errors or GLMMs with binomial errors with tick family as a fixed effect and mouse ID as a random effect. To test whether there were significant differences among the 40 mice, the 5 life history traits were modelled as LMs or GLMs with mouse ID as a fixed effect. As before, the weights of the engorged larvae and the weights of the unfed nymphs were log10-transformed.

Correlations between the life history traits of *I. ricinus*

For this correlation analysis, there were six variables: the number of engorged larvae that were recovered from each mouse and the five life history traits of the immature *I. ricinus* ticks. The five life history traits of the immature ticks included (i) engorged larval weight (mg), (ii) unfed nymphal weight (mg), (iii) larva-to-nymph moulting success (%), (iv) larva-to-nymph moulting time (days), and (v) immature tick survival (%). For each of these six variables, the mean was calculated for each of the 40 mice. The pairwise correlations were calculated for each of the 15 pairs of variables across the 40 mice.

Results

Effect of egg bleaching on the microbiota of *I. ricinus* larvae

The *I. ricinus* larvae that hatched from the eggs washed with bleach had a significantly lower bacterial load than the control larvae that hatched from the eggs washed with water (Figure 2; $c^2 = 25.359$, df = 1, p = 0.0000005). The log10(*16S rRNA*/ *calreticulin*) ratio in the larval ticks of the control group (mean ratio = 6.61, 95% CI = 1.78–24.61) was 28 times higher than the bleach treatment group (mean ratio = 0.24, 95% CI = 0.06–0.88). The bleach treatment was therefore highly effective at reducing the microbiota in the larval ticks (Figure 2).

B. afzelii induced a strong IgG antibody response in the infected mice

One of the control mice died during the study, so that the realized sample sizes were 19 uninfected control mice and 20 *B. afzelii*-infected mice. The ability of the ELISA to detect *B. afzelii* infection in the mice was unambiguous (Figure 3) because there was no overlap in absorbance between the control group (range = 637 to 1,692 absorbance units (AU)) versus the infected group (range = 10,756 AU to 12,572 AU). According to the ELISA, all of the 20 mice challenged with infected nymphs became infected with *B. afzelii* strain NE4049 and all of the 19 mice challenged with uninfected nymphs remained uninfected (Figure 3). The mean absorbance level of the infected mice (11,749 AU) was significantly higher (12.9x) compared to the control mice (912 AU; independent samples t-test: t = 43.88, df = 37, p < 0.000001).

Prevalence of *B. afzelii* infection in unfed *I. ricinus* nymphs

A total of 289 unfed nymphs were tested for *B. afzelii* via qPCR. Of the 289 nymphs, 106 nymphs had fed as larvae on uninfected control mice and 183 nymphs had fed as larvae on infected mice. The prevalence of *B. afzelii* infection in the nymphs that fed as larvae on the infected mice (71.0% = 130/183) was significantly higher (25.1x) compared to the nymphs that fed as larvae on the uninfected control mice (2.8% = 3/106; proportion test: $c^2 = 122.97$, df = 1, $p < 0.000001$). The 3 nymphs that fed as larvae on uninfected mice, but that tested positive for *B. afzelii* on the qPCR are false positives (these 3 nymphs had high Cq values of 39.62, 39.74, 40.22; see section 3 of the ESM). Data on the variation in host-to-tick transmission of *B. afzelii* among the 20 infected mice is given in section 4 of the ESM.

Relationship between engorged larval weight and unfed nymphal weight

The engorged larval weights and unfed nymphal weights were both measured for a sample of 653 immature *I. ricinus* ticks. Weights were taken for an additional 89 engorged larvae and 414 unfed nymphs, so that the total sample sizes were 742 engorged larvae and 1067 unfed nymphs, respectively. According to the simple linear regression model, the log10-transformed engorged larval weight was a highly significant predictor of the log10-transformed unfed nymphal weight, and the former explained 72.71% of the variation in the latter (Figure 4; regression: $F_{1, 651} = 1738$, $p < 0.000001$; correlation: r = 0.854, t = 41.859, df = 652, $p < 0.000001$). Given that the engorged larvae and unfed nymphs were weighed independently and more than 10 weeks apart, the strong relationship between these two variables indicates that our immature tick weight measurements are highly reliable. For this paired sample of 653 immature ticks, the mean weight of the engorged larvae (438.7 mg) was 2.4x greater than the weight of the resultant unfed nymphs (180.8 mg), and this difference was highly significant (paired t-test: t = -229.53, df = 652, $p < 0.000001$). These results show that the engorged larvae lost a lot of weight during the larva-to-nymph moulting process (i.e., due to water loss and the digestion and excretion of faecal material). We found no evidence that *B. afzelii* infection in the mouse influenced the efficiency with which the engorged larval weight was converted into unfed nymphal weight (see section 5 of the ESM).

A previous study had shown that *I. scapularis* larvae that took larger blood meals had a higher probability to acquire *B. burgdorferi* sensu stricto (ss) [49]. For the subset of *I. ricinus* larvae that fed on the 20 infected mice, we tested whether the size of the larval blood meal influenced the probability that the larvae acquired *B. afzelii*, but we found no evidence for this hypothesis (see section 6 of the ESM).

Larva-to-nymph survival, moulting success, and moulting time

Of the 1739 engorged larvae monitored, the mean larva-to-nymph moulting success over 74 days was 91.9% (1598/1739), and the mean immature tick survival over 74 days was 84.5% (1470/1739). Of the 141 larvae that did not moult, 134 died before reaching the moult, and the other 7 had not moulted after

74 days. Of the 1739 engorged larval ticks, the moulting time (days) was recorded for 1529 individuals. The median larva-to-nymph moulting time was 40 days (range = 38 days to 54 days).

