Rickettsia spp. and its Effects on the Physiology and Behaviour of *Dermacentor Variabilis*

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Abstract

The genus *Rickettsia* is home to a number of species of endobacteria that infect vertebrates as well as invertebrates, some of these species being pathogenic. *Rickettsia* bacteria are transmitted to mammals by *Dermacentor variabilis* ticks in North America, but while pathogenic effects in mammals have been well described, the effect of the bacteria in ticks is less well explored. Experiments were performed to determine how the physiology and behaviour of the *Dermacentor variabilis* (Say) (Arachnida: Ixodidae) are affected by infection with *Rickettsia* bacteria. DNA analysis was used to determine the presence of *Rickettsia* genus in ticks. The supercooling point (SCP), fat content, and temperature preference were compared between infected and uninfected ticks. Infection incidence with *Rickettsia* spp. was 45.8% in males, 46.4% in unengorged females and 48.3 % in engorged females. Eggs were more resistant to freezing, followed by larvae, then adult ticks. There was no change in the temperature preference between males *vs*. engorged females, and females *vs*. engorged females. The presence of *Rickettsia* spp. did not significantly affect supercooling point, fat content and water content. However, there was a difference between infected and uninfected females, both engorged and unengorged, in temperature preference. We conclude that in both, engorged and unengorged female ticks, *Rickettsia* bacteria affect their behaviour but not physiology.

Keywords: *Dermacentor variabilis*, supercooling point, *Rickettsia,* temperature preference.

1. Introduction

Ticks are blood-feeding ectoparasites capable of transmitting pathogens (Estrada-Pena et al., 2013). Tick-vectored diseases were first documented in the nineteenth century (Smith & Kilbourne, 1893), and in the twentieth century ticks were found to be vectors of such human bacterial diseases as tick relapsing fever (Dutton & Todd, 1905), Rocky Mountain Spotted Fever (RMSF) (Ricketts, 1909), tularemia (Francis, 1929), and Lyme borreliosis (Johnson et al., 1984, Sperling & Sperling, 2009). Ixodid ticks are considered the most effective of the tick disease vectors because of their anatomy and their life cycle, which feature longer feeding periods and painless bites (Parola & Raoult, 2001). Several Ixodid tick genera transmit Rickettsia bacteria of the spotted fever group in North America: *Haemaphysalis*, *Rhipicephalus*, *Amblyomma*, and *Dermacentor* (Thorner et al., 1998). *Dermacentor variabilis* (Say) ticks are considered hosts, reservoirs and vectors of Rickettsiae bacteria (McDade & Newhouse, 1986).

1.1 Dermacentor Variabilis and Rickettsial Disease Distribution

The respective genospecies of the Rickettsiae group have distinctive geographic distribution, with some geographic overlap present (Wood & Artsob, 2012). The wide distribution of the spotted fever group *Rickettsiae* has likely been aided by migratory birds spreading the ticks infected with the bacteria. (Elfving et al., 2010). *Rickettsia* bacteria are transmitted transovarially (from mother to offspring) and transstadially (in between life stages) (Dantas-Torres, 2007). Infection can also start in uninfected tick larvae after feeding on infected mammals (McDade & Newhouse, 1986), depending on how long the larvae feed and on the number of *Rickettsia* bacteria in the blood. The infection starts in the epithelial cells of the midgut, where the *Rickettsia* spp. multiply and enter the hemocoel. Then they start invading other tissues including the salivary glands and ovaries (McDade & Newhouse, 1986).

Dermacentor variabilis, the American Dog tick, is an ectoparasite that carries bacteria of the *Rickettsia* spp. responsible for Rocky Mountain Spotted Fever (RMSF) (Estrada-Pena & Jongejan, 1999). Vasculitis is the primary outcome of Rickettsial infections. RMSF affects the lining of the blood vessels and causing them to leak. Knowledge regarding the ability of *Dermacentor variabilis* ticks to obtain, keep, and transmit the bacterial species that cause RMSF are vital to understanding the epidemiology and pathology of the disease. *Dermacentor variabilis* is found predominantly east of the Rocky Mountains in the United States and Canada (Chan & Kaufman, 2008), and is especially common in Atlantic Canada and Nova Scotia (Wood & Artsob, 2012). *Rickettsia rickettsii* was detected in *Dermacentor variabilis* ticks collected on the south shore of Nova Scotia between 1976-1980 (Wood & Artsob, 2012).

