University of South Bohemia, Faculty of Science

Exploration of the tick-Borrelia molecular interactions by employing the transcriptomic approaches

Ph.D. Thesis

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České Budějovice, January 2021

This thesis should be cited as:

Mahmood, S. 2021: Exploration of the tick-Borrelia molecular interactions by employing the transcriptomic approaches. Ph.D. Thesis, in English – 108 pp., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Annotation: Along with climate change and increased sharing of habitat, ticks are coming into more frequent contact with humans. The hard tick *Ixodes scapularis* and *Ixodes ricinus* are known disease vectors in Northern America and Europe, respectively. Along with many other pathogenic microorganisms, these ticks spread *Borrelia* sp. by ectoparasitic blood feeding. *Borrelia afzelii* is the major European Lyme disease pathogen spread by *I. ricinus*. Our study focuses on differential gene expression in *I. ricinus* salivary gland and midgut, induced in the nymphal stage by *B. afzelii* infection. Tick genes upregulated by infection are considered to play essential roles for the acquisition, persistence, and transmission of Borrelia. We have determined 32,897 full length sequences of tick mRNA from *B. afzelii* infected/noninfected tick salivary glands and the whole body. In addition, we have obtained MACEseq (Massive Analysis of cDNA Ends) from both midgut and salivary glands while the nymphs were non-infected or infected with *B. afzelii* during three different phases of blood-feeding. From the MACE database, we obtained 250-500 bp 3'-end sequences with raw quantitative expression values. Total reads, unique sequences and protein coding tick genes from midgut samples were 38,199,641, 88,825 and 24,276, and from salivary gland were 74,651,134, 93,096 and 26,179, respectively. After filtering, using several criteria, expression was validated by qPCR. Hence, the validated genes may most likely interact with Borrelia in its acquisition, persistence, or transmission to the vertebrate host. In our study, RNA interference approaches and vaccination were implemented in order to investigate the impact of upregulated tick midgut and salivary gland genes on Borrelia transmission to C3H mice.

Funding: This work is funded by GACR 17-27393S, GACR 17-27386S, GACR 13-12816P, GACR 13-27630P, the European Union FP7 project Antidote (grant agreement number 602272), AZV NU20-05-00396, GAJU 04-039/2019/P, and Institute of Parasitology (BC, CAS), University of South Bohemia, and Ministry of Education (ČR).

Declarations

I hereby declare that this thesis is based on my work, and all other sources of information have been acknowledged. I hereby declare that, in accordance with Article 47b of Act No.111/1998 in the valid wording, I agree with the publication of my Ph.D. thesis, in full to be kept in the Faculty of Science archive, in electronic form in the publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree with the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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This thesis originated from a partnership of Faculty of Science (University of South Bohemia) and the Institute of Parasitology (Biology Centre of the CAS), supporting the study of tick transcriptomics and prevention strategies of Lyme disease transmission**.**

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Acknowledgement

First, I would like to remember my parents Prof. Mohammad Sayeed and Mrs. Khadiza Akter, they raised me, taught me everything, and made me who I am.

I would like to thank RNDr. Ondřej Hajdušek, Ph.D. for believing me at the first moment. He inspired and trained me. He supervised my doctoral research and patiently assisted in manuscript preparation and dissertation writing. I would also like to express my gratitude to RNDr. Radek Šíma, Ph.D. due to his remarkable roles. Their contribution together have made my research completed. Besides, I am grateful to RNDr. Petr Kopáček, CSc., RNDr. Veronika Urbanová, Ph.D., RNDr. Marie Jalovecká, Ph.D., Ryan O. M. Rego, Ph.D., and Dr. Abdoallah Sharaf for their cooperation, suggestion and help. I would also like to acknowledge the outstanding and exceptional contribution from Ing. Gabriela Loosová, Jan Erhart, Adéla Palusová (Harcubová) and Zuzana Šmejkalová. I like to mention Terka, Mirka, Hanka, Verča, and Romana for their friendly company while sharing the same office room. I would also like to give thanks to Professor John D. Brooker for his assistance in language correction of this thesis.

Very special acknowledgement to my wife Dr. Asma Akter and our son Aleef Mahmood. Asma's contribution and supports are extensively vast. Without her strong assistance and encouragement, I never could reach here. Also, it is my honor and pleasure to express my heartful gratitude to uncle Hossain and uncle Shafi for their devoted and unconditional love for me. Their contribution in my excellent childhood with lots of memories will never be fade. I will always be grateful to them.

Lastly, I would like to remember all of my friends and families in the Czech Republic and in Bangladesh including Choto, Adib, Zinia, Zehadee and many others. Thank you everyone for your support in your own way and for enriching my life with happiness.

Table of Contents

List of Figures

List of Tables

1. General Introduction

Ticks are arachnids in the order Parasitiformes (Déruaz *et al.*, 2008). Mites and ticks belong to the subclass Acari. Ticks are external parasites, living by blood-feeding on mammals, birds, reptiles, and amphibians. These are vectors of several diseases that affect both humans and other animals. Since a few woodland animals such as amphibians, birds, and reptiles utilize ticks for food, the tick is therefore an excellent ecosystem biomarker (Stutsman, 2019).

Of the three families of ticks, Nuttalliellidae is made up of only a single species, *Nuttalliella namaqua*. The remaining two families contain the hard ticks (Ixodidae) and the soft ticks (Argasidae) (Horak, Camicas and Keirans, 2002). Tick species are distributed globally (Magnarelli, 2009), but they are much more abundant in places with warm and humid climates. Minimum moisture in the air is a requirement for metamorphosis, and hatching from eggs demands a higher temperature (Merino *et al.*, 2013). Ticks are responsible for transmitting several diseases through blood-feeding to domesticated animals, causing significant economic loss every year. Moist sandy soil with hardwood trees is ideal for tick infestation, and the most predictable indicator of a dense tick population is the presence of many deer in the forest (Magnarelli, 2009).

Ixodes ricinus, known as the 'Castor Bean Tick' covers a broad geographic region in the EU from Portugal to Russia and from North Africa to Scandinavia. This wide geographic distribution demonstrates that this tick species can survive under various environmental conditions. Due to weather change, this tick is reportedly found more frequently at higher altitudes and latitudes (Bugmyrin *et al.*, 2013). For sucking host blood with its hypostome, the tick cuts a hole through the host epidermal layer and uses an anticoagulant to prevent the clotting of blood at the feeding site to ensure that feeding remains uninterrupted (Hidano *et al.*, 2014; Kazimírová and Štibrániová, 2013).Tick saliva components also prevent several host immune responses (Kotál *et al.*, 2015). Ticks locate hosts by detecting an animal's breath or body odours or by sensing body heat, moisture, and vibrations.

Ixodes ticks require three hosts, and their life cycle takes at least one year to complete. Around 1,000 eggs are laid on the ground by an adult female tick.

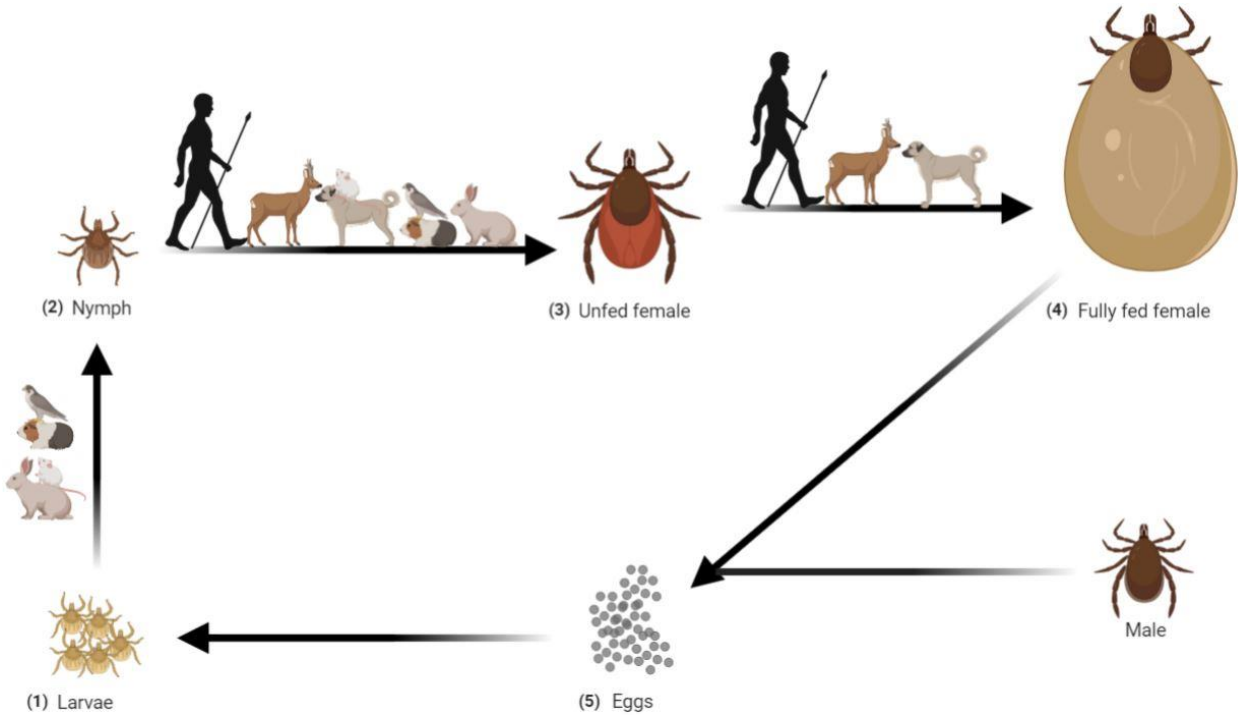

Figure 1: **Life cycle of *Ixodes* ticks in different hosts and Borrelia transmission**. When larvae emerge at summer (1), they feed primarily on small mammals and birds. After feeding, larvae detach from the host and molt to nymphs on the ground the following spring (2), feeding on larger hosts and then molt to adults in the autumn (3). Female adults attach to larger hosts, feed and lay eggs, while males do not feed on blood (4, 5).

Unlike other insects, the tick adult has four pairs of legs, as do other arachnids, and the body is not segmented. The mouthparts are used to attach to the host body and to suck blood using two palps, two chelicerae, and one hypostome. Palps function as the sensory organ, while chelicerae cut the skin to insert the anchoring organ, the hypostome. The tick also secretes 'cement' like proteins from the salivary gland to facilitate the stronger attachment of mouthparts to the host skin (Trimnell *et al.*, 2005). Soft cuticles with internal folds make the hard tick surface flexible, enabling them to feed blood in amounts up to 100 times more than their original body weight.

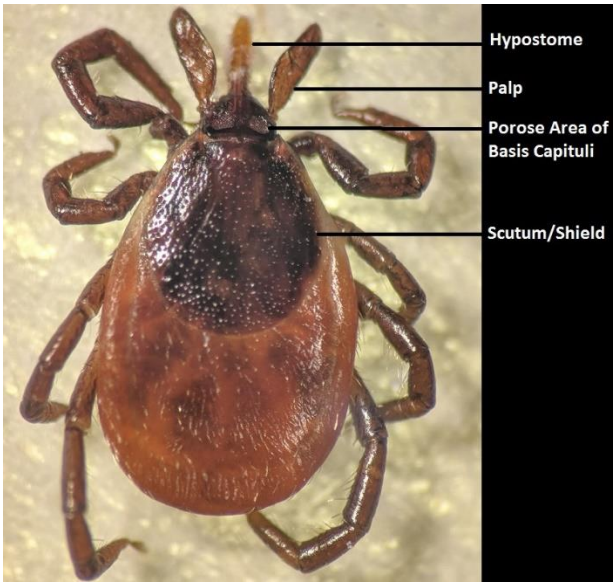

Figure 2: ***Ixodes* tick external anatomy (dorsal view)** (*Anatomy of a Female Deer Tick*, n.d.)

1.1 Midgut impacts on Borrelia

Within the internal anatomy, the salivary gland, midgut, and haemolymph are the most notable organs. In the fed tick, the midgut is the largest organ in the body. It consists of a central ventriculus and numerous caeca covering all parts of the body cavity. Thus there are anterior, lateral, and posterior caeca. The midgut wall consists of an epithelium and a thin layer of elongated smooth muscle cells (Tarnowski and Coons, 1989; Matsuo *et al.*, 2003). The epithelial cells are reported to consist of at least two basic types, undifferentiated and digestive. The digestive cells are believed to develop from the undifferentiated cells during feeding, whereupon they ingest food materials (albumin, haemoglobin) by various types of endocytosis. An adult female can uptake an enormous amount of host blood and the ultrastructure of the midgut is designed to adjust to this feeding phenomenon (Matsuo *et al.*, 2003). Endocytosis is reserved for fluids, and macromolecules that attach to vesicles or pits on the plasma membrane. This process internalizes food particles by forming endosomes. While erythrocytes are lysed intraluminally, all digestion of the blood meal is strictly intracellular. Absorption and intracellular digestion are complex events that are coordinated with changes in the physiological state of the tick, e.g., feeding, molting, mating, repletion, and oviposition, etc. The nature of the intracellular digestive process of haemoglobin is

essential for understanding the ability of diverse microbes to survive in the tick body and to disseminate infection in the tissues (Tarnowski and Coons, 1989; Caperucci, Bechara and Camargo Mathias, 2010). The absence of a tick extracellular digestion system strongly contrasts with all other blood-feeding arthropods (Graça-Souza *et al.*, 2006).

1.2 Salivary gland as the exit point

The female Ixodid salivary gland consists of a large number of acini of three different types (type I to type III). Type-I acini contain four distinct cell types: a single central lamellate cell, multiple peripheral lamellate cells, peritubular cells, and one circumlumenal cell. Like epithelial cell types, in type II and III acini, 7–9 different glandular cells are divided into six categories enclosing secretory granules (Fawcett, Binnington and Voigt, 1986; Sonenshine and Roe, 2013). The single and luminal cell, also called the Cap or myoepithelial cell (Krolak *et al.*, 1982; Meredith and Kaufman, 1973), covers the edges of the surface on the luminal side of type II and III acini, and is more often described as having a web-like structure. This facilitates contractions which expels the acinar contents into connecting ducts while the tick feeds (Krolak *et al.*, 1982; Coons *et al.*, 1994; Šimo *et al.*, 2014). During feeding, most acinar cells undergo marked hypertrophy, resulting in a total increase in the mass of the salivary gland (Šimo *et al.*, 2013; Fawcett, Binnington and Voigt, 1986). In particular, uptaken fluid hugely expands the lumen of type III acini (Mateos-Hernandéz *et al.*, 2020, Kim *et al.*, 2014, Fawcett *et al.*, 1981;), while in type II acini, the lumen remains relatively small, but the cell bodies enlarge during blood-feeding (Binnington, 1978; Walker *et al.*, 1985).

The tick salivary gland mediates diverse functions that ensure the tick's biological success during both on and off-host states. This vital organ has a role in moisture absorption from the unsaturated atmosphere, removal of excess fluid to concentrate nutrients from uptaken blood meal, and production of the cement protein that helps tick to remain attached firmly through the hypostome in the host skin. The critical role of the tick salivary gland is to secrete different biologically active molecules that facilitate the acquisition of tick-borne pathogens.

The preservation of water is crucial for ticks since ticks are susceptible to desiccation, especially during the fasting period. Here, type I acini play an essential role in absorbing atmospheric moisture. These acini cells produce a Na^+, K^+, and Cl^- -rich hygroscopic solution, which becomes diluted by absorbing water from the air and is then swallowed back into the body. (Needham *et al.*, 1986; Knulle and Rudolph, 1982; Needham, Rosell and Greenwald, 1990; Gaede and Knülle, 1997; Sonenshine and Roe, 2013). The absorbent property of salivary gland type I acini has been proved recently by Kim *et al.* (2016) using fluorescent microscopic techniques.

Ixodid ticks are unique among all other blood-feeding arthropods for their long duration of attachment to the host skin, depending on tick species and their life stages. To maintain homeostasis during feeding, excess fluid containing ions are excreted back to the feeding site via the salivary gland after crossing the midgut wall and haemocoel (Sauer *et al.*, 1995; Banajee *et al.*, 2015). In *Dermacentor andersoni,* females excrete 96% of the sodium and 74% of the water back to the host via the same route as blood-feeding, and Kaufman and Philips reported this in 1973. From the surrounding haemolymph, water and electrolytes are thought to gain access to the salivary gland through the type III acini epithelium layer, as demonstrated in several studies (Fawcett *et al.*, 1981; Meredith and Kaufman; 1973). This hypothesis was supported in a recent study where the sodium-potassium pump (Na/K-ATPase) involved in forming the sodium-rich primary saliva was confirmed within the epithelial cells of all three kinds of salivary gland acini (Kim *et al.*, 2016). Argasid ticks use a different strategy to eliminate excess water from their body with the help of the coxal gland, and this is a unique characteristic of this tick family (Kim *et al.*, 2014).

A cement cone is apparently produced to protect the mouthparts of ixodid ticks during their blood-feeding, helping the tick to remain attached to their host without difficulty. This is also vital in order to resist the host immune system and to feed smoothly (Kern *et al.*, 2011). Not all, but a majority of *Ixodes* ticks use this cement cone for securing their feeding (Mans, 2013; Kern *et al.*, 2011). Lipids, polymerized and hardened glycine-rich proteins, and carbohydrates are the main components of the cement complex produced from type II and III salivary gland acini (Chinery, 1973; Jaworski *et al.*, 1992). However, *I. ricinus*, *Ixodes holocyclus* and *Nuttalliella namaqua* do not produce this cement protein (Barker and Walker, 2014; Suppan *et al.*, 2018). Multiple serine

protease inhibitors and metalloproteases were also found by proteomic analyses of the cement cone from *Amblyomma americanum* (Bullard *et al.*, 2016). These are interesting feeding support molecules due to their antigenic properties and are considered as promising anti-tick vaccine candidates (Bishop *et al.*, 2002; Shapiro, *et al.*, 1987; Mulenga *et al.*, 1999).

2. Tick as a disease vector

In 1970, *Babesia microti*, an intraerythrocytic parasite, was first described in an otherwise healthy woman (Western *et al.*, 1970). Shortly after that, *B. microti* was identified in white-footed mice (*Peromysus leucopus*) and *I. scapularis*; experimental studies later confirmed that *I. scapularis* nymphs were capable of transmitting the protozoa *B. microti* (Spielman, 1976; Piesman and Spielman, 1980).

Lyme disease was first observed in 1975 in the United States of America as a new form of inflammatory arthritis (Steere, Hardin & Malawista, 1977). In 1982, a spirochete, later named *Borrelia burgdorferi* (Johnson, Schmid and Hyde, 1984), was identified as the etiological agent and was shown to be transmissible by *I. scapularis* (Schachat *et al.*, 2017; Burgdorfer *et al.*, 1982). Although numerous small mammals and birds have been identified as reservoirs of *B. burgdorferi* sensu stricto (s.s.), the white-footed mouse is among the most important reservoir in the eastern United States (Donahue, Piesman and Spielman, 1987; Mather *et al.*, 1989; Lane, Piesman and Burgdorfer, 1991; LoGiudice *et al.*, 2003). In the following year, for the first time in Europe, morphologically and antigenically similar spirochetes were detected in *Ixodes dammini* (Burgdorfer, 1984).

Human granulocytic anaplasmosis, described initially as human granulocytic ehrlichiosis (*Ehrlichia phagocytophila*), was first identified between 1990 and 1993 in six patients from northern Minnesota and Wisconsin, presenting with acute febrile illnesses. The timing of onset of cases was consistent with the host-seeking activity of *I. scapularis* and *Dermacentor variabilis* (Goodman *et al.*, 1996). In 1996, *I. scapularis* was experimentally confirmed as a vector of *E. phagocytophila*, and *P. leucopus* was shown to be a competent reservoir (Mather *et al.*, 1989). In 2001, this intraleukocytic bacterium (*E. phagocytophila*) was renamed *Anaplasma phagocytophilum (*Dumler *et al.*, 2001*)*.

Powassan virus, a flavivirus, was first recognized in 1958 as a human pathogen when it was isolated from a child who died of encephalitis (Dumler *et al.*, 2001). *Ixodes marxi*, *Ixodes cookei*,

and *Ixodes spinipalpis* were implicated as enzootic vectors of Powassan virus in the 1960s (Doughty, Yawetz and Lyons, 2017; Fatmi, Zehra and Carpenter, 2017), more than 30 years before the vector competence was demonstrated for *I. scapularis* (Costero and Grayson, 1996). *I. scapularis* is considered the primary bridging vector of Powassan virus to humans (Costero and Grayson, 1996; Telford *et al.*, 1997; Ebel, 2010).

In 2011, a novel obligate intracellular Gram-negative bacterium, found in *I. scapularis* from Minnesota and Wisconsin, and later described as *Ehrlichia muris eauclarensis (Pritt et al., 2017),* was recognized to cause ehrlichiosis in humans (Pritt *et al.*, 2011). *I. scapularis* was demonstrated experimentally to be a vector of *E. muris eauclarensis* (Karpathy *et al.*, 2016; Lynn *et al.*, 2017). It supports earlier reports of natural infection in *I. scapularis* from Minnesota and Wisconsin (Pritt *et al.*, 2011; Telford III, 2011; Stromdahl *et al.*, 2014). *E. muris eauclairensis* has been detected in naturally infected white-footed mice collected in these two US states (Castillo *et al.*, 2015), and reservoir competence was demonstrated in the laboratory (Lynn *et al.*, 2017).

Borrelia miyamotoi, a relapsing fever spirochete, was first described in *Ixodes persulcatus* in Japan (Fukunaga *et al.*, 1995). In 2001, the ability of *I. scapularis* to transmit *B. miyamotoi* while feeding, and to pass spirochetes transovarially, was demonstrated under laboratory conditions (Fukunaga *et al.*, 1995). A decade later, *B. miyamotoi* was recognized as a human pathogen in a report of 46 cases from Russia (Fukunaga *et al.*, 1995). Shortly after that, the first recognized case of relapsing fever caused by *B. miyamotoi* in North America was described in an 80-year-old woman from New Jersey (Fukunaga *et al.*, 1995). The first large case series from the northeastern United States revealed that the peak onset of illness occurred from July through August, one month later than for Lyme disease, anaplasmosis, and babesiosis, and thus corresponds with the peak host-seeking activity of larval rather than nymphal *I. scapularis* ticks (Krause and Barbour, 2015; Molloy *et al.*, 2015). Although white-footed mice support short-lived infections of *B. miyamotoi* transmissible to feeding ticks, they likely play a role in the amplification of infections (Scoles *et al.*, 2001; Barbour *et al.*, 2009). For this pathogen, the transovarial transmission may be the primary route of enzootic maintenance (Bunikis and Barbour, 2005; Barbour *et al.*, 2009; Crowder *et al.*, 2014; Wagemakers *et al.*, 2015).

Until 2016, when *B. mayonii* was described and recognized as a causative agent of Lyme disease in Minnesota and Wisconsin (Pritt, Mead, *et al.*, 2016; Pritt, Respicio-Kingry, *et al.*, 2016), *B. burgdorferi* s.s. had been considered the sole agent of Lyme disease in the United States. *B. mayonii* has been detected in field-collected *I. scapularis* from Minnesota and Wisconsin (Pritt, Mead, *et al.*, 2016), and vector competence has been demonstrated under laboratory conditions (Dolan *et al.*, 2016). *B. mayonii* also was isolated from white-footed mice and an American red squirrel (*Tamiasciurus hudsonicus*) in Minnesota, but reservoir competence has not yet been demonstrated experimentally (Johnson *et al.*, 2017).

3. Lyme Disease

Lyme disease (LD) is an increasing global public health concern due to climate change and human activities (Dumic and Severnini, 2018; Van Hout, 2018). LD is caused by spirochetes of the *B. burgdorferi* sensu lato (s.l.) species complex, which are transmitted by infected *Ixodes* ticks (Rudenko *et al.*, 2011). Prevention of transmission is highly reliant on tick control, including the use of tick repellents. There are no vaccines presently available to prevent human infection, although several vaccines have been shown to reduce transmission and the clinical manifestations of illness in dogs (Chomel, 2015). *B. burgdorferi* spirochetes are acquired by larval ticks when feeding on reservoir hosts; they persist in the tick midgut through the molt into nymphs, subsequently migrating to the salivary glands (Caimano *et al.*, 2016). During nymph feeding, *B. burgdorferi* is transmitted to a vertebrate host. The pathogen is capable of adapting to species-specific environments, including available nutrient resources and immune responses.

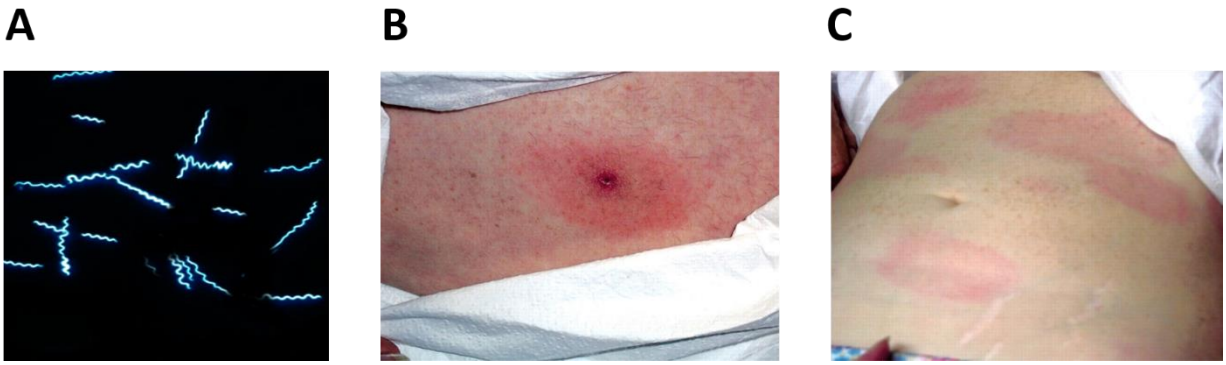

Figure 3: **A)** Borrelia spirochetes under the darkfield microscope, x400 (Tatum, 2020), **B** and **C)** Tick bite and erythema migrans (Shimamura, Maeda and Gocho, 2018)

In the USA and Europe, LD is the most transmitted tick-borne disease. The onset of LD is divided into three stages. These are early localized, early disseminated, and late. The first stage of the disease is identified by the red ring-like expanding rash called Erythema migrans (EM) at the site or near the tick bite. Other symptoms that may be observed at this stage include flu-like symptoms, headache, fever, myalgia, arthralgia, etc. The majority of patients experience only the symptoms of early and localized disease. Approximately 20% of patients may develop early disseminated disease, with some symptoms like multiple EM. Other symptoms of disseminated LD are arthralgia, lymphadenopathy, myalgia, ophthalmic conditions, palsies of the cranial nerves

(especially CN-VII), and lymphocytic meningitis, etc. Moreover, cardiac manifestations such as myocarditis, conduction abnormalities, and/or pericarditis may appear. The most common symptom of late disease is arthritis, which usually affects large joints, especially the knees (Bransfield, 2018; Cervantes, 2018).

The diagnosis is not always straightforward, as many patients are not able to notice a tick bite, especially the nymphal tick. However, in endemic areas, people who might think they received a recent tick bite, without having the typical rash, should start treatment without confirming a positive case.

B. burgdoferi s.s. has a very particular tendency to affect the joints. *B. garinii* is found in Europe and is responsible for causing *white* matter encephalitis. *B. afzelli* is located primarily at the site of infection and has an affinity for the skin. There are many *Ixodes* subspecies that may transmit the Borrelia spirochete. In the USA, the rate of Lyme borreliosis reporting is about 40 per 100,000 population. Over the last two decades in Europe, around 360,000 patients have been reported to acquire LD (*Ixodes Ricinus - Factsheet for Experts*, 2014). The possibility of transmission increases during the late spring, summer, and early autumn.

3.1 Pathophysiology

The most common and first presentable sign of LD is the EM rash. It is found in 70% to 80% of victims and appears at the tick bite site or at least in the nearby area as an expanding, erythematous skin lesion, which is generally 5 cm in diameter or larger. The rash may present as homogeneous erythema or as a targetoid appearance. The display of inflammation takes place within two weeks after the tick bite. If it remains untreated, pathogen dissemination may lead to other common symptoms, including early arthritis in up to 30%, neurologic instances in 10% to 15%, or cardiac involvement in 1% to 2% of Borrelia positive patients (Norris, 2018).

3.2 Histopathology

Histology of EM is nonspecific, generally showing a perivascular cellular infiltration, and consists of plasma cells, histiocytes, and lymphocytes. Rarely, neutrophils and mast cells are identified. A biopsy may show a local reaction at the site of EM, where eosinophilic infiltrates occur. Spirochetes may be identified using silver stains or antibody-labeling. Usually, very few spirochetes are found in the infected tissues of LD patients (Moguelet, 2007).

3.3 Physical impact

Localized Lyme arthritis is identified by EM occurring within two weeks of tick exposure in a pestilence area. The medical diagnosis for early zoonotic disease with em includes other skin conditions like tinea and nummular eczema. If not treated within the localized stage, patients may develop early disseminated or late disease manifestations. Early neurologic zoonotic disease symptoms are facial (CN-VII) palsy, radiculopathy, or lymphocytic meningitis. Cardiac involvement includes myopericarditis and typically presents with Stokes-Adams syndrome. LD is mono- or pauciarticular, generally involving large joints, most commonly the knee, and occurring months after the initial tick bite (Smith *et al.*, 2011)
If the symptoms don't seem to be specific, one should consider other infections transmitted by ticks like *B. microti*. Co-infection is reported for 10% of LD patients (Berghoff, 2013).

Stage 1: Early localized disease that will present with EM and a low fever. The uniform rash occurs at the location of the tick bite, sometimes burning or itching, or might be asymptomatic. The rash tends to expand for some days, and the concentric ring is also visible. If this stage is left untreated, the inflammation will usually persist for 2-3 weeks. About 20% may have recurrent occurring of the rash, and multiple lesions may also appear. At the same time, flu-like symptoms may also be present. The fever is low grade and should be related to myalgia, neck stiffness, and headache. Visual problems include eye redness and tearing. About 30% of LD patients with the primary symptom of rash don't have other complications (Murray and Shapiro, 2010; Skar and Simonsen, 2017).