Effect of *B. afzelii* infection and egg washing treatment on the life history traits of immature *I. ricinus* ticks

We found no significant effects of *B. afzelii* infection in mice, egg washing treatment, or their interaction on the engorged larval weights or unfed nymphal weights (Table 1; see section 7 of the ESM). The results remained the same when the explanatory variable of mouse infection status was replaced with tick infection status (see section 8 of the ESM). No significant effects of *B. afzelii* infection in mice, egg washing treatment, or their interaction were found on larva-to-nymph moulting success, larva-to-nymph moulting time, or immature tick survival (Table 1); the only exception was the effect of *B. afzelii* infection in mice on the larva-to-nymph moulting time (Figure 5; Table 1; $c^2 = 16.510$, df = 1, $p < 0.0001$). The mean larva-to-nymph moulting time for the ticks that fed on the infected mice (41.4 days) was 1.0 day faster (2.4% faster) than the ticks that fed on the uninfected control mice (42.4 days). The effect of *B. afzelii* infection remained significant after using a non-parametric independent two-samples Wilcoxon test (W = 359542, $p < 0.00001$; see section 9 of the ESM) and after Bonferroni correction for multiple comparisons (i.e., $p = 0.050/5 = 0.010$).

Effects of tick family and mouse ID on the life history traits of immature *I. ricinus* ticks

We found significant effects of mouse ID and tick family on all five life history traits, with the exception of the effect of tick family on larva-to-nymph moulting success (for details see section 10 of the ESM). For the engorged larval weight (log10-transformed), tick family, mouse ID, and the residuals accounted for 8.0%, 5.8%, and 86.3% of the variance, respectively. Similarly, for the unfed nymphal weight (log10-transformed), tick family, mouse ID, and the residuals accounted for 7.9%, 4.0% and 88.1% of the variance, respectively. Thus, the majority of the variance in immature tick weight occurred at the level of the individual ticks and remains unexplained.

Correlations between the life history traits of immature *I. ricinus* ticks

There were five significant correlations between the six variables, which included the the number of engorged larvae that were recovered from each mouse and the five life history traits of the immature *I. ricinus* ticks (Figure 6). There was a negative correlation between the number of engorged larvae collected and survival of immature ticks ($r = 0.438$, $p = 0.005$). The remaining 4 pairwise correlations were positive: (i) survival of immature ticks and larva-to-nymph moulting success ($r = 0.782$, $p < 0.0001$), (ii) larva-to-nymph moulting time and engorged larval weight ($r = 0.575$, $p = 0.0002$), (iii) larva-to-nymph moulting time and unfed nymphal weight ($r = 0.471$, $p = 0.0025$), and (iv) engorged larval weight and unfed nymphal weight ($r = 0.895$, $p < 0.0001$; see also Figure 4).

Power analysis to determine the minimal detectable effect size

For each of the five life history traits of the immature *I. ricinus* ticks, we conducted a retrospective power analysis to determine the minimal effect size that our sampling effort could have detected (see section 11 of the ESM). This power analysis showed that our sampling effort had a power >80% to detect a treatment effect (of *B. afzelii* infection or egg bleaching) that either increased or decreased tick phenotype by 10% for each of the five life history traits. For moulting time, engorged larval weight, and unfed nymphal weight, our sampling effort had a power >80% to detect a treatment effect (of *B. afzelii* infection or egg bleaching) that either increased or decreased tick phenotype by ~5%. In summary, our sampling effort was more than sufficient to detect small positive or negative effects of *B. afzelii* infection or egg bleaching on tick phenotype.

Discussion

Effects of *B. afzelii* on the life history traits of *I. ricinus*: Our study found that the Lyme disease pathogen *B. afzelii* reduced the larva-to-nymph moulting time of its principal tick vector *I. ricinus* by 2.4% (i.e., 1.0 day) under laboratory conditions. In contrast, we found no evidence that infection with *B. afzelii* influenced the other four life history traits of immature *I. ricinus* ticks under laboratory conditions. Similarly, we found no evidence that reduction of the larval microbiota via the egg bleaching treatment influenced these same life history traits. The five life history traits investigated in this study describe the transition of engorged larvae to unfed nymphs over the first 2.5 months of the tick life cycle, and they are expected to influence the population ecology of *I. ricinus* and hence the R_0 of *B. afzelii*[1-3]. The sample size differed among the five life history traits and ranged from 742 to 1739 immature ticks that had fed as larvae on 40 different mice. Our power analyses demonstrated that our sampling effort was more than sufficient to detect small positive or negative effects (changes of 5% to 10%) of either *B. afzelii* infection or the egg bleaching treatment on each of the five life history traits. Thus, under the laboratory conditions used in this study, neither *B. afzelii* infection nor reduction of the larval microbiome had major effects (i.e., >10% difference) on the fitness of immature *I. ricinus* ticks.

Importance of controlled experimental infections: Our study shows the importance of using experimental infections to test whether infection with *B. burgdorferi* sl influences the life history traits of *Ixodes* ticks. A number of studies that reported effects of *B. burgdorferi* sl on *Ixodes* phenotype (e.g., behaviour, body size, fat content, survival) used ticks that had been naturally infected in the field [28,30-34]. This approach cannot control for other confounding factors that influence tick phenotype, such as tick age, vertebrate host species that provided the larval blood meal, quality of the host blood meal, presence of other microbes, etc. For example, vertebrate host species differ in their ability to transmit *B. burgdorferi* to feeding larval ticks [50-53], and in blood meal quality, which influences variation in larva-to-nymph moulting success [54]. Together, these two phenomena can produce significant associations between tick infection status and tick life history traits, which are then misinterpreted as demonstrating that the former causes variation in the latter.