1.2 Physiology and Behaviour of Ticks

While ticks are generally resistant to freezing and starvation, they can also be sensitive to environmental factors such as temperature and humidity (Needham & Teel, 1991), with sensitivity varying across species (Sonenshine & Mather, 1994; Thorner et al., 1998). These factors influence tick locomotion and behaviour, such that ticks often choose habitats and location in response to variation in humidity and temperature (Sonenshine & Mather, 1994). Ticks prefer a relative humidity of approximately 80% in order to avoid desiccation (Gray et al., 2009). These factors can also influence the timing of the life cycle. For example, Yano et al. (1987) showed that when temperatures were low, engorged *Haemaphysalis longicornis* females took a longer time to lay eggs, and larvae and nymphs took longer to moult.

Cold hardiness and Supercooling Point (SCP): *Dermacentor* adult ticks differ from larvae and nymphs in terms of seasonal activity, implying a difference in their cold-hardiness (Meyer-Konig, 1999; Dorr & Gothe, 2001). Several studies have examined the supercooling point (SCP; the lowest temperature limit for survival) of different tick species, including *Dermacentor marginatus* (Dorr & Gothe, 2001), *Ixodes ricinus* and *Argas reflexus* (Dautel & Knulle, 1997, 1998), *Amblyoma americanum* (Needham et al., 1996), and *Ixodes uriae* (Lee & Baust, 1987). These studies demonstrated that eggs and larvae freeze at the lowest temperatures, followed by nymphs and then adults.

Previous research has shown that infection can influence the physiology and behaviour of Ixodid ticks. In some cases, the bacteria allow the tick to better survive the winter, thereby increasing the likelihood of successful transmission of the bacteria. For example, *Ixodes ricinus* ticks infected with *Borrelia* bacteria were more resistant to harsh environments, such as desiccation, and survived longer than uninfected ticks. They also exhibited less movement than uninfected ticks (Hermann et al., 2013). *Ixodes ricinus* ticks use the fat in their body while questing for hosts. Therefore, it can be inferred that the fat content in a hard tick's body such as *Dermacentor variabilis* can provide information on how long it can still quest for a host.

The purpose of this study was to examine the effect of infection with *Rickettsia* spp. on the physiology and behaviour of *Dermacentor variabilis* ticks, specifically fat content, supercooling point (i.e. the lowest temperature limit for survival), and temperature preference. It was hypothesized that infected ticks will be larger, with more fat and more water in their bodies, and that they will be more freeze tolerant, in order to promote bacterial transmission, as was found by Hermann et al. (2013) for *Ixodes ricinus* infected with *Borrelia* bacteria. We inferred that infected ticks would choose lower temperature, although no previous studies have examined the temperature preference in ticks.

2. Materials and Methods

2.1 Sample Collection and Identification

Dermacentor variabilis ticks were acquired from veterinary hospitals and clinics in Nova Scotia, and through donations from the public within local communities. Additional field sampling was conducted by flagging in Nova Scotia (Burnside, Lunenburg, and Timberlea). To obtain eggs for the life stage trials, several engorged female ticks were permitted to lay eggs. Engorged ticks were kept in a closed container at room temperature with 100% humidity. *Dermacentor variabilis* ticks were separated into three groups: Males, unengorged females hereafter referred to as females, and engorged females.

