Adaptation to environmental stress in the lone star tick, *Amblyomma americanum*: osmoregulation, thermoregulation, and microbiome

by

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B.S., Universidad Michoacána, MX, 2010
M.Sc., Universidad Michoacána, MX, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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Abstract

Ticks are the arthropod vectors with the widest range of harbored pathogens. Ticks can survive for extended periods of time, often more than a year, without dietary nutrition during their *off*-host periods under stressful environmental conditions. In addition, during the time, ticks can maintain water balance in dry environment. Here, we report that the lone star ticks, *Amblyomma americanum*, can actively drink water, which greatly increases their survival rates. Significance of the ticks drinking environmental water is brought up in this study; importance of direct water drinking in the tick survival and implications of the water drinking in acquisition of environmental organic and inorganic constituents. In the Chapter 2, I found that ticks directly drinking water is necessary for their survival. It was also demonstrated that ticks drinking certain inorganic components or a bacterium leads to tick death, which offers a new avenue for tick management by the delivery of toxic agents through drinking water.

Tick water homeostasis physiology was further expanded to the study of the dermal secretion physiology and its relevance in terms of thermoregulation and dehydration in Chapter 3. Dermal secretion, analogous to the vertebrate sweat, occurs through type II dermal glands located under the cuticle layer, which are exclusive to Metastriate ticks, including *Amblyomma*. In this research, I found that contact of a heat probe on the tick body can induce the secretory response. The dermal secretion was induced at as low as 35^{o}C, while an exhaustive dermal secretion induced by 52^{o}C resulted in tick lethality in 24-hour. We demonstrated that a role of dermal secretion is evaporative cooling that shortens the time for cooling the body 1-1.2 seconds faster than those of the ticks which did not secrete. In a further in depth study for understanding the mechanisms of the secretion, I found that the dermal secretion is triggered by serotonin. Ouabain, Na/K-ATPase blocker, suppresses the serotonin-mediated dermal secretion. A candidate serotonin receptor

mediating the dermal secretion was identified as *A. americanum* serotonin receptor 1A (Aame5-HT1A) in a transcriptomics study.

The study for understanding the impacts and potential applications of environmental water drinking physiology was further expanded to a microbiome of *A. americanum* females collected in eastern Kansas in Chapter 4. The 16S rRNA sequencing results showed the dominant endosymbiotic genera *Coxiella* and *Rickettsia* (>95%) with other operational taxonomic units (OTUs) presenting typical soil bacterial taxa, implying that the environmental bacteria are frequently acquired by the tick, presumably, through the water drinking.

This study has uncovered the physiological mechanisms that expose a vulnerable tick physiology that can be targeted in development of tick control measures. Delivery of tick-specific toxic reagent disrupting the osmoregulation or pathogenic agents inducing bacterial dysbiosis may provide new routes for development of tick management strategies.

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Major Professor
Yoonseong Park

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List of Symbols

α	alpha, used in gene names and statistics
β	beta, used in gene names
C	Celsius
χ	chi, used in statistics
Δ	delta, used to show a fold change in transcript levels
°	degrees
F	Fahrenheit
μ	micro, 10^{-6}
M	molar
±	plus or minus
&	and

List of Abbreviations

µl	microliter(s)
µM	micromolar
5HT	serotonin receptor
16S	16S ribosomal RNA gene
ΔCt	delta CT: method for calculating fold change in expression relative to reference gene
ΔΔCt	delta-delta CT: method for calculating fold change in expression relative to reference gene and calibrator treatment
A	*Amblyomma* (only when followed by "*americanum*")
Aame	*Amblyomma americanum*
Ae	*Aedes*
Acal	*Aplysia californica*
An	*Anopheles*
AMP	antimicrobial peptide
ANOVA	analysis of variance
ATPase	adenosine triphosphate hydrolases
BLAST	basic local alignment search tool
bp	base pair(s)
Bt	*Bacillus thuringiensis*
B	*Borrelia*
B	body
°C	temperature in degrees Celsius
cAMP	cyclic adenosine 3',5'-cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
CEA	critical equilibrium activity
cGMP	cyclic guanosine 3',5'-cyclic monophosphate
CC	carcass
CFU	colony forming units
CFP	cyan fluorescent protein
Cgig	*Crassostrea gigas*
CNS	central nervous system
Ct	cycle threshold
D	*Dermacentor*
DAPI	4′,6′-diamino-2-phenylindole
DENV	dengue virus
Dmel	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
DTN	dityrosine network