Effect of *B. burgdorferi* on larva-to-nymph moulting time of *Ixodes* ticks: Our study found that *B. afzelii* infection in the mouse reduced the larva-to-nymph moulting time of the engorged larvae by 2.4% (i.e., 1.0 days) under laboratory conditions. Although the effect size was modest, it was highly significant ($p < 0.0001$). Faster larva-to-nymph development rates enhance the fitness of both *I. ricinus* and *B. afzelii*[1-3]. In nature, larvae acquire their blood meal during the summer and then moult into unfed nymphs; most of these nymphs overwinter and quest the following spring, but some nymphs will quest later that same fall [55]. The decision of whether to quest that same fall or the following spring depends on the timing of the larval blood meal and the larva-to-nymph development rates [55]. If *B. afzelii* can speed up larva-to-nymph development so that nymphs can quest that same fall rather than the following spring, it would increase the R_0 of *B. afzelii* and Lyme disease [1-3]. Future studies should investigate whether *B. afzelii* speeds up the larva-to-nymph moulting time under ecologically relevant conditions.

Effect of *B. burgdorferi* on moulting success and survival of immature *Ixodes* ticks: We found no effect of *B. afzelii* infection on larvae-to-nymph moulting success or immature tick survival. The engorged larvae were kept under favourable environmental conditions (high humidity and at room temperature), which may be a limitation if the effects of *B. afzelii* infection on tick phenotype are more visible under harsher environmental conditions. Studies using field-collected *I. ricinus* ticks found that nymphs infected with *B. burgdorferi* sl have higher survival under stressful conditions of humidity and temperature compared to uninfected nymphs [32], and that *B. burgdorferi* sl-infected nymphs survive better under cold temperatures and high-frequency temperature variations [33]. A study on another tick-borne bacterium, *Anaplasma phagocytophilum* found that infection reduced the molting success and survival of immature *I. scapularis* ticks under laboratory conditions [5]. In contrast, another study found that *A. phagocytophilum* increased the expression of a tick antifreeze glycoprotein, which enhanced survival of engorged larvae under cold temperature conditions [56]. These contradictory results demonstrate that the environmental conditions may influence the effect that tick-borne pathogens have on the life history traits of their tick vectors.

Effects of *B. afzelii* on body size of immature *I. ricinus* ticks: We found no effect of *B. afzelii* infection on the body size of immature *I. ricinus* ticks. Our results contradict previous studies that found that immature *Ixodes* ticks infected with *B. burgdorferi* sl were larger than uninfected ticks [34,35,49]. A study on field-collected *I. ricinus* nymphs found that infected individuals had a larger body size and higher energy reserves compared to uninfected individuals [34]. Similarly, an experimental study with bank voles, *B. afzelii*, and *I. ricinus* found that nymphs that had acquired *B. afzelii* during the larval blood meal from infected animals were heavier than the corresponding uninfected nymphs [35]. This result led the authors to suggest that *B. afzelii* manipulates the larvae to take larger blood meals [35]. Another experimental study with mice, *B. burgdorferi* ss, and *I. scapularis* reversed this chain of causality and found that larvae that take a larger blood meal from infected mice are more likely to acquire *B. burgdorferi* ss [49]. In the first scenario, the pathogen is the cause of the difference in the size of the larval blood meal between infected

and uninfected vectors, whereas in the second scenario, the variation in the size of the larval blood meal causes the variation in infection status. Our study found no evidence for either scenario.

Scope of selection for manipulation: Hematophagous (blood-feeding) insects and ticks differ dramatically in their biology (e.g., motility, host seeking, number and duration of blood meals, etc.) and the scope of any potential manipulation by a pathogen is therefore expected to differ between the two types of vectors [1]. In mosquito-malaria systems, the malaria parasite manipulates the mosquito vector to increase its biting rate, which enhances the parasite transmission at the expense of mosquito fitness [57-59]. In contrast, in hard ticks of the genus *Ixodes*, each stage takes one blood meal from a single host, and there is no evidence that tick-borne pathogens can manipulate their tick vectors to take multiple blood meals. Following their acquisition during the larval blood meal, the interests of the tick-borne pathogen and the immature tick are largely the same, as both require the engorged larva to moult into a nymph, survive, and take a nymphal blood meal in order to complete their life cycles. One exception is when the infected *Ixodes* nymph chooses a vertebrate host that is not competent to harbor the tick-borne pathogen [29]. Another exception is that if there are any energetic costs of sexual development in the nymph (i.e., male and female nymphs are different and can be distinguished after the nymphal blood meal), the tick-borne pathogens would prefer the reallocation of all these energetic resources towards questing and finding a host. In summary, there are more reports of adaptive manipulation for insect-borne pathogens compared to tick-borne pathogens [8] and one potential explanation is that the life cycle of hard ticks is not very conducive to manipulation because each stage is strongly selected to complete a single blood meal on a single host.

Effect of microbiome dysbiosis on life history traits of immature *I. ricinus* ticks: The bleach treatment of the eggs reduced the abundance of the bacterial microbiota (as measured by the *16S rRNA* gene copy number) in the *I. ricinus* larvae by a factor of 28 [44]. In insects, smearing bacteria over the surface of newly deposited eggs is a common extracellular route of transferring symbionts from mothers to their offspring [60]. In a previous study on the same sample of ticks, analysis of the *16S rRNA* gene diversity suggested that the egg bleaching treatment also changed the composition of the larval microbiota; the relative abundance of bacteria presumably associated with the egg surface (e.g., *Pseudomonas*) decreased, whereas the relative abundance of endosymbiotic bacteria (e.g., *Candidatus* Midichloria mitochondrii) increased [44]. Despite the strong reduction of the larval microbiota, the egg bleach treatment did not have any effect on the life history traits of the immature *Ixodes* ticks. This result contradicts a study that manipulated the microbiota of *I. scapularis* larvae by allowing engorged females to lay their eggs in sterile tubes [25]. In that study, dysbiosed *I. scapularis* larvae took larger blood meals than control larvae [25]. Our results are similar to studies on *I. ricinus* and *I. pacificus* that found that injection of antibiotics into engorged females had no effect on their reproductive fitness [38,39]. In other tick species that carry nutritional symbionts, such as *Coxiella*-like endosymbionts and *Francisella*-like endosymbionts that provide B vitamins, antibiotic treatments reduced tick fitness (e.g., moulting rate, body weight, fecundity, larval survival) and induced physical abnormalities [15-20]. Normal tick phenotypes were recovered after combining the antibiotic treatment with an artificial blood meal that contained the missing B vitamins