Stage 2: Usually, 3-12 weeks is necessary to progress the disease into the 2nd stage after the initial feature. Symptoms such as neurological disorder, fever, cardiac complications, and muscle pain may appear. Cornea inflation with eye pain may also occur. Joints like wrist and ankle and especially the knee might exhibit tightness and pain. These symptoms may last up to 12-20 weeks. 20% of LD patients may develop nerve neuropathy and meningitis (Halperin, 2015; Skar and Simonsen, 2017).

Stage 3: Later stages of LD patients may occur even years after a tick bite. The features of this final stage include rheumatological and neurological problems such as arthritis and cognitive deficits along with cardiac involvement (Skar and Simonsen, 2017).

3.4 Evaluation

Treatment can be started as the first preference without a proper diagnosis if the patients are from an endemic area or display the classical EM rash. But not everyone develops a rash, so diagnosis should not be ignored. A serological test is not an appropriate test at the very beginning of an infection when the patients develop the classic bull's eye rash. At this stage, treatment usually starts, depending on the patient's symptoms and travel history. Later, a two-step serological diagnostic test is recommended: EIA (enzyme immunoassays) or IFA (immunofluorescence assay) followed by Western blot. (Paparone and Paparone, 2018; Trayes, Savage and Studdiford, 2018; Yeung and Baranchuk, 2018).

3.5 Treatment

Disease progression and the patient's age are critical factors for specifying treatment. Doxycycline is recommended for ten days for patients older than eight years displaying early infection. Amoxicillin is given to younger aged patients for two weeks in the early stages. Parenteral antibiotics with longer time courses may be needed for patients with severe symptoms such as carditis, encephalitis, arthritis, meningitis, etc. (Synopsis: Lyme Disease in Canada – A Federal Framework, 2017; Antony, 2018; Patton and Phillips, 2018). Patients with cardiac complications should always be in a monitoring system with ECG.

3.6 Prognosis and complications

Treatment with the proper antibiotic is sufficient to eradicate spirochete infection if it is used at the early stage of LD. However, the situation might get more complicated in some cases, e.g., the antibiotic doesn't work as expected, infection is diagnosed in the later stage, a concomitant condition with other tick-borne diseases occurs from the same tick bite, immune-compromised patients, etc. Moreover, after successful treatment with antibiotics, 5% of patients are diagnosed with post-treatment symptoms like fatigue, muscle ache, and joint pain (Skar and Simonsen, 2019).

4. The causative agent 'Borrelia'

B. burgdorferi s.s. and *Borrelia afzelii* are pathogenic microorganisms responsible for LD via the bite of the hard tick *I. scapularis* and *I. ricinus* in USA and Europe, respectively. This type of spirochete could be a tick-borne obligate parasite whose preferred natural reservoir is tiny mammals and birds; it doesn't cause disease in these natural hosts. When the parasite infects a person, Lyme borreliosis may develop. The established dissemination route for *B. burgdorferi* s.l. is through these hard ticks (Tilly, Rosa & Stewart, 2008).

4.1 Morphology and genome

B. burgdorferi s.l. is an atypical Gram-negative bacteria (Meriläinen *et al.*, 2015), 10-20 µm long and 0.3 µm in diameter (Hyde, 2017). Two lipid membranes make up the periplasmic cylinder (Barbour and Hayes, 1986) and flagellar filaments are imbedded in this periplasmic space (Kudryashev *et al.*, 2009). In addition to the function of motility, flagella play an important role in maintaining the cell shape (Motaleb *et al.*, 2000). Unlike other gram-negative bacteria, the Borrelia cell envelope doesn't contain lipopolysaccharide (LPS) (Takayama, Rothenberg and Barbour, 1987), and instead, it has glycolipids which are highly reactive immunologically (Ben-Menachem *et al.*, 2003).

All strains of *B. burgdorferi* s.l. contain a conserved linear chromosome and several linear and circular plasmids (Brisson *et al.*, 2012). Chromosomal proteins generally function in providing basic structural support, nutrient uptake, the glycolytic pathway, chemotaxis, and motility to ensure the survival of Borrelia within different host systems (Fraser *et al.*, 1997; Paulsen *et al.*, 2000; Charon *et al.*, 2012). In contrast, plasmid proteins are mainly responsible for establishing infection and transmission. Each Borrelia strain contains a different number of plasmids, but plasmids with crucial genes are quite common throughout the strains. For example, circular plasmid cp26, often found in every Borrelia isolates, encodes OspA, which is upregulated in the mammalian host at early stages of infection and is important for transmission (Wilske *et al.*, 1986; Marconi *et al.*, 1993; Sadziene *et al.*, 1993; Tilly *et al.*, 2006; Radolf and Caimano, 2008). Linear plasmid lp54 is also present universally in *B. burgdorferi* s.l. (Casjens *et al.*, 1995) and encodes ospA, ospB, and decorin binding proteins that are crucial for tick-mouse cycles (Promnares *et al.*,

2009). Neither the chromosome nor the plasmids contain genes for nucleotide, fatty acid, or amino acid synthesis involved in citric acid cycle and oxidative phosphorylation (Von Lackum and Stevenson, 2005; Radolf *et al*., 2012). This scenario clearly explains the long evolutionary co-existence with hosts, where Borrelia does not synthesize its own building blocks, but rather takes up these nutrients from the host blood.

4.2 Difference between *B. afzelii* and *B. burgdorferi* s.s.

The *I. scapularis* / *B. burgdorferi* s.s. system has been intensively studied by several researchers comparing with our disease transmission model, the *I. ricinus* / *B. afzelii* system (Pospisilova *et al.* 2019). The total number of *B. afzelii* was traced through *I. ricinus* metamorphic phases and during different feeding stages here. Also antibody-stained Borrelia was visualized in various tick tissues during feeding. Significant differences were found between US and European LD transmission system, and these are briefly mentioned below.

- After three days of feeding, the number of *B. afzelii* in the tick midgut were reduced by about ten-fold, whereas *B. burgdorferi* multiplied more than ten times during 15 hours of feeding (De Silva and Fikrig, 1995).

- *B. afzelii* took a shorter time to establish a systemic infection in a mammalian host than *B. burgdorferi* (Crippa, Rais and Gern, 2002). This outcome suggests *B. afzelii* leaves tick midgut earlier.

- Transmission of *B. burgdorferi* through the tick salivary gland is a well-established and proven route (Kurokawa *et al.*, 2020). But there is another unconfirmed hypothesis in the case of the *I. ricinus* / *B. afzelii* system. This route was suggested by Pospisilova *et al.* (2019) for *B. afzelii* transmission and was called as ‘active reverse migration’. Here the Borrelia travels from the tick midgut to the mouthparts for transmission instead of crossing the midgut barrier and invading the salivary gland. This theory was strongly supported by the lack of any trace of Borrelia in the salivary gland by microscopy over the different feeding stages.

5. Tick-Borrelia interaction

Multi-dynamic interactions occur between ticks, hosts, and pathogens in both the tick and host environments. These can be regarded as a continuous war of all against all. When a tick ingests host blood, haemoglobin is digested and detoxified in the tick gut (Sojka *et al.*, 2013; Franta *et al.*, 2010; Franta *et al.*, 2010), and proteases of host or pathogenic origin are neutralized (Buresova *et al.*, 2009). Tick-borne pathogens remain in the midgut and then migrate via the haemocoel during transmission (Hajdušek *et al.*, 2013). On the way, they invade the salivary glands and use salivary proteins for effective transmission. For example, the midgut proteins TROSPA (tick receptor for OspA) and Ixofin3D (Ixodidae fibronectin type III domain-containing tick gut protein) bind to *Borrelia* spirochetes and facilitate midgut colonization and subsequent pathogen transmission to the host (Pal *et al.*, 2004; Narasimhan *et al.*, 2014). Proteins of the Salp15 (salivary protein 15)-like multigene family are produced in the tick salivary glands. This Salp15 binds to *Borrelia* spirochetes to modulate host immunity, thus facilitating infection of the host (Wang *et al.*, 2014). Tick saliva is secreted into the host and suppresses the local host immune responses. The resulting host immunosuppression facilitates host infection (Liu & Bonnet, 2014; Kazimírová & Štibrániová, 2013; Kotsyfakis, Horka, Salat, & Andersen, 2010). Because tick salivary secretions are the primary mediators of host immunosuppression or immunomodulation, salivary composition plays a crucial role in the transmission of tick-borne pathogens. It represents an important topic of interest to researchers in the field (Wikel, 2013).

5.1 Borrelia acquisition

Ixodid ticks not only serve as vectors for infectious agents but also as natural reservoirs and amplifiers of these agents (Korenberg, 1999). In unfed ticks, *B. burgdorferi* s.s. spirochetes are present at the apical surface of the midgut diverticula epithelium (Movert *et al.*, 2013). The process of tick attachment and feeding stimulates the replication and migration of these agents (De Silva and Fikrig, 1995; Gern *et al.*, 1996), facilitating their transmission into the host skin. The process of acquisition from the host to the tick, however, is less well understood. *B. burgdorferi* spirochetes have a strong dermatographism, and the skin is the most consistent site of infection during chronic stage (Barthold *et al.*, 1991). However, spirochetes appear to be focally distributed within the skin

of infected mice, with other areas being spirochete-free (Barthold *et al.*, 1991). This would suggest that acquisition by the vector might be a hit-or-miss phenomenon, yet a very high percentage of ticks acquire the infection when randomly placed on infected hosts (Piesman, 1991). When assessing the burden of spirochetes at the skin–tick interface during acquisition, it was consistently found that the number of spirochetes at each time point was lower than non-tick attachment sites on the same animal. This phenomenon was seen during both early (two weeks) and late (eight weeks) infections. The introduction of a non-specific inflammatory stimulus, suture material, did not have the same effect as a tick bite, suggesting that a local inflammatory stimulus does not kill spirochetes. Furthermore, it has been shown that tick saliva influences the host immune response and facilitates the transmission and acquisition of vector-borne pathogens (Nuttall, 1998; Wikel, 1999). The low copy number of *fla* (encodes for flagellin) might be as a result of *B. burgdorferi* migration out of a tick attachment site. Still, it has been shown that during their transmission from infected nymphs to uninfected hosts, spirochetes tend to stay at the tick attachment site for several days before dissemination (Spielman *et al.*, 1992). On the other hand, during acquisition, spirochetes migrate from infected hosts into uninfected ticks, facilitating the natural transmission cycle. Analysis of the feeding ticks at time points relative to their attachment reveals a concomitant increase in spirochetes. Copy number of the flagellin target within ticks, combined with the copy number within the skin at the tick site, and compared with the non-tick site, revealed no significant difference. These findings suggest that spirochetes are active at the tick attachment site after being stimulated by the tick bite (saliva) and are actively acquired by the tick. Others have shown that co-feeding infected ticks with uninfected ticks on a host stimulates acquisition of *B. burgdorferi* spirochetes by the uninfected ticks. These findings suggest the existence of a factor in tick saliva that attracts spirochetes to the tick feeding site and potentiates their transmission (Ogden, Nuttall and Randolph, 1997; Patrican, 1997), supporting the findings of active participation of *B. burgdorferi* spirochetes during tick acquisition.

5.2 Borrelia transmission

I. ricinus ticks transmit *B. afzelii* as the LD agent, *A. phagocytophilum* as the human granulocytic ehrlichiosis agent, and *B. microti,* which causes human babesiosis. Anaplasma and Babesia infect the salivary glands of the tick, hence are poised to exit the tick vector during feeding. On the other hand, *B. afzelii* establishes the colony in the tick midgut with the help of a protein-protein interaction involving the tick midgut protein TROSPA and the spirochete lipoprotein OspA (Figlerowicz *et al.*, 2013). The commencement of feeding induces the spirochete to trigger preparation for migration from the midgut through the haemolymph to the salivary glands. The salivary gland is the established port of exit to the vertebrate host while the ticks are having a blood meal (Kurokawa *et al.*, 2020). There is a growing understanding of this phenomenon through studies of tick salivary gland transcriptome and research on its dynamic influence on pathogen transmission. The tick salivary gland plays multiple essential functions during both on- and off-host periods and represents a key route in the transmission of the tick-borne pathogen. The physiological activity and unique morphology of this tissue are intimately associated with adaptation of the tick to the parasitic lifestyle.

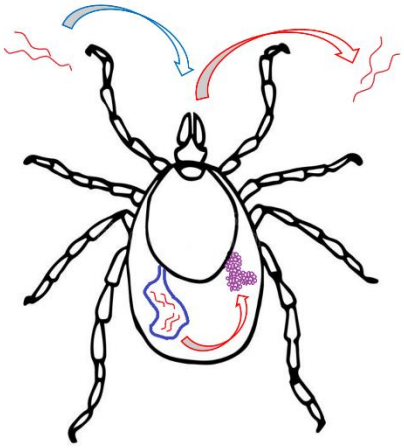

Figure 4: **Borrelia transmission during a tick bite**. During the larval stage, Borrelia is acquired from infected host blood. Later, at the next instar feeding, Borrelia travels from the tick midgut to the next host through the salivary gland.

To date, there is no comprehensive research data on the Borrelia infected tick midgut-specific transcriptome. It is still a mystery therefore, how Borrelia interacts with tick midgut proteins during fasting, feeding, or different phases of growth and migration to the mammalian host through the salivary gland. Borrelia is well known to interact with host proteins, including arthropod and mammalian proteins during its life cycle. Simultaneously, it needs to disseminate and cope with different temperatures, pH, nutritional richness, and with other factors in different hosts.

5.3 Importance for anti-tick vaccination approach for Lyme Disease

Anti-tick vaccination is an attractive and feasible alternative for the control of tick infestations and pathogen infections, as it is one of the most environment-friendly methods. By targeting the vector using a vaccination approach, several tick-borne diseases can be controlled simultaneously (Almazán, Kocan, Blouin, & De La Fuente, 2005; Brossard, 1998; J de la Fuente *et al.*, 1998). Since vector-borne pathogens use tick proteins to establish their infection in tick tissues, targeting the pathogens in the vector by blocking transmission or targeting the tick protein itself is an innovative and promising idea to control vector-borne diseases (Havlíková *et al.*, 2009). Moreover, it takes more than 24 hours for Borrelia to be infectious started from tick feeding, so there is a window of time to neutralize tick proteins that are associated with Borrelia activation for infecting mammalian host (Kurokawa *et al.*, 2020). However, selecting suitable antigens as the vaccine candidate is the primary constraint to successful vaccine development.

5.3.1 Finding suitable antigen candidate

Tick antigens are being identified as vaccine candidates using high throughput screening technologies that allow rapid, systematic, and global antigen screening, providing a comprehensive approach for selecting candidate vaccine antigens (Almazán *et al.*, 2003; Antunes *et al.*, 2012). RNA interference (RNAi) is one of the most used reverse genetics approaches for screening a vaccine candidate (de la Fuente *et al.*, 2010; Almazán *et al.*, 2010; Kocan, Blouin, & De La Fuente, 2011) before capillary feeding (Almazán, Blas-Machado, *et al.*, 2005; Canales, Almazán, Naranjo, Jongejan, & de la Fuente, 2009; Gonsioroski *et al.*, 2012; Rodriguez-Valle, Vance, Moolhuijzen, Tao, & Lew-Tabor, 2012). Using functional genomics approaches, Antunes *et al.* (2012) identified differentially expressed genes in *B. bigemina*-infected *Riphicephalus* ticks. Other methods such as protein arrays (Manzano-Román *et al.*, 2012) and yeast surface displays (Schuijt *et al.*, 2011) have also been proposed for the identification and characterization of antigens that elicit anti-tick immunity.

5.3.2 Transcriptomics for finding vaccine candidates

Transcriptomics is the study of the transcriptome, which is the complete set of transcripts in a cell, both in terms of type and quantity. As it concerns gene expression at the RNA level, comparison between transcriptomes allows the identification of genes that are differentially expressed in distinct cell populations or responses to different treatments. High-throughput approaches in transcriptomics have facilitated the description of gene expression dynamics throughout infection states by various pathogens and during tick feeding. Furthermore, high-throughput technologies are useful for investigating the effects of other biological factors such as sex or the developmental stages of different tick tissues. Some of the new high-throughput techniques have been recently used to study transcriptomes. One of them is Next-Generation Sequencing (NGS). Compared to classical Sanger sequencing, NGS platforms such as Illumina or 454 have provided much more in-depth transcriptome coverage and has made them prioritized tools for quantitative analysis of gene expression dynamics in different tick tissues (Chmelař *et al.*, 2016). The main advantage of NGS-based transcriptomics lies in its ability to describe transcription dynamics quantitatively. In our study, we have used MACE, and *de novo* RNAseq technique developed by GenXPro GmbH (Germany). In MACE only a short fragment (250bp to 500bp) is sequenced from the 3'end of mRNA. Since each fragment is count as one read in MACE, this method does not have the biasness based on the length. Moreover, it has the advantage of tracking even rare transcripts, without the involvement of very deep sequencing.

Different treatments upregulate different genes in order to cope with a new situation or for assisted induction. The idea behind our research is that ideally Borrelia most likely induces and upregulates tick midgut and salivary gland proteins to be facilitated in regard of acquisition and transmission. So, identifying these upregulated tick genes may open new research possibilities for revealing the dynamics of tick-Borrelia interactions. Quantitative transcriptomics is an appropriate tool to identify such important genes, whose encoded proteins might be targeted as potential anti-tick vaccine candidates. Gene discovery from tick tissues by classical Sanger sequencing and NGS are compared in Table 1. This table is inspired from the review article of Chmelař *et al.* (2016) with the new addition of recent findings as well, including our works.

Table 1: **Comparison between classical Sanger sequencing and two NGS platforms** (454 and illumina); [a]Abbreviations: AF – adult female, whole body; HC- hemoytes; MG- midgut; ML- mixed library from several tissues; SG- salivary gland; FS- female synganglion; Lar.- larvae; Nym- nymph; WB- whole body

Year	Seq. Method	Tissue	Species	Total Reads	Avg. Read Length	Good Quality EST	Uniq. Seq.	Ref.
2005	Sanger	SG[a]	*Ixodes pacificus*	-	487	1068	557	(Francischetti *et al.*, 2008)
2006	Sanger	SG	*Ixodes scapularis*	8150	-	7476	3020	(Ribeiro, *et al.*, 2006)
2007	Sanger	SG	*Dermacentor andersoni*	1440	600	1299	762	(Alarcon-Chaidez *et al*., 2007)
2007	Sanger	ML[a]	*Rhipicephalus microplus*	42512	-	-	13643	(M. Wang, Guerrero *et al*., 2007)
2008	Sanger	SG	*Amblyomma cajannense*	1920	472	1754	1234	(Batista *et al.*, 2008)
2008	Sanger	MG[a]	*Dermacentor variabilis*	2304	-	1679	835	(Anderson *et al*., 2008)
2008	Sanger	SG	*Ixodes ricinus*	2304	503	1881	1274	(Chmelar *et al.*, 2008)
2008	Sanger	SG	*Ornithodoros coriaceus*	-	-	1089	726	(Francischetti *et al.*, 2008)
2008	Sanger	SG	*Ornithodoros parkeri*	-	-	1529	649	(Francischetti *et al.*, 2008)
2009	Sanger	SG	*Amblyomma americanum*	-	-	3868	2002	(Aljamali *et al.*, 2009)
2010	Sanger	SG	*Rhipicephalus sanguineus*	-	-	2034	1024	(Anatriello, Riberiro, *et al.*, 2010)
2010	454	AF[a]	*Dermacentor variabilis*	233335	203	-	38683	(Jaworski *et al.*, 2010)
2011	Sanger	SG	*Hyalomma marginatum rufipes*	-	-	2084	1167	(Francischetti *et al*., 2011)

2011	454	SG	*Amblyomma maculatum*	1626969	-	190646	15814	(Karim, Singh and Ribeiro, 2011)
2011	454	AF	*Ixodes ricinus*	60186	227	-	-	(Carpi *et al.*, 2011)
2012	Sanger	Lar.	*Rhipicephalus microplus*	-	-		775	(Heekin *et al.*, 2012)
2012	Sanger	SG	*Antricola delacruzi*	-	-	1147	923	Ribeiro, J.M. *et al.*, 2012
2013	Sanger	ML	*Amblyomma americanum*	20256	-	15390	12319	(Gibson *et al.*, 2013)
2013	Sanger	MG	*Rhipicephalus microplus*	5000	-	4054	1628	(Heekin *et al.*, 2013)
2013	Illumina	Nym.	*Ixodes ricinus*	162000000	101			(Vayssier-Taussat *et al.*, 2013)
2013	454	SG	*Ixodes ricinus*	441381	518	93331	34560	(a Schwarz *et al.*, 2013)
2013	Illumina	SG	*Ixodes ricinus*	67703183	90	269600	34560	(a Schwarz *et al.*, 2013)
2013	Illumina	-	*Haemaphysalis Longicornis*	53690000			23330	(Figlerowicz *et al.*, 2013)
2014	Illumina	SG	*Amblyomma americanum*	18800000	-		17593	(Radulović *et al.*, 2014)
2014	Illumina	Lar.	*Dermacentor reticulatus*	21677414	207	18946	3808	(Villar *et al.*, 2014)
2014	Illumina	SG + MG	*Ixodes ricinus*	585000000	-	198504	25808	(Schwarz *et al.*, 2014)
2014	454	SG	*Amblyomma cajennense*	67677	-	-	4604	(Garcia *et al.*, 2014)
2014	454	SG	*Amblyomma parvum*	104817	-	-	3796	(Garcia *et al.*, 2014)
2014	454	SG	*Amblyomma triste*	442756	-	-	11240	(Garcia *et al.*, 2014)
2014	454	SG	*Ixodes ricinus*	778598	379		24539	(Liu *et al.*, 2014)
2014	Illumina	FS[a]	*Ixodes scapularis*	34520330	68	41249	8160	(Egekwu, N. *et al.*, 2014)
2014	Illumina	FS	*Ixodes scapularis*	117900476	72	30838	17660	(Egekwu, N. *et al.*, 2014)

2014	454	FS	*Ixodes scapularis*	394946	268	20630	2692	(Egekwu, N. *et al.*, 2014)
2015	454	HC[a]	*Ixodes ricinus*	926596	498	-	15716	(Kotsyfakis *et al.*, 2015)
2015	Illumina	HC	*Ixodes ricinus*	49328982	148	-	15716	(Kotsyfakis *et al.*, 2015)
2015	Illumina	SG	*Amblyomma americanum*	344909378	101		5792	Karim *et al.*, 2013)
2015	Illumina	SG	*Haemaphysalis flava*	162912848	100	70542	54357	(Xu *et al.*, 2015)
2015	Illumina	SG + MG	*Ixodes ricinus*	268914130	-	-	25808	(Kotsyfakis *et al.*, 2015)
2015	Illumina	SG	*Ixodes scapularis*	28000000	101	-	11105	(Ayllón *et al.*, 2015)
2015	Illumina	MG	*Ixodes scapularis*	26000000	101	-	12651	(Ayllón *et al.*, 2015)
2015	Illumina	Nym.	*Ixodes scapularis*	31000000	101	-	16083	(Ayllón *et al.*, 2015)
2015	Illumina	AF	*Ixodes scapularis*	3700000	-	-	9134	(Lewis *et al.*, 2015)
2015	Illumina	SG	*Rhipicephalus pulchellus*	241229128	-	-	50460	(Saito and Sadoshima, 2016)
2016	Sanger	AF	*Ixodes scapularis*	17400000	-	570637	20486	(Gulia-Nuss *et al.*, 2016)
2019	Tn-Seq	Nym.	*Ixodes scapularis*					(Phelan *et al.*, 2019)
2020	Illumina	Nym. (WB)	*Ixodes ricinus*	329111102	-	32897	-	(Trentelman *et al.*, 2020)
2020	Illumina (MACE)	Nym. (SG)	*Ixodes ricinus*	75651134	-	-	-	(Trentelman *et al.*, 2020)
2020	Illumina (MACE)	Nym. (MG)	*Ixodes ricinus*	38199641	-	-	-	(Mahmood *et al.*, 2021)

The most extensive Sanger sequencing analysis revealed 13643, 12319 and 20486 unique transcripts in mixed libraries from *R. microplus* (Wang *et al.*, 2007), *A. americanum* (Gibson *et al.*, 2013) and *I. scapularis* respectively, where generally the usual number of unique sequences in

similar projects is around 1000. NGS-based transcriptome projects, by contrast, can produce hundreds of thousands of assembled contigs (Schwarz *et al.*, 2013) and over 50000 unique transcripts. On average, around 16000 individual transcripts have been discovered in NGS projects on ticks, around 10-fold higher than Sanger sequencing in terms of the identification of novel transcripts, and about 100–200-fold higher in terms of total contigs.

5.4 Literature review of tick upregulated genes

For years, researchers have been investigating tick midgut and salivary gland genes that are upregulated in the presence of Borrelia. Revealing tick–Borrelia interactions is always the primary research strategy for controlling several tick-borne diseases. Upregulated tick genes during Borrelia infection might have three types of functional possibilities: responsible for tick immune boost against Borrelia; allowing Borrelia to use tick resources for nutrients; and to be in a mutualistic relationship including helping Borrelia in acquisition and/or transmission. Below there is a list of tick upregulated candidates in the midgut and salivary gland in the presence of different Borrelia species (Table 2).

Table 2: **Upregulated tick midgut, salivary gland and haemolymph proteins due to Borrelia infection**; Abbreviations: HL- haemolymph; MG- midgut; SG- salivary gland, UF – unfed, FF - fully fed; *D. vari- Dermacentor variabilis, I. scap.- Ixodes scapularis, B. Burg.- Borrelia burgdorferi*, ; pF vs PI- Partial fed vs pre-injected, Inf – infected, G. pig - guinea pig,

Accession (Protein)	Accession (Nt)	Putative Description	Host	Stage	Tissues	Bacteria	Method, UpReg times	Reference
	AY333958	Thioredoxin Peroxidase	*I. ricinus* - Female	FF	HL, SG, MG	*B. burg*. Inf - G. pig	Subtractive hybridization	(Rudenko *et al.*, 2005)
	AY333959	Thioredoxin Peroxidase	*I. ricinus* - Female	FF	HL, SG, MG	*B. burg*. Inf - G. pig	Subtractive hybridization	(Rudenko *et al.*, 2005)
AAP85376.1	AY321308	Dolichyl phosphate glucosyltransferase	*I. ricinus* - Female	FF	SG, MG	*B. burg*. Inf - G. pig	Subtractive hybridization	(Rudenko *et al.*, 2005)
ABK62871.1	AY335442	Defensin precursor	*I. ricinus* - Female	FF	MG	*B. burg*. Inf - G. pig	Subtractive hybridization	(Rudenko *et al.*, 2005)
	AY323234	ML domain	*I. ricinus* - Female	FF	MG	*B. burg*. Inf - G. pig	Subtractive hybridization	(Rudenko *et al.*, 2005)
AOG18169.1		TROSPA	*I. scap.* - Nymph	UF	MG	*B. burg*. Inf - C3H	Up ~2 times, qRT-PCR	(Pal *et al.*, 2004)
ALQ28602.1		Dystroglycan (ISDLP)	*I. scap.* - Nymph	FF	MG	*B. burg*. Inf - C3H	ImageJ	(Coumou *et al.*, 2016)
		Lysozyme-like peptide (15 KDa)	*D. vari.* - Female	pF vs PI	HL	*B. burg*. Hemocoelic Inoculation	SDS-PAGE (Up - 22%)	(Johns, Sonenshine and Hynes, 2000)
		Peptide (5.8 KDa)	*D. vari.*	pF vs PI	HL	*B. burg.* Hemocoelic Inoculation	SDS-PAGE	(Johns, Sonenshine and Hynes, 2000)
		Varisin (Tick defensin), 5.1 KDa	*D. vari.*	pF vs PI	HL	*B. burg.* Hemocoelic Inoculation	SDS-PAGE	(Sonenshine *et al.*, 2002)
	Contig-123	Secreted protein (5.3 kDa)_1	*I. scap.* - Nymph	FF	SG	*B. burg.* Inf-Hamsters	EST clustering, Up 25 times	(Ribeiro *et al.*, 2006)
	Contig-122	Secreted protein (5.3 kDa)_2	*I. scap.* - Nymph	FF	SG	*B. burg.* Inf-Hamsters	EST clustering, Up 8 times	(Ribeiro *et al.*, 2006)

	Contig-125	Secreted protein (5.3 kDa)_3	*I. scap.* - Nymph	FF	SG	*B. burg.* Inf-Hamsters	EST clustering, Up 7 times	(Ribeiro *et al.*, 2006)
	Contig-332	Secreted protein (5.3 kDa)_4	*I. scap.* - Nymph	FF	SG	*B. burg.* Inf-Hamsters	EST clustering, Up 11 times	(Ribeiro *et al.*, 2006)
	Contig-18	Secreted Basic tail protein	*I. scap.* - Nymph	FF	SG	*B. burg.* Inf-Hamsters	EST clustering, Up 3.7 times	(Ribeiro *et al.*, 2006)
	Contig-17	Secreted Basic tail protein	*I. scap.* - Nymph	FF	SG	*B. burg.* Inf-Hamsters	EST clustering, Up 3.2 times	(Ribeiro *et al.*, 2006)
	Contig-330	Truncated HBP	*I. scap.* - Nymph	FF	SG	*B. burg.* Inf-Hamsters	EST clustering, Up 10 times	(Ribeiro *et al.*, 2006)
	AF209914.1	Salp15	*I. scap.* - Nymph	66h post FF	SG	*B. burg.* Inf-C3H	qPCR	(Ramamoorthi *et al.*, 2005)
		tHRF	*I. scap.* - Nymph	24h, 48h, 72h	SG	*B. burg.* Inf-C3H	DIGE, Immunoblots Up 2.5 times	(Dai *et al.*, 2010)
	ISCW022329	StAR protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
	ISCW009548	Hypothetical secreted protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC10497.1	ISCW019584	PHGPx	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC02092.1	ISCW016022	Cytochrome C oxidase subunit I	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 10	(Narasimhan *et al.*, 2017)
EEC09635.1	ISCW007844	Alcohol dehydrogenase	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC11360.1	ISCW020757	Cytochrome C Oxidase III	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 10	(Narasimhan *et al.*, 2017)
EEC05517.1	ISCW004817	Exocyst complex component 6B	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC04054.1	ISCW003205	Hypothetical protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC08933.1	ISCW005926	Scapularisin-5	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)

EEC14912.1	ISCW011165	3-Oxoacyl [acyl-carrier-protein] reductase	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC16586.1	ISCW012492	Hypothetical protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC10497.1	ISCW019584	PHGPx	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC08989.1	ISCW018900	Hypothetical protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC19124.1	ISCW014515	Secreted salivary protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC00748.1	ISCW015983	Aspariginyl peptidase/legumain	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC16219.1	ISCW012432	Alcohol dehydrogenase	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC02991.1	ISCW003426	Glutathione S-Transferase	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC17896.1	ISCW012239	Salp9pac	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
AQX36323.1	KY629420	Protein a with Reeler domain (PIXR)	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC08997.1	ISCW018909	Putative secreted protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC05372.1	ISCW003983	Oxysterol binding protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC10688.1	ISCW007199	Villin/gelsolin domain-containing protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC10483.1	ISCW007338	Erlin-2	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC04704.1	ISCW017045	Catenin-beta-like ARM protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)

EEC09407.1	ISCW008141	Salp26A	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC19294.1	ISCW013959	Putative secreted salivary protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC01220.1	ISCW024071	Secreted salivary gland protein	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
EEC18389.1	ISCW014240	Metallophosphatase domain	*I. scap.* - Nymph	FF	MG	*B. burg.*	Subtractive hybrid, Up ≥ 5	(Narasimhan *et al.*, 2017)
ADZ96258.1		TRE31	*Ixodes* spp.		MG	*B. burg.*		(Kurokawa *et al.*, 2020)
AIJ19439.1		Ixofin3D	*Ixodes* spp.		MG	*B. burg.*		(Kurokawa *et al.*, 2020)
ADU86241.1		STAT	*Ixodes* spp.			*B. burg.*		(Kurokawa *et al.*, 2020)
EEC14202.1		Toll	*Ixodes* spp.			*B. burg.*		(Kurokawa *et al.*, 2020)
EEC12280.1		Myd88	*Ixodes* spp.			*B. burg.*		(Kurokawa *et al.*, 2020)
EEC10543.1		DUOX	*Ixodes* spp.			*B. burg.*		(Kurokawa *et al.*, 2020)
EEC04515.1		Peroxidase	*Ixodes* spp.			*B. burg.*		(Kurokawa *et al.*, 2020)

6. Article: 1

Mahmood, S. *et al.* (2021) 'Identification of Tick Ixodes ricinus Midgut Genes Differentially Expressed During the Transmission of Borrelia afzelii Spirochetes Using a Transcriptomic Approach', *Frontiers in Immunology*. Frontiers, 11, p. 3731. doi: 10.3389/fimmu.2020.612412.