DUOX	dual oxidases
E. chaffeensis	*Ehrlichia*
E. coli	*Escherichia*
ELV-1	elevenin
ExpDec	exponential decay
F	*Fransicella*
GFP	green fluorescent protein
HG	hindgut
hrs	hours
HRP	horseradish peroxidase
Hsap	*Homo sapiens*
HTS	high throughput sequencing
IgG	immunoglobulin G
IHC	immunohistochemistry
Isca	*Ixodes scapularis*
K^+	Potassium
KNO_3	Potassium nitrate
KH_2PO_4	Potassium phosphate monobasic
MEGA	molecular evolutionary genetics analysis
MG	midgut
min	minute(s)
MIP	mioinhibitory peptide
ml	milliliter(s)
ML	maximum likelihood
mm	millimeter
mM	millimolar: one millimole per liter
MT	malpighian tubules
MUSCLE	multiple sequence comparison by Log-expectation
Myes	*Mizuhopecten yessoensis*
Na^+	Sodium
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NGS	normal goat serum
NJ	neighbor joining
ng	nanograms(s)
nm	nanometer
nM	nanomolar
nl	nanoliter(s)
ORF	open reading frame
OTU	operational taxonomic unit

OV	ovary
p	p-value: the probability of a statistical measure
P	*Pseudomonas*
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline-Triton x-100
Pcan	*Pomacea canaliculata*
pH	a measure of acidity and alkalinity of a solution
PM	peritrophic matrix: structure surrounding food bolus
PRRs	pattern recognition receptors
Ptep	*Parasteatoda tepidariorum*
qPCR	quantitative real-time polymerase chain reaction
R	*Rhipicephalus*
RDP	ribosomal database project
RH	relative humidity
Rh123	rhodamine123
RNA	ribonucleic acid
ROI	region of interest
RPS4	ribosomal protein S4 gene
Rsan	*Rhipicephalus sanguineus*
RT	room temperature
S.	*Staphylococcus*
sec	seconds
SEM	standard error of mean
SG	salivary glands
SNAP	N-(acetyloxy)-3-nitrosothiovaline
sp.	species
SRA	sequence read archive
STARI	southern tick associated rash illness
t	t-statistic: test statistic distribution, used for parametric two-sample t-tests
TSA	transcript shotgun assembly (bioinformatics), trypticase soy agar (microbiology)
UNCLAS	unclassified
UPGMA	unweighted pair group method with arithmetic mean
Vdes	*Varroa destructor*
V-ATPase	vacuolar type- adenosine triphosphate hydrolase

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Dedication

I would like to dedicate this dissertation to my husband, Dario Zuniga-Chavez for always believing in me and for his constant support and encouragement, and to my mentor; Yoonseong Park, for his guidance and patience throughout my program and for always looking out for my best interests.

Chapter 1 - Literature review and Dissertation structure

Abstract

Ticks are the arthropod vector with the widest range of harbored pathogens, and they are capable of surviving under stressful environmental conditions. One of the evolutionary outcomes of these obligatory hematophagous arthropods is their ability to survive for extended periods of time without a bloodmeal during their off-host periods. Water conservation biology and heat tolerance have allowed the ticks to thrive even under high temperatures and low relative humidity. They are also broadly distributed globally, making them highly successful arthropods. Although water drinking biology has been early reported in Ixodid ticks, only recently we have learned that ticks can actively drink environmental water, which allows hydration. This knowledge opens a new avenue for tick management through the delivery of toxic agents into drinking water. Ticks' drinking behavior may also provide the opportunity to manipulate the tick microbiome. Related to water acquisition are water excretion processes, which occur primarily due to disturbance (dermal excretion) and other natural imminent water losses like waste excretion. Here, we evaluated the impact of water drinking in ticks and its implications for tick survival and management (Chapter 2), the dermal excretion physiology and its relevance in terms of dehydration (Chapter 3), and the characterization of the bacterial community of *A. americanum* as a means to identify environmental bacteria with potential for tick toxicity (Chapter 4). Further, the implications of ingestion of toxic agents through a combination of dehydration by dermal excretion for tick management are discussed.

Introduction

Ixodida ticks: Taxonomic position and life cycle

Ticks, which are obligate blood-feeding ectoparasites, are one of the most important arthropod groups that vector pathogens to people and other animals worldwide (1, 2). In the United States, ticks are the number one arthropod vector of diseases to animals and humans (3, 4). Ticks are taxonomically positioned in Class Arachnida; Subclass Acari; Superorder Parasitiformes. Further taxonomic subdivisions of ticks (Order Metastigmata) include Families Ixodidae (hard ticks) and Argasidae (soft ticks), which include species of human and veterinary importance. Ixodidae, which contains the main vector species, comprises more than 700 species (4-6). Ixodidae

ticks are subdivided into two groups: Prostriata, containing one genus (*Ixodes*), and Metastriata, containing 11 genera, including *Amblyomma*, *Dermacentor*, and *Rhipicephalus* (5).