[19,61,62]. Egg bleaching is not expected to have an impact on these *Coxiella*-like and *Francisella*-like endosymbionts because these obligate intracellular bacteria are exclusively transmitted through the egg cytoplasm [19,61,62]. In summary, a successful and dramatic reduction of the microbiota inherited via egg smearing had no meaningful impact on the life history traits of immature *I. ricinus* ticks.

Density-dependent mortality of immature *I. ricinus* ticks: We found evidence for density-dependent mortality of immature *I. ricinus* ticks. Mice were infested with a standardized number of *I. ricinus* larvae, but differences in larval attachment and/or mouse grooming behaviour [63] led to variation in the number of engorged larval ticks that were recovered among mice. There was a strong negative correlation between the number of engorged larvae that were recovered for each mouse and the probability of immature tick survival. Density-dependent mortality occurs when the host develops anti-tick immunity so that the fitness of engorged larvae decreases over successive infestations [64-66]. However, the mice were only infested once in the present study, and the mechanism underlying this negative density dependence is therefore unclear. Nevertheless, density-dependent mortality of immature ticks on vertebrate hosts is an important mechanism regulating *Ixodes* tick populations [67-69]. We also found strong positive correlations between larva-to-nymph moulting time and the weights of the engorged larvae and unfed nymphs. This result suggests that larger larval blood meals take longer to digest, which increases the duration of the larva-to-nymph moult but results in a larger unfed nymph. The effects were relatively modest; for example, two engorged larvae with weights of 400 mg and 500 mg will moult in 40.5 days and 46.3 days, respectively.

Effects of mouse ID and tick family: We found highly significant effects of tick family and mouse ID on almost all of the life history traits of the immature *I. ricinus* ticks. Variance component analysis showed that when tick family and mouse ID are combined, they account for 14% and 12% in the total variance of engorged larval weight and unfed nymphal weight, respectively. The factors underlying differences among tick families include genetics, nutritional condition of the mother tick, quality of the blood meal, microbiome of the mother tick, clutch size, and other factors. Similarly, despite the fact that the BALB/c mice were acquired from a commercial breeder, they still differ in body size, nutritional condition, skin microbiota, stress levels, and other factors. Regardless of the reason for the phenotypic variation among larvae from different families and that feed on different mice, studies that do not account for these sources of variation can produce biased results. For the statistical analysis of these data, it is essential to use mixed effects models that estimate and thereby control the variance in tick phenotype that is due to tick family and mouse ID.

Conclusion

In summary, we found that infection with *B. afzelii* reduced the larva-to-nymph moulting time of engorged *I. ricinus* larvae, but the effect was modest (2.4% reduction). In contrast, we found no evidence that infection with *B. afzelii* influenced the other four life history traits of immature *I. ricinus* ticks including engorged larval weight, unfed nymphal weight, larva-to-nymph moulting success, and immature tick

survival. Although the egg bleaching treatment was highly effective at reducing the abundance of non-pathogenic bacteria in *I. ricinus* larvae, there was no evidence that this highly successful dysbiosis influenced the five life history traits of the immature *I. ricinus* ticks. Under the environmental conditions of this experimental infection study, *B. afzelii* had no meaningful effect on its own R_0 value.

Declarations

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Author contributions: EM, OD, OP and MJV conceived and designed the study. EM, AS, and AB conducted the experiment and performed the molecular work. GH, PTH and MJV analysed the data. GH and MJV wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests: The authors declare that they have no competing interests.

Data availability: The raw data for this study will be provided as Excel files.

Approval for animal experiments: The authors complied with the ARRIVE guidelines. This study followed the Swiss legislation on animal experimentation. The commission that is part of the "Service de la Consommation et des Affaires Vétérinaires (SCAV)" of the canton of Vaud, Switzerland evaluated and approved the ethics of this study. The SCAV of the canton of Neuchâtel, Switzerland issued the animal experimentation permits for the study (NE04-2014) and for the maintenance of the *I. ricinus* tick colony on vertebrate hosts at the University of Neuchâtel (NE05-2014).

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Tables

Table 1. Statistical analyses of the five life history traits of the immature *I. ricinus* ticks. The five life history traits include engorged larval weight, unfed nymphal weight, larva-to-nymph moulting success, larva-to-nymph moulting time, and immature tick survival. LMMs and GLMMs were used to test the effects of mouse infection status (I), egg washing (E), and their interaction (I:E) on the five life history traits. Shown are the results from the type II log-likelihood ratio test to determine the statistical significance of the explanatory variables. The row headers refer to the life history trait, the explanatory variable, the degrees of freedom (df), the Chi-square statistic (c^2), and the p-value (p).