6.1 Introduction:

Tick midgut proteins specifically interact with spirochetes and these interactions are predictable since the spirochetes remain in the midgut for a long time. RNA interference has been used to determine whether these interactions facilitate the transmission to the vertebrate host. Possible Borrelia-interacting steps include their replication in the midgut, survival in the presence of mammalian host blood, escape from the midgut, migration through the haemocoel, and entry into salivary glands. In this study, we have shown different interaction dynamics between the spirochete and tick midgut proteins by differential gene expression following blood-feeding and infection by Borrelia. These studies have produced new insights into the dynamics of the complicated interaction between the tick and the spirochete and, during feeding, how these interactions influence the spirochete and its ultimate transmission to the host. This complete study model may also be a powerful tool that might be used to investigate other vector-pathogen interactions in terms of public health importance.

frontiers in Immunology

ORIGINAL RESEARCH
published: 04 February 2021
doi: 10.3389/fimmu.2020.612412

Identification of Tick *Ixodes ricinus* Midgut Genes Differentially Expressed During the Transmission of *Borrelia afzelii* Spirochetes Using a Transcriptomic Approach

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OPEN ACCESS

Edited by:
Alicja Elzbieta Tabor, The University of Queensland, Australia

Reviewed by:
Fengliang Jin, South China Agricultural University, China
Benjamin Makepeace, University of Liverpool, United Kingdom

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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *30 September 2020*
Accepted: *17 December 2020*
Published: *04 February 2021*

Citation:
Mahmood S, Sima R, Urbanova V, Trentelman JJA, Krezdorn N, Winter P, Kopacek P, Hovius JW and Hajdusek O (2021) Identification of Tick Ixodes ricinus Midgut Genes Differentially Expressed During the Transmission of Borrelia afzelii Spirochetes Using a Transcriptomic Approach. Front. Immunol. 11:612412. doi: 10.3389/fimmu.2020.612412

Lyme borreliosis is an emerging tick-borne disease caused by spirochetes *Borrelia burgdorferi* sensu lato. In Europe, Lyme borreliosis is predominantly caused by *Borrelia afzelii* and transmitted by *Ixodes ricinus*. Although *Borrelia* behavior throughout tick development is quite well documented, specific molecular interactions between *Borrelia* and the tick have not been satisfactorily examined. Here, we present the first transcriptomic study focused on the expression of tick midgut genes regulated by *Borrelia*. By using massive analysis of cDNA ends (MACE), we searched for tick transcripts expressed differentially in the midgut of unfed, 24h-fed, and fully fed *I. ricinus* nymphs infected with *B. afzelii*. In total, we identified 553 upregulated and 530 downregulated tick genes and demonstrated that *B. afzelii* interacts intensively with the tick. Technical and biological validations confirmed the accuracy of the transcriptome. The expression of five validated tick genes was silenced by RNA interference. Silencing of the uncharacterized protein (GXP_Contig_30818) delayed the infection progress and decreased infection prevalence in the target mice tissues. Silencing of other genes did not significantly affect tick feeding nor the transmission of *B. afzelii*, suggesting a possible role of these genes rather in *Borrelia* acquisition or persistence in ticks. Identification of genes and proteins exploited by *Borrelia* during transmission and establishment in a tick could help the development of novel preventive strategies for Lyme borreliosis.

Keywords: *Borrelia afzelii*, *Ixodes ricinus*, transcriptome, tick, midgut, RNAi, massive analysis of cDNA ends (MACE)

INTRODUCTION

Lyme borreliosis is an emerging human disease, occurring predominantly in temperate regions of the northern hemisphere (1, 2). It is caused by spirochetes *Borrelia burgdorferi* sensu lato and is spread by ticks from the genus *Ixodes*. In Europe, ~65,000 new cases are reported annually (3). However, the real prevalence is substantially higher due to under-reporting (4). In North America,

the transmission cycle primarily involves the spirochete *B. burgdorferi* sensu stricto and the tick *Ixodes scapularis*. In Europe, the disease is caused by several *Borrelia* species and is transmitted by related tick species, *Ixodes ricinus* and *Ixodes persulcatus*. The early disease typically manifests itself with a bulls-eye rash on the skin, called *erythema migrans*. The spirochetes then disseminate throughout the body to diverse tissues and are associated with arthritis, neurological symptoms, and dermatitis (5). Prompt antibiotic treatment usually cures the disease and symptoms. Despite several promising trials (6–9), a vaccine against human Lyme borreliosis is not currently available and prevention mainly depends on avoiding tick bites (10).

Ixodes ricinus is the most common tick in Europe and is typically found in humid sheltered environments and forests, mainly from early spring until late fall. It is a three-host tick, where all developmental stages (larva, nymph, and adult female) must feed on the host blood to undergo molting into the next instar. *B. afzelii* is the dominant spirochete in Europe (11). *Borrelia* enter the tick gut when the larvae feed on an infected mouse. The spirochetes then multiply and are transstadially maintained in the tick through the molts (12). The nymph's ability to survive without feeding for years contributes to stabilization of *Borrelia* prevalence in the reservoir host population. Because of their small size, the tick nymphs are considered to be the most critical tick stage for human infections (13). During engorgement, which typically lasts for two to four days, the spirochetes continuously migrate from the tick into the host. An interval between 24 and 48 h after tick attachment is considered the most critical time for transition of *B. afzelii*. Although *Borrelia* can already be detected in the skin on the first day of feeding, this early spirochetal population cannot initiate a systemic infection (12). Unlike *B. burgdorferi* s.s. in *I. scapularis* (14), which migrate through the hemolymph and salivary glands into the host, *B. afzelii* probably infect the host directly from the midgut of *I. ricinus* (12).

The segmented tick midgut is well adapted to accommodate an enormous volume of host blood. Unlike other blood-feeding arthropods, digestion in ticks occurs intracellularly (15), so extracellular pathogens are not directly exposed to the harsh effects of secreted proteases. Despite this, the tick midgut is still a relatively sterile environment (16), maintained presumably by combining active components of the blood and tick immune molecules. Adaptations of *Borrelia* spirochetes inhabiting the tick midgut are still not satisfactorily explained. However, it has been documented that during tick colonization, *Borrelia* change expression of their genes (17). For instance, the main surface protein outer surface protein A (OspA) is preferentially expressed within the tick midgut and is downregulated during transmission of the spirochete to the vertebrate host (18). The tick receptor for OspA (TROSPA), is a midgut protein identified in *Ixodes scapularis*, ensuring adherence of *B. burgdorferi* to the midgut surface. Expression of *trospa* is significantly upregulated in *Borrelia*-infected nymphs. Moreover, the silencing of *trospa* expression reduces colonization and transmission of the pathogen (19). Another example of this co-adaptation is *Borrelia*-induced overexpression of the tick salivary protein 15 (Salp15) necessary for *Borrelia* survival in the host (20). *Borrelia*-infected nymphs have also been shown to accumulate significantly more fat reserves (21) to better survive unfavorable temperatures and humidities (22). These examples point to the existence of delicate gene interactions between *Borrelia* spirochetes and the tick.

Here we show that midgut cells of infected nymphs before, during, and after feeding on the vertebrate host react to *B. afzelii*. By employing the MACE transcriptomic method, we were able to identify, in total, 1,083 *Borrelia*-responding tick midgut genes. Silencing of tick uncharacterized protein (GXP_Contig_30818) by RNA interference reduced transmission of *Borrelia* spirochetes from the tick to the host, whereas silencing of several other candidate tick genes had no effect. This suggests that these genes may have a role in processes associated with acquisition rather than transmission of *Borrelia*, and persistence in the vector.

MATERIAL AND METHODS

Biological Material

Adult females of *I. ricinus* were collected by flagging in a forest near Ceske Budejovice and kept at 95% humidity, 24°C, and 15/9 daylight settings. The adult ticks were fed on a single guinea pig. The laid eggs were preserved to hatch separately to form individual populations, each coming from a single female. For the transcriptomics purposes, the larvae from three populations were mixed together to scale up the number of ticks and then divided into two groups to prepare for infected and uninfected nymphs (**Figure 1**). Prior to feeding, a half of 6–8 week old C3H/HeN mice (Charles River Laboratories, GER) were infected with *B. afzeli* CB43 (23) by subcutaneous injection of 0.2 ml of culture (approximately 10^6 spirochetes). Mouse infection was checked by PCR on ear punctures taken 3 weeks after injection. The *Borrelia*-infected nymphs were obtained by feeding the larvae on *Borrelia*-infected mice. Uninfected nymphs were obtained by feeding the larvae on uninfected C3H/HeN mice. The resulted nymphs, molted 4–6 weeks after repletion, were rested for 2 weeks and used in these experiments. The prevalence of *Borrelia* infection in nymphs was checked by PCR and reached >90%. All experiments were carried out according to the animal protection law of the Czech Republic (§17, Act No. 246/1992 Sb) with the approval of CAS (approval no. 79/2013). The experiments with *Borrelia* were performed in BSL2 conditions.

Tick Dissection and RNA Extraction

The *Borrelia*-infected nymphs were divided into three groups (MACE 1,3,5), as well as the uninfected nymphs, which were also divided into three groups (MACE 2,4,6). The nymphs of MACE groups 1 and 2 remained unfed. The nymphs of MACE groups 3 and 4 were forcibly removed from the naïve 6–8 weeks old C3H/HeN mice at 24 h after attachment. The nymphs of MACE groups 5 and 6 were allowed to feed on the naïve 6–8 weeks old C3H mice until repletion (around 72 h). All tick were surface-sterilized by washing in 3% H_2O_2, 70% ethanol, and distilled

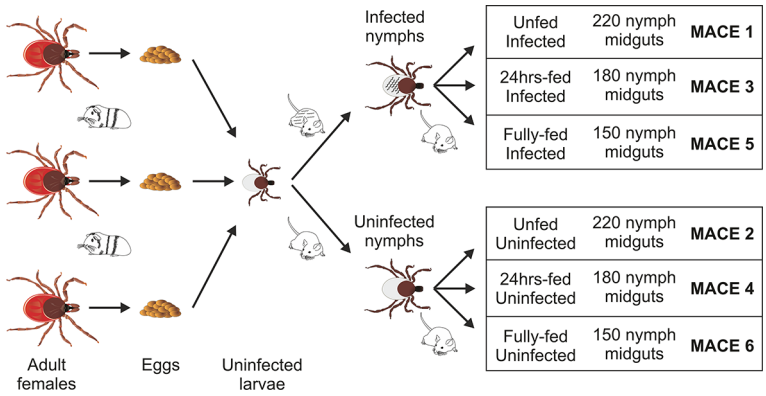


FIGURE 1 | Scheme of sample preparation for massive analysis of cDNA ends (MACE) analysis. The uninfected larvae, originating from three individual females fed on a single guinea pig, were mixed and fed on *B. afzelii*-infected or uninfected mice. The nymphs then were fed on uninfected mice and dissected for midguts (150–220 nymphs for each group) at the three indicated time points. The MACE analyses were performed on six different RNA pools (MACE 1-6).

water (30 seconds each wash). The nymphs were dissected for midguts [pools of: 220 unfed nymphs (MACE 1,2), 180 24 h-fed nymphs (MACE 3,4), and 150 fully fed nymphs (MACE 5,6)] under the stereomicroscope (Olympus) on wax dishes with diethyl pyrocarbonate (DEPC)-treated cold phosphate buffered saline (PBS) and then transferred in RA1 buffer (NucleoSpin miRNA Kit, Macherey-Nagel, GER) supplemented with β-mercaptoethanol (Sigma-Aldrich). Before extraction, the midguts were homogenized in an insulin syringe. Total RNA (including miRNA) was extracted using the above extraction kit by following the manufacturer's protocol ("small+large" protocol). The concentration of RNA was measured by NanoDrop ND-1000 (Thermo Fisher Scientific), its consistency was checked on an agarose gel, and stored at −80°C until further use.

MACE Analysis

The massive analysis of cDNA ends (MACE) was performed as previously described (24) using the GenXPro MACE Kit (GenXPro) according to the manufacturer's protocol (www.genxpro.net). The isolated RNA was subjected to an additional DNAse I treatment and its quality was assessed on an Agilent 2100 Bioanalyzer. First and second-strand cDNA synthesis was then performed, initiated from biotinylated oligo dT primers. The cDNA was fragmented randomly by ultrasonication, resulting in fragments with an average size of 300bp as determined by an Agilent 2100 Bioanalyzer. The biotinylated 3′ cDNA ends were bound to a streptavidin matrix, while the remaining fragments were eliminated through the washing step. Then, the p5 "TrueQuant" sequencing adapter was ligated to the unbound end of the fragments using tailed Illumina p5 and p7 oligonucleotides as primers. The quality of the final library was determined using an Agilent 2100 Bioanalyzer. The next-generation single-end sequencing of the 5' cDNA fragments was performed on an Illumina HiSeq2000 sequencer. To remove the PCR bias, all duplicate reads detected by the GenXPro in-house TrueQuant technology were removed from the raw datasets. In addition, low-quality sequence nucleotides and poly(A)-tails were clipped off using Cutadapt (25). Overlapping sequencing reads were then assembled into contigs. The reads were aligned to different reference sequences using NovoAlign (www.novocraft.com/products/novoalign/), resulting in "GXP_Contigs" (sequences derived from our previously published nymphal RefSeq database (Bioproject PRJNA657487), "Contigs" (*I. ricinus* sequences were derived from NCBI nuccore and the BioProjects 177622, 230499, 34667, and 183509), and "noHITAssemblies" (assemblies of MACE sequences that could not be mapped to sequences from the existing BioProjects or our own RefSeq database). The contigs of the assemblies were annotated further by BLASTX to either the SwissProt or Trembl database (www.uniprot.org). Contigs that did not match to one of these databases were annotated by BLASTN to all "*Ixodes*" mRNA sequences available in the NCBI database, against the "nt" (nucleotide collection from GenBank, RefSeq, TPA, and PDB) of NCBI, or the *I. scapularis* genome (NW_002505054). Only uniquely mapped reads were accepted for the quantification of the MACE tags. Finally, gene expression was normalized per million reads and tested for differential gene expression between the different conditions using the DEGSeq R/Bioconductor package (26) (R package version 1.16.0.). The final table was produced as an Excel file (**Supplemental Table 1**).

In Silico Analysis

The selection of *Borrelia*-upregulated and downregulated genes at different time points was performed using the MACE Excel file according to these selection criteria: 1) the transcript was annotated (e-value ≤ $10E^{-6}$) in the *I. ricinus* genome PRJNA270959, the *I. scapularis* genome PRJNA314100, or in all *Ixodes* sequences available in NCBI; 2) "noHitAssemblies" contigs were removed from the analysis because of no homologies with tick sequences (no hits, host contaminants, and short-length sequences); 3) to select upregulated genes: fold change upregulation of expression in the infected *vs.* uninfected

group was set to ≥ 5 and expression in the infected group to ≥ 5 transcripts per million; selection of downregulated genes was done *vice versa* (expression in the uninfected *vs*. infected group was set to ≥ 5 and expression in the uninfected group to ≥ 5 transcripts per million). The selected candidate sequences were translated into proteins (DNASTAR) and screened for the presence of a signal sequence by SignalP 5.0 (www.cbs.dtu.dk/services/SignalP/) and for cellular localization by DeepLoc-1.0 (www.cbs.dtu.dk/services/DeepLoc/).

Technical and Biological Validation of the MACE Analysis

An aliquot of RNA from each MACE analysis was used for the technical validation of MACE results. For biological validation, we prepared 10 genetically distinct larval populations of *I. ricinus* ticks coming from wild-captured adult females fed on a guinea pig (**Supplemental Figure 1**). Each of the batches of larvae was divided in half and fed on *B. afzelii* CB43-infected or uninfected 6–8 weeks old C3H/HeN mice (Charles River Laboratories, GER) mice. The resulting infected and uninfected nymphs were then fed on naïve mice for 0h, 24h, and until replete (fully fed), midguts were dissected (for each group and time point pools of: 50 unfed nymphs, 20 24h-fed nymphs, and 10 fully fed nymphs (equal number of females and males), and RNA was extracted following the methods and time points used for the MACE analysis. Then, the RNA was reverse transcribed into cDNA (0.5µg RNA per 20µl reaction; random hexamers) using the Transcriptor High-Fidelity cDNA Synthesis Kit (Roche) and diluted 20-times in sterile water. Gene-specific qRT-PCR primers were designed in Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and verified by PCR using cDNA prepared from a mix of infected nymphs at different time points. Gene expression in technical and biological replicates was measured by quantitative real-time PCR (qRT-PCR) using a LightCycler 480 (Roche) and SYBR green chemistry, as described previously (27) and primers listed in **Supplemental Table 2**. Relative expression was normalized to *I. ricinus* elongation factor (GU074769) and ferritin 1 (AF068224, data not shown) using the mathematical model of Pfaffl (28).

RNA Interference and Its Effect on Nymph Feeding and Development

To prepare the gene-specific dsRNA, 200–600bp long gene fragments were amplified from *I. ricinus* cDNA and cloned into the pll10 vector with two T7 promoters in reverse orientations (29), using primers listed in **Supplemental Table 2** and containing additional restriction sites ApaI and XbaI. The dsRNA was synthesized as described previously (30). The dsRNA (3 µg/µl) was injected through the coxa of the third pair of legs into the hemocoel of nymphs (32 nl) using Nanoinject II (Drummond). After 3 days of rest in a humid chamber at room temperature, the nymphs (20 nymphs per mouse, 3 mice per group) were fed on BALB/c mice (Velaz, CR). The level of gene silencing was checked by qRT-PCR in a mix of five fully fed nymphs and compared to the dsGFP control group. For each group, we recorded feeding success, length of feeding, the weight of individual nymphs after feeding, and molting into adults (took approximately 2 months; recorded every 2 weeks until molting in the dsGFP control group reached 80%).

Borrelia-Transmission Experiments

Borrelia afzelii CB43-infected nymphs were prepared as described previously (31). The infected nymphs were injected with 32nl of gene-specific dsRNA or dsGFP (control), rested for 3 days, and fed on the uninfected 6-weeks old C3H/HeN mice (five nymphs per mouse, 5–8 mice per group) in plastic cylinders attached to the murine back. Detached engorged nymphs were weighed. The level of *Borrelia* infection in each mouse was measured the second week after tick detachment by qRT-PCR using DNA isolated from an ear biopsy and normalized to the number of mouse genomes (actin). Three weeks after tick detachment, mice were sacrificed and the numbers of *Borrelia* in the ear, urinary bladder, and heart tissue were determined by qRT-PCR as reported previously (12).

Statistical Analysis

For biological validations, feeding experiments, and transmission experiments, statistical significance of differences were analyzed using GraphPad Prism 8.0 (GraphPad Software, CA) employing the One-way ANOVA Kruskal-Wallis test or the non-parametric Mann-Whitney test and $P < 0.05$ (∗), $P < 0.01$ (∗∗), or $P < 0.001$ (∗∗∗) were considered as significant. If not further specified, all results were expressed as the mean ± standard error (SEM).

RESULTS

MACE Analysis

Initially, we measured differences in gene expression of *Borrelia*-infected ticks by employing the MACE technology, where high throughput sequencing of cDNA fragments provides a high resolution of gene expression and can reveal expression of low-abundance transcripts, compared to standard RNA sequencing (24, 32). We pooled more than 150 nymph midguts from each stage of tick feeding to minimize variations in gene expression. Being aware of intra-species genetic variation of wild-captured ticks, we limited the transcriptomes to the mixed population of nymphs originating from only three tick females (**Figure 1**). During the preparation of ticks for the transcriptomes and biological validations, we did not observe any adverse effects of the *Borrelia* infection on tick survival, fitness, or feeding, as demonstrated by body weights of fully fed infected nymphs compared with uninfected controls (**Supplemental Figure 2**). As a result, we obtained a total of 38,199,641 raw reads from the six cDNA MACE libraries. By mapping these sequences to our previously sequenced RefSeq library [containing 32,897 high-quality GXP contigs; Bioproject PRJNA657487 (33)] and the public *Ixodes* genomic and transcriptomic databases, we identified in each MACE library, on average, 17,257 GXP contigs and 1,302 additional tick genome/transcriptome contigs (gi|contigs absent from the RefSeq database) (**Supplemental Table 3**). Overall, in the midgut transcripts, we

observed a total of 24,276 tick genes. This number is in line with the 26,179 transcripts identified in our previous MACE transcriptomic project of the nymph *I. ricinus* salivary glands (33) and lower than the total number of genes described in the tick *I. scapularis* genome project (32,572 protein-coding genes) (34).

Identification of the Differentially Expressed Genes

To sort the database for genes upregulated or downregulated in the presence of *B. afzelii*, we defined a transcript as differentially expressed when the fold change was ≥ 5 (log2fold change ≤ −2.3 or ≥ 2.3) and the p-value ≤ 0.05. This primary selection led to the identification of 553 upregulated and 530 downregulated unique genes (**Figure 2**). Interestingly, in the group of fully fed nymphs (**Figure 2C**), we identified the largest number of *Borrelia*-upregulated genes (fold change > 1) and the highest ratio between upregulated and downregulated transcripts. Then, to produce a slimmed list of the differentially regulated genes, potentially confirmable by qRT-PCR in technical and biological validations, we selected transcripts with a fold change ≥ 5 and expression ≥ 5 transcripts per million in the infected (for upregulated genes) or uninfected (for downregulated genes) groups. By applying these criteria, we obtained a list of 118 upregulated and 96 downregulated genes (**Figure 3A**), of which 34, 49, and 55 genes were upregulated by infection at unfed (UF), 24h-fed (24-h), and fully fed (FF) stages, respectively. Conversely, 38, 33, and 30 genes were downregulated. Interestingly, five genes were upregulated, and one gene downregulated in all three time points (**Supplemental Tables 4**–**7**). The genes encode potentially secreted proteins (SignalP) containing a signal sequence [labeled as "SP(Sec/SPI)"] or intracellular proteins (labeled as "OTHER"). We did not observe any pattern in the prediction of subcellular localization (DeepLoc). The full list contained extracellular proteins, as well as proteins localized to the cytoplasm, mitochondrion, nucleus, or lysosome. Most of the proteins were predicted to be soluble, although the list also contained several membrane proteins (e.g., receptors, channels, glycoproteins). In summary, we identified 214 tick genes with various functions and localizations, highly differentially expressed in the presence of *B. afzelii*, suggesting a significant interaction of the tick midgut tissue with the spirochetes.

Technical and Biological Validations of MACE

We confirmed the expression of several differentially regulated genes arising from the MACE analysis by technical and biological validations. We focused only on genes from our upregulated candidate list as only these could be later silenced by RNA interference and tested in our *B. afzelii*-transmission model. We selected 46 candidates (from various time points) with homologous sequences present in the genomic databases of *I. ricinus* and *I. scapularis* (for the selection criteria see *Methods*). For 33 of these genes, we were able to design gene-specific PCR primers and for 22 of these genes, these primers worked well in a standard PCR assay. Their expression was then validated in technical and biological validations by qRT-PCR. All 22 candidate genes passed the technical validation and were proven to be upregulated at specific time points (**Figure 3**). Gene expression levels in 10 genetically distinct *I. ricinus* populations of nymphs were then determined to validate these candidate genes biologically. Through this strict validation level, seven genes passed, representing 32% of the 22 pre-selected genes. Of these, four candidates were shown to be overexpressed at the same time point compared to MACE, while the other three genes were overexpressed at other time points. The seven gene sequences represented: 1) cytosolic iron-sulfur protein assembly protein CIAO1 homolog (GXP_Contig_7059), 2) uncharacterized protein (GXP_Contig_30818), 3) BTB domain-containing protein (GXP_Contig_6657), 4) cytochrome p450 cyp2 subfamily protein (GXP_Contig_26946), 5) solute carrier organic anion transporter family member (GXP_Contig_29696), 6) cyclin-D-binding Myb-like transcription factor 1 (GXP_Contig_16121), and 7) Kolobok-5 tv protein (GXP_Contig_1931). All transcripts encoded intracellular proteins without predicted signal sequences (SignalP) and were predicted for various cellular localizations (DeepLoc).

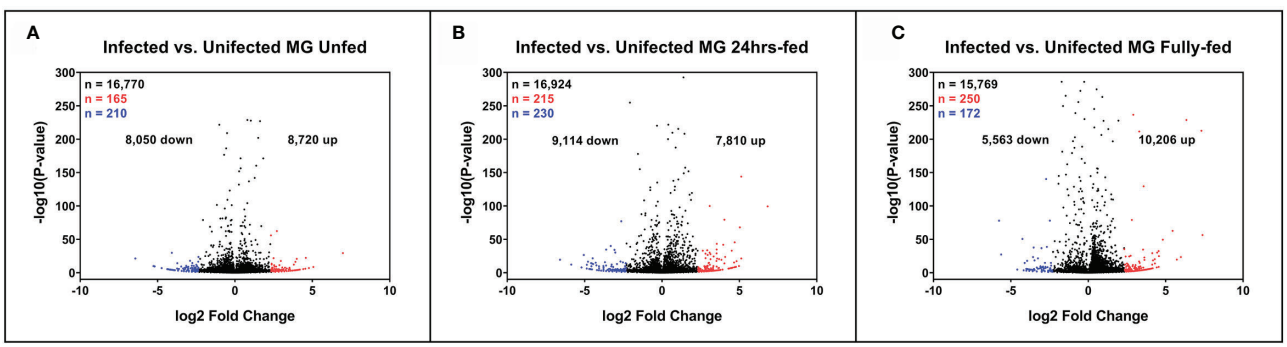
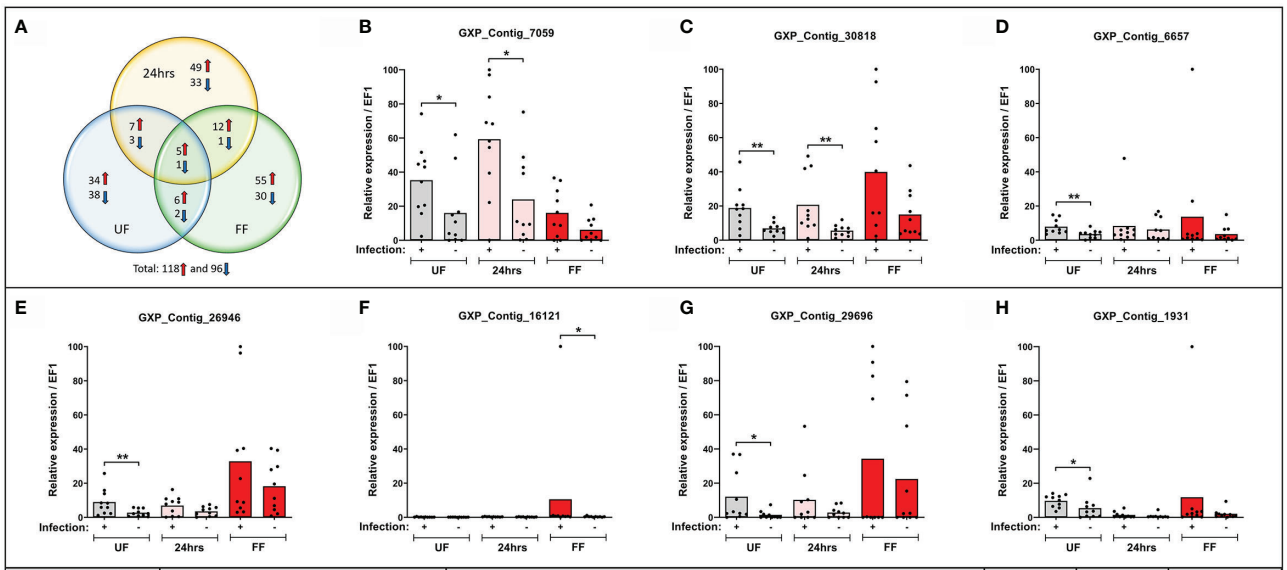


FIGURE 2 | Expression of tick midgut genes is altered in the presence of *Borrelia afzelii*. Volcano plots showing differentially expressed tick transcripts analyzed by MACE at individual time points. **(A)** Unfed nymphs **(B)** Nymphs fed for 24 h **(C)** Fully fed nymphs. n = number of differentially expressed transcripts. Total differentially expressed transcripts (black), upregulated transcripts (red; p-value ≤ 0.05 and log2 fold change ≥ 2.3), and downregulated transcripts (blue; p-value ≤ 0.05 and log2 fold change ≤ −2.3). up = total upregulated transcripts, down = total downregulated transcripts, MG, midgut.