2.2 Thermopreferendum

Dermacentor variabilis males, females, and engorged females were tested in a thermopreferendum, a device to measure temperature preference (Figure 1). Ticks were placed in the middle of a rectangular plaster tray (38.7 cm x 26 cm x 1.91 cm) that varied in temperature from one end to the other, ranging from 10 to 50°C. Each tick was allowed to walk in any direction towards its preferred temperature; when it stopped, the temperature at that spot was measured using an electronic thermometer (K-thermocouple thermometer, Hanna). Elements of the tick's behavior (how fast the tick was walking, at what temperatures it turned around, and to which end of the

thermopreferendum it was moving towards) were also recorded. The relative humidity in the rectangular plaster tray was kept near 100% at all times by keeping the plaster wet. The ticks took an average of 30 seconds (range = x to y) to choose their preferred temperature. The temperature preference of each tick was calculated as the average value from 10 sequential trials.
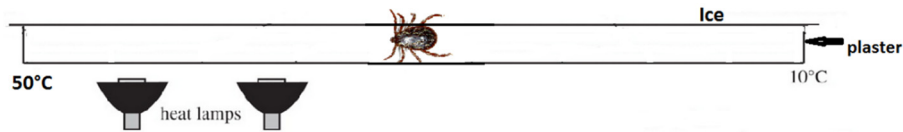

Figure 1. A schematic representation of the thermopreferendum apparatus made with plaster. The range of temperature on the plaster was 10°C to 50°C

2.3 Supercooling Point (SCP) Determination

Cooling equipment: The freezing experiments used either a cooling bath (VWR International, model 1197P) or a Dewar flask containing liquid nitrogen, depending on the developmental stage of the tick being tested. The cooling bath reduced the temperature to -22°C, which was sufficient to freeze adults and nymphs, but not eggs and larvae. Therefore, eggs and larvae were frozen using the liquid nitrogen contained in a Dewar flask. A thermocouple (Vernier, Omega) used in the Dewar flask was attached to a 30 cm metal rod with tape. A Styrofoam board was used to suspend the equipment and keep the thermocouple from falling into the liquid nitrogen.

The ticks were attached to the thermocouple by their scutum with a thin layer of petroleum jelly, which freezes without crystallization and functions as an effective glue to hold samples in place. After the ticks were attached to the thermocouples, they were placed inside the holding apparatus in a cooling bath (VWR International, model 1197P) and the temperature decreased at a rate of 1°C per minute. The temperature of crystallization was then recorded. The SCP determination was done using Vernier thermocouples connected to a converter box, a Labpro sensor interface, and then to a computer. A Vernier thermistor connected to the same interface was placed in the cooling bath to measure the temperature of the environment (i.e., the ethylene glycol in the cooling bath). Loggerpro 3.8.6 software recorded the temperatures of both the thermistor and the thermocouples. The SCP was detected in the Loggerpro software based on observation of a spike representing an abrupt increase in temperature, i.e. release of the heat of fusion, followed by a gradual regular decrease in temperature as latent heat returned to sensible heat.

2.4 Fat Quantification

The method for fat content quantification was adapted from Hermann et al. (2013). Ticks were weighed to the nearest 0.2 mg using an ultra-microbalance (Mettler Toledo, Switzerland) kept at a controlled atmospheric pressure and temperature. Each tick was then cut in half longitudinally and both halves were weighed together to check for weight loss during cutting and each half was then weighed separately. One half was used in the experiment and the other half was stored at -20°C for DNA extraction.

The half tick chosen for fat analysis was desiccated in an oven at 70°C for 24 hours, reweighed and then immersed in chloroform for 48 hours, with the chloroform being changed every 24 hours to prevent saturation with fat. The tick half was then dried at 70°C for 24 hours and weighed to get the fat-free and water-free mass.