Life history trait	Explanatory variable	df	c^2	p
Engorged larval weight	I:E interaction	1	0.414	0.520
Engorged larval weight	Mouse infection status	1	0.135	0.714
Engorged larval weight	Egg washing	1	0.522	0.470
Unfed nymphal weight	I:E interaction	1	1.131	0.288
Unfed nymphal weight	Mouse infection status	1	0.195	0.659
Unfed nymphal weight	Egg washing	1	0.249	0.618
Moulting success	I:E interaction	1	0.081	0.775
Moulting success	Mouse infection status	1	0.288	0.592
Moulting success	Egg washing	1	0.037	0.847
Moulting time	I:E interaction	1	0.648	0.421
Moulting time	Mouse infection status	1	16.510	<0.0001
Moulting time	Egg washing	1	0.071	0.790
Survival	I:E interaction	1	0.176	0.675
Survival	Mouse infection status	1	0.630	0.427
Survival	Egg washing	1	0.225	0.635

Figures

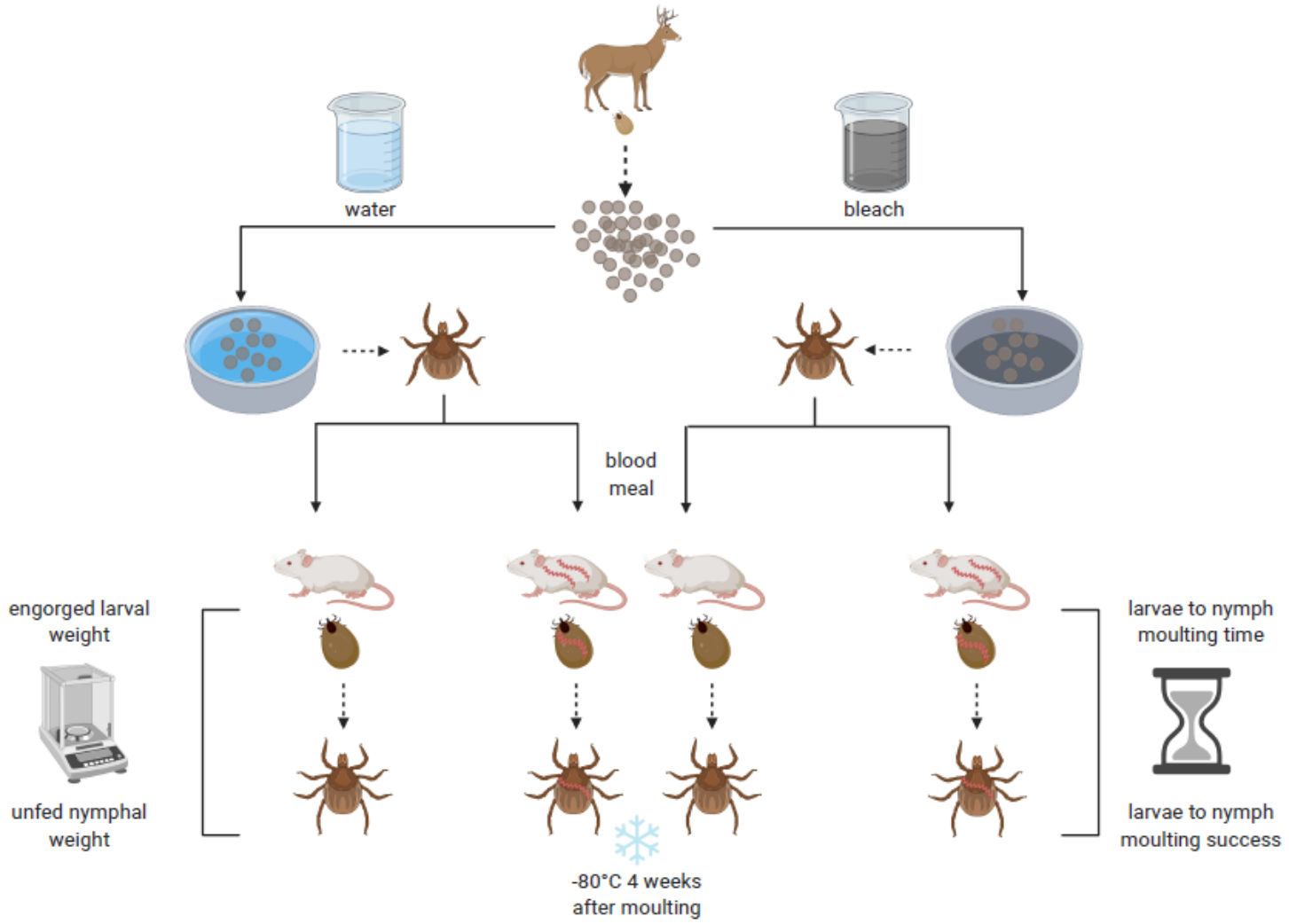

Figure 1

Experimental design. Engorged female I. ricinus ticks (n = 10) were collected from roe deer captured in the Chizé forest, France and laid their eggs in the laboratory. Each of the 10 egg clutches was split into two batches and rinsed with either 10% bleach (n = 10 batches) or distilled water (n = 10 batches) and hatched into larvae. Larvae for each of the 20 batches (10 tick families x 2 egg washing treatments) were split into two subgroups of ~100 larvae (total of 40 subgroups) that were fed on either an uninfected control mouse (n = 20) or a B. afzelii-infected mouse (n = 20). For each of the 40 mice, up to 60 engorged larvae were collected and placed in individual Eppendorf tubes to moult into nymphs. The larva-to-nymph moulting success and survival of these engorged larvae (n = 1739) was surveyed over a period of 74 days. For each of the 40 mice, up to 20 engorged larvae were randomly selected to be weighed as engorged larvae and again as unfed nymphs (4 weeks after the larva-to-nymph moult). A subset of ~400 nymphs was frozen at −80°C for future molecular analyses. A subset of ~300 of these nymphs was tested for B. afzelii infection using qPCR.