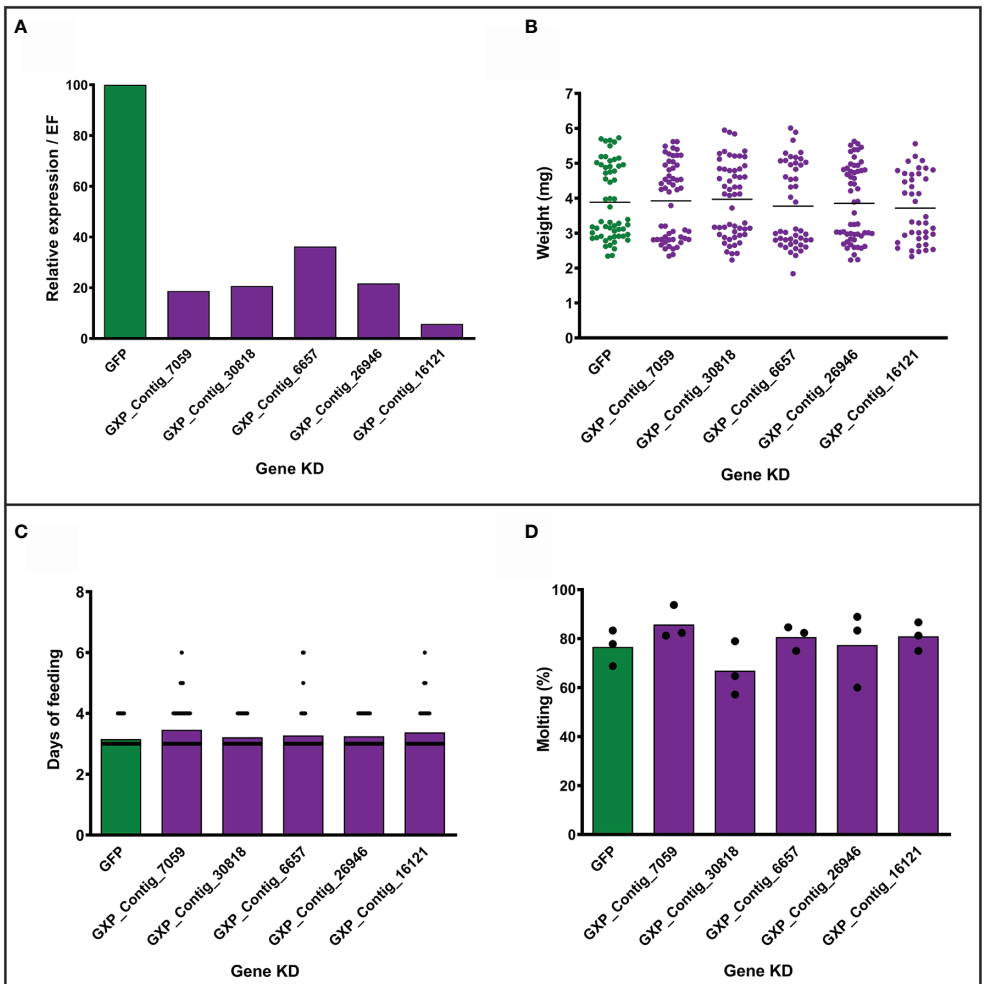


Database of upregulated transcript	Contig	Putative description	Technical validation	Biological validation	Timepoint of upregulation
UF	GXP_Contig_7059	Cytosolic iron-sulfur protein assembly protein CIAO1 homolog	YES	YES	UF, 24hrs
UF	GXP_Contig_31514	Hypothetical protein	YES	NO	
UF	GXP_Contig_30818	Uncharacterized protein	YES	YES	UF, 24hrs
UF	GXP_Contig_5839	Glycine-rich protein	YES	NO	
24hrs	gi|241123951|ref|XM_002403994.1|	Hypothetical protein	YES	NO	
24hrs	GXP_Contig_8911	Glycine-rich protein	YES	NO	
24hrs	GXP_Contig_6131	Pla2 bee venom like a sub-family of phospholipase a2	YES	NO	
24hrs	GXP_Contig_2889	Salivary lipocalin	YES	NO	
24hrs	GXP_Contig_28121	WC protein	YES	NO	
24hrs	GXP_Contig_7109	Enoyl-coa hydratase/isomerase	YES	NO	
24hrs	GXP_Contig_30557	Diacylglycerol acyltransferase dgat	YES	NO	
FF	GXP_Contig_16607	Putative DNA-bridging protein baf	YES	NO	
FF	GXP_Contig_6657	BTB domain-containing protein	YES	YES	UF
FF	GXP_Contig_25185	Uncharacterized protein	YES	NO	
FF	GXP_Contig_26946	Cytochrome p450 cyp2 subfamily protein	YES	YES	UF
FF	GXP_Contig_476	RNA-directed dna polymerase from mobile element jockey-like protein	YES	NO	
FF	GXP_Contig_29696	Solute carrier organic anion transporter family member	YES	YES	UF
FF	GXP_Contig_29976	Solute carrier organic anion transporter family member	YES	NO	
FF	GXP_Contig_16121	Cyclin-D-binding Myb-like transcription factor 1	YES	YES	FF
UF+24hrs+FF	GXP_Contig_21561	Secreted salivary gland peptide	YES	NO	
UF+24hrs	GXP_Contig_1931	Kolobok-5 tv	YES	YES	UF
24hrs+FF	GXP_Contig_1305	Salivary secreted peptide	YES	NO	

FIGURE 3 | Expression of selected transcripts can be verified by technical and biological qRT-PCR validations. (Upper) **(A)** Venn diagram of the top-score differentially expressed *B. afzelii*-infected nymph midgut transcripts (fold change ≥ 5 fold and expression ≥ 5 transcripts per million). The upregulated transcripts are marked by a red arrow, downregulated by a blue arrow. **(B–H)** qRT-PCR profiles (relative expression) of seven biologically validated transcripts were significantly upregulated by *B. afzelii* infection (Mann-Whitney test). The biological validations were carried out on 10 individual tick populations. Each dot represents expression in a single nymph population. In each graph, cDNA with the highest expression was set as 100. The tick *elongation factor* was used as a housekeeping gene. (Lower) Summary table of the validated transcripts. In total, 22 transcripts from different time points of feeding (see *Results* for the selection criteria) were analyzed by the technical and biological validations. UF, unfed; 24hrs, fed for 24hrs; FF, fully fed. $P < 0.05$ (*), $P < 0.01$ (**).

RNA Interference and *Borrelia*-Transmission

To assess the role of the stimulated genes in transmission of *Borrelia*, we employed the method of RNA interference and injected nymphal ticks individually with five different gene-specific dsRNAs designed against the previously biologically validated transcripts. Before the transmission experiments with infected nymphs, we tested the effect of silencing in uninfected nymphs. The genes were successfully silenced in the fully fed nymphs to expression levels ranging from 6 to 36% comparing to the dsGFP control (**Figure 4A**). We did not observe any significant impact on feeding success, duration of feeding, tick weight after feeding, or molting of nymphs to adults (**Figures 4B, D**). We then performed the silencing in infected nymphs. Initially, we tested



Gene	Ear week 2	Ear week 3	Heart week 3	Urinary bladder week 3	Cumulative
GFP control	9 / 13	12 / 13	12 / 13	12 / 13	12 / 13
GXP_Contig_7059	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5
GXP_Contig_30818	**2 / 13**	10 / 13	10 / 13	**7 / 13**	10 / 13
GXP_Contig_6657	8 / 13	11 / 13	12 / 13	11 / 13	12 / 13
GXP_Contig_26946	4 / 5	5 / 5	5 / 5	5 / 5	5 / 5
GXP_Contig_16121	4 / 5	5 / 5	5 / 5	5 / 5	5 / 5

FIGURE 4 | Effect of gene silencing by RNA interference on nymph feeding and *Borrelia afzelii* transmission. (Upper) Silencing of five tick genes in uninfected nymphs. **(A)** Evaluation of the silencing level by qRT-PCR (each group represents a mix of five fully fed nymphs). **(B)** Weights of individual fully fed nymphs. Each dot represents a single tick. **(C)** Duration of nymph feeding. **(D)** Molting success of fully fed nymphs into adults (percentage of molted nymphs fed on each mouse; biological triplicates). (Lower) Summary table of two transmission experiments with the gene-silenced *B. afzelii*-infected nymphs. Numbers indicate total qRT-PCR positive/total mouse tissues during the infection (ear week 2) and after mice scarification (week 3). dsGFP was used as a negative control. A decrease of positivity by >25% is highlighted in red.

transmission with five mice per group. Silenced genes associated with the blocking of transmission of *B. afzelii* in at least one mouse, were further tested with an additional eight mice per group. Similarly, as observed with the uninfected nymphs, gene silencing did not affect tick feeding (**Supplemental Figures 3A, B**). The transmission of *B. afzelii* from the tick to the mouse was not noticeably blocked after the silencing of GXP_Contigs_7059, _6657, _26946, and _16121 (**Figure 4**). The number of spirochetes in deeper mouse tissues, as measured by qRT-PCR, was also not significantly altered (**Supplemental Figures 3C–F**). Interestingly, in the group with silenced uncharacterized protein (GXP_Contig_30818), the progress of infection in mice was delayed (only 15% of ears were *Borrelia*-positive by the second week compared to 70% in control), which was then reflected in a

reduction of *Borrelia* prevalence in the ear (3rd week), heart, and urinary bladder by 23, 23, and 46%, respectively (**Figure 4**).

DISCUSSION

The hypothesis that parasites actively modify the physiology and behavior of their hosts to enhance transmission is an intriguing and well-documented phenomenon in many species of living organisms (35). However, evidence of manipulation of ticks by *Borrelia* spirochetes is still mostly unknown. In this study, we have revealed differential gene expression in the midgut of *I. ricinus* nymphs infected with the Lyme borreliosis spirochete *B. afzelii* before, during, and after blood-feeding. This study represents the first transcriptome produced from ticks focusing on midgut genes stimulated by *Borrelia*. Previous transcriptomic studies described differential gene expression in salivary glands (33, 36), or used alternative approaches for such gene identifications (19, 20, 37–42).

The motile *Borrelia* enters the tick when larvae or nymph feed on an infected reservoir host. The spirochetes are attracted to the feeding site by the tick proteins secreted into the saliva (43). During the acquisition phase, the ingested spirochetes change their gene expression and multiply in the tick midgut contents (12, 44) to successfully infect the vector. After tick molting, the midgut appears empty. The midgut walls are localized close to each other, and the peritrophic matrix, a layer consisting of glycoproteins bound to the chitin network, is absent. In these harsh conditions of limited nutrients, which can last for months or years, the spirochetes switch into their "sleeping mode" and can be found attached to the midgut cell wall. OspA, a membrane lipoprotein produced by the *Borrelia*, was shown to bind the tick TROSPA protein present on the surface of *I. scapularis* midgut cells (19). *Trospa* was the first tick gene recognized as upregulated by the presence of *Borrelia* in the unfed nymph. Surprisingly, we were not able to identify *trospa* in our RefSeq database nor the recent TSA databases of *I. ricinus* available at NCBI. However, this gene has previously been sequenced from *I. ricinus* (NCBI: EU034646) (45), indicating that in *I. ricinus*, *trospa* was probably expressed to a limited level.

It is unknown how *Borrelia* spirochetes change expression of the tick midgut genes and how these modifications help *Borrelia* multiply and persist in the gut lumen. Using the MACE method on unfed midguts we have identified 210 downregulated and 165 upregulated tick genes as a result of infection (p-value ≤ 0.05 and log2 fold change ≤ −2.3 or ≥ 2.3). We found that *mitochondrial carboxypeptidase* (V5HK70) and *cytochrome C oxidase subunit VIa* (a component of the respiratory complex IV, XM_002435666) were downregulated > 8 fold in expression, indicating possible suppression of energy metabolism in unfed infected ticks. Among the highly upregulated genes, we identified several peritrophins and chitinases, constituents of the peritrophic matrix. However, the peritrophic matrix is formed in *I. ricinus* > 18h after the beginning of feeding (46), meaning that mRNAs of these genes could be pre-synthesized to accelerate the formation of the peritrophic matrix after the initiation of feeding. Alternatively, these proteins could be involved in establishing and maintaining other chitin structures such as tracheae, which supplement the midgut tissue with oxygen.

Ticks do not receive any nutrients from the environment, and the blood-feeding represents a significant milestone in their life cycles. The *Borrelia* spirochetes residing in the tick midgut become activated by a mechanism that is not completely clear [probably by nutrients in the blood, temperature, pH (47), osmolarity (48)] and thereby accelerate the expression of genes necessary for their transmission and survival in the vertebrate host. In the case of *B. burgdorferi* sensu stricto, the number of spirochetes multiplies from several hundred in an unfed nymph to a hundred thousand in a fully fed nymph (49). Next, *B. burgdorferi* migrate to the basolateral surface of the midgut epithelium, cross the basal membrane, and enter the hemocoel and salivary glands to infect the host through the secretion of saliva (14). However, *B. afzelii* appears to behave differently. These spirochetes do not multiply during feeding, but their numbers reduce continuously, possibly by direct traversal of the spirochetes from the midgut into the host (12). Importantly, spirochetes of *B. afzelii* have not been found to infect the salivary glands. In addition, and in contrast to *B. burgdorferi*, the number of *B. afzelii* spirochetes dramatically decreases over the next few months after molting (50). Bontemps-Gallo et al. previously showed that physicochemical parameters such as the level of oxygen, osmolality, and oxidative stress, affect growth and motility differently in these two genetically distinct bacterial species (51). Consistent with this, from 42 previously identified tick *Borrelia*-responsive genes (19, 20, 37–42) (including *tre31*, *isdlp*, *pixr*, *stat*, etc.), in our databases we found only *duox* (52) and *alcohol dehydrogenase* (42) being upregulated more than two-fold, further supporting the behavioral differences between *B. burgdorferi* and *B. afzelii*.

The primary purpose of this study was to identify tick proteins suitable for developing new anti-tick therapies. Ideally, such candidates should be abundantly expressed during feeding and targeted to the tick midgut wall or secreted from the cells into the midgut content in order to be accessible to antibodies or drugs present in the host blood. It was demonstrated that *B. afzelii* enters the host skin within 24h of attachment, but this population of spirochetes is not infectious. This means that the *Borrelia* need >24h for activation in the tick midgut to become infectious. We identified several tick genes altered in expression at the 24h time point. We do not know if this response was evoked explicitly by the *Borrelia* to gain an advantage during transmission, reflecting ongoing host modifications, or was induced by the tick as a reaction (immune) against the spirochetes.

We observed that at the fully fed time point, the number of upregulated genes were almost doubled when compared to the downregulated genes. We hypothesized that this overexpression was evoked by *Borrelia* during feeding to alter the tick physiology in order to transmit the spirochetes from the tick midgut into the host. To test the necessity of this upregulation, we silenced five previously biologically validated tick genes by RNA interference and tested the ability of nymphs to transmit *Borrelia*. All these candidates were predicted to be intracellular proteins, and many of them were transcription factors, so we tested whether silencing of these genes could block the expression of their downstream-regulated genes. We observed that the silencing of GXP_Contig_30818 caused the absence of *Borrelia* infection in the ear the second week after tick detachment (the beginning of infection) in 85% of mice (11 of 13).

This delay in onset of disease probably triggered a further decrease in *Borrelia* prevalence in the ear (week 3) and destination tissues, heart, and urinary bladder. This transcript encodes yet uncharacterized protein with predicted nuclear localization. The expression of genes possibly regulated by this protein deserves further attention. The silencing of other genes did not affect *Borrelia* transmission. Therefore, we propose that upregulation of these genes is necessary for processes other than transmission, possibly for the acquisition and persistence of *Borrelia*. Additionally, in the transcripts upregulated during feeding, and similar to the unfed stage, we more often identified genes connected with synthesis and reconstruction of the peritrophic matrix (e.g., peritrophins and chitinases), whose expression has been previously shown to influence spirochete colonization of ticks (53).

We believe that this work will enable further identification and characterization of the tick midgut proteins necessary for acquisition, persistence, and transmission of *B. afzelii* from *I. ricinus*. In our MACE transcriptomic database, we found, in total, 55 *Borrelia*-stimulated, well expressed, and secreted or cell membrane-associated midgut proteins. We assume that some of these candidate proteins are necessary for *Borrelia* activation and transmission and that blocking of these proteins by a specific vaccine or a drug will contribute to the development of novel therapies against Lyme borreliosis.

DATA AVAILABILITY STATEMENT

The dataset presented in this study is deposited as **Supplementary Table 1** in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal protection law of the Czech Republic (§17, Act No. 246/1992 Sb) with the approval of CAS (approval no. 79/2013).

AUTHOR CONTRIBUTIONS

RS, JT, PK, JH, and OH designed the experiments. SM, RS, VU, and OH prepared the infected nymphs, performed the dissections, isolated RNA, and accomplished the feeding and transmission experiments. NK and PW did the MACE analysis and annotations. SM analyzed the transcriptomic data and performed the technical and biological validations. SM and OH wrote the manuscript with an input from all co-authors. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Czech Science Foundation grant no. 20-05736S, the European Union FP7 project Antidote (grant agreement number 602272), and by the Centre for Research of Pathogenicity and Virulence of Parasites (no. CZ.02.1.01/0.0/0.0/16_019/0000759), funded by the European Regional Development Fund (ERDF) and Ministry of Education, Youth, and Sport, Czech Republic (MEYS).

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Gabiela Loosova, Jan Erhart, Adela Palusova, and Zuzana Smejkalova.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.612412/full#supplementary-material

Supplementary Figure 1 | Scheme of sample preparation for biological validations. Ten populations of uninfected larvae, each originating from a single female fed on a guinea pig, were fed on *B. afzelii*-infected or uninfected mice. The nymphs then were fed on uninfected mice and dissected for midguts (10-50 nymphs for each group) at the three indicated time points.

Supplementary Figure 2 | *Borrelia* infection does not affect tick feeding or final weights of the fully-fed nymphs. Each group of nymphs was comprised of females (higher weights) and males (lower weights). Each dot represents a single nymph. The data in each group contain a collection of 20 individual feedings (in total 360 infected and 339 uninfected nymphs). INF = infected nymphs, UNINF = uninfected nymphs. The horizontal bar indicates a mean. n.s. = not significant (Mann-Whitney test).

Supplementary Figure 3 | Effect of gene silencing by RNA interference on nymph feeding and *B. afzelii* transmission.**(A)** Weights of individual fully-fed nymphs. Each dot represents a single tick. **(B)** Duration of nymph feeding. **(C–F)** The absolute number of *B. afzelii* in individual mouse tissues measured by qRTPCR. Two genes with no detectable *B. afzelii* in the heart tissue from the silencing Experiment 1 (left) were once more tested in the silencing experiment 2 (right). dsGFP was used as a negative control.

Supplementary Table 1 | A list and expression of all Ixodes ricinus nymph midgut genes identified in individual MACE transcriptomes. UF = unfed, 24hrs = fed for 24 hours, FF = fully-fed, INF = B. afzelii-infected nymphs, UNINF = uninfected nymphs.

Supplementary Table 2 | List of primers. Restriction sites for ApaI/XbaI are underlined.

Supplementary Table 3 | Raw reads and mapped contigs obtained after the sequencing of MACE libraries. Raw reads mapped to our previously sequenced RefSeq library (Bioproject PRJNA657487) were labeled as GXP sequences. Sequences absent from the RefSeq library, but present in other Ixodes tick genomes and transcriptomes, were labeled as gi|. UF = unfed, 24hrs = fed for 24 hours, FF = fully-fed, INF = infected nymphs, UNINF = uninfected nymphs.

Supplementary Table 4 | A list of Ixodes ricinus nymph midgut genes upregulated in the presence of Borrelia afzelii at three different timepoins of feeding. n.c. = not calculated.

Supplementary Table 5 | A list of Ixodes ricinus nymph midgut genes co-upregulated by Borrelia afzelii at different timepoints. n.c. = not calculated.

Supplementary Table 6 | A list of Ixodes ricinus nymph midgut genes downregulated in the presence of Borrelia afzelii at three different timepoins of feeding. n.c. = not calculated.

Supplementary Table 7 | A list of Ixodes ricinus nymph midgut genes co-downregulated by Borrelia afzelii at different timepoints. n.c. = not calculated.

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Conflict of Interest: NK and PW were employed by GenXPro GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6.2 Supplementary materials (Article 1):

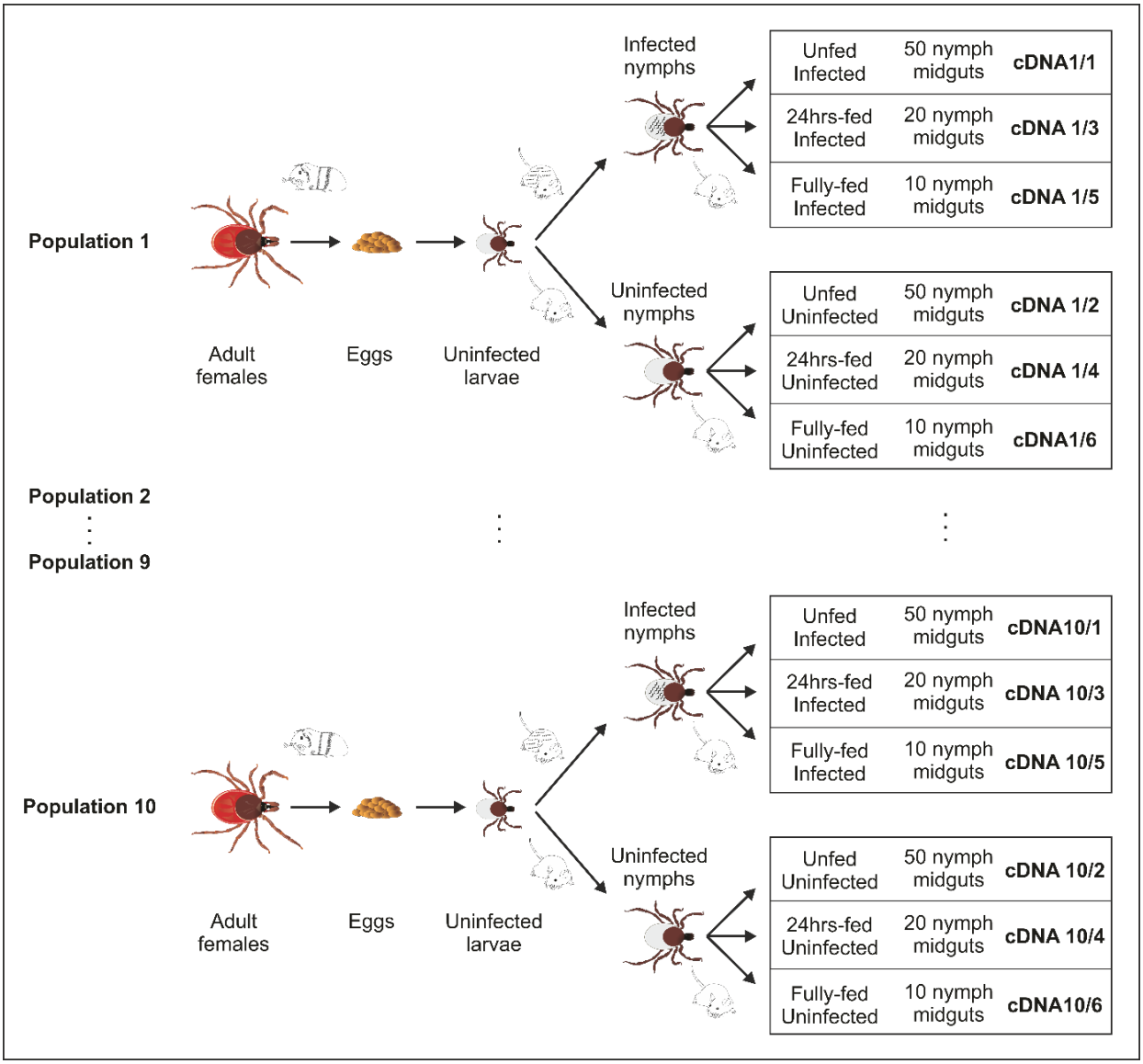


Figure 5: **(Supplemental Figure) Scheme of sample preparation for biological validations.** Ten populations of uninfected larvae, each originating from a single female fed on a guinea pig, were fed on *B. afzelii*-infected or uninfected mice. The nymphs then were fed on uninfected mice and dissected for midguts (10-50 nymphs for each group) at the three indicated time points.

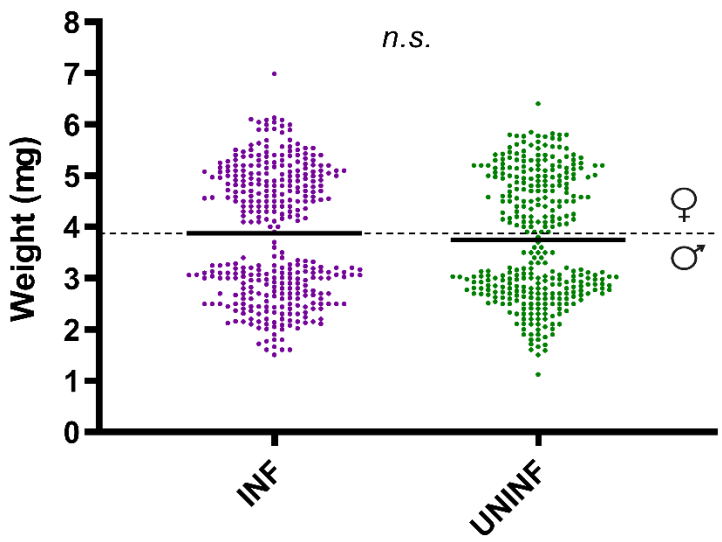
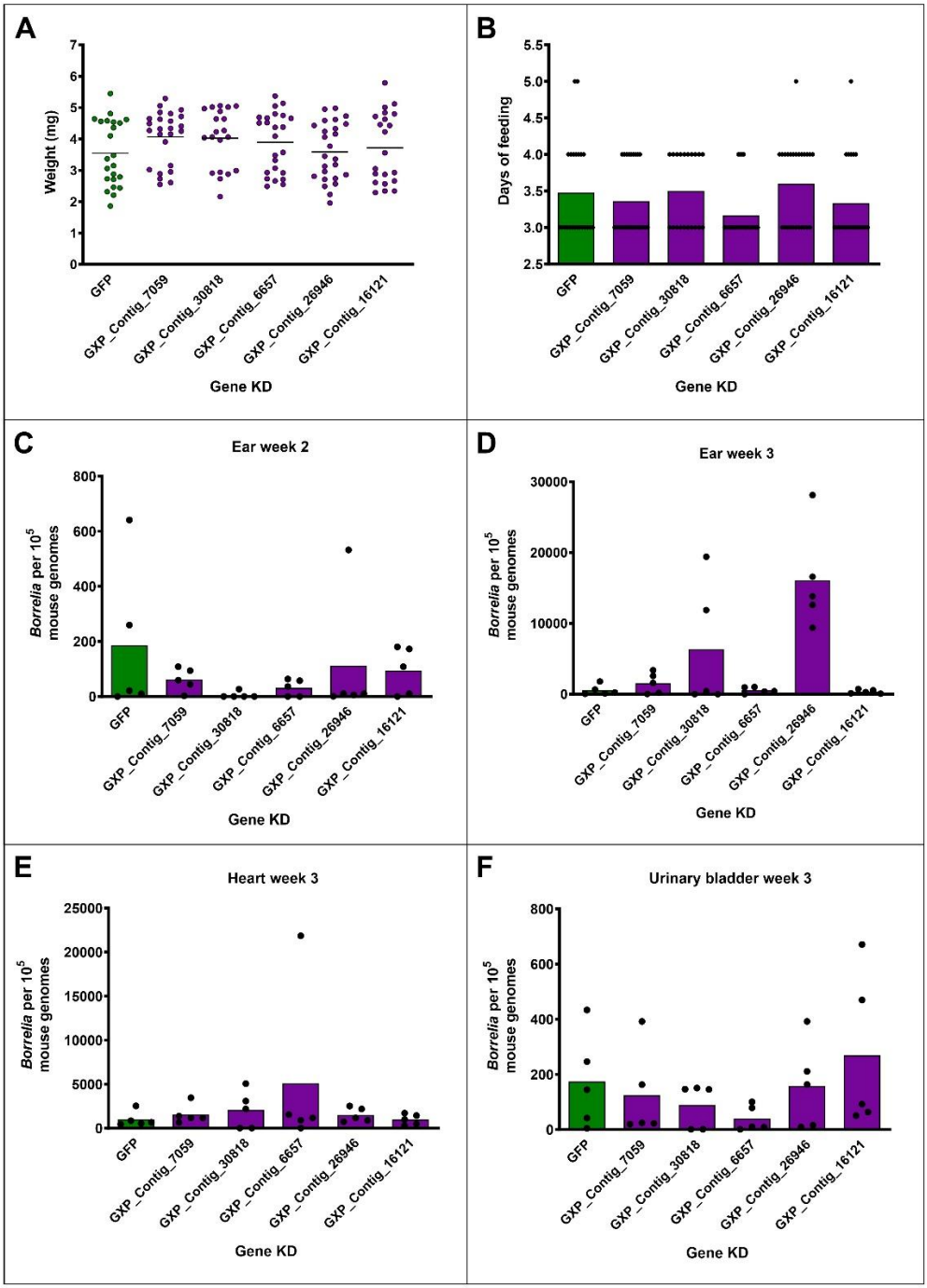


Figure 6: **(Supplemental Figure) Borrelia infection does not affect tick feeding or final weights of the fully-fed nymphs.** Each group of nymphs was comprised of females (higher weights) and males (lower weights). Each dot represents a single nymph. The data in each group contain a collection of 20 individual feedings (in total 360 infected and 339 uninfected nymphs). INF = infected nymphs, UNINF = uninfected nymphs. The horizontal bar indicates a mean. n.s. = not significant (Mann-Whitney test).