2.5 DNA Testing

DNA Extraction: Each frozen half-tick was crushed using pestles or glass rods, which were rinsed with 90% ethanol between samples to avoid contamination. A 200μL of glass milk digestion buffer (Silica 325 mesh with double distilled water) was added to each sample along with 3μL of the proteinase K enzyme (20 mg/mL). The mixture was shaken (200 rpm) for at least eight hours at 55°C. 150μL of sodium iodide and 50μL of each of the tissue digests were transferred to each of the wells of a 96 well filter plate. The 50μL of the glass milk solution was transferred to the wells containing the samples and then the plates were put on a shaker (300 rpm) for one minute at room temperature. The supernatant was then removed using a vacuum suction; this process was repeated two more times without the glass milk. 200μL of wash buffer was transferred to each well and the plate was vacuum dried and placed in an oven at 75°C for 2 hours. A new 96 well collection plate was placed below the filter plate, and 125μL of 1 X TE (10mM Tris HCl, PH 8, and 0.1 mM EDTA) was transferred to each well containing samples.

The plates were then incubated for 5 minutes and centrifuged at 4000g to elute the DNA from each of the wells. The eluted DNA was transferred from the collection plate to Eppendorf tubes.

Polymerase Chain Reaction: Polymerase chain reaction (PCR) was performed using the oligonucleotide forward and reverse primers, Tz-15 5'-TTCTCAATTCGGTAAGGGC-3' and Tz-16 5'-ATATTGACCAGTGCTATTTC-3', as described in Tzianabos et al. (1989). These primers were designed to amplify a 246-base pair portion of the *17kDa antigen* gene of *Rickettsia rickettsia* but detects all *Rickettsia* spp. belonging to the spotted fever group (Tzianabos et al., 1989). PCR reaction conditions were a 5 minute denaturation step at 95°C, 40 cycles of: 94°C for 30 seconds, 52°C for 30 seconds, 66°C for 45 seconds followed by 72°C for five minutes and holding at 4°C. Amplicons were imaged following electrophoresis for 1 hour at 100V on a 2% (w/v) agarose 0.5X TRIS-Borate EDTA (TBE) gel pre-stained with Gel Green (Biotium), 10,000X in DMSO and imaged under ultraviolet illumination.

2.6 Statistical Analysis

ANOVA followed by post-hoc t-tests were used to test the significance of sex differences with respect to SCP, temperature preference, water and fat content. Pearson Correlation analysis was used to determine whether there was a correlation between size of *Dermacentor variabilis* and each of: supercooling point, temperature preference, water content, and fat content. Unpaired two-tailed Students t-tests were used to test for significant differences in infection with *Rickettsia* spp. with respect to SCP, temperature preference, water, and fat content.

3. Results

3.1 Infection Rate

The rate of infection with *Rickettsia* spp. in *Dermacentor variabilis* ticks, determined by PCR of the *17kDa antigen* gene from *Rickettsia* genus was approximately 50 percent: 45.8% for males, 46.4% for females and 48.3 % for engorged females 48.3%.

3.2 Total Body Weight

Not surprisingly, there was a significant difference in total wet body weight between females and engorged females (t (x) = y, p =1.8E-10)) as well as between males and females (t(x)=y, p= 2.1E-05. Infected males (6.75 ± 2.03 mg) had weights that were not significantly different from uninfected males (6.72 ± 1.81 mg) (t (x) = y, p =0.566)). Nor was the weight of infected females (8.91 ± 2.35 mg) or engorged females (33.17 ± 14.6) statistically different from uninfected females and engorged females, (9.28 ± 3.47 mg) and (31.11 ± 17.95 mg) respectively, (t (x) = y, p =0.289)). Thus, infection status did not affect total wet body weight.

3.3 Supercooling Point (SCP)

Eggs and larvae freeze at lower temperatures than adults (Figure 2). Infected males had similar supercooling points (-15.3°C ± 3.2) to uninfected males (-14.9°C ±5.7) (t (x) = y, p =0.61)). The same trend was seen in females, where infected females (-13.1°C ± 3.06) exhibited similar SCP to uninfected females (-13.8°C ± 2.3) (t (x) = y, p =0.27)) (Figure 2). Supercooling points were also similar between infected (-12.4°C ± 2.51) and uninfected engorged females (-13.1°C ± 2.29) (t (x) = y, p =0.32)). Therefore, there was no significant effect of Rickettisal infection status on SCP (t (x) = y, p >0.05)).