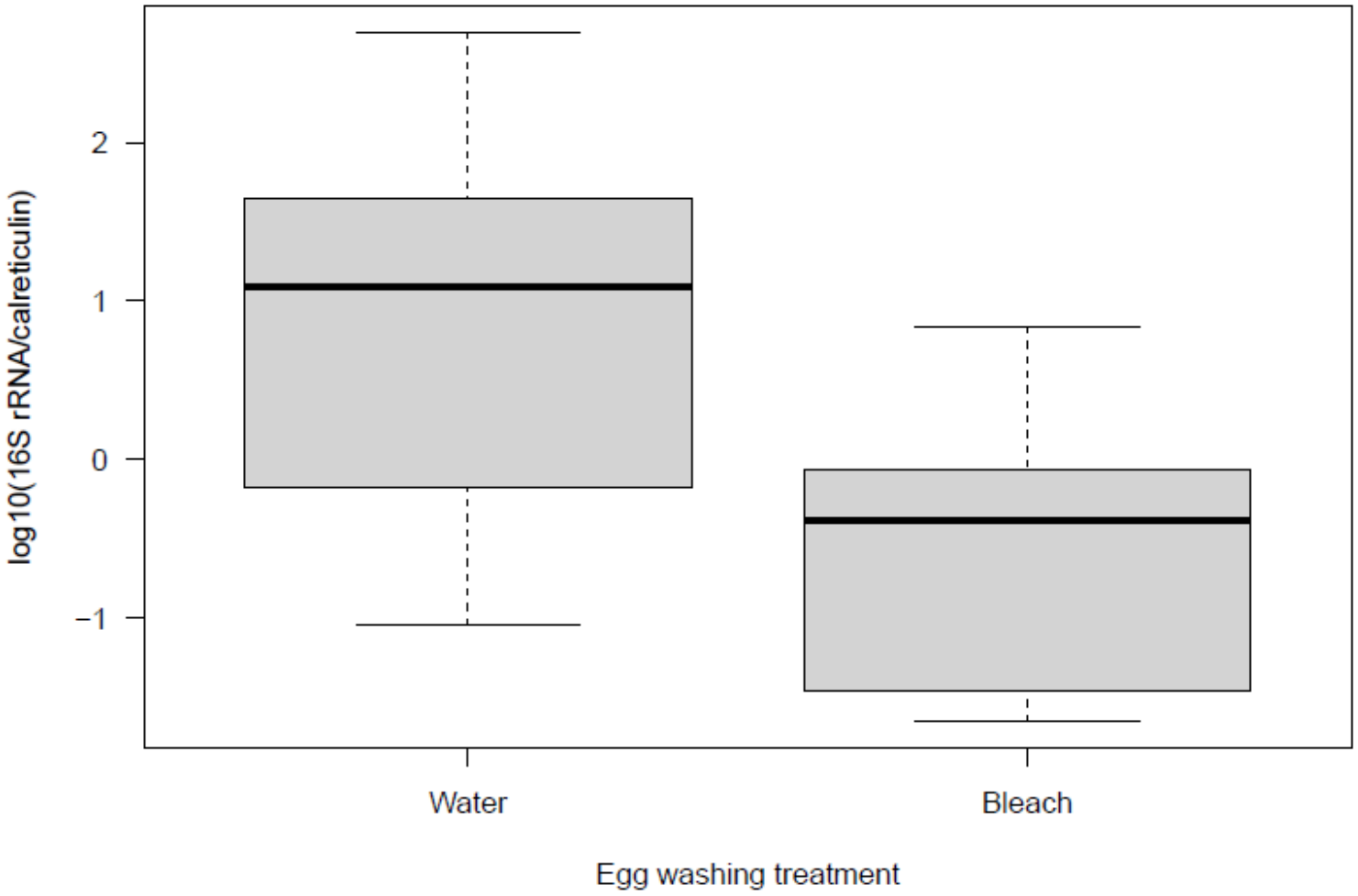

Figure 2

The egg bleaching treatment reduced the microbiota of the I. ricinus larvae. The abundance of the tick-associated non-pathogenic bacteria is given by the log10-transformed ratio of the bacterial 16S rRNA gene copy number to the tick calreticulin gene copy number. This ratio was 28 times higher for the I. ricinus larvae that hatched from the eggs washed with water compared to the larvae that hatched from the egg washed with bleach, and this difference was statistically significant ($p = 0.0000005$). The boxplots show the median (black line), 25th and 75th percentiles (edges of the box), minimum and maximum values (whiskers), and outliers (open circles).

Figure 3

B. afzelii induces a strong antibody response in the infected mice compared to the uninfected control mice. The total absorbance values were obtained from an ELISA that measures the strength of the IgG antibody response against B. afzelii. The mean absorbance level of the infected mice (11,749 AU) was 12.9x higher compared to the control mice (912 AU), and this difference was statistically significant ($p < 0.000001$). The boxplots show the median (black line), 25th and 75th percentiles (edges of the box), minimum and maximum values (whiskers), and outliers (open circles).