EXPERIMENT 1



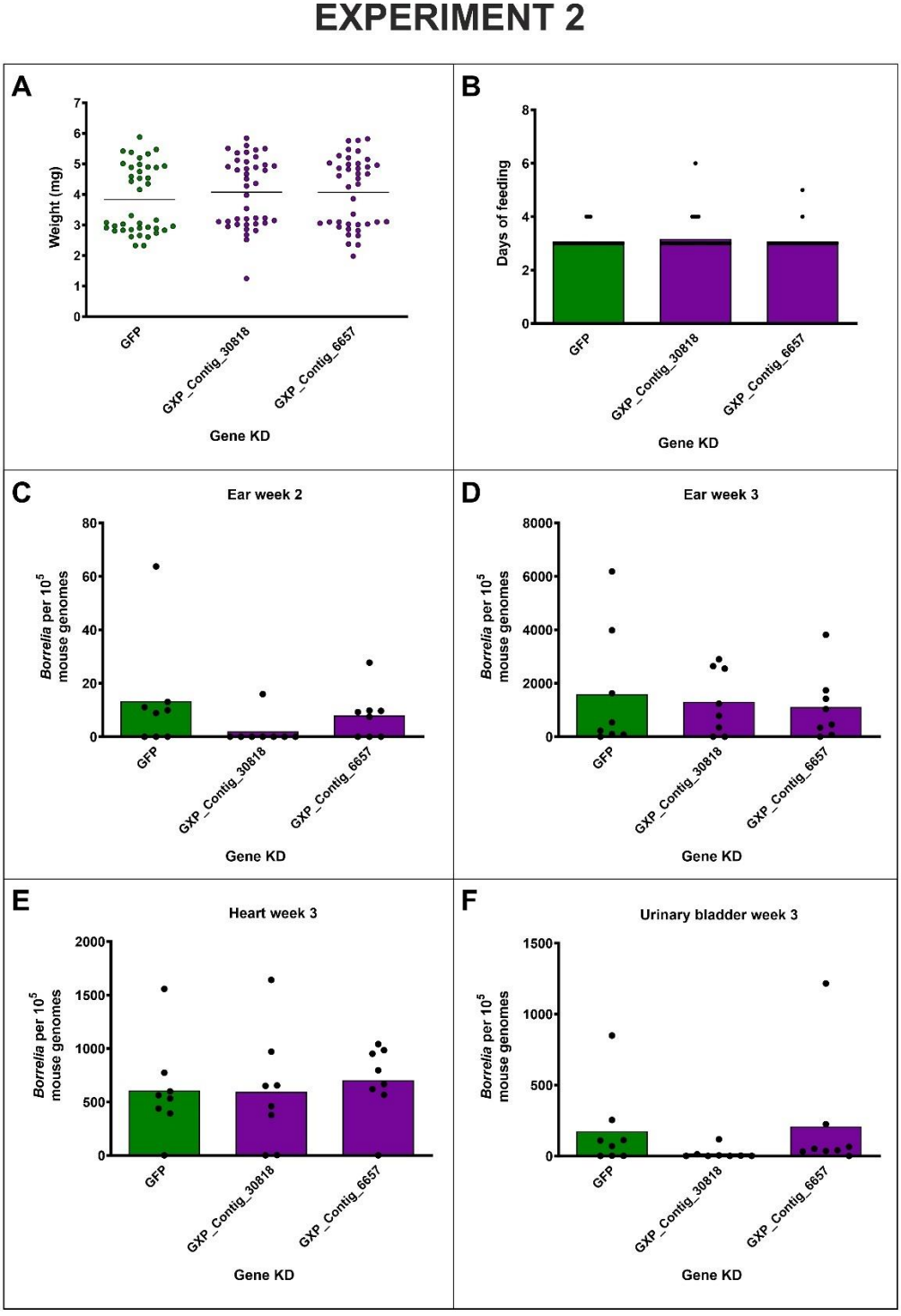


Figure 7: **(Supplemental Figure) Effect of gene silencing by RNA interference on nymph feeding and *B. afzelii* transmission.** (A) Weights of individual fully-fed nymphs. Each dot represents a single tick. (B) Duration of nymph feeding. (C-F) The absolute number of *B. afzelii* in individual mouse tissues measured by qRT-PCR. Two genes with no detectable *B. afzelii* in the heart tissue from the silencing Experiment 1 (left) were once more tested in the silencing Experiment 2 (right). dsGFP was used as a negative control.

Table 3: **(Supplemental Table) List of primers.** Restriction sites for ApaI/XbaI are underlined.

Method	**Target**	**Name**	**Sequence (5´-->3´)**
RNAi	GXP_Contig_7059	IR998	ATGGGCCCTGGCGTCCTGAGCACTTTCG
		IR999	ATTCTAGAATGCACGAAAGAGACGATGG
	GXP_Contig_30818	IR996	ATGGGCCCGAAGGGCGGGGAAAATGTGC
		IR997	ATTCTAGAAACAATTGAAATGCAGCCCG
	GXP_Contig_6657	IR988	ATGGGCCCCGGTCAGTAGCTTGACCTCC
		IR989	ATTCTAGAAGGTCTCTTCGCTCCCATCG
	GXP_Contig_26946	IR1168	ATGGGCCCCCTGCTCTCCTATCTCGTGG
		IR1119	ATTCTAGACTCGAATCCACTATATTGGG
	GXP_Contig_16121	IR990	ATGGGCCCTGCAGTCGATCAGCAGCACC
		IR991	ATTCTAGATTTCCACACAGTTTTCATCC
qRT-PCR	GXP_Contig_7059	IR788	TAACTGCCGGGAGAGATAGC
		IR789	CTTTATTCGAGTCGCCCTTG
	GXP_Contig_31514	IR752	GTAATCCGGCCAAATGACAC
		IR753	TTGCCCTAATACATGCACCA
	GXP_Contig_30818	IR756	CTTGAGAATGTGGCACAACG
		IR757	ATCCACGCAAGTTGACACAA
	GXP_Contig_5839	IR754	ACCTACGGCGACTACGCTAC
		IR755	TGAGGAAGTTGTTGGATGTGA
	gi|241123951|ref|XM_002403994.1|	IR810	AGCTACGGTGGACACTACGG
		IR811	ATCCTTCCGACAGCAGATCA
	GXP_Contig_8911	IR824	CATGGTTTCGGAGGACACTT
		IR825	GCTTGTTCTTCCATGGTCGT
	GXP_Contig_6131	IR804	GCCAAACGGTTTAACGACAT
		IR805	TATCGTTGGTGGTGTCCAGA
	GXP_Contig_2889	IR790	ATATTGCAACACGACGCAAA
		IR791	TTATACTGCGGGGGTTTCAG
	GXP_Contig_28121	IR806	AATATGCACAGGCTGCACAC
		IR807	TGCTACCAGAGGCCGAAATA
	GXP_Contig_7109	IR820	CGCAGAAGAAGATCCAGGAG
		IR821	TCGATGAGTTGTTCGCACTC
	GXP_Contig_30557	IR822	GTGCCCACAGTCTGATGAAG
		IR823	CCAGCTCACTTGCACCTTTA
	GXP_Contig_16607	IR890	GCTGGTACAGAAAGGCTTCG
		IR891	ACGTCCTTCATCCACTCGAC
	GXP_Contig_6657	IR896	ATCTACTTCGGGCGTGTTTG
		IR897	ACCTCATCCGTTTTCATTGC
	GXP_Contig_25185	IR886	GCATCGGTTAAAATCGGAAA
		IR887	GCAAGCGGAATCTTCATTGT
	GXP_Contig_26946	IR892	ACAACATAATCGGCCTGTCC
		IR893	ACCGTTGGGTGAAAACAAAA
	GXP_Contig_476	IR900	GCATCACCCCACTCACTCTT
		IR901	GCTGATCCTTGTGGAGCTG
	GXP_Contig_29696	IR888	CGAATTGTACAAGCGTCAGC
		IR889	CACTTTCACCTGGGCTGTTT
	GXP_Contig_29976	IR946	GCTCATGTAGGGGAGCCATA

		IR947	CGCGGTTTTACAGTAGCACA
	GXP_Contig_16121	IR910	TGCAGGAAATTCATGACACC
		IR911	CGCAAGCATAGGATCAGACA
	GXP_Contig_21561	IR748	ACTTGGATGGAACGCTCAAG
		IR749	GCCGATGACCAAGGAGTTTA
	GXP_Contig_1931	IR758	TGCCAAAAGACTCCGAACTT
		IR759	GCTAGGATCCTGCGTTCTTG
	GXP_Contig_1305	IR914	TCCAGTGGAAGTTGAAGGAAA
		IR915	TGCCTTCTTAAGACGCGAAT
	Tick *actin*	IR526	CGACATCAAGGAGAAGCTCTG
		IR527	GTCGGGAAGCTCGTAGGAC
	Tick *elongation factor*	IR524	ACGAGGCTCTGACGGAAG
		IR525	CACGACGCAACTCCTTCAC
	Tick *ferritin 1*	IR522	GACTTCCTGGAGGGCAACTA
		IR523	ATTCGGACAGCTCCTTGATG
	Mouse *actin* (Dai *et al.*, 2009)	MM-ACT-F	AGAGGGAAATCGTGCGTGAC
		MM-ACT-R	CAATAGTGATGACCTGGCCGT
		MM-ACT-PROBE	CACTGCCGCATCCTCTTCCTCCC
	Borrelia spp. *flagellin* (Schwaiger, Peter and Cassinotti, 2001)	IR1345	AGCAAATTTAGGTGCTTTCCAA
		IR1346	GCAATCATTGCCATTGCAGA
		Fla Probe1	TGCTACAACCTCATCTGTCATTGTAGCATCTTTTATTTG
PCR	*Borrelia* spp. *flagellin* (Schwaiger, Peter and Cassinotti, 2001)	IR1345	AGCAAATTTAGGTGCTTTCCAA
		IR1346	GCAATCATTGCCATTGCAGA

Table 4: **(Supplemental Table) Raw reads and mapped contigs obtained after the sequencing of MACE libraries.** Raw reads mapped to our previously sequenced RefSeq library (Bioproject PRJNA657487) were labeled as GXP sequences. Sequences absent from the RefSeq library, but present in other *Ixodes* tick genomes and transcriptomes, were labeled as gi|. UF = unfed, 24hrs = fed for 24 hours, FF = fully-fed, INF = infected nymphs, UNINF = uninfected nymphs.

Library	Number of raw reads	GXP	gi| (other *Ixodes* genomes and transcriptomes)
MACE 1 (UF INF)	4,765,625	17,261	1,255
MACE 2 (UF UNINF)	8,905,738	17,800	1,278
MACE 3 (24hrs INF)	9,248,649	18,179	1,354
MACE 4 (24hrs UNINF)	4,836,146	17,068	1,293
MACE 5 (FF INF)	5,826,650	17,757	1,363
MACE 6 (FF UNINF)	4,616,833	15,478	1,266
Total	**38,199,641**		

Table 5: **(Supplemental Table) A list of *Ixodes ricinus* nymph midgut genes upregulated in the presence of *Borrelia afzelii* at three different time-points of feeding.** n.c. = not calculated.

Unfed	Transcripts per million		Fold	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected	upregulation	SignalP	DeepLoc	Type
GXP_Contig_492	26.0	0.2	125.7	OTHER	Cytoplasm	Soluble
gi|241574685|ref|XM_002403355.1|	6.9	0.2	33.5	SP(Sec/SPI)	Extracellular	Soluble
gi|241712765|ref|XM_002413414.1|	5.7	0.2	27.4	OTHER	Mitochondrion	Soluble
GXP_Contig_13584	19.7	0.8	23.9	OTHER	Nucleus	Soluble
GXP_Contig_20597	19.7	1.2	15.9	OTHER	Nucleus	Soluble
GXP_Contig_2194	15.5	1.0	15.0	OTHER	Lysosome/Vacuole	Soluble
GXP_Contig_26924	11.5	0.8	14.0	OTHER	Cytoplasm	Soluble
GXP_Contig_24155	7.1	0.6	11.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_30937	6.3	0.6	10.2	OTHER	Extracellular	Soluble
GXP_Contig_21561	7.8	0.8	9.4	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_5294	5.5	0.6	8.8	OTHER	Nucleus	Soluble
GXP_Contig_1961	8.6	1.0	8.3	OTHER	Extracellular	Soluble
GXP_Contig_5839	5.0	0.6	8.1	OTHER	Extracellular	Soluble
GXP_Contig_7059	21.0	2.7	7.8	OTHER	Nucleus	Membrane
GXP_Contig_31514	13.6	1.9	7.3	OTHER	Mitochondrion	Soluble
GXP_Contig_3467	5.9	0.8	7.1	OTHER	Cytoplasm	Soluble
GXP_Contig_17955	5.9	0.8	7.1	OTHER	Cytoplasm	Soluble
GXP_Contig_3621	5.9	0.8	7.1	OTHER	Cytoplasm	Soluble
GXP_Contig_9968	5.7	0.8	6.9	OTHER	Cytoplasm	Soluble
GXP_Contig_18088	85.0	13.0	6.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_2703	6.7	1.0	6.5	OTHER	Cytoplasm	Soluble
GXP_Contig_30818	11.8	1.9	6.3	OTHER	Nucleus	Soluble
GXP_Contig_5550	6.3	1.0	6.1	OTHER	Mitochondrion	Soluble
gi|241998155|ref|XM_002433676.1|	7.3	1.2	5.9	OTHER	Mitochondrion	Soluble
GXP_Contig_8541	12.2	2.1	5.9	SP(Sec/SPI)	Cytoplasm	Soluble
GXP_Contig_809	15.5	2.7	5.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_1328	31.5	5.6	5.6	OTHER	Lysosome/Vacuole	Soluble

Unfed	Transcripts per million		Fold upregulation	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected		SignalP	DeepLoc	Type
GXP_Contig_23546	10.1	1.9	5.4	OTHER	Lysosome/Vacuole	Membrane
GXP_Contig_22588	7.8	1.4	5.4	OTHER	Extracellular	Soluble
GXP_Contig_24982	10.9	2.1	5.3	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_10940	18.3	3.5	5.2	OTHER	Nucleus	Soluble
GXP_Contig_31392	20.4	3.9	5.2	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_26955	7.3	1.4	5.1	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_1931	90.2	18.0	5.0	OTHER	Cytoplasm	Soluble

Fed for 24hrs	Transcripts per million		Fold upregulation	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected		SignalP	DeepLoc	Type
GXP_Contig_1305	83.4	2.4	34.7	OTHER	Cytoplasm	Soluble
GXP_Contig_1065	11.9	0.3	34.7	OTHER	Extracellular	Soluble
GXP_Contig_14191	39.2	1.2	32.6	OTHER	Extracellular	Soluble
GXP_Contig_7974	5.4	0.2	31.3	OTHER	Nucleus	Soluble
GXP_Contig_14140	9.7	0.3	28.2	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_6131	26.6	1.0	25.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_492	8.6	0.3	25.2	OTHER	Cytoplasm	Soluble
GXP_Contig_13584	19.4	0.9	22.6	OTHER	Nucleus	Soluble
gi|241633467|ref|XM_002408636.1|	14.5	0.9	16.9	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_16847	50.5	3.1	16.4	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_28121	8.4	0.5	16.4	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_1842	26.6	1.7	15.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_27109	12.2	0.9	14.3	OTHER	Nucleus	Soluble
GXP_Contig_5550	13.3	1.0	12.9	OTHER	Mitochondrion	Soluble
GXP_Contig_24292	14.1	1.2	11.8	OTHER	Nucleus	Soluble
gi|241123951|ref|XM_002403994.1|	29.9	2.6	11.6	OTHER	Mitochondrion	Membrane
GXP_Contig_18990	23.8	2.1	11.6	OTHER	Cytoplasm	Soluble
gi|442761696|gb|GADI01000801.1|	6.0	0.5	11.6	OTHER	Extracellular	Soluble
GXP_Contig_1931	19.8	1.7	11.5	OTHER	Cytoplasm	Soluble

Fed for 24hrs	Transcripts per million		Fold upregulation	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected		SignalP	DeepLoc	Type
GXP_Contig_26204	11.8	1.0	11.4	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_29823	292.2	26.4	11.1	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_26017	17.3	1.7	10.1	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_17308	6.9	0.7	10.0	OTHER	Endoplasmic reticulum	Membrane
gi|604928396|gb|GBBN01023646.1|	5.1	0.5	9.8	OTHER	Extracellular	Soluble
GXP_Contig_21561	15.0	1.7	8.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_32490	21.0	2.4	8.7	OTHER	Extracellular	Soluble
GXP_Contig_8571	8.8	1.0	8.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_15547	78.5	9.3	8.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_10940	23.1	2.7	8.4	OTHER	Nucleus	Soluble
GXP_Contig_14694	11.2	1.4	8.2	OTHER	Cytoplasm	Soluble
GXP_Contig_1328	11.0	1.4	8.0	OTHER	Lysosome/Vacuole	Soluble
GXP_Contig_7109	8.0	1.0	7.7	OTHER	Peroxisome	Membrane
GXP_Contig_2889	21.2	2.7	7.7	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_21622	7.9	1.0	7.6	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_30557	6.5	0.9	7.6	OTHER	Mitochondrion	Membrane
GXP_Contig_8911	27.3	3.6	7.6	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_4595	5.2	0.7	7.5	OTHER	Cytoplasm	Soluble
GXP_Contig_7865	6.4	0.9	7.5	OTHER	Extracellular	Soluble
GXP_Contig_10386	7.4	1.0	7.2	OTHER	Nucleus	Soluble
GXP_Contig_1377	18.1	2.6	7.0	OTHER	Extracellular	Soluble
GXP_Contig_26641	24.0	3.4	7.0	OTHER	Plastid	Membrane
gi|442761556|gb|GADI01000871.1|	7.2	1.0	7.0	OTHER	Extracellular	Soluble
GXP_Contig_9976	5.8	0.9	6.8	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_28830	8.0	1.2	6.6	OTHER	Extracellular	Soluble
gi|442760824|gb|GADI01001237.1|	29.8	4.8	6.2	OTHER	Mitochondrion	Membrane
GXP_Contig_18072	5.6	1.0	5.5	OTHER	Extracellular	Soluble
GXP_Contig_836	6.2	1.2	5.1	OTHER	Mitochondrion	Soluble
GXP_Contig_7213	7.9	1.5	5.1	OTHER	Cytoplasm	Soluble
GXP_Contig_8041	5.2	1.0	5.0	OTHER	Nucleus	Soluble

Fully-fed	Transcripts per million		Fold upregulation	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected		SignalP	DeepLoc	Type
GXP_Contig_18990	36.1	0.2	166.4	OTHER	Cytoplasm	Soluble
GXP_Contig_23096	137.5	0.9	158.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_492	13.7	0.2	63.3	OTHER	Cytoplasm	Soluble
GXP_Contig_21763	11.5	0.2	52.8	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_16847	30.5	1.1	28.2	SP(Sec/SPI)	Extracellular	Soluble
gi|241712765|ref|XM_002413414.1|	15.4	0.7	23.6	OTHER	Mitochondrion	Soluble
GXP_Contig_3268	5.1	0.2	23.4	OTHER	Cytoplasm	Soluble
GXP_Contig_10940	20.2	0.9	23.3	OTHER	Nucleus	Soluble
GXP_Contig_21561	18.9	0.9	21.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_31030	226.0	10.4	21.7	OTHER	Extracellular	Soluble
GXP_Contig_14833	195.4	9.1	21.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_9976	8.5	0.4	19.7	OTHER	Endoplasmic reticulum	Membrane
gi|684282621|gb|GBIH01000469.1|	12.8	0.7	19.6	OTHER	Plastid	Membrane
GXP_Contig_13584	11.8	0.7	18.1	OTHER	Nucleus	Soluble
GXP_Contig_9778	19.0	1.1	17.6	OTHER	Extracellular	Soluble
GXP_Contig_348	7.6	0.4	17.5	OTHER	Nucleus	Soluble
GXP_Contig_26204	13.4	1.1	12.4	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_25185	16.0	1.3	12.3	OTHER	Nucleus	Soluble
gi|241780171|ref|XM_002400047.1|	95.6	8.0	11.9	OTHER	Mitochondrion	Soluble
GXP_Contig_947	5.1	0.4	11.7	SP(Sec/SPI)	Cell membrane	Membrane
GXP_Contig_8165	25.2	2.2	11.6	OTHER	Mitochondrion	Membrane
GXP_Contig_4595	6.7	0.7	10.3	OTHER	Cytoplasm	Soluble
GXP_Contig_476	10.8	1.1	10.0	OTHER	Cytoplasm	Soluble
GXP_Contig_21296	6.5	0.7	10.0	OTHER	Extracellular	Soluble
GXP_Contig_23259	6.4	0.7	9.8	OTHER	Cytoplasm	Soluble
GXP_Contig_1305	167.6	17.1	9.8	OTHER	Cytoplasm	Soluble
GXP_Contig_15228	12.7	1.3	9.7	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_1336	6.2	0.7	9.5	OTHER	Lysosome/Vacuole	Soluble
GXP_Contig_22663	5.5	0.7	8.5	OTHER	Cytoplasm	Soluble
GXP_Contig_16121	5.5	0.7	8.5	OTHER	Nucleus	Soluble
GXP_Contig_30507	9.0	1.1	8.3	OTHER	Extracellular	Soluble

Fully-fed	Transcripts per million		Fold upregulation	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected		SignalP	DeepLoc	Type
GXP_Contig_29696	7.0	0.9	8.1	OTHER	Cell membrane	Membrane
GXP_Contig_29976	6.9	0.9	8.0	OTHER	Cell membrane	Membrane
GXP_Contig_5875	5.1	0.7	7.8	OTHER	Cytoplasm	Soluble
GXP_Contig_16607	210.2	27.9	7.5	OTHER	Nucleus	Soluble
GXP_Contig_5550	9.6	1.3	7.4	OTHER	Mitochondrion	Soluble
GXP_Contig_30201	7.9	1.1	7.3	OTHER	Cell membrane	Membrane
GXP_Contig_20207	71.8	10.2	7.1	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_6657	23.1	3.5	6.7	OTHER	Cytoplasm	Soluble
GXP_Contig_8375	7.0	1.1	6.5	OTHER	Cytoplasm	Soluble
GXP_Contig_13506	5.5	0.9	6.4	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_23087	6.8	1.1	6.3	OTHER	Extracellular	Soluble
GXP_Contig_24914	23.0	3.7	6.3	OTHER	Extracellular	Soluble
GXP_Contig_29628	8.0	1.3	6.2	SP(Sec/SPI)	Nucleus	Soluble
GXP_Contig_27659	9.3	1.5	6.1	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_30156	5.3	0.9	6.1	OTHER	Cytoplasm	Soluble
GXP_Contig_21378	11.6	1.9	5.9	OTHER	Nucleus	Soluble
GXP_Contig_29823	487.6	84.3	5.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_19564	9.8	1.7	5.7	OTHER	Nucleus	Soluble
GXP_Contig_26946	13.5	2.4	5.7	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_32390	8.2	1.5	5.4	OTHER	Mitochondrion	Soluble
GXP_Contig_8981	25.4	4.8	5.3	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_23904	12.5	2.4	5.3	SP(Sec/SPI)	Endoplasmic reticulum	Soluble
GXP_Contig_22867	12.3	2.4	5.2	OTHER	Cytoplasm	Soluble
GXP_Contig_13189	39.0	7.8	5.0	SP(Sec/SPI)	Extracellular	Soluble

Table 6: **(Supplemental Table) A list of *Ixodes ricinus* nymph midgut genes co-upregulated by *Borrelia afzelii* at different timepoints**. n.c. = not calculated, Inf = infected, Uninf = uninfected.

	Transcripts per million						Fold upregulation		
Unfed, 24h, Fully-fed	**Unfed**		**Fed for 24hrs**		**Fully-fed**				
Gene ID (RefSeq)	**Inf**	**Uninf**	**Inf**	**Uninf**	**Inf**	**Uninf**	**Unfed**	**Fed for 24hrs**	**Fully-fed**
GXP_Contig_492	26.0	0.2	8.6	0.3	13.7	0.2	125.7	25.2	63.3
GXP_Contig_13584	19.7	0.8	19.4	0.9	11.8	0.7	23.9	22.6	18.1
GXP_Contig_21561	7.8	0.8	15.0	1.7	18.9	0.9	9.4	8.8	21.8
GXP_Contig_5550	6.3	1.0	13.3	1.0	9.6	1.3	6.1	12.9	7.4
GXP_Contig_10940	18.3	3.5	23.1	2.7	20.2	0.9	5.2	8.4	23.3

	Transcripts per million				Fold upregulation	
Unfed, 24h	**Unfed**		**Fed for 24hrs**			
Gene ID (RefSeq)	**Inf**	**Uninf**	**Inf**	**Uninf**	**Unfed**	**Fed for 24hrs**
GXP_Contig_492	26.0	0.2	8.6	0.3	125.7	25.2
GXP_Contig_13584	19.7	0.8	19.4	0.9	23.9	22.6
GXP_Contig_21561	7.8	0.8	15.0	1.7	9.4	8.8
GXP_Contig_5550	6.3	1.0	13.3	1.0	6.1	12.9
GXP_Contig_1328	31.5	5.6	11.0	1.4	5.6	8.0
GXP_Contig_10940	18.3	3.5	23.1	2.7	5.2	8.4
GXP_Contig_1931	90.2	18.0	19.8	1.7	5.0	11.5

Unfed, Fully-fed	Transcripts per million				Fold upregulation	
	Unfed		Fully-fed			
Gene ID (RefSeq)	Inf	Uninf	Inf	Uninf	Unfed	Fully-fed
GXP_Contig_492	26.0	0.2	13.7	0.2	125.7	63.3
gi|241712765|ref|XM_002413414.1|	5.7	0.2	15.4	0.7	27.4	23.6
GXP_Contig_13584	19.7	0.8	11.8	0.7	23.9	18.1
GXP_Contig_21561	7.8	0.8	18.9	0.9	9.4	21.8
GXP_Contig_5550	6.3	1.0	9.6	1.3	6.1	7.4
GXP_Contig_10940	18.3	3.5	20.2	0.9	5.2	23.3

24h, Fully-fed	Transcripts per million				Fold upregulation	
	Fed for 24hrs		Fully-fed			
Gene ID (RefSeq)	Inf	Uninf	Inf	Uninf	Fed for 24hrs	Fully-fed
GXP_Contig_1305	83.4	2.4	167.6	17.1	34.7	9.8
GXP_Contig_492	8.6	0.3	13.7	0.2	25.2	63.3
GXP_Contig_13584	19.4	0.9	11.8	0.7	22.6	18.1
GXP_Contig_16847	50.5	3.1	30.5	1.1	16.4	28.2
GXP_Contig_5550	13.3	1.0	9.6	1.3	12.9	7.4
GXP_Contig_18990	23.8	2.1	36.1	0.2	11.6	166.4
GXP_Contig_26204	11.8	1.0	13.4	1.1	11.4	12.4
GXP_Contig_29823	292.2	26.4	487.6	84.3	11.1	5.8
GXP_Contig_21561	15.0	1.7	18.9	0.9	8.8	21.8
GXP_Contig_10940	23.1	2.7	20.2	0.9	8.4	23.3
GXP_Contig_4595	5.2	0.7	6.7	0.7	7.5	10.3
GXP_Contig_9976	5.8	0.9	8.5	0.4	6.8	19.7

Table 7: **(Supplemental Table) A list of *Ixodes ricinus* nymph midgut genes downregulated in the presence of *Borrelia afzelii* at three different timepoins of feeding.** n.c. = not calculated.