3.4 Thermopreferendum

In contrast, there was a significant difference in temperature preference between infected and uninfected females (t (x) = y, p =0.006)), both females and engorged females (t (x) = y, p =0.05)) (Figure 3). Feeding status and sex do not affect temperature preference.

3.5 Body Composition

In general, tick bodies are composed of approximately 60% water, 9% fat, and 31% other components. Water content was similar between infected (55.8 ± 8.80 %) and uninfected males (50.4 ±13.2 %), (t (x) = y, p =0.92). Water content was also similar between infected females (56.7 ± 9.9%) and uninfected females (54.1 ± 10.4 %), (t (x) = y, p =0.88) as well as infected engorged females (62.3 ± 6.31%) and uninfected engorged females (62.0 ± 5.15%), (t (x)=y, p=0.86). Thus, there was no significant effect of infection with *Rickettsia* spp. on water content in *Dermacentor variabilis.* Similarly, there was no difference in fat content between infected and uninfected males (t (x) = y, p =0.45), females (t (x) = y, p =0.82) or engorged females (t (x)=y, p=0.35) (Figure 4). Thus, the percent body composition (percent water, percent fat, percent residue) of the different *Dermacentor variabilis* groups did not differ depending on infection status.
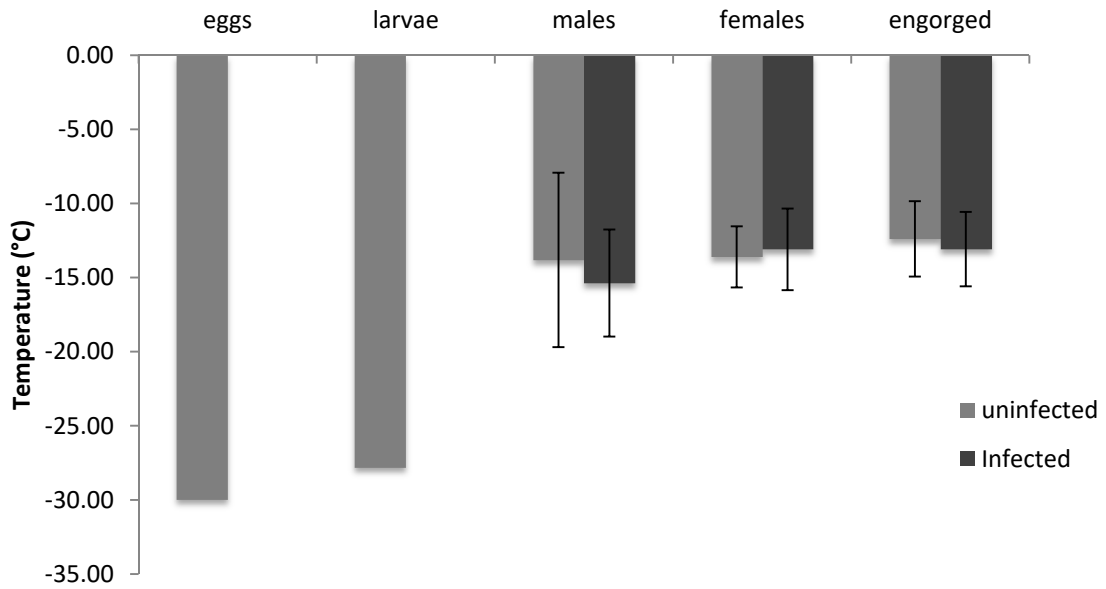
Figure 2. Supercooling points (°C) of the different *Dermacentor variabilis* life stages from eggs to adults and the supercooling points of the adults based on their sex and infection status
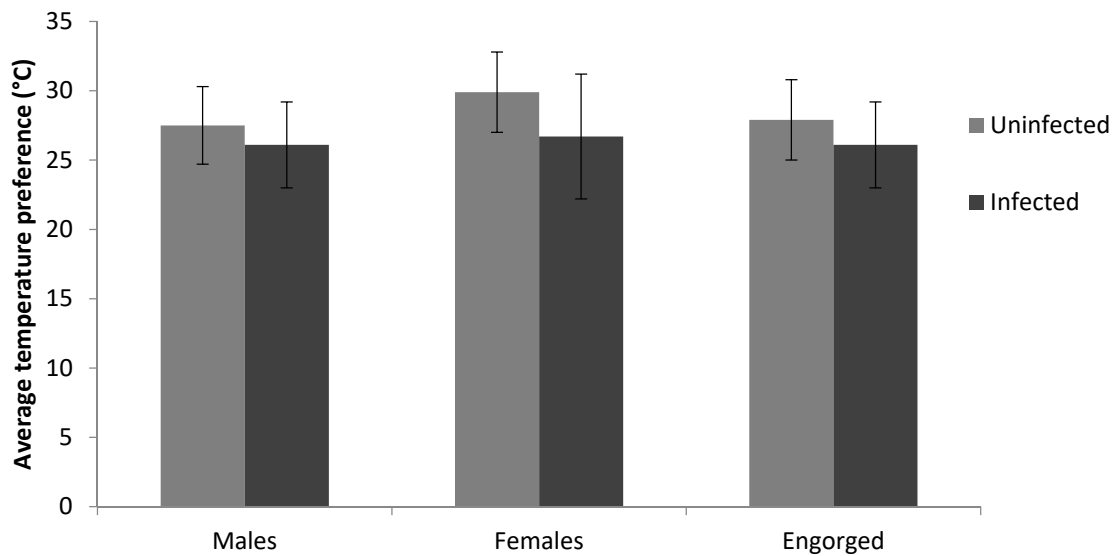
Figure 3. Average temperature preference of *Dermacentor variabilis males,* females and engorged females according to infection status with *Rickettsia* bacteria
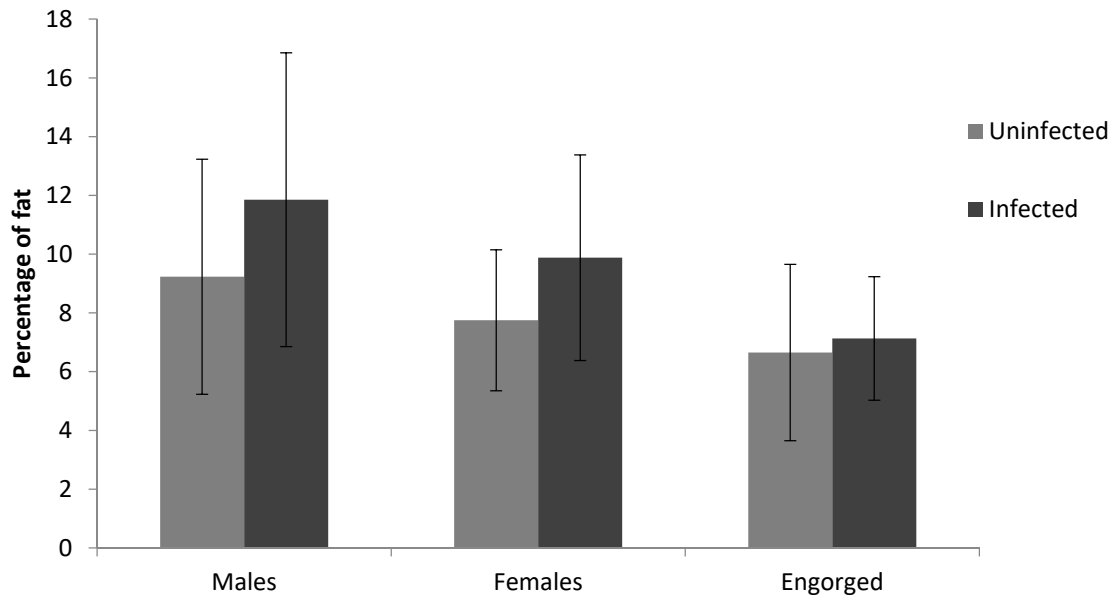
Figure 4. The percentage of fat in *Dermacentor variabilis* ticks according to infection status with *Rickettsia* spp. bacteria