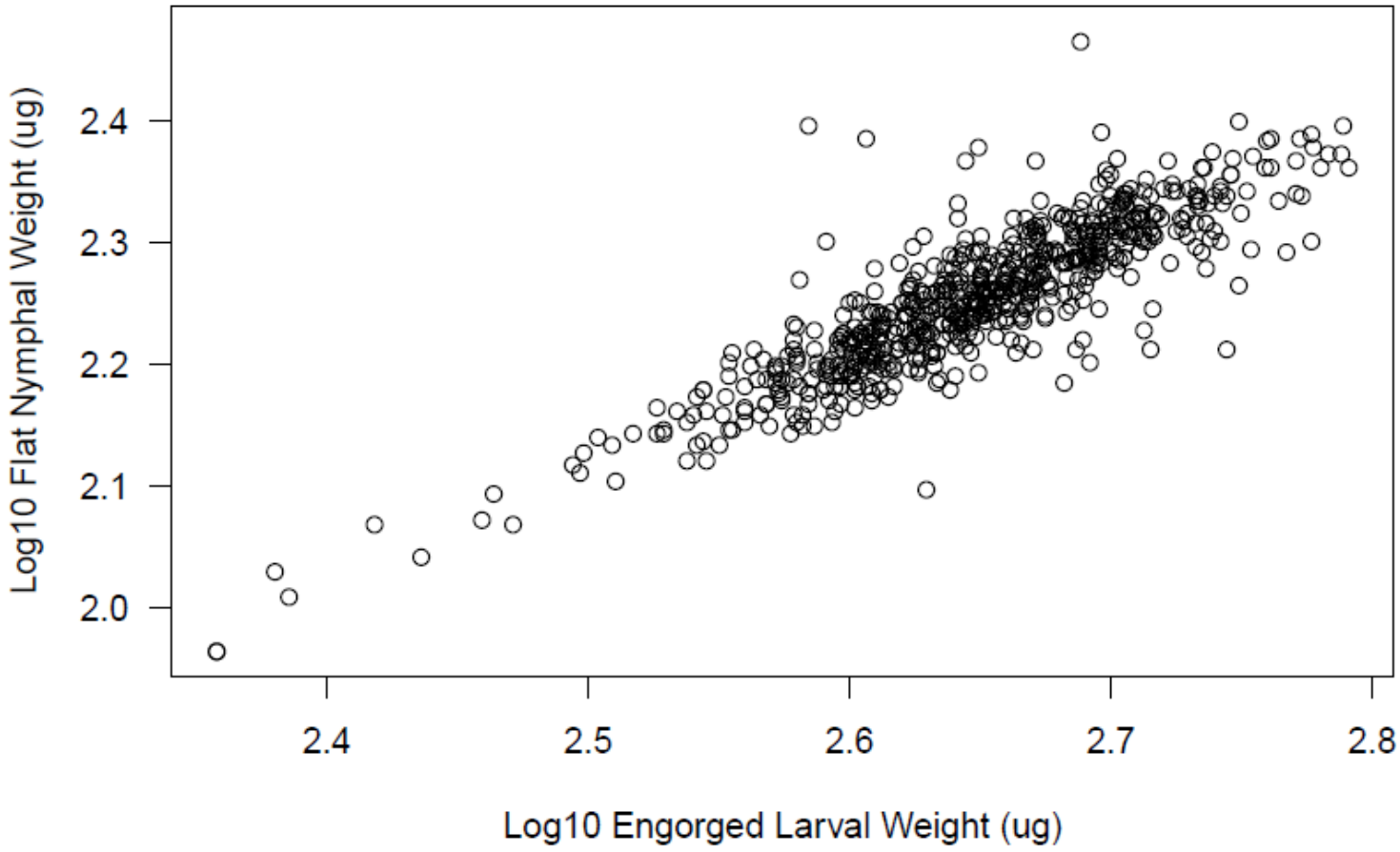

Figure 4

The weight of the unfed nymphs depends on the weight of the engorged larvae. The engorged larval weights and unfed nymphal weights were measured ~10 weeks apart for a sample of 653 immature I. ricinus ticks. The positive relationship between the log10-transformed unfed nymphal weights (g) and the log10-transformed engorged larval weights (g) is highly statistically significant (r = 0.854, t = 41.859, df = 652, p < 0.000001).