Unfed	Transcripts per million		Fold	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected	downregulation	SignalP	DeepLoc	Type
GXP_Contig_18754	0.2	18.2	86.6	OTHER	Mitochondrion	Soluble
GXP_Contig_4053	0.2	7.7	36.4	OTHER	Mitochondrion	Soluble
GXP_Contig_31679	0.2	5.6	26.6	OTHER	Extracellular	Soluble
GXP_Contig_18650	1.7	28.3	16.9	OTHER	Mitochondrion	Soluble
GXP_Contig_15199	0.4	5.6	13.3	OTHER	Nucleus	Soluble
GXP_Contig_13657	1.0	13.9	13.2	OTHER	Nucleus	Soluble
GXP_Contig_32014	0.4	5.2	12.3	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_24469	0.8	9.3	11.1	OTHER	Extracellular	Soluble
GXP_Contig_5178	0.6	6.4	10.2	OTHER	Nucleus	Soluble
GXP_Contig_17851	0.8	7.9	9.4	OTHER	Golgi apparatus	Membrane
GXP_Contig_9503	0.6	5.8	9.2	OTHER	Nucleus	Soluble
GXP_Contig_20266	1.3	11.2	8.9	OTHER	Mitochondrion	Soluble
gi|242002135|ref|XM_002435666.1|	1.0	9.1	8.7	OTHER	Mitochondrion	Membrane
gi|241604142|ref|XM_002405772.1|	0.6	5.2	8.2	OTHER	Plastid	Soluble
GXP_Contig_9269	0.8	6.8	8.1	SP(Sec/SPI)	Cytoplasm	Soluble
GXP_Contig_12704	1.3	10.1	8.0	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_32885	2.9	21.9	7.5	OTHER	Nucleus	Soluble
GXP_Contig_17177	1.0	7.7	7.3	OTHER	Nucleus	Soluble
GXP_Contig_26180	1.3	8.7	6.9	OTHER	Nucleus	Soluble
GXP_Contig_8549	1.9	13.0	6.9	OTHER	Cytoplasm	Soluble
GXP_Contig_23374	2.7	18.4	6.7	OTHER	Mitochondrion	Membrane
GXP_Contig_14484	1.3	8.5	6.7	OTHER	Mitochondrion	Soluble
GXP_Contig_17696	0.8	5.6	6.7	OTHER	Lysosome/Vacuole	Membrane
gi|604926323|gb|GBBN01025717.1|	0.8	5.6	6.7	OTHER	n.a.	n.a.
GXP_Contig_19798	2.1	13.2	6.3	OTHER	Plastid	Soluble

Unfed	Transcripts per million		Fold	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected	downregulation	SignalP	DeepLoc	Type
GXP_Contig_5735	1.5	9.1	6.2	OTHER	Cytoplasm	Soluble
GXP_Contig_6305	1.0	6.4	6.1	OTHER	Cytoplasm	Soluble
GXP_Contig_31957	1.0	6.2	5.9	OTHER	Nucleus	Soluble
GXP_Contig_6121	1.9	11.0	5.8	OTHER	Cytoplasm	Soluble
GXP_Contig_5149	1.3	7.2	5.7	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_4181	2.9	16.5	5.6	OTHER	Cytoplasm	Soluble
GXP_Contig_30736	1.0	5.8	5.5	OTHER	Cytoplasm	Soluble
GXP_Contig_31917	1.3	6.8	5.4	OTHER	Cytoplasm	Soluble
GXP_Contig_9046	2.5	13.6	5.4	OTHER	Cell membrane	Membrane
GXP_Contig_5757	4.8	25.0	5.2	OTHER	Mitochondrion	Soluble
GXP_Contig_7628	1.7	8.7	5.2	OTHER	Plastid	Membrane
GXP_Contig_11744	7.1	36.6	5.1	OTHER	Cytoplasm	Soluble
GXP_Contig_19796	2.1	10.8	5.1	OTHER	Mitochondrion	Soluble

Fed for 24hrs	Transcripts per million		Fold	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected	downregulation	SignalP	DeepLoc	Type
GXP_Contig_1294	0.1	10.8	96.5	OTHER	Cytoplasm	Soluble
GXP_Contig_28871	0.1	6.5	58.2	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_7016	0.4	14.9	33.3	OTHER	Extracellular	Soluble
GXP_Contig_22325	0.2	6.5	29.0	OTHER	Nucleus	Soluble
GXP_Contig_17851	0.3	9.3	27.5	OTHER	Golgi apparatus	Membrane
GXP_Contig_3315	0.3	8.1	23.9	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_17077	0.2	5.3	23.6	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_634	0.3	7.7	22.9	OTHER	Cytoplasm	Soluble
GXP_Contig_18449	0.6	12.5	22.3	OTHER	Mitochondrion	Membrane

Fed for 24hrs	Transcripts per million		Fold	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected	downregulation	SignalP	DeepLoc	Type
GXP_Contig_27415	0.3	7.4	21.9	OTHER	Mitochondrion	Soluble
GXP_Contig_3620	0.4	8.8	19.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_20839	0.3	6.4	18.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_15080	0.3	5.7	16.8	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_12893	0.4	6.2	13.8	OTHER	Extracellular	Soluble
GXP_Contig_30746	0.8	10.1	12.9	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_15345	0.6	6.9	12.2	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_6295	1.9	23.3	12.2	OTHER	Cytoplasm	Soluble
GXP_Contig_30729	2.9	29.0	9.9	OTHER	Cytoplasm	Soluble
GXP_Contig_22296	0.7	6.5	9.7	OTHER	Mitochondrion	Soluble
GXP_Contig_29730	0.9	8.6	9.6	OTHER	Plastid	Soluble
GXP_Contig_7304	0.7	6.0	8.9	OTHER	Nucleus	Membrane
GXP_Contig_25934	3.1	26.4	8.4	OTHER	Extracellular	Soluble
gi|242002135|ref|XM_002435666.1|	2.9	23.7	8.1	OTHER	Mitochondrion	Membrane
GXP_Contig_30474	1.3	10.1	7.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_4156	2.2	14.4	6.4	SP(Sec/SPI)	Cytoplasm	Soluble
GXP_Contig_17314	11.5	70.9	6.2	OTHER	Endoplasmic reticulum	Membrane
GXP_Contig_994	2.5	14.2	5.8	OTHER	Cytoplasm	Soluble
gi|604927107|gb|GBBN01024934.1|	1.0	5.5	5.4	n.a.	n.a.	n.a.
GXP_Contig_30475	1.8	9.4	5.3	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_17086	1.7	8.8	5.2	OTHER	Nucleus	Soluble
GXP_Contig_22307	2.7	13.7	5.1	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_13761	2.8	14.2	5.1	OTHER	Cytoplasm	Soluble
GXP_Contig_14484	1.2	6.2	5.0	OTHER	Mitochondrion	Soluble

Fully-fed	Transcripts per million		Fold	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected	downregulation	SignalP	DeepLoc	Type
gi|442756582|gb|GADI01003358.1|	0.9	47.0	54.3	OTHER	Mitochondrion	Soluble
gi|604928396|gb|GBBN01023646.1|	0.3	16.0	49.5	n.a.	n.a.	n.a.
GXP_Contig_17101	1.7	32.9	19.0	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_15650	0.5	9.3	17.2	OTHER	Cytoplasm	Soluble
GXP_Contig_2019	0.8	11.7	15.5	OTHER	Nucleus	Soluble
GXP_Contig_31364	1.4	16.7	11.9	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_21098	2.4	27.5	11.6	OTHER	Cytoplasm	Soluble
GXP_Contig_11561	1.2	13.2	11.1	SP(Sec/SPI)	Cell membrane	Membrane
GXP_Contig_16844	0.5	5.4	10.0	OTHER	Mitochondrion	Membrane
GXP_Contig_14827	35.1	341.1	9.7	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_12704	1.7	16.5	9.5	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_15492	0.9	7.4	8.5	OTHER	Nucleus	Soluble
GXP_Contig_25640	0.6	5.4	8.3	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_8527	0.8	6.3	8.3	OTHER	Mitochondrion	Soluble
GXP_Contig_8849	3.8	30.8	8.1	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_26802	0.9	6.7	7.8	OTHER	Extracellular	Soluble
GXP_Contig_22558	1.0	7.4	7.6	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_26822	0.8	5.6	7.4	SP(Sec/SPI)	Peroxisome	Membrane
gi|241574685|ref|XM_002403355.1|	1.8	12.8	7.0	OTHER	Extracellular	Soluble
gi|242002135|ref|XM_002435666.1|	1.1	7.4	6.8	SP(Sec/SPI)	Mitochondrion	Membrane
GXP_Contig_11904	0.9	5.8	6.8	OTHER	Plastid	Soluble
GXP_Contig_17042	20.2	133.2	6.6	OTHER	Extracellular	Soluble
GXP_Contig_12220	5.7	36.6	6.4	SP(Sec/SPI)	Extracellular	Soluble
GXP_Contig_3718	1.2	7.4	6.2	OTHER	Endoplasmic reticulum	Soluble
GXP_Contig_11182	853.4	4888.6	5.7	OTHER	Cell membrane	Membrane
GXP_Contig_25202	14.6	81.4	5.6	OTHER	Cell membrane	Membrane

Fully-fed	Transcripts per million		Fold	In-silico peptide prediction		
Gene ID (RefSeq)	Infected	Uninfected	downregulation	SignalP	DeepLoc	Type
GXP_Contig_12260	1.0	5.4	5.6	OTHER	Cytoplasm	Soluble
GXP_Contig_26842	1.2	6.5	5.5	OTHER	Mitochondrion	Soluble
GXP_Contig_15225	2.8	14.9	5.3	OTHER	Extracellular	Soluble
GXP_Contig_1745	2.1	10.8	5.3	OTHER	Cytoplasm	Soluble

Table 8: **(Supplemental Table) list of *Ixodes ricinus* nymph midgut genes co-downregulated by *Borrelia afzelii* at different timepoints**. n.c. = not calculated, Inf = infected, Uninf = uninfected

	Transcripts per million						Fold downregulation		
Unfed, 24h, Fully-fed	**Unfed**		**Fed for 24hrs**		**Fully-fed**				
Gene ID (RefSeq)	**Inf**	**Uninf**	**Inf**	**Uninf**	**Inf**	**Uninf**	**Unfed**	**Fed for 24hrs**	**Fully-fed**
gi|242002135|ref|XM_002435666.1|	1.0	9.1	2.9	23.7	1.1	7.4	8.7	8.1	6.8

	Transcripts per million				Fold downregulation	
Unfed, 24h	**Unfed**		**Fed for 24hrs**			
Gene ID (RefSeq)	**Inf**	**Uninf**	**Inf**	**Uninf**	**Unfed**	**Fed for 24hrs**
GXP_Contig_17851	0.8	7.9	0.3	9.3	9.4	27.5
gi|242002135|ref|XM_002435666.1|	1.0	9.1	2.9	23.7	8.7	8.1
GXP_Contig_14484	1.3	8.5	1.2	6.2	6.7	5.0

	Transcripts per million				Fold downregulation	
Unfed, Fully-fed	**Unfed**		**Fully-fed**			
Gene ID (RefSeq)	**Inf**	**Uninf**	**Inf**	**Uninf**	**Unfed**	**Fully-fed**
gi|242002135|ref|XM_002435666.1|	1.0	9.1	1.1	7.4	8.7	6.8
GXP_Contig_12704	1.3	10.1	1.7	16.5	8.0	9.5

	Transcripts per million				Fold downregulation	
24h, Fully-fed	**Fed for 24hrs**		**Fully-fed**			
Gene ID (RefSeq)	**Inf**	**Uninf**	**Inf**	**Uninf**	**Fed for 24hrs**	**Fully-fed**
gi|242002135|ref|XM_002435666.1|	2.9	23.7	1.1	7.4	8.1	6.8

7. Article: 2

Trentelman, J. J. A. *et al.* (2020) 'A combined transcriptomic approach to identify candidates for an anti-tick vaccine blocking B. afzelii transmission', *Scientific Reports*. Nature Research, 10(1). doi: 10.1038/s41598-020-76268-y.

7.1 Introduction:

To understand the feeding biology of ticks and the transmission of tick-borne pathogens, we must first describe the composition of tick saliva and the role of salivary proteins in the infection process. Such attempts would assist in the description of pharmacologically important compounds that may act as protective antigens in the development of anti-tick vaccines or agents whose cognate antibody responses represent serological biomarkers for exposure to ticks. Initial proteomic studies that addressed the composition of tick saliva were reported in the first decade of the twenty-first century (Francischetti *et al.*, 2008; Madden, Sauer and Dillwith, 2004; Narasimhan *et al.*, 2007). Transcript and protein profiling in the tick salivary gland have subsequently been applied to describe different tick feeding and developmental stages, sex differences and to highlight interspecies differences. Comparative analyses of tick salivary glands have decoded changes in molecular expression according to tick sex, life stage or behavior (Díaz-Martín *et al.*, 2013; Ribeiro *et al.*, 2006; Anatriello *et al.*, 2010), as well as according to the presence of parasites such as bacteria, protozoa and viruses (Liu and Bonnet, 2014). It is important to note that only a small number of salivary proteins have been functionally annotated, with putative function being verified for fewer than 5% of salivary proteins (Francischetti, Sa-Nunes, *et al.*, 2009). These studies have led to the discovery of multiple vital factors that contribute to successful tick feeding and facilitate the evasion of pathogen transmission by protecting the host immune and haemostatic defenses.

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A combined transcriptomic approach to identify candidates for an anti-tick vaccine blocking *B. afzelii* transmission

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Ixodes ricinus **is the vector for** ***Borrelia afzelii*****, the predominant cause of Lyme borreliosis in Europe, whereas** ***Ixodes scapularis*** **is the vector for** ***Borrelia burgdorferi*** **in the USA. Transcription of several** ***I. scapularis*** **genes changes in the presence of** ***B. burgdorferi*** **and contributes to successful infection. To what extend** ***B. afzelii*** **influences gene expression in** ***I. ricinus*** **salivary glands is largely unknown. Therefore, we measured expression of uninfected vs. infected tick salivary gland genes during tick feeding using Massive Analysis of cDNA Ends (MACE) and RNAseq, quantifying 26.179 unique transcripts. While tick feeding was the main differentiator,** ***B. afzelii*** **infection significantly affected expression of hundreds of transcripts, including 465 transcripts after 24 h of tick feeding. Validation of the top-20** ***B. afzelii*****-upregulated transcripts at 24 h of tick feeding in ten biological genetic distinct replicates showed that expression varied extensively. Three transcripts could be validated, a basic tail protein, a lipocalin and an ixodegrin, and might be involved in** ***B. afzelii*** **transmission. However, vaccination with recombinant forms of these proteins only marginally altered** ***B. afzelii*** **infection in** ***I. ricinus*****-challenged mice for one of the proteins. Collectively, our data show that identification of tick salivary genes upregulated in the presence of pathogens could serve to identify potential pathogen-blocking vaccine candidates.**

Ixodes ticks are small parasitic arthropods that feed on the blood of vertebrate hosts. They are three host-ticks; their lifecycle consists of four life stages, egg, larva, nymph and adult, where the latter three each parasitizes different hosts. Ticks needs to feed on blood of their hosts to obtain the nutrients and energy to develop into their next life stage or for successful reproduction. They do so by penetrating the skin of their host with their hypostome and, depending on the life stage, stay attached for 3–10 days to complete their blood meal. This feeding behavior presents a large window of opportunity for tick-borne pathogens to be transmitted to the host. Ticks are therefore only second to mosquitoes as the most important arthropod vectors for human disease. In contrast to the USA where *Ixodes scapularis* is the tick species most notorious for human disease[1], in Europe, *Ixodes ricinus* is the tick that most affects human health[2]. *I. ricinus* is a vector for viruses, bacteria and protozoan parasites, and as such can cause a wide range of diseases, including tick-borne encephalitis, relapsing fever, anaplasmosis, babesiosis and most notably Lyme borreliosis.

Lyme borreliosis, also referred to as Lyme disease, is the most prevalent *I. ricinus*-borne disease; in Europe alone, over 65,000 cases of Lyme borreliosis are reported every year and some expect it to be 2–3 times higher due to underreporting[3]. Lyme borreliosis is caused by bacteria belonging to *Borrelia burgdorferi* sensu lato (s.l.) group and in Europe, *Borrelia afzelii* has the highest incidence rate. In humans, it is associated with (chronic)

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	R1 Reads	R2 Reads	Total Reads
Uninfected I. ricinus nymphs	37,960,637	37,960,637	75,921,274
B. afzelii-infected I. ricinus nymphs	40,784,777	40,784,777	81,569,554
B. afzelii-infected I. ricinus whole body	44,465,827	44,465,827	88,931,654
Uninfected I. ricinus whole body	41,339,909	41,339,909	82,688,620
Total	164,555,551	164,555,551	329,111,102

Table 1. Summary of RNA sequencing reads after cleaning.

cutaneous manifestations of Lyme borreliosis[4]. *B. afzelii* is acquired by the larval tick during its first blood meal, it can survive in the tick to later life stages and can be transmitted with each following blood meal. However, given their smaller size nymphal ticks are less easy to be identified (visually and sensationally) than adult ticks and they are therefore considered to be the most clinically relevant life stage with regard to human disease[5]. It is commonly accepted that *B. burgdorferi* s.l. transmission starts approximately 16–36 h after attachment of the tick, transmission of *B. afzelii* starts earlier than *B. burgdorferi* sensu stricto (s.s.)[6,7]. In spite of this, it has been shown in a mouse model that *B. afzelii*-infected ticks need to feed for longer than 24 h to establish infection. *B. afzelii* is presumably transmitted through the saliva of the feeding tick, although alternative routes of infection have been proposed[8].

The saliva of the tick is crucial for the long period of attachment and the successful completion of the blood meal. It contains proteins that interfere with host defense mechanisms through for instance immunosuppressive, anticomplement or antihemostatic roles. Indeed, animals repeatedly infested with ticks have antibodies against tick saliva and display so-called tick-immunity; ticks are less able to feed and/or are rejected[9–11]. As the host defense mechanisms are also essential to prevent and contain infection, these tick salivary gland proteins (TSGPs) greatly increase the odds of successful infection of the vertebrate host by *B. burgdorferi* s.l.-infected ticks, as it has been shown most notably for *I. scapularis*[12–16]. As a consequence, anti-tick immunity also protects against *B. burgdorferi* s.l. infection via tick bites and it has been shown that this anti-tick immunity can be transferred by serum[17–20]. These observations show the potential of anti-tick vaccines, by targeting tick proteins, specifically TSGPs, one could prevent tick feeding and/or pathogen transmission. Neutralization of specific TSGPs by antibodies indeed reduced *B. burgdorferi* s.s. infection *in vivo*[18,19]. As with all biological processes, the expression, translation and secretion of TSGPs is a dynamic process. The expression of these TSGPs is highly upregulated during the tick feeding process[21–24], but it is also known that infection with *B. burgdorferi* s.s. induces alterations in gene expression that contribute to the successful infection of the host[13–15,25–29]. Based on their properties, TSGPs can be divided in large multi-gene families that have distinct functionalities, as reviewed before[30]. These multi-gene families are thought to be the result of gene duplication early in evolution[31].

We used a combined transcriptomic approach to gain insight into the transcriptional changes within the salivary glands of *I. ricinus* during the complex interplay between the tick, the host and the pathogen. The respective strengths of both Massive Analysis of cDNA Ends (MACE) and RNAseq were combined to identify tick transcripts and subsequent processes influenced by *B. afzelii*. Gene expression in salivary glands of *I. ricinus* nymphs in different stages of feeding (unfed, 24 h and fully fed) and in different states of infection with regards to *B. afzelii* (infected and uninfected) were analyzed and characterized to identify specific TSGPs upregulated in *B. afzelii*-infected salivary glands. TSGPs upregulated in *B. afzelii*-infected salivary glands and those biologically validated were tested as *B. afzelii*-blocking anti-tick vaccines.

Results

RNA sequencing. In order to obtain long sequences to serve as our own framework for the annotation of the ensuing MACE analyses, RNA was prepared simultaneously for the construction of both the MACE and RNA sequencing libraries. Salivary gland and whole body RNA was isolated from *B. afzelii* CB43-infected *I. ricinus* nymphs and uninfected *I. ricinus* nymphs from the same parental lineage fed for 0, 24 or 72 h. RNA was pooled for all time points of *B. afzelii*-infected salivary glands, uninfected salivary glands, *B. afzelii*-infected whole body and uninfected whole body tick samples to obtain four cDNA libraries for RNAseq. The resulting cDNA libraries were used for paired-end sequencing and resulted in a total of 329,111,102 reads (Table 1) to be used for analysis, after elimination of duplicates and quality trimming. From these reads, 32,897 high quality contigs could be assembled. These formed our Master Reference exome (Master Reference) for the MACE analyses and represent an unprecedented source of *I. ricinus* sequence information.

MACE analysis. MACE was chosen as a quantitative tool as sequencing of the polyA captured cDNA molecules will result in one short read per molecule. In contrast, with RNAseq, one cDNA molecule will result in multiple reads. As such, MACE is excellent for detailed quantification of gene expression and has proven to be able to identify even low expressed genes[32,33]. RNA from salivary glands extracted at each time point (unfed, 24 h fed and fully fed) of *B. afzelii* CB43-infected (ISG) or uninfected (NISG) were used to prepare a total of six cDNA libraries for MACE. A total of 74.651.134 sequencing reads were processed (Table 2) and mapped against our Master Reference or assembled de novo and subsequently annotated against SwissProt, Trembl and the NCBI nucleotide database. Annotation resulted in the identification of a total of 93,096 unique transcripts of which on average 250–500 bp were covered by the MACE reads. We focused on transcripts that had more than one

Library	Reads
ISG0h	12,954,933
ISG24h	15,916,749
ISGFF	12,701,261
NISG0h	12,449,936
NISG24h	9,553,944
NISGFF	11,074,311
Total	74,651,134

Table 2. Summary of MACE reads after cleaning.

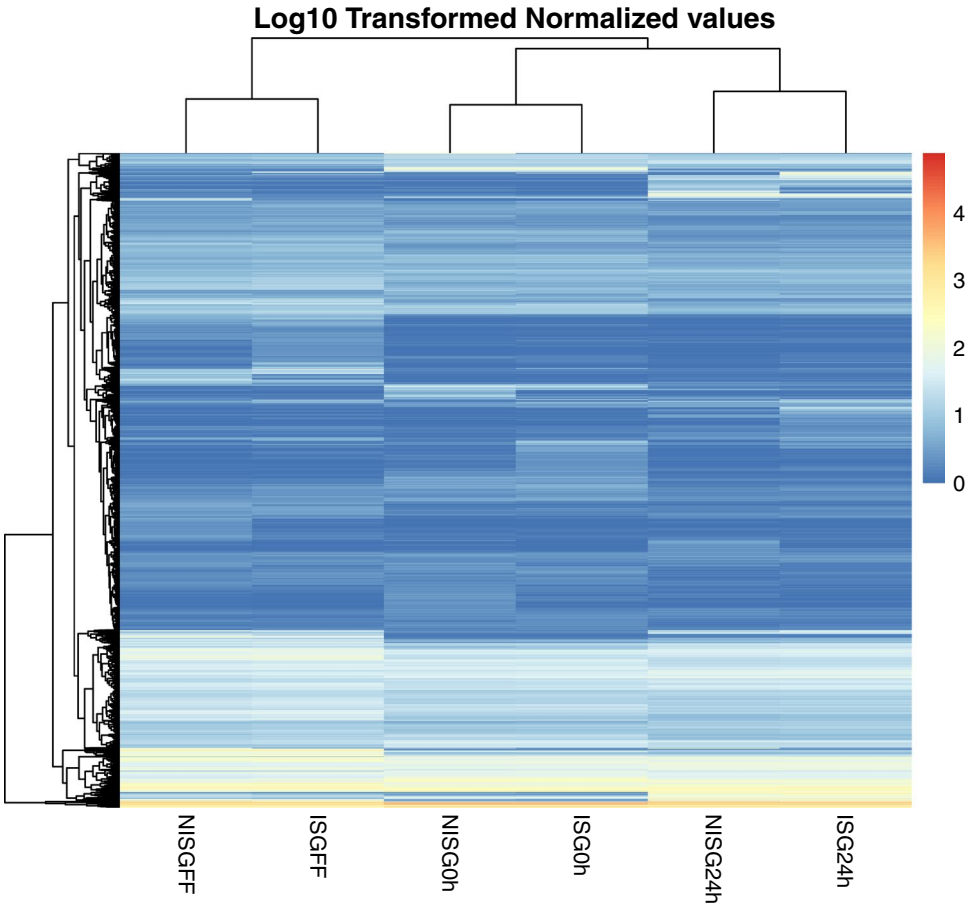


Figure 1. Unsupervised hierarchial cluster analyses of gene expression. Heatmap of log 10 transformed normalized reads illustrating gene expression of nymphal *I. ricinus* uninfected salivary glands (NISG) and *B. afzelii*-infected salivary glands (ISG) that were unfed (0 h), fed for 24 h (24 h) or fully engorged (FF). Each condition is represented in a single column. Gene expression is illustrated by color code, the color scale ranges from blue for low normalized reads to red for very high normalized reads.

normalized read (read per million reads) in at least one of the MACE libraries to reduce the background signals. As a result, the number of transcripts used for further analysis was 26,179 unique transcripts.

Differential gene expression. An unsupervised hierarchical clustering of all transcripts was performed (Fig. 1). From this analysis, it becomes clear that salivary gland gene expression was mostly affected by the stage of the feeding process. Differences in gene expression were most pronounced between early time points, 0 and 24 h fed, versus 72 h fed tick salivary glands (1790 and 1665 differentially expressed transcripts, respectively). Transcripts were considered to be differentially expressed when the change in gene expression was 4 times lower or 4 times higher, the corresponding p value $< 1^{-50}$. Although gene expression was largely driven by the feeding status of the ticks, *B. afzelii* infection also altered gene expression (Fig. 2); in *B. afzelii*-infected unfed salivary glands (ISG0), 60 transcripts were upregulated and 110 transcripts were downregulated. In 72 h-fed *B. afzelii*-

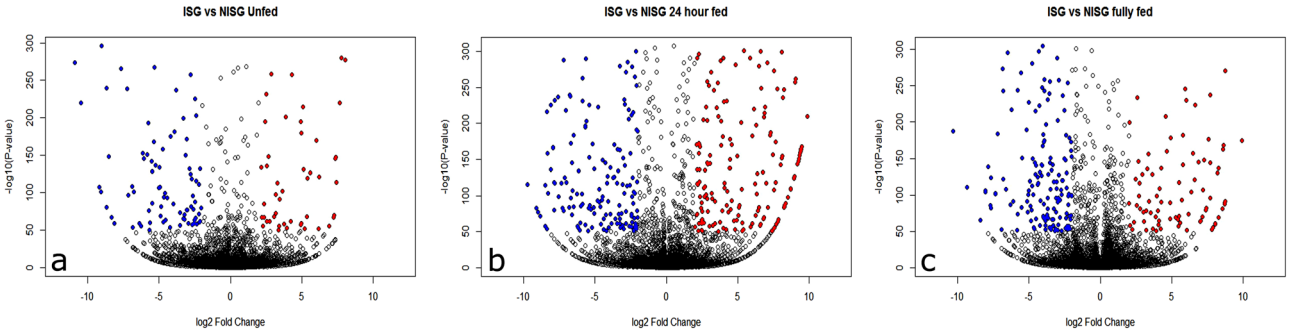


Figure 2. Volcano plot of comparison in salivary gland gene expression upon *B. afzelii* infection. (**a**) Relative gene expression in ISG vs NISG at 0 h. (**b**) Relative gene expression in ISG vs NISG at 24 h. (**c**) Relative gene expression in fully fed ISG vs NISG. Red dots are significantly upregulated genes, blue dots are significantly down regulated genes (log_2fold < −2 or > 2 and the corresponding p value < 1^{-50}).

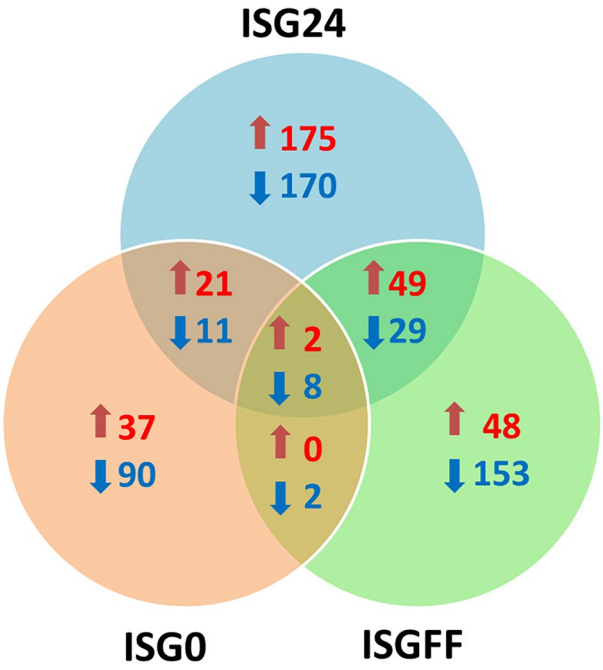


Figure 3. VENN diagram depicting differentially expressed tick salivary transcripts upon *B. afzelii infection* and their behaviour through time. Transcripts that are differentially up-regulated (> 2log2fold change in normalized reads, p < 1e−50) are depicted in red. Transcripts that are differentially down-regulated (> −2log2fold change in normalized reads, p < 1e−50) are depicted in blue.

infected salivary glands (ISGFF) 99 transcripts were upregulated, while 192 were downregulated. Interestingly, most transcripts were differentially expressed upon 24 h feeding in *B. afzelii*-infected salivary glands (ISG24); 247 transcripts were upregulated and 218 were downregulated. Only a fraction of the genes were upregulated or downregulated at all time points (Fig. 3). Overall, *B. afzelii* infection influenced the expression of 795 unique salivary gland transcripts (> 2log2fold change or < -2log2fold change, $p < 1e^{-50}$); 332 unique transcripts were up-regulated in one or more time points, whereas 463 unique transcripts were down-regulated in one or more time points. Interestingly, most transcripts that were affected by infection in a single time point only, were differentially expressed at 24 h of tick feeding (345 genes; 175 upregulated, 170 downregulated).

Thus, although differential gene expression in *I. ricinus* salivary glands was mostly driven by tick feeding, *B. afzelii* also influenced gene expression in *I. ricinus* salivary glands, and mostly at 24 h of tick feeding.

Characterization of *B. afzelii*-induced differentially expressed tick salivary gland genes. To provide more insight into the possible biological functions of the differentially expressed tick salivary gland transcripts, these were assigned to known tick protein families. To this end, the corresponding contigs were aligned (blastx) to contigs of a previously described *I. ricinus* bioproject[23], in which genes were eloquently assigned to different families of tick proteins. Our contigs that had a match with contigs from the previously described

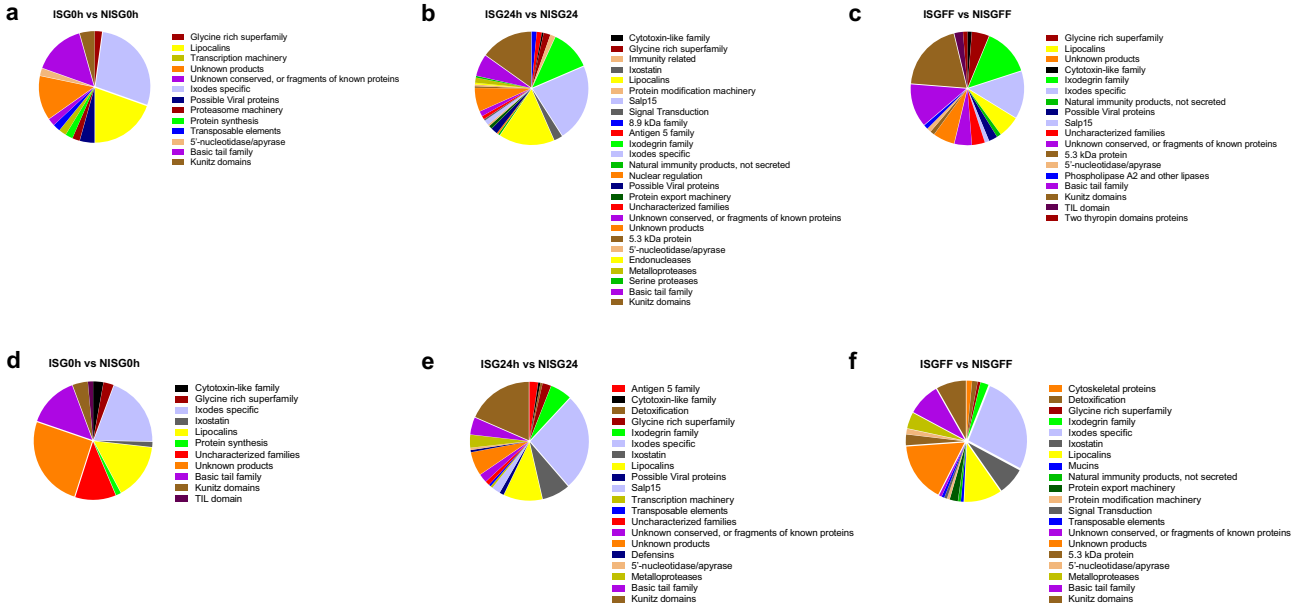


Figure 4. Distribution of differentially expressed transcripts over tick protein families for each time point. (**a**, **b**, **c**) Distribution of up-regulated transcripts (>2 log2 fold change, $p < 1^{-50}$) over tick protein families in *B. afzelii*-infected salivary glands of unfed (ISG0h vs NISG0h), 24 h (ISG24h vs NISG24h) and 72 h (ISGFF vs NISGFF) fed nymphs respectively. (**d**, **e**, **f**) Distribution of down-regulated transcripts (< −2 log2 fold change, $p < 1^{-50}$) over tick protein families in *B. afzelii*-infected salivary glands of unfed (ISG0h vs NISG0h), 24 h (ISG24h vs NISG24h) and 72 h (ISGFF vs NISGFF) fed nymphs respectively.

bioproject, with an Expect value below 0.00001, were assigned to the respective tick protein family[23]. Using this strategy, 81% of the differentially expressed transcripts could be annotated to a tick protein family.