The life cycle of hard ticks consists of four stages: one non-feeding stage (egg), and three feeding stages (larva, nymph, and adult). All ticks are required to feed on a host in order to molt into the next stage (7), which allows ticks to explore multiple hosts throughout their lifetime, an important factor for vectoring pathogens. During their lifecycle, ticks can be one-host, two-host, or three-host ticks (5), although the majority of hard ticks fall under the three-host category. The life cycle of soft ticks differs from hard ticks in that soft ticks require multiple feedings in the same stage. Associated with two or more instar nymphal moltings, this pattern is referred to as the multihost life cycle (5).

The three-host tick life cycle

In the three-host life cycle, ticks are required to feed from three different hosts (or three different attachments) in order to complete their life cycle. The cycle starts with the attachment of the larvae to the first host, which is usually small vertebrate. The larvae drop off from the host after feeding and later molt into nymphs (Figure 1.1). The unfed nymphs climb on vegetation in search of a second host. This behavior is known as questing. Once ticks find and attach to a second host, they feed and drop off once more to molt into adults and repeat this cycle for a third host. After ticks complete their last drop off as adults from the third host, they lay eggs and die. The adult stage can last for several months depending on host availability, and the overall tick life cycle varies from months to even years depending on climate conditions and tick species (5). After attachment, male and females mate on the host and although males ingest significantly less blood than female ticks, most metastriate males require a blood meal for sperm maturation (8). Once the tick female is fully engorged, it drops and lays eggs on the ground or leaf litter, which can take several days (5, 9). Ixodid ticks have only one gonotrophic cycle. One single female can lay up to several thousands of eggs at once, with the highest recorded amount being 36,206 eggs laid by *Amblyomma variegatum* (10).

Ixodidae as disease vectors

Ticks are recognized as harboring the greatest range of pathogens among arthropod vectors, making them the most competent vectors next to mosquitoes (1). Ticks can vector such pathogens

as bacteria, viruses, protozoans, and helminths (11). These pathogens spread tularemia, human and canine ehrlichiosis, Lyme disease, Rocky Mountain spotted fever, babesiosis, human anaplasmosis, tick-borne encephalitis, and many other diseases to animals and humans (Table 1.1) (1, 2, 12, 13). Ticks acquire pathogens primarily through ingestion during feeding, not by contact, as the tick cuticle serves for protection against microbial challenges (11). Upon ingestion, pathogens must overcome the tick's biological barriers (including the midgut and its microbiota, as well as hemocoel) in order to migrate from the midgut to the salivary glands (14), from which they can be transmitted to another host during the next blood feeding (15).

Certain interactions between tick physiology and pathogens during the infection cycle have been established. Tick saliva has important immunomodulatory effects; containing anti-inflammatory molecules, it can suppress the immune and inflammatory responses from the host to permit prolonged feeding (16-18). In the infection of *Borrelia burgodorferi* (causative agent of Lyme disease) and its vector (*Ixodes scapularis*), studies have shown that specific salivary proteins such as Salp15 and Salp20 bind to lipoproteins of the pathogen, which help the bacteria evade the immune mechanisms of the vertebrate host (19, 20). Invasion of the tick by a pathogen is a complex process that involves the specific components in ticks' immunity pathways (21). Nonetheless, the study of pathogen-microbiome-tick immune system interactions are of great interests for blocking pathogen transmission in ticks and also for the development of transmission blocking vaccines.

Table 1.1. Hard tick species and their associated diseases (modified from Dantas-Torres et al., 2012 and CDC, 2018)