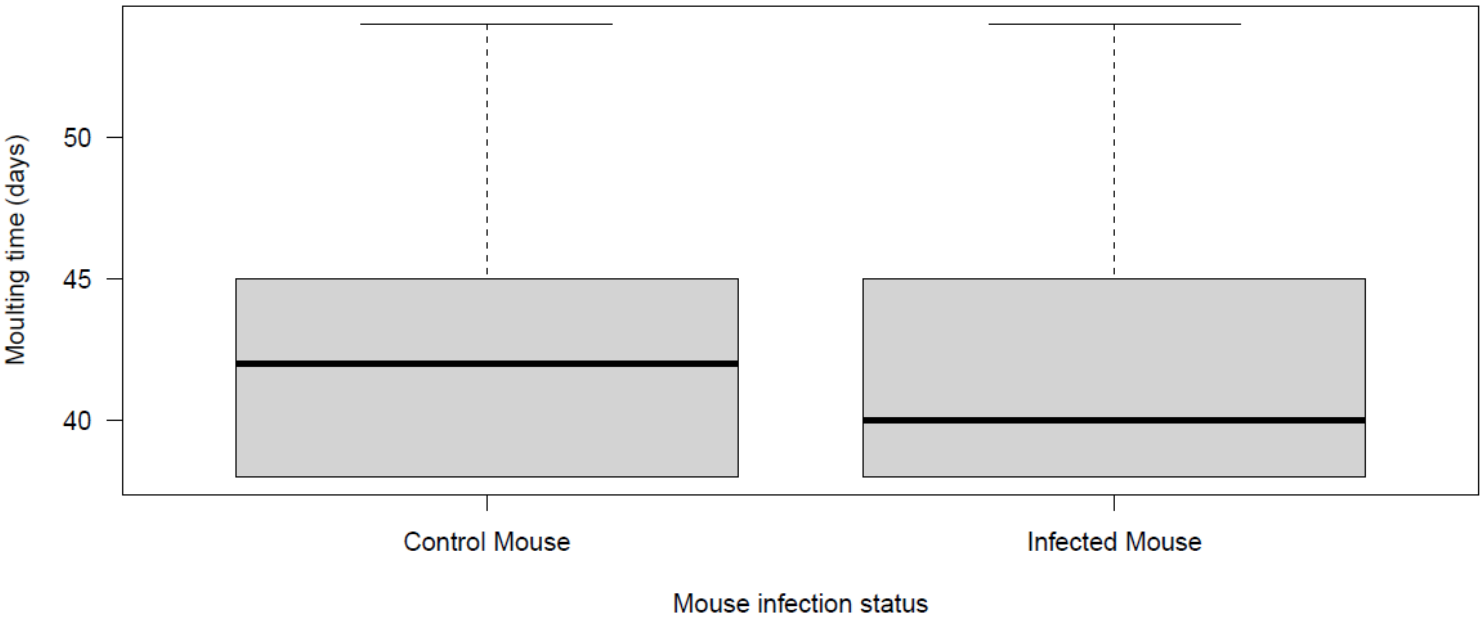

Figure 5

The B. afzelii infection status speeds up the moulting rates of engorged larvae into unfed nymphs. The mean larva-to-nymph moulting time (days) for the larvae that fed on B. afzelii-infected mice (mean = 41.3 days; median = 40 days; n = 776 ticks) was 1.0 day faster compared to the larvae that fed on the uninfected control mice (mean = 42.3 days; median = 42 days; n = 753 ticks), and this difference was significant ($p < 0.0001$). The larva-to-nymph moulting time was monitored every 2 days over the time period from 38 to 54 days after the start of the larval blood meal. The boxplots show the median (black line), 25th and 75th percentiles (edges of the box), minimum and maximum values (whiskers), and outliers (open circles).

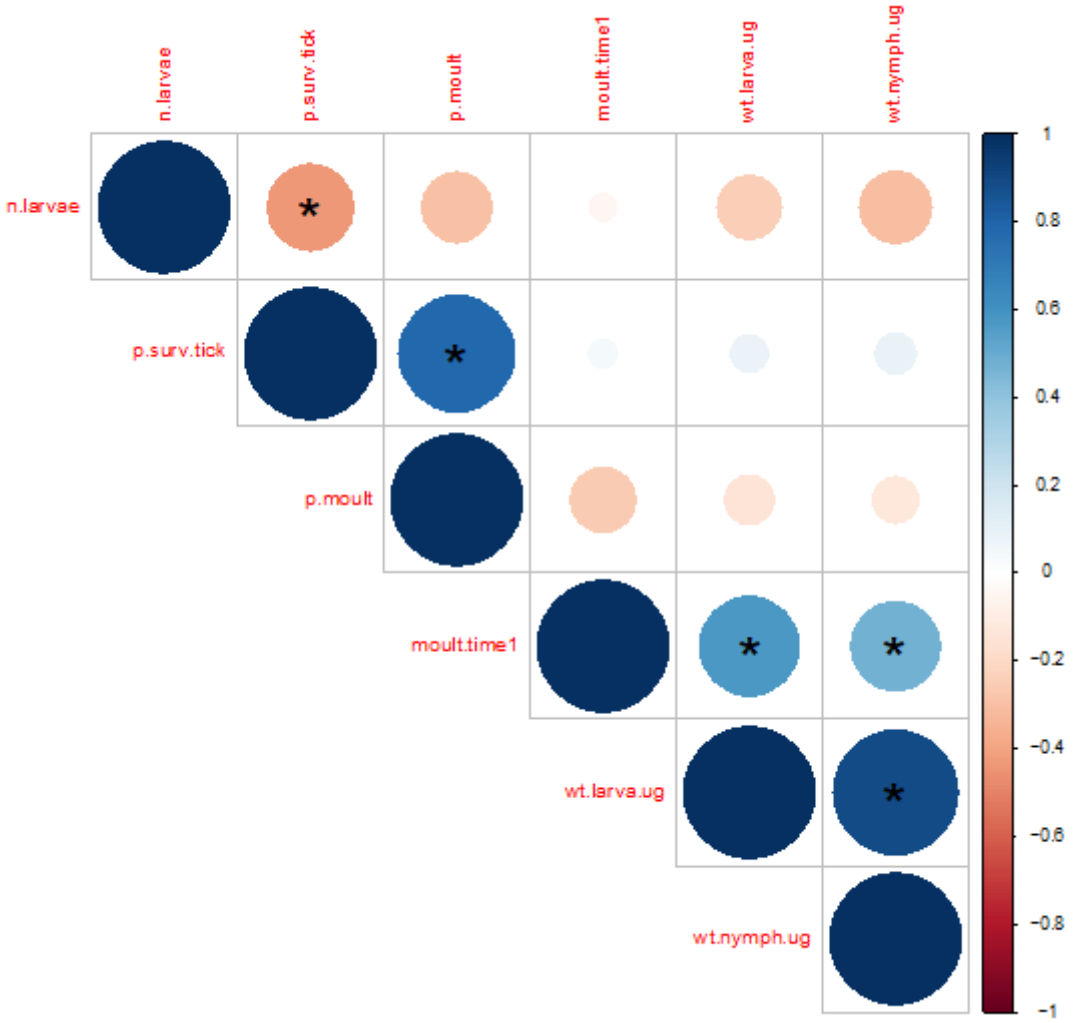

Figure 6

Correlations between the life history traits of immature I. ricinus ticks. The 6 variables shown include (i) the number of engorged larvae that were recovered after feeding on the mouse (n. larvae), (ii) immature tick survival (p.surv.tick), (iii) larva-to-nymph moulting success (p.moult), (iv) larva-to-nymph moulting time (moult.time1), (v) weight of engorged larvae (wt.larva.ug), and (vii) weight of unfed nymphs (wt.nymph.ug). The correlations were based on the means of the life history traits of the immature ticks

for each of the 40 mice. Positive correlations are shown in blue and negative correlations are shown in red. The size of the circle indicates the magnitude of the correlation coefficient. Statistically significant correlations are indicated by the asterisks.

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