The functional annotation was limited to the main classes, only the classes of enzymes, antimicrobial peptides and protease inhibitor domains were divided into subclasses. Transcripts belonging to the glycine-rich superfamily, lipocalins, *Ixodes* specific family, and kunitz domain inhibitor family accounted for most of the transcripts upregulated by *B. afzelii* infection at any given time point (Fig. 4). Some tick protein families were only upregulated in ISG24h; most notably those related to immunity (1.46% of the upregulated transcripts at 24 h), ixostatin (2.44%), signal transduction related transcripts (0.49%), 8,9 kDa family (1.46%), antigen 5 family (1.46%), protein export machinery (0.98%), protein modification machinery (0.49%), metalloproteases (1.46%) and serine proteases (0.49%). Other families were upregulated at both ISG24h and ISGFF, those time points at which the tick is feeding and transmission of *B. afzelii* is taking place. Among these upregulated transcripts, members of the ixodegrin family (11.71% and 13.75% respectively) and Salp15 family (1.46% and 1.25% respectively) members were observed. In addition, although a few transcripts were upregulated in ISG0h (0.35% of upregulated transcripts), a marked increase of upregulated transcripts belonging to the kunitz domain inhibitor family were observed in ISG24h (15.12%) and ISGFF (20%) as well. Most of the transcripts upregulated at ISG24h belonged to the kunitz domain inhibitor (15.12%), ixodegrins (11.71%), Ixodes specific (22.44%) and lipocalin (16.10%) families.

Regarding the transcripts downregulated in infected salivary glands, the families affected at all time points were the glycine rich superfamily, *Ixodes* specific, lipocalins, kunitz domain families and transcripts that are considered as unknown products (with no homology to known sequences). Downregulated only in ISG24 were transcripts belonging to the Antigen 5 family (2.38% of the downregulated transcripts), Salp15 (2.38%), defensins (0.6%) and transcription machinery (0.6%).

Next to transcripts that were present in both uninfected and infected salivary glands, some transcripts could exclusively be detected in infected salivary glands, of which those only expressed in ISG24h are depicted in Supplemental Fig. 1. These transcripts were associated with the TIL—(Trypsin Inhibitor like cysteine rich) domain (4.17% of the transcripts only expressed in ISG24h compared to NISG24h), lipocalin (12.5%), Salp15 (4.17%), ixodegrin (16.67%), *Ixodes* specific (16.67%), ixostatin (8.33%) and, particularly, Kunitz domain families (33.33%).

Overall, *B. afzelii* was shown to affect *I. ricinus* salivary gland expression of transcripts encoding proteins belonging to multiple tick proteins families. Interestingly, we observed unique expression, as well as up-regulation and down-regulation, of transcripts within certain tick protein families, most notably *Ixodes* specific, lipocalins, basic tail protein, ixodegrin, kunitz domain inhibitor and ixostatin tick protein families.

Selection of vaccine candidates; technical and biological validation. Tick salivary transcripts upregulated upon infection with *B. afzelii* might be important for transmission of *B. afzelii* and/or subsequent successful infection of the vertebrate host. Therefore, proteins encoded by transcripts that were highly upregulated in ISG24 were considered as potential candidates for a *Borrelia* transmission blocking vaccine. Significantly

a

Gene ID	Protein family	reads ISG24	reads NISG24	log2FoldChange reads	Technical validation	Biological validation	Upregulation in number of biological samples	Average log2fold increase of biological samples
Gene 1	***Basic tail family***	***5174,66***	***12,90***	***8,65***	***Passed***	***Failed***	***6/10***	***4,46***
Gene 2	***Lipocalins***	***2234,62***	***299,36***	***2,90***	***Passed***	***Passed***	***8/10***	***5,09***
Gene 3	Basic tail family	1787,10	3,09	9,17	Passed	Failed	1/10	-1,34
Gene 4	Ixodegrin family	1560,53	45,63	5,10	Passed	Failed	1/10	-2,41
Gene 5	Basic tail family	1544,10	4,16	8,54	Passed	Failed	6/10	3,55
Gene 6	***Lipocalins***	***1364,60***	***73,24***	***4,22***	***Passed***	***Passed***	***7/10***	***5,58***
Gene 7	Lipocalins	1190,87	90,51	3,72	Passed	Failed	4/10	-2,41
Gene 8	Ixodes specific P32 family	1071,89	45,63	4,55	Passed	Failed	4/10	-1,38
Gene 9	Ixodes specific WC family	571,22	1,07	9,07	Passed	Failed	2/10	-4,20
Gene 10	Antigen 5 family	547,49	2,45	7,80	Passed	Failed	0/10	N/A
Gene 11	Nuclear regulation	546,79	62,58	3,13	Passed	Failed	4/10	-0,84
Gene 12	Ixodes 8-cys family	543,97	0,96	9,15	Passed	Failed	5/10	3,76
Gene 13	***Ixodegrin family***	***509,24***	***31,02***	***4,04***	***Passed***	***Passed***	***8/10***	***7,50***
Gene 14	Ixodes specific WC family	497,15	0,64	9,60	Passed	Failed	2/10	2,18
Gene 15	Lipocalins	102,03	2,35	5,44	Passed	Failed	1/10	1,98
Gene 16	Kunitz domain	186,14	0,75	7,96	Passed	Failed	1/10	-0,59
Gene 17	Basic tail family	102,60	14,29	2,84	Failed	Failed	0/10	N/A
Gene 18	Lipocalins	461,27	76,55	2,59	Passed	Failed	5/10	3,42
Gene 19	Unknown	250,94	1,71	7,20	Passed	Failed	1/10	-1,64
Gene 20	Ixodes specific WC family	532,14	7,57	6,14	Failed	Failed	0/10	N/A

b

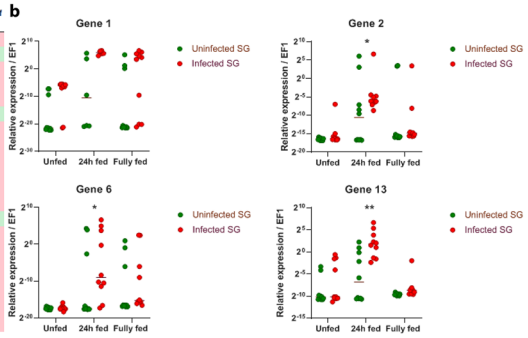


Figure 5. Technical and biological validation of top 20 vaccine candidates. (**a**) Top 20 genes highly upregulated in infected tick salivary glands at 24 h after onset feeding, (> 2 log2 fold change ISG24h vs NISG24h, $p < 1 \times 10^{-50}$) were considered potential *Borrelia* transmission blocking vaccine candidates. Top 20 was ranked based on expression levels in infected salivary glands at 24 h as determined by MACE. Of the 10 biologically distinct tick pools used for biological validation, the number of pools that showed upregulation of the respective transcript are indicated as well as the average log2fold difference in all 10 tick pools. (**b**) Gene expression profiles of biologically validated Genes 1, 2, 6 and 13 in the salivary glands of 10 biologically distinct tick pools as determined by RT-qPCR. Elongation factor 1 alpha was used as a reference gene. Lines indicated median expression values. Significantly upregulated transcripts are indicated by * (Friedman test paired analysis, Dunn's multiple comparison $p < 0.05$).

upregulated genes (> 2 $\log_2$, $p < 1 \times 10^{-50}$) were ranked based on expression levels determined by MACE and the 20 most abundantly expressed transcripts were selected for technical and biological validation. Primers were designed based on the nucleotide sequence identified by MACE and qRT-PCR was performed on the cDNA used for MACE (technical validation) or cDNA from tick pools derived from 10 genetically distinct ticks (biological validation). Technical validation showed that expression levels determined by qRT-PCR could confirm the MACE results for nearly all transcripts (Fig. 5), underscoring the robustness and accuracy of our approach. However, biological validation using cDNA from 10 genetically distinct tick pools showed marked variability in gene expression of the selected transcripts. Of the 20 selected transcripts, 3 genes were significantly upregulated in *B. afzelii*-infected tick salivary glands in most of the 10 genetically distinct tick pools; Gene 2, Gene 6 and Gene 13 (Fig. 5). In silico analysis showed that Gene 6 and Gene 2 are in fact highly similar; their sequence analysis showed 86% similarity at the amino acid level and Gene 6 appears to have a deletion compared to Gene 2. All 3 significantly upregulated transcripts encode a signal sequence and are likely to encode secreted proteins. Although not significantly upregulated at 24 h after the onset of feeding in the biological validation, Gene 1 was considered to be an interesting candidate. Gene 1 was only detected in 6 out of 10 tick pools, but in these tick pools Gene 1 was highly expressed upon infection at 24 h (Fig. 5). In addition, Gene 1 also encoded a signal sequence and showed a high degree of homology to basic tail proteins, although there were no conserved domains that might indicate possible functions of the encoded protein. Gene 2 and 6 were putative lipocalins and contain predicted histamine binding domains. Gene 13 was classified as a putative ixodegrin, containing a prokineticin domain and was part of the colipase-like superfamily. As Gene 2 and 6 were highly similar, Gene 1, 2 and 13 were selected for cloning and recombinant protein production in *E. coli*. For the selected targets the amino acid sequence, the predicted protein model, conserved domains and other characteristics are shown in Table 3.

Transmission and vaccination studies. Preliminary RNAi studies, with successful knock down of Gene1, 2, 6 and 13, in *B. afzelii*-infected nymphs fed on a small number of mice (n = 3), did not show a significant reduction of tick feeding or *B. afzelii* infection (Supplemental Fig. 2). This result indicated that the absence of transcripts by itself was not enough to affect *B. afzelii* transmission. We next focused on vaccination studies where antibody–antigen interactions and complexes can lead to multiple effector mechanism that can block transmission. To this end, mice were vaccinated with recombinant proteins of Gene 1, 2, 13 or a combination of these antigens and subsequently challenged with *B. afzelii*-infected nymphal ticks. Vaccination was shown to be successful; antigen-specific total IgG levels could be detected after vaccination (Supplemental Fig. 3), although antibody levels against recombinant Gene 2 were significantly lower as compared to the other antigens (Mann–Whitney test, $p < 0.05$). Vaccination with recombinant Gene 1 significantly reduced the number of infected mice tissues as determined by qPCR and although the number of mouse tissues infected as determined by culture was also lower, this effect was not significant nor was there a difference in the cumulative number of mice that were infected (Chi-square, $p < 0.05$) (Table 4). For all other experimental groups, including the cocktail vaccination, no significant differences were observed in the spirochetal loads of the tissues nor in the number of infected mice (Supplemental Fig. 3).

Discussion

To our knowledge, this is the first time that the relationship between *B. afzelii* and nymphal *I. ricinus* on the total transcript level of salivary glands is studied. In the current study, two different gene quantification tools have been combined to provide an unprecedented insight into the transcriptome of *I. ricinus* salivary glands. RNAseq

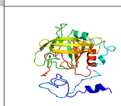
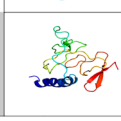
	Amino acid sequence	Predicted protein structure	Protein family	Domain	Signal peptide	N - glycosylation sites	O - glycosylation sites	Transmembrane helices	GPI anchor	MHC I binding	MHC II binding	Linear B cell epitopes
Gene 1	MMGLTGITLVLSLAIFFGSVAADDCRNGTRPSSQNDREGCDPICWNESTRSYDCQYFFTDGVECFYNNGDKGLCQNGECHLTTDTRVPSNHDGDTPVTTKKPKQKKKPKPKKSHGKSKKE		Basic tail protein	None	Yes, AA1:22	1	4	1 AA1-22, signal peptide	No	6 high binding, 15 low binding peptides (mouse)	7 high binding, 23 low binding peptides (mouse)	5 predicted epitopes (average 16AA)
Gene 2	MKLVLSLAIFVCGVFLETYGSTSTTTRPLGNSGSTTTTKPKGTPSARMMTTVPPEEDPSKYKEQNATRVIEMNNATQWVKWWTYNVTDLSSDSLQCEFRFVMKKITPTNYSLQYRYRSGNWVNTINETLVLENLGPHGSSNDMYFQSRTPHVGPTPHNLVCYSNYMNCTVLRIPMPNQGGRHCDLWANNLTLSQETPDDCLNKFEYCNTTQIYRVFYPNCTNENMHFSLV		Lipocalin	Histamine binding	Yes, AA1:20	7	12	0	No	44 high binding, 112 low binding peptides (mouse)	14 high binding, 118 low binding peptides (mouse)	10 predicted epitopes (average 9AA)
Gene 13	MKTFCLALALTVIVTALLADVTAGFIGQVPVLPPGVVAPPPRDVCDSCSAYAKCKNGTCCLQSRSRSGFGYSAICKPLGLIKGEECSVAPTKGDDYHGHCPCSAGLTCRDIKNNRHYCVPRK		Ixodegrin	Prokinectin	Yes, AA1:23	1	3	1 AA1-28, signal peptide	No	8 high binding, 31 low binding peptides (mouse)	24 low binding proteins (mouse)	2 predicted epitopes (average 46AA)

Table 3. In silico analysis of validated transcripts selected for vaccination studies. Amino acid sequences encoded on the transcripts Gene 1, 2 and 13 as determined by the ExPASy Translate tool. Protein structures were predicted using Phyre2 web portal and although confidence in the predicted model was low for Gene 1 (32% of residues modelled at > 90% confidence, 55% of the sequence is predicted disordered), confidence in the predicted model was good for Gene 2 and 13 (73% and 67% of residues modelled at > 90% confidence respectively. Proteins sequences were subsequently scanned for domains with InterProScan. Signal peptide, O- and N-glycosylation sites were predicted based on amino acid sequence by SignalP 5.0 server, NetOGlyc 4.0 Server and NetNGlyc 1.0 server, respectively. HTHMM v2.0 server was used to predict transmembrane helices and GPI-SOM to predict GPI-anchor. MHC class I and II binding peptides were predicted using NetMHCpan-4.1 and NetMHCIIpan-4.0 and linear B cell epitopes with BepiPred Linear Epitope Prediction 2.0. Columns colored in red are negative and those in blue are positive, while the others are neutral.

	Skin[1]	Bladder[1]	Skin[2]	Bladder[2]	Heart[2]	Joint[2]	Cumulative
PBS	5/6	4/6	6/6	6/6	6/6	5/6	6/6
Recombinant Gene 1	3/6	3/6	2/6*	2/6*	2/6*	2/6*	5/6
Recombinant Gene 2	4/6	4/6	4/6	4/6	4/6	4/6	4/6
Recombinant Gene 13	4/6	4/6	4/6	4/6	4/6	5/6	5/6
Recombinant Gene 1 + Gene 2 + Gene 13	5/6	4/6	4/6	4/6	5/6	5/6	6/6

Table 4. Number of *Borrelia*-infected mice as determined for each organ. *Borrelia* infection as determined by culture[1] or qPCR[2] and shown as number of positive mice/total mice. Cumulative infection was calculated as the number of mice that were positive in at least one of the organs either by culture or qPCR. Significance was calculated compared to the PBS groups and significant differences are indicated by * (Chi-square, $p = 0.04$).

is a powerful technique to obtain accurate and qualitative sequence information of transcripts, but fragmentation of the RNA molecules and sequencing of all fragments could lead to a bias in the quantification of longer transcripts[34]. MACE, on the other hand, only targets sequences from the 3′ end of the sequence by capturing the RNA fragment containing the pol-A tail. As a result, sequence information might be partial (i.e. not providing sequence information of the whole gene sequence), but it provides a high resolution gene expression analysis, even revealing differential expression of low-abundant transcripts, which are beyond the scope of RNAseq or microarrays[35]. In addition, the TrueQuant method increases the reliability of quantification by eliminating PCR bias[36]. By combining RNAseq and MACE, the complete sequence information provided by RNAseq results in increased mapping accuracy of MACE reads, strengthening the highly accurate quantification by MACE. RNAseq analysis was performed using pooled RNA from nymphal *I. ricinus* salivary gland and whole bodies fed for different time points (0, 24 or fully fed), with or without *B. afzelii* infection, resulting in 32,897 high-quality contigs, which is similar to or higher than the number of transcripts reported by previous RNAseq projects[37–39]. MACE resulted in the quantification of 26.179 transcripts selected for further analysis. Technical validation by qRT-PCR using the MACE cDNA libraries, to determine the expression profiles of the 20 most abundantly expressed *B. afzelii*-induced *I. ricinus* salivary glands transcripts, corroborated the MACE expression profiles and clearly validated our findings.

As described previously, our results confirm that the feeding process greatly affected gene expression in tick salivary glands[37–39]. Although the feeding process is the main differentiator of gene expression, MACE analysis showed that the expression of hundreds of transcripts is significantly affected by *B. afzelii* infection. This could have multiple underlying mechanisms that are not mutually exclusive; firstly, the transcripts could be part of the tick immune response to *Borrelia* infection. Secondly, the expression could be altered by *Borrelia* infection to increase survival in the tick. Thirdly, the transcripts could be affected by *Borrelia* infection to increase transmission through saliva and infectivity in the mammalian host. Interestingly, only a few transcripts were upregulated in *B. afzelii*-infected salivary glands of unfed ticks. This fits the general assumption that there are few to none spirochete in the salivary glands at this time point as has been observed for *B. burgdorferi s.s.* as they are located in the midgut and still have to migrate to the salivary glands upon onset of feeding[40]. In addition, the expression

of hardly any transcript is affected in all three time points (0, 24 and FF), making it unlikely that the identified upregulated transcripts are involved in the tick immune response against *B. afzelii*. Most of the *B. afzelii*-induced differentially expressed transcripts were observed 24 h after onset of tick feeding. This coincides with the time point that *B. burgdorferi* s.l. is thought to have found its way into the tick saliva and starts to be transmitted to the host[7]. Indeed, transmission experiments using the same experimental model that we have previously used, has shown that removal of *B. afzelii*-infected ticks after 24 h of tick feeding blocks successful infection of the host[41–43].

As described above, it is known that certain *I. scapularis* TSGPs are upregulated upon *B. burgdorferi* infection and that some of these proteins are to be beneficial for the transmission success of the spirochete. However, this is the first study to investigate whether and to what extend *B. afzelii* influences gene expression in the salivary glands of nymphal *I. ricinus* ticks. With 465 transcripts differentially expressed at 24 h after onset feeding, the MACE analysis indicates that *B. afzelii* infection has an extensive effect on gene expression. The majority of transcripts upregulated in *Borrelia*-infected SG at 24 h belong to the kunitz domain inhibitor, ixodegrins, *Ixodes* specific and lipocalin protein families. Kunitz domain inhibitors are one of the largest families of secreted salivary gland proteins. These proteins have one or multiple kunitz domains that inhibit activity of specific proteases, most of which are involved in the coagulation pathway[30,44]. Ixodegrins are cysteine rich proteins that have a RGD or KGD domain, which can bind to integrins; transmembrane receptors that mediate cell–cell and cell–extracellular matrix interactions and as such can have multiple functions. Ixodegrins can block the interaction of integrins with their other ligands and block downstream processes. For instance, binding of ixodegrins to αIIbβ3 of activated platelets, prevents fibrinogen–platelet interaction and platelet aggregation[45]. Integrins, as they are transmembrane receptors, are also involved in immunity. For instance, macrophage 1 antigen, more recently known as complement receptor 3 (CR3), is an integrin present on polymorphonuclear leukocytes and binds fibrinogen which leads to macrophage adhesion and activation. Interestingly, CR3 interacts with uPAR, which has been shown to be important for the clearance of *Borrelia* and CR3 also binds *Borrelia* directly to polymorphonuclear leukocytes[28,46–49]. As the ligands of most ixodegrins are currently unknown, it might be possible that some could protect *Borrelia* from the host's immune response. Ixodes specific protein family comprises several smaller families and only some members of the Isac protein subfamily have been characterized; these tick proteins interfere with the complement cascade[50–52]. The complement cascade is an important line of defense against *B. burgdorferi* s.l.. Although sensitivity for complement-mediated killing varies between *B. burgdorferi* s.l. genospecies and *B. afzelii* is particular complement resistant, complement leads to opsonophagocytosis of *B. burgdorferi* by immune cells and in antibody-dependent complement-mediated killing[53,54]. Thus, it is possible that the proteins upregulated in *B. afzelii*-infected *I. ricinus* salivary glands at 24 h after feeding facilitate both *B. afzelii* transmission from the tick to the host or successful infection of the host. However, as transcripts belonging to the same protein family are both upregulated and downregulated, the characterization of the majority of proteins in each protein family is poor or non-existent, and the same TSGP can exert multiple functions, it is difficult to appreciate the exact biological role of the different families of tick proteins in *B. afzelii* transmission or infection.

One of the aims of this study was to identify possible pathogen transmission blocking anti-tick vaccine targets. Previous studies have shown that antibodies against *I. scapularis* TSGPs not only interfere with tick feeding, but antibodies induced after 24 h of tick feeding could also partially protect against *B. burgdorferi* infection[11,17–19,55]. In search for potential vaccine targets to block *B. afzelii* transmission by *I. ricinus*, the 20 most abundantly expressed transcripts upregulated in *B. afzelii*-infected *I. ricinus* salivary glands at 24 h after onset feeding were validated in 10 biological and genetically distinct replicates. Three transcripts—encoding 2 unique proteins—were significantly upregulated in *B. afzelii*-infected salivary glands across the 10 biological samples; Gene 2, Gene 6 and Gene 13. Despite the fact that we, in line with previously published tick transcriptome studies[21,23,24], pooled salivary glands of hundreds of ticks to obtain enough RNA for both RNAseq and MACE, we were only able to biologically validate three out of the 20 selected abundantly-expressed *I. ricinus* transcripts. This suggests that there is substantial biological variation, either in transcript sequence or expression and underscores that it is critical to consider this variation, especially when selecting vaccine candidates. Ideal vaccine candidates are highly conserved and expressed in multiple biological replicates, which is the case for the three selected transcripts. Gene 2 and 6 proved to encode the same protein, a putative lipocalin with predicted histamine binding domains. Lipocalins are one of the largest and most diverse protein families in ticks. Despite their diversity in amino acid sequence, they all have a barrel structure that creates a fold and facilitates the binding of hydrophobic ligands. The targets of lipocalins are as diverse as the protein family itself; lipocalins can target inflammation, acquired immunity and the complement system. As Gene 2 and 6 have histamine binding domains, they appear to belong to the first category. Histamine release by host cells induce inflammation at the tick bite site; hence, Gene 2 and 6 could inhibit inflammation by binding histamine. Gene 13 is characterized as a putative Ixodegrin, has a prokineticin domain and is part of the colipase-like superfamily. As described above, Ixodegrins are cysteine-rich proteins and although the function for most of these proteins is unknown, there are some that act as antiplatelet inhibitors and they might affect innate immunity[30,46,47,56,57]. In addition, although overall not significantly upregulated in *B. afzelii*-infected salivary glands at 24 h, Gene 1 was highly expressed in the tick pools in which the transcript could be detected and therefore evaluated as a vaccine candidate. In silico analysis showed that Gene 1 showed a high degree of homology to basic tail proteins and has a very basic carboxy terminus or tail, which is one of the key features of this protein family. It is thought that the basic tail might help binding to anionic phospholipids expressed at the surface of activated platelets and mast cells and can interfere in the functioning of the subsequent host processes[30,58]. Indeed, several TSGPs belonging to the basic tail protein family have been described to interfere with complement or coagulation (TSLPI, Salp14, Ixonnexin and Salp-9pac)[15,16,44,59–61]. These processes have been proven to be important for tick feeding and *B. burgdorferi* survival in the host[15,16,61]. Although there are no conserved domains to directly pinpoint possible effector functions of Gene 1, the presence of a basic tail and upregulation in *B. afzelii*-infected salivary glands indicate that this TSGP could facilitate *B. afzelii* transmission or survival in the mammalian host. Thus, in silico analysis indicated that

the three selected targets could very well be involved in the manipulation of the host defense mechanisms that are essential for tick feeding and/or survival of *B. afzelii* in the host. However, a preliminary RNAi experiment, in which the four validated transcripts encoding the three targets were successfully silenced, did not reveal an essential role for the identified TSGPs in tick feeding or *B. afzelii* transmission.

In line with these findings, vaccination with recombinant forms of Gene 2 or 13 did not reduce tick feeding nor *B. afzelii* transmission to the host after challenge with *B. afzelii*-infected *I. ricinus* nymphs, compared to control mice. In silico analysis showed that all antigens are predicted to have peptides that can bind to MHC class I and class II, and are linear epitopes for B cells. Vaccination indeed did induce high antibody titers for recombinant Gene 13, confirming immunogenicity. For recombinant Gene 2, antibody levels are relatively low despite predicted immunogenicity and although the purified antigen seem to contain *E. coli* residue that could interfere with the immune response, we consider it unlikely that these trace amounts of contaminants have interfered with the antigen specific immune response (Supplemental Fig. 3). It is therefore unclear what explains the modest antibody titers. Interestingly, vaccination with recombinant Gene 1 significantly induced a robust antibody response and reduced the number of infected tissues in mice as determined by qPCR. However, vaccination with Gene 1 did not protect against infection; the cumulative number of infected mice as determined by qPCR and culture was similar for recombinant Gene 1 vaccinated and control animals. This modest effect was not observed in mice vaccinated with all three antigens. This might be explained by interference of the other two antigens with the immune response against recombinant Gene 1 upon vaccination or tick-challenge. In general, other vaccination platforms or different ways of producing the tick antigens as recombinant proteins might lead to improved vaccine efficacy. Indeed, a recent publication showed the importance of glycosylation of tick saliva proteins in tick immunity against *I. scapularis*[9]. Therefore, one could argue that the fact that we produced the selected tick antigens in an *E. coli* expression system, and the resulting absence of posttranslational modifications such as glycosylation, are responsible for the low observed vaccine efficacy. Whether vaccination with Gene 1 produced in an Eukaryotic expression system would increase vaccine efficiency remains to be investigated.

To conclude, in this study, using two independent next generation sequencing techniques, we clearly show that *B. afzelii* affects *I. ricinus* salivary glands gene expression during tick feeding, and that the uniquely expressed, as well as up- and downregulated tick transcripts upon *B. afzelii* infection encode proteins belonging to the same tick protein families. Four transcripts encoding three different proteins were shown to be robustly upregulated in *B. afzelii*-infected *I. ricinus* salivary glands at 24 h. Of these three proteins, only recombinant Gene 1 altered *B. afzelii* infection when tested as a transmission blocking anti-tick vaccine in the current set-up and although it did not prevent infection, it could still be an interesting antigen for further optimization, for example as part of a multivalent vaccine or produced in a different expression system. In addition, future research could focus on determining the function of these proteins in either the tick or the host.

Material and methods

Infection of ticks with *Borrelia afzelii*, tick feeding and RNA extraction. *I. ricinus* ticks were obtained from the BC ASCR tick colony and were free of *Borrelia*, *Babesia*, and *Anaplasma,* as determined by PCR[62,63]. To obtain non-infected and *B. afzelii*-infected ticks, clean *I. ricinus* larvae—a mixture of the offspring from three individual adult females—were fed on naive or *B. afzelii* strain CB43 syringe-inoculated 6–8 weeks old C3H/HeN mice (Charles River Laboratories, Sulzfeld, Germany). Larvae were collected and allowed to molt to nymphs in a climate chamber with a humidity of about 95%, temperature 24 °C and day/night period set to 15/9 h. Infection rates for infected ticks were assessed by qPCR and ticks were used when infection rates were higher than 90%. Resulting non-infected and *B. afzelii*-infected ticks (4 to 6 weeks after molting) were fed for 0 h (220 nymphs per infection state, 440 total), 24 h (180 nymphs, 380 total) or to repletion (150, 300 total) on naive 6–8 weeks old C3H/HeN mice and dissected under a dissection microscope. Salivary glands were collected and total (small and large) RNA was extracted using a NucleoSpin miRNA kit (MACHEREY–NAGEL, Dürren, Germany) according to the manufacturer's instructions and stored at − 80 °C until further use. All tick and animal experiments were approved by the BC ASCR animal ethical committee (Animal protection laws of the Czech Republic No. 246/1992 Sb., Ethics approval No. 79/2013). All experiments were performed in accordance with relevant guidelines and regulations.