4. Discussion

The infection prevalence of 45.8% and 46.4 % for *Rickettsia* spp. in male and female *Dermacentor variabilis* ticks, respectively, was found in the tick population from southern Nova Scotia, Canada. Our study paralleled the investigation of *Rickettsia parkeri* in *Amblyomma maculatum* ticks in Fairfax County, Virginia in 2011, which showed a 41.1% infection rate with no sex difference (Formadel et al., 2011). Similar infection prevalence in males and females is not unexpected because both sexes feed on the same hosts, which will be either infected or uninfected and arise from the same population of infected *versus* uninfected females.

Our studies show that infection with *Rickettsia* spp. influences the behaviour but not the physiology of *Dermacentor variabilis.* There was a significant effect of infection with *Rickettsia* spp. on temperature preference, but not on size, SCP, water content, and fat content. In contrast, feeding status influenced the physiology but not behaviour of *Dermacentor* variabilis; there was a significant difference in body size between females and engorged females regardless of infection status.

This study found no significant effect of infection upon body size of *Dermacentor variabilis*, suggesting that *Rickettsiae* bacteria do not play a role in the weight gain/loss in *Dermacentor variabilis.* Infection status also did not influence supercooling point. This result is consistent with the research done by Hermann & Gern (2010) who showed that ticks infected with *Borrelia* are more resistant to inconsistent temperature and humidity environments even though *Dermacentor variabilis* is a different tick species and has different bacteria species.

In our experiments, ticks were kept at 100% relative humidity at all times. Water is vital for *Dermacentor variabilis* because they are sensitive to desiccation, and when the water content in their body decreases they descend from their questing position into the leaf litter to acquire water (Hermann et al., 2013). There was no influence of infection on water content in *Dermacentor variabilis* regardless of sex or *Rickettsia* infection status, contrary to our predictions, which were based on the findings of Hermann et al. (2013). Analysis of fat content revealed that infected *Dermacentor variabilis* males have about 10 % more fat than uninfected males in their body. Although not statistically significant, this result is similar to the 12 % difference found by Hermann et al. (2013) for nymphs of *Ixodes ricinus* infected with *Borrelia* spp. As Hermann et al. (2013) suggested, the higher fat content in infected ticks might be due to an increase in the blood meal size, faster digestion, or decreased activity.

In contrast to the lack of response of physiological metrics to Rickettsial infection status, there was a significant effect of infection on temperature preference in *Dermacentor variabilis*; infected females and engorged females preferred lower temperature than uninfected ones. One might speculate that the bacteria might have a role in

lowering energy expenditure in *Dermacentor variabilis*. This finding is also consistent with research done by Hermann (2013), who found that *Borrelia*-infected ticks had higher survival rates and decreased activity.

In order to understand the behaviour of bacteria causing human diseases, as well as the vectors that carry them, it is important to consider the biological differences between species (Dantas-Torres, 2007). Our analysis is based on the genus *Rickettsia,* which includes 20 different species that are part of the spotted fever group. Since different species of *Rickettsia* may have different effects on tick physiology, identification of the most prevalent *Rickettsia* species present in these *Dermacentor variabilis* ticks might be valuable. In this study, we examined several characteristics related to the morphology, physiology and behavior of *Dermacentor variabilis* to see how those characteristics were affected by infection with *Rickettsia* spp. bacteria. Overall, there was an effect of feeding status on the physiology and an effect of the presence of *Rickettsia* spp. on behaviour. This is an important finding because it provides insights on *Dermacentor vatriabilis* host-bacteria interactions and how these factors may influence disease risk.

Acknowledgements

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