RNA sequencing. For RNA sequencing, we created four separate RNA-Seq libraries; infected salivary glands (RNA from 550 *B. afzelii*-infected nymphs at 0, 24 h and fully fed were pooled), uninfected salivary glands (RNA from 550 uninfected nymphs at 0, 24 h and fully fed were pooled), 45 *B. afzelii*-infected whole body fully fed nymphs and 45 uninfected whole body fully fed. The RNAseq libraries were generated using the "NEBNextUltra directional RNA-Seq" (NEB, Ispawich, USA) protocol, as described by the manufacturer and based on the method previously published[64]. In short, mRNA was captured from 5 µg of total RNA using Oligo dT(25) beads. The purified mRNA was randomly fragmented in a Zn^{2+} solution and first strand synthesis was performed using random hexamers. Second strand synthesis was performed using a dNTP mixture in which dTTP was exchanged with dUTP and P5-P7-Y-adapers were ligated. The second strand was eliminated prior to PCR using dUTPase. Subsequently, a PCR was performed using 14 cycles. The final products were analyzed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and product sizes ranged from 200 to 800 bp, with a major peak at 450 bp. Finally, the products were sequenced on an Illumina HiSeq2000 machine (Illumina, Inc., San Diego, CA, USA) using 2 × 100 bp. Overlapping sequencing reads were de novo assembled into GXP_Contigs with TrinityRNAseq (Version: v2.2.0[65]). Further assembly output refining resulted in 32,897 high quality Contigs used as a reference database. The obtained sequences were uploaded to GenBank (Bioproject PRJNA657487).

MACE analysis. Essentially, MACE analysis was performed as previously described (Nold-Petry et al. Mueller et al.) using the GenXPro MACE kit (GenXPro, Frankfurt am Main, Germany) and according to the manufacturer's protocol. Briefly, 1 µg of obtained large and small tick salivary gland RNA from 550 *B. afzelii* CB43-infected or 550 non-infected ticks fed for 0, 24 or approximately 72 h (fully fed) were subjected to an additional DNAse treatment to remove all DNA. Quality was assessed on an Agilent 2100 Bioanalyzer and no or only negligible degradation products were observed. Next, first and second strand cDNA synthesis was performed starting from biotinylated oligo dT primers. The cDNA was fragmented randomly by ultrasonication resulting in fragments with an average size of 300 bps as determined by an Agilent 2100 Bioanalyzer. The biotinylated 3′ cDNA ends were bound to a streptavidin matrix and all other fragments were eliminated through washing. To the unbound end of the fragments, a p5 "TrueQuant" sequencing adapter included in the MACE kit was ligated and a PCR was performed, using tailed Illumina p5 and p7 oligonucleotides as primers, in order to obtain a library of fragments suitable for Next Generation Sequencing on an Illumina Hiseq2000 machine. The Quality of the final library was determined using an Agilent 2100 bioanalyzer. Single end sequencing of the products produced the sequence-information of the 5′ side of the bound cDNA fragment. To remove PCR-bias, all duplicate reads detected by the in house TrueQuant technology were removed from the raw datasets. In addition, low quality sequence nucleotides and poly(A)-tails were clipped off using cutadapt[66]. The reads were thereafter aligned to different reference sequences using Novoalign (Novocraft Technologies, Selangor, Malaysia).The main reference for the Novoalign alignment was the outcome of the RNASeq de novo assembly, described in the RNASeq section. Additionally a de novo assembly of MACE sequences that could not be mapped to sequences from the Master Reference (RNASeq) using TrinityRNAseq (Version: v2.2.0 [65]) was performed. Subsequently, the contigs of the assemblies, "Master Reference" and "noHitAssembly" were annotated further by BLASTX to first the SwissProt and hereafter Trembl database "Arachnida" proteins[67]. Additional blastn analyses were performed for all Contigs against all "Ixodes" mRNA sequences available at the NCBI database, nucleotide collection from GenBank (RefSeq, TPA and PDB), *Ixodes scapularis* genome (PRJNA314100), *Ixodes ricinus* genome (PRJNA270959) and against sequences from a previous published *I. ricinus* salivary gland transcriptome[23] submitted to Genbank (PRJNA177622). The e-value threshold for BLASTX and BLASTN was 0.00001. Only uniquely mapped reads were accepted for quantification of the MACE tags. Finally, the expression was normalized and tested for differential gene expression between the different conditions using the DEGSeq R/Bioconductor package[67]. Only transcripts with at least 1 normalized read in one of the libraries were used for analysis (Supplemental file 1).

Allocation of genes to tick protein families. For more functional insight, the transcripts were allocated to tick protein families based on sequence homology. In short, gene sequences of the Master Reference were aligned to proteins from a previous bioproject[39] using blastx. Transcripts were considered to belong to a specific tick protein family if the e-value with their respective protein hit from the Bioproject Number PRJNA177622 was below 0.0001.

Technical and biological validation. An aliquot of total RNA from each time point analyzed by MACE was used to make cDNAs (Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland)) for qRT-PCR technical validation of the MACE results. For biological validations, *Borrelia afzelii*-infected (Infection rates were assessed by qPCR and ticks were used when infection rates were higher than 90%) and uninfected nymphal *I. ricinus* ticks derived from 10 distinct egg batches laid by adult female ticks collected from the wild, were fed on mice for different time points. RNA was isolated from the salivary glands and subsequent cDNA was prepared for the individual time points. Then, gene-specific primers appropriate for unambiguous PCR confirmation of gene expression in *Borrelia*-infected nymphs at the time interval 24 h and the genes upregulated by feeding, were designed using Primer3 software (Supplemental Table 1). qRT-PCR was used to evaluate expression of the selected genes in technical and biological samples.

In silico analysis. The encoded protein sequence for Gene 1, 2 and 13 were determined from the transcripts nucleotide sequences using the ExPASy translate tool[68]. Proteins sequences were subsequently scanned for domains with InterProScan[69] and a predicted protein model was built using the Phyre2 web portal[70]. Signal peptide, O- and N-glycosylation sites were predicted based on amino acid sequence by SignalP 5.0 server[71], NetOGlyc 4.0 Server[72] and NetNGlyc 1.0 server[73], respectively. HTHMM v2.0 server[74] was used to predict transmembrane helices and GPI-SOM to predict GPI-anchor[75]. MHC class I and II binding peptides were predicted using NetMHCpan-4.1[76] and NetMHCIIpan-4.0[76] and linear B cell epitopes with BepiPred Linear Epitope Prediction 2.0[77].

Expression and purification of recombinant proteins. Transcripts were cloned by overlapping PCR from previously designed artificial genes and cloned as NcoI-SalI fragments into the pHIS-parallel 2 expression vector[78]. For Gene 1 forward primer (FW) CGCCATGGGAGACGATTGCAGAAACGGAACTAGA and reverse primer (RV) CGGTCGACTAGTACGTTTTCCCTTCCTTAATTATTTTCTGTG was used. For Gene 2 CGCCATGGGATCTACAAGTACTACTACCCATCCAGTG (FW) and CGGTCGACTACACCAAGGAAA AGTGCATATTCTCGTT (RV) and for Gene 13 CGCCATGGGACAGGTACCAGTGTTTCCCCCTGG (FW) and CGGTCGACTATTTCCTTGGCACGCAAATATGTCTG (RV) were used. Clones were induced with 1 mM Isopropyl-β-D-thiogalactoside (IPTG) for 16 h at 20 °C in E. coli BL21 C41(DE3). The bacterial cells were then lysed and centrifuged. The expressed insoluble proteins were extracted from the inclusion bodies with the following protocol. The pellets were thoroughly homogenized in Phosphate Buffered Saline (PBS); 2% Triton X-100 followed by an incubation at 37 °C for 30 min with shaking. The samples were ultracentrifuged at 96,000 g for

30 min and the pellets were homogenized again in PBS and incubated at 37 °C for 30 min with shaking. After a second ultracentrifugation, the pellets were homogenized in PBS; 7 M urea. The denatured proteins were dialyzed to 2 M urea overnight.

Preliminary RNA interference study. Silencing of the gene candidates by RNA interference (Genes 1, 2, and 13) was done as described previously (1). The Borrelia-infected nymphs were injected with 0.32 nl of dsRNA, rested for three days, and fed on C3H mice (5 nymphs per mouse, infection rate of ticks > 90%). The level of silencing was checked by qRT-PCR on a mix of five fully-fed nymphs per group and compared to the GFP control; expression of gene 3 was reduced by 92%, expression of gene 2 was reduced by 98%, expression of gene 13 was reduced by 99% and for gene 1 expression was reduced by 68% (primers can be found in Supplemental Table 1). The mice were screened for infection by qRT-PCR in a skin, heart, and urinary bladder, as described below.

Vaccination-transmission studies and infection parameters. Pathogen-free C3H/HeN mice (Charles River Laboratories) were used for the vaccination transmission experiments. Six mice per group were vaccinated with either PBS, recombinant Gene 1, Gene 2, Gene 13 or all three recombinant proteins injected subcutaneously at different sites. 20 µg of antigen was emulsified in Complete Freund's Adjuvant (Sigma-Aldrich, St. Louis, MO, USA) to 100 µl total volume for prime vaccination at day 0. For booster vaccinations at day 14 and 28, 20 µg of antigen were emulsified in Incomplete Freund's Adjuvant, 100 µl total volume. 2 weeks after the last vaccination, mice were challenged with 5 *B. afzelii*-infected *I. ricinus* nymphs (infection rate > 90%) which were allowed to feed to repletion. Before each vaccination and the tick challenge, mouse blood was collected. 3 weeks after tick infestation, mice were sacrificed and organs were collected for culture and qPCR. Half of the mouse bladder and a part of the tick bite site were cultured in BSK medium (Amsterdam UMC, AMC, The Netherlands).

Total spirochete load in mouse tissues was determined by qPCR, which targeted a fragment of the *flagellin* gene (154 bp). DNA was isolated from individual murine tissues (ear, skin, heart, and urinary bladder) using a NucleoSpin tissue kit (Macherey–Nagel) according to the manufacturer's protocol. The reaction mixture contained 12.5 µl of FastStart universal probe master (Rox) (Roche), 10 pmol of primers FlaF1A and FlaR1, 5 pmol of TaqMan probe Fla Probe1[8,8], 5 µl of DNA, and PCR water up to 25 µl. Quantification of murine β-actin was performed using MmAct-F and MmAct-R primers and a MmAct-P TaqMan probe[14]. The following amplification program was run on a LightCycler 480 (Roche) for both targets: 95 °C for 10 min, 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The spirochete burden in murine tissues was expressed as the number of spirochetes per 10^5 murine β-actin copies.

Antibody responses. Total antigen-specific IgG levels were determined by ELISA. ELISA plates (Thermo Scientific) were coated with full length proteins at 0.05 µg/well in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After washing with PBS, containing 0.05% Tween 20, the plates were incubated with blocking buffer (10% of fetal calf serum (FCS, Biowest) in PBS) for 1 h. Mouse sera were added at 1:5600 dilution and incubated for 2 h at room temperature. After washing, goat anti-mouse total IgG conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch) was added (1:1000 dilution) in blocking buffer and incubated for 1 h at room temperature. The plates were then extensively washed and incubated with KPL SureBlue substrate. The reaction was stopped with 2N H_2SO_4. Absorbance (450 nm) was immediately measured using a BioTek Synergy HT multi-detection microplate reader.

Received: 17 April 2020; Accepted: 17 September 2020
Published online: 18 November 2020

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Acknowledgements

This project has received funding from the European Union's Seventh Programme for research, technological development and demonstration under Grant Agreement No. 602272, of which JWH was the scientific coordinator. OH, RS, VU and PK were also supported from two additional sources: Czech Science Foundation Grant No. 20-05736S and Centre for Research of Pathogenicity and Virulence of Parasites (No. CZ.02.1.01/0.0/0.0/16_019/0000759), funded by the European Regional Development Fund (ERDF) and Ministry of Education, Youth, and Sport, Czech Republic (MEYS). CIC bioGUNE thanks the Spanish ministry of Science and Innovation (MCI) for the Severo Ochoa Excellence accreditation (SEV-2016-0644). Diego Barriales is the recipient of a MCI FPI fellowship. We thank Estibaliz Atondo, Leticia Sampedro, Jasmin Ersoz and Miguel Angel Pascual-Itoiz for their excellent technical assistance.

Author contributions

J.W.H. was the scientific coordinator. N.K. and P.W. designed and performed RNAseq and MACE sequencing and annotation. H.S., K.T., J.M.B., N.K. and J.J.A.T. were involved in transcriptome analysis. V.U., R.S., O.H., S.M. and P.K. performed tick dissections, validations and RNAi-transmission experiments. R.S., O.H., P.K., M.J.K., J.T.C., D.B., J.A., J.J.A.T. and J.W.R. were involved in vaccination-transmission experiments. R.S., .O.H., P.W., P.K., J.A., N.K., J.J.A.T. and J.W.H. designed the experiments. J.J.A.T. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-76268-y.

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7.2 Supplementary materials (Article 2):

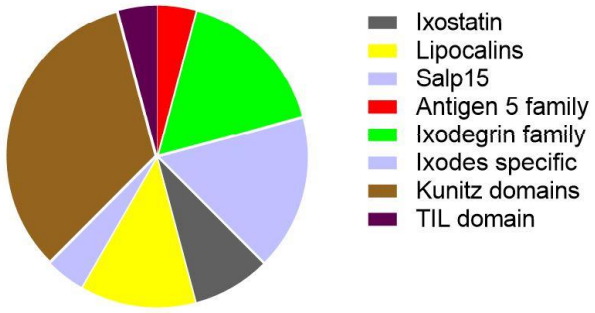

Figure 8: **(Supplemental Figure) Distribution of transcripts only expressed in 24 hours fed *B. afzelii* infected salivary glands over tick protein families.** Distribution of transcripts up-regulated in *B. afzelii* infected salivary glands at 24h only (>2 log2 fold change ISG24h vs NISG24h, $p<1^{-50}$).

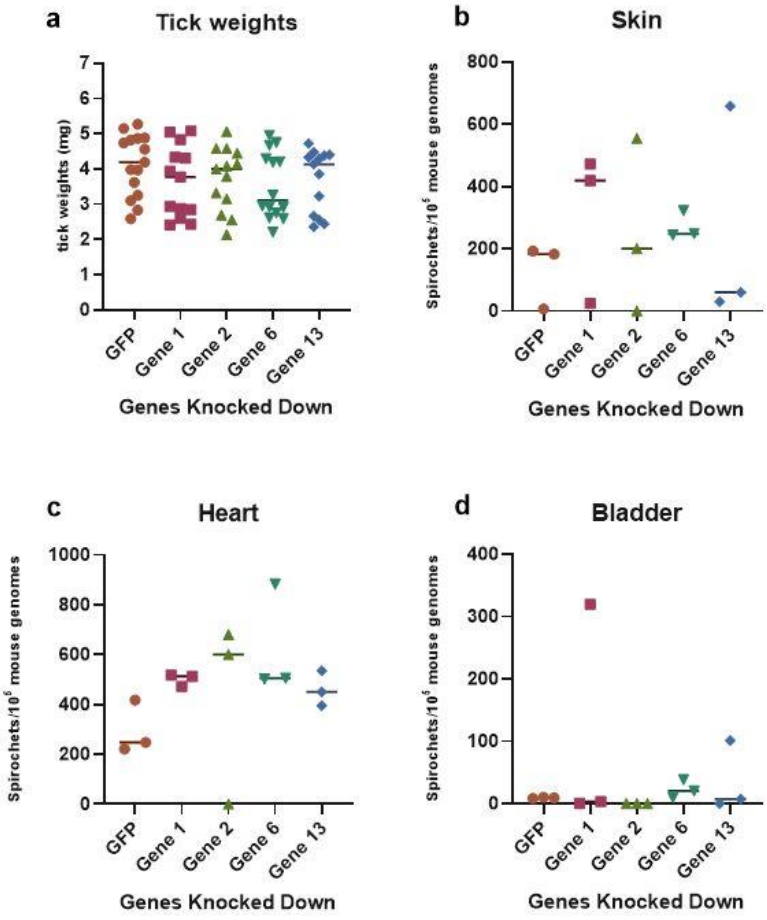

Figure 9: **(Supplemental Figure) Tick weights and *B. afzelii* loads in mouse tissue after gene knock down by RNAi. a)** Engorged tick weights. **b-d)** *B. afzelii* loads in mouse tissues 3 weeks after tick infestation were determined by qPCR using probes targeting *Borrelia* flagellin and mouse β-actin. Loads were calculated as number of spirochetes/10^5 mouse genome.

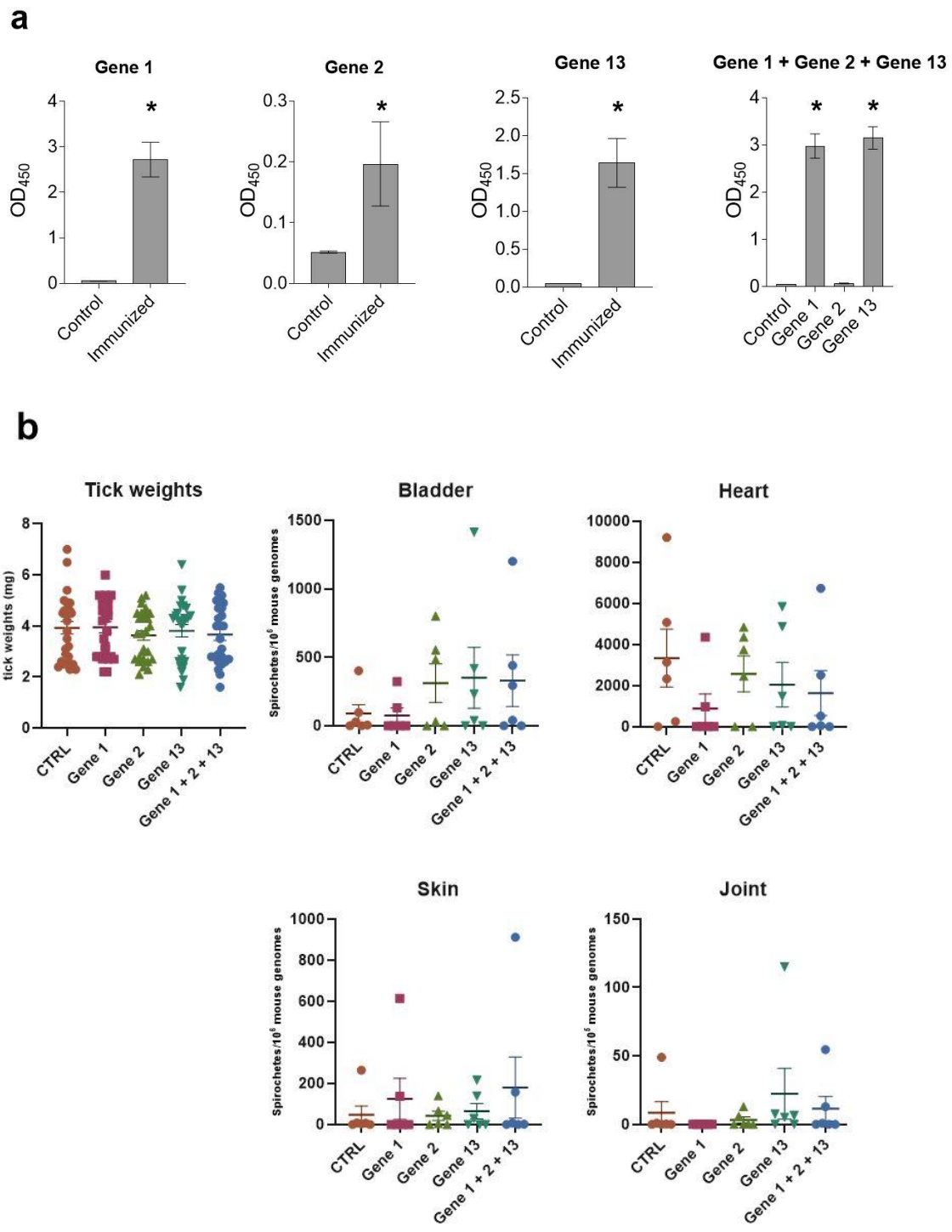

Figure 10: (**Supplemental Figure) Antibody titers, tick weights and *B. afzelii* loads in mouse tissues after vaccination. a) Antigen specific total IgG levels at challenge.** Antigen specific total IgG levels were determined by ELISA mouse sera (1:25600 dilution) collected at the moment of challenge (t=42). Columns represent mean OD's and error bars show the standard error of the mean. Statistical significant increases in IgG levels compared to control are indicated by * (Mann-Whitney, $P < 0.05$). **b) Tick weights and *B. afzelii* loads in mouse tissues.** Engorged ticks were weighed after drop off. *B. afzelii* loads in mouse tissues 3 weeks after tick infestation were determined by qPCR using probes targeting *Borrelia* flagellin and mouse β-actin. Loads were calculated as number of spirochetes/10^5 mouse genome.

Table 9: **(Supplemental Table)** Primers used for biological validation.

Gene ID	Contig	Sequence Forward Primer	Sequence Revers Primer	Product size (bp)
Gene 1	GXP_Contig_15155	CGATACACGTGTTCCCTCAA	TGCTTCGGCTTTTTAGTGGT	63
Gene 2	GXP_Contig_17077	CGCACAATCTCGTGCTGTAT	CTTGATTTGGCATTGGGATT	69
Gene 3	GXP_Contig_14523	ACAATGGTGATGGGGGTTTA	TGCTTCGGCTTTTTAGTGGT	112
Gene 4	GXP_Contig_14792	GTGTTTCGGAAGCTGAACCT	GGCTTTTTCTTGCAAGTTCG	130
Gene 5	GXP_Contig_33156	ACCGACGGTGTAGAATGCTT	TAGTGGTCGCAGGTGTATCG	124
Gene 6	gi|442749986|gb|GADI01006655.1|	CGGCAGTAACTCTTTGGAGTG	CCACGACGTTCAAAATCTTTC	147
Gene 7	GXP_Contig_1065	AGTCAAGGAGGACGCCATT	TGCTCGGCTGTAGAATTAGGA	146
Gene 8	GXP_Contig_8342	TGTAAAAATGGCACCTGCTG	TCTGCCGACGTAGATGTCAC	135
Gene 9	gi|442752832|gb|GADI01005232.1|	GAGATGGCCAGATTGAAAGG	TGCAGCCTGTGCATATAAGG	114
Gene 10	GXP_Contig_28730	GGGATGTGGCTACAGCGTAT	GGGTGGTTTGACGTTGTCTT	106
Gene 11	GXP_Contig_16607	AGGCTGGTACAGAAAGGCTTC	GCATACGTCCTTCATCCACTC	106
Gene 12	gi|442750666|gb|GADI01006315.1|	AAAGCCGAAAGAGTTCATGG	TGGAGAACCGTTTTTATCGTG	133
Gene 13	GXP_Contig_5703	AGACATATATGCGTGCCAAGG	GGCTAACCATCTGAGCGTTT	100
Gene 14	gi|442757522|gb|GADI01002888.1|	CTGCCTCCCGGAATAATATG	CATCAGGTGTGCAGCCTAAG	100
Gene 15	GXP_Contig_11536	GCAGACAATGTAGCCTGTGG	CAGCGTCCAGGGTTATCAAT	118
Gene 16	gi|442754780|gb|GADI01004258.1|	CTTCGGCAGTGTGTGTCATT	CCAGTGTATTTCTGGCAACG	139
Gene 17	GXP_Contig_17807	TTTCATCGATGCCCTTTTCT	CCATCCTCGTAGATCCCGTA	119
Gene 18	GXP_Contig_25165	CGGTTCCCAACCTAATGCTA	CCTTTTCTCACCCACAGCTC	145
Gene 19	noHitAssembly_71081	AATCCGGGCAGTATTTTGG	TCAACCTTGCTGCCATGA	105
Gene 20	noHitAssembly_67398	CTTGCACTGTCGTGCGTTAT	AGTGTAGCCTTCGCATTGGT	138

8. Conclusion

Different tick tissues such as midgut, salivary glands, haemocytes, and ovaries have specific effects on tick physiology and pathogen transmission. Depending on demand, Borrelia may induce differential expression of a particular gene. Many researchers nowadays are therefore focusing on the transcriptomics of different tick tissues to determine their relationship with tick-borne pathogens, especially Borrelia (A. Schwarz *et al.*, 2013; Liu *et al.*, 2014; Cramaro *et al.*, 2015; Kotsyfakis, Kopáček, Franta, J. H. F. Pedra, *et al.*, 2015; Kotsyfakis, Schwarz, Erhart & J. M. C. Ribeiro, 2015). To obtain a broad and clear view, we acquired the transcriptome of *I. ricinus* by sequencing and assembling transcripts from the salivary gland and midgut under different conditions defined by feeding status and the presence of the Borrelia spirochete.

The 3' end sequences provided by MACE is a complete match to our *de novo* transcriptome assembly (Trentelman *et al.*, 2020) with very few exceptions. We focused separately on the salivary gland and midgut for identifying genes upregulated in the presence of Borrelia. Therefore, each analysis had a relatively large level of specificity. This indicates that it is vital to compare and combine the different sources of data to obtain the complete description of the transcriptome of the species. A second factor is the time-dependence of gene expression during the blood meal, as shown in previous studies on *I. ricinus* (Kotsyfakis, Schwarz, Erhart & J. M. C. Ribeiro, 2015). The experimental design for the salivary transcriptome had only a single time point at 24 hours after starting nymphal feeding (24h). In contrast, all three feeding time points (unfed, 24h and fully fed) have been used to identify upregulated tick midgut genes during infection with Borrelia. For the salivary gland, the time-point 24h is essential because Borrelia begins transmission from tick to mammalian host just after that time. Borrelia indeed is assisted by many known and unknown salivary gland proteins and non-coding RNAs, along with the saliva itself at that particular feeding phase (Bensaoud, Hackenberg and Kotsyfakis, 2019). Therefore this unique time-point based on feeding was chosen for salivary gland transcriptome.

On the other hand, the midgut plays complicated roles in terms of both pathogen acquisition and transmission. After taking the infected blood meal during the larval stage, Borrelia stays in the tick midgut by establishing a very stable colony. That’s why it is crucial to study the unfed stage to understand the acquisition and persistence. At approximately 24 hours after the start of feeding,

Borrelia starts its journey from the midgut to the next host. At this time, the spirochaete has to cross the peritrophic membrane and epithelial layer in order to invade the salivary gland through haemolymph; hence this time point for midgut tissue transcriptomics is the most important in order to identify transmission-blocking vaccine candidates.

Lipocalins and ixodegrin protein families are significantly upregulated in *I. ricinus* salivary gland in the presence of *B. afzelii* during 24h. Lipocalins are barrel-shaped proteins and have already been reported to be upregulated in the presence of the LD spirochete (Valdés *et al.*, 2016). It makes sense that this salivary gland protein family is upregulated because it plays essential roles in tick feeding and in transmitting Borrelia to the mammalian host (Beaufays *et al.*, 2008; Šimo *et al.*, 2017). Ixodegrin is a protein belonging to a large family called disintegrins. It functions as an inhibitor of platelet aggregation. A previous study (Francischetti *et al.*, 2009) showed that ixodegrin prevents blood clotting, so the tick can feed on blood smoothly. Thus, we can see that a mutual relationship has been established between Borrelia and the tick. Although

these interactions are favoring tick for uninterrupted feeding, full length recombinant protein-based vaccination approaches with Freund's adjuvant could not significantly stop Borrelia transmission from tick to C3H mice (Trentelman *et al.*, 2020).

On the other hand, seven *I. ricinus* midgut genes were also upregulated at different feeding time points in the presence of Borrelia infection. These genes are mainly uncharacterized transcription factors and genes related to the electron transport chain. Transcription factors are modulators of gene expression. Proteins in demand are therefore strongly induced by such transcription factors in the presence of Borrelia; this may be advantageous to either Borrelia alone, or to both parasite and vector. Cytochrome, which is known as its involvement in the electron transport chain, was also upregulated (cytochrome p450). Previously Mansfield *et al.* (2017) showed cytochrome C was upregulated in the presence of Anaplasma and Tick-Borne Encephalitis Virus, possibly playing a role in the tick JAK-STAT immune pathway and leading the cell into apoptosis. On the other hand, cytochrome C oxidase subunit VIa was downregulated in MACE, possibly due to metabolic inactiveness. In our study, we have shown that knock-down cytochrome p450 induced the transmission of significantly more Borrelia in murine ear at the third week of tick repletion. In contrast, knock down of an uncharacterized protein (GXP_Contig_30818) decreased the

dissemination rate of Borrelia throughout the body and reduced significantly the spirochete load in the urinary bladder.

We also briefly studied peritrophic membrane chitin-binding proteins that share chitin deacetylase domains. Borrelia remains in close contact with the peritrophic matrix within the lumen of the tick gut. Chitin deacetylase (CDA) is one of the proteins connecting chitin fibrils that form a highly structured lattice and strengthens the peritrophic membrane (Kariu *et al.*, 2013). From our MACE database, we observed that the presence of Borrelia increased the expression of CDAs roughly by 3 fold, and among these, three CDAs were also significantly upregulated in our biological validation (GXP_Contig_1655, 5371, and 17512). This is indicative of an interactive relationship between the peritrophic membrane and spirochete. Either Borrelia signals the tick midgut epithelial cells to secrete more CDAs or the tick produces higher levels of these CDAs as a reflex reaction in order to increase innate immunity. Previous studies confirmed the acquisition, persistence and transmission of *B. burgdorferi* s.s. being influenced by the reverse genetic approaches (RNAi, active mouse immunization, and passive immunization by injecting antisera) with different CDAs (Kariu *et al.*, 2013; Yang *et al.*, 2020). In similar fashion to other candidate genes (Mahmood *et al*., 2021), we silenced the CDAs (GXP_Contig_1655, 5371, and 17512) and checked the impact on *B. afzelii*. Unlike previous studies with *B. burgdorferi* s.s., we obtained mixed results on *B. afzelii* acquisition and transmission, making it more difficult to determine the precise role of CDA's. This outcome suggests different functions of CDAs in distinct tick species, or that different Borrelia species respond in different ways to CDA silencing.

After validation of upregulation, we performed transmission experiments with immunization (Trentelman *et al.*, 2020) and RNA interference approaches (Trentelman *et al.*, 2020; Mahmood *et al*., 2021). None of the upregulated candidates from the salivary gland or midgut could completely stop Borrelia transmission. This suggests that these genes might help the pathogen against immune cells, or they are upregulated due to the presence of a pathogen in order to control its replication. Other midgut candidates may be involved in pathogen acquisition or persistence. Although the experimental results are not unambiguous, we have identified several genes that warrant further investigation regarding the pathogen-vector relationship. For future immunological studies, we will examine the effectiveness of different types of vaccines and different adjuvants.

We may also include experiments with various immunological assays in order to visualize and optimize vaccine efficacy.

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Ph.D. Thesis, 2021

Printed in the Czech Republic by D Print
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