Tick Groups and Species	Associated Pathogens	Found in the Americas
Metastriata		
Amblyomma americanum	*Borrelia lonestari, Ehrlichia chaffeensis, Francisella tularensis, Rickettsia parkeri, Rickettsia rickettsii*	North, South, and Central America
Amblyomma aureolatum	*R. rickettsii*	North, South, and Central America
Amblyomma cajennense	*Candidatus R. amblyommii, Rickettsia honei, R. rickettsii*	North, South, and Central America
Amblyomma coelebs	*Candidatus R. amblyommii*	
Amblyomma hebraeum	*Ehrlichia ruminantiuma, Rickettsia africae, Theileria mutans*	
Amblyomma maculatum	*E. ruminantiumb, Hepatozoon americanumb, R. parkeri*	
Amblyomma neumanni	*Candidatus R. amblyommii*	
Amblyomma ovale	*Hepatozoon canis*	
Amblyomma triste	*R. parkeri*	
Amblyomma variegatum	*E. ruminantiumb, R. africae, T. mutans, Thogoto virus (THOV), Bhanja virus (BHAV), Crimean–Congo hemorrhagic fever virus (CCHFV)*	North America
Dermacentor andersoni	*Anaplasma marginale, F. tularensis, R. rickettsii, Colorado tick fever virus (CTFV), Powassan encephalitis virus (POWV)*	North, South, and Central America
Dermacentor auratus	*Rickettsia sibirica*	
Dermacentor marginatus	*Babesia canis, Coxiella burnetii, F. tularensis, R. sibirica, Rickettsia slovaka, BHAV, Omsk hemorrhagic fever virus (OHFV)*	
Dermacentor nuttalli	*R. sibirica*	

Tick Groups and Species	Associated Pathogens	Found in the Americas
Dermacentor reticulatus	*A. marginale, Babesia caballi, B. canis, C. burnetii,*	
Dermacentor silvarum	*F. tularensis, Rickettsia helvetica, R. sibirica, R. slovaka, OHFV*	
Dermacentor variabilis	*Rickettsia heilongjiangensis, R. sibirica*	North, South, and Central America
Rhipicephalus bursa	*B. burgdorferi, F. tularensis, R. helvetica, Louping ill virus (LIV), TBEV*	North America
Rhipicephalus microplus	*A. phagocytophilum, B. microti, B. burgdorferi, POWV*	North America
Rhipicephalus sanguineus	*A. marginale, Anaplasma ovis, A. phagocytophilum, Babesia bigemina, Babesia ovis, BHAV, CCHFV*	North, South, and Central America
Rhipicephalus turanicus	*A. marginale, B. bigemina, B. bovis, Theileria equi*	
Haemaphysalis concinna	*A. marginale, F. tularensis, R. rickettsii*	
Haemaphysalis flava	*Anaplasma phagocytophilum, F. tularensis, R. sibirica, tick-borne encephalitis virus (TBEV)*	
Haemaphysalis longicornis	*Rickettsia japonica*	North America
Haemaphysalis punctata	*Babesia gibsoni, Babesia ovata, Theileria buffeli, R. japonica, POWV*	
Haemaphysalis spinigera	*A. phagocytophilum, Babesia major, Babesia motasi, C. burnetii, T. buffeli, BHAV, CCHFV, TBEV*	
Haemaphysalis turturis	*Kyasanur forest disease virus (KFDV)*	
Hyalomma anatolicum	*KFDV*	North America
Hyalomma asiaticum	*Theileria annulata, Theileria lestoquardi, CCHFV*	
Hyalomma marginatum	*T. annulata, R. sibirica subsp. mongolitimonae, BHAV*	North America
Hyalomma truncatum	*Rickettsia aeschlimannii, T. annulata, BHAV, CCHFV*	

Tick Groups and Species	Associated Pathogens	Found in the Americas
Prostriata		
Ixodes cookei	*R. sibirica subsp. mongolitimonae, BHAV, CCHFV*	North America
Ixodes hexagonus	*POWV*	
Ixodes holocyclus	*Borrelia burgdorferi sensu lato*	
Ixodes ovatus	*Rickettsia australis*	
Ixodes pacificus	*R. japonica*	North America
Ixodes persulcatus	*A. phagocytophilum, B. burgdorferi*	North America
Ixodes ricinus	*B. burgdorferi., OHFV, TBEV*	North America
Ixodes scapularis	*A. phagocytophilum, Babesia divergens, B. microti,*	North America

The lone star tick

The lone star tick (*Amblyomma americanum*) belongs to the family *Ixodidae* and is further classified in the Metastriata group. It is characterized as a three-host tick since it feeds on three different hosts throughout its life cycle (Figure 1-1). This tick is widely distributed in the United States, primarily in the Midwest (22). The lone star tick vectors pathogens including *Fransicella tularensis* (causative agent of Tularemia) and *Ehrlichia chaffeens*is and *E. ewingii* (causative agents of human and canine Ehrlichiosis and viruses, including the heartland virus) (23-25). In addition, this tick is known to cause conditions such as southern tick associated rash illness (STARI), the specific cause of which is unknown. It is also known to trigger an allergy to red meat, which is caused by a specific glycan (alpha-gal) present in the tick's salivary glands (26, 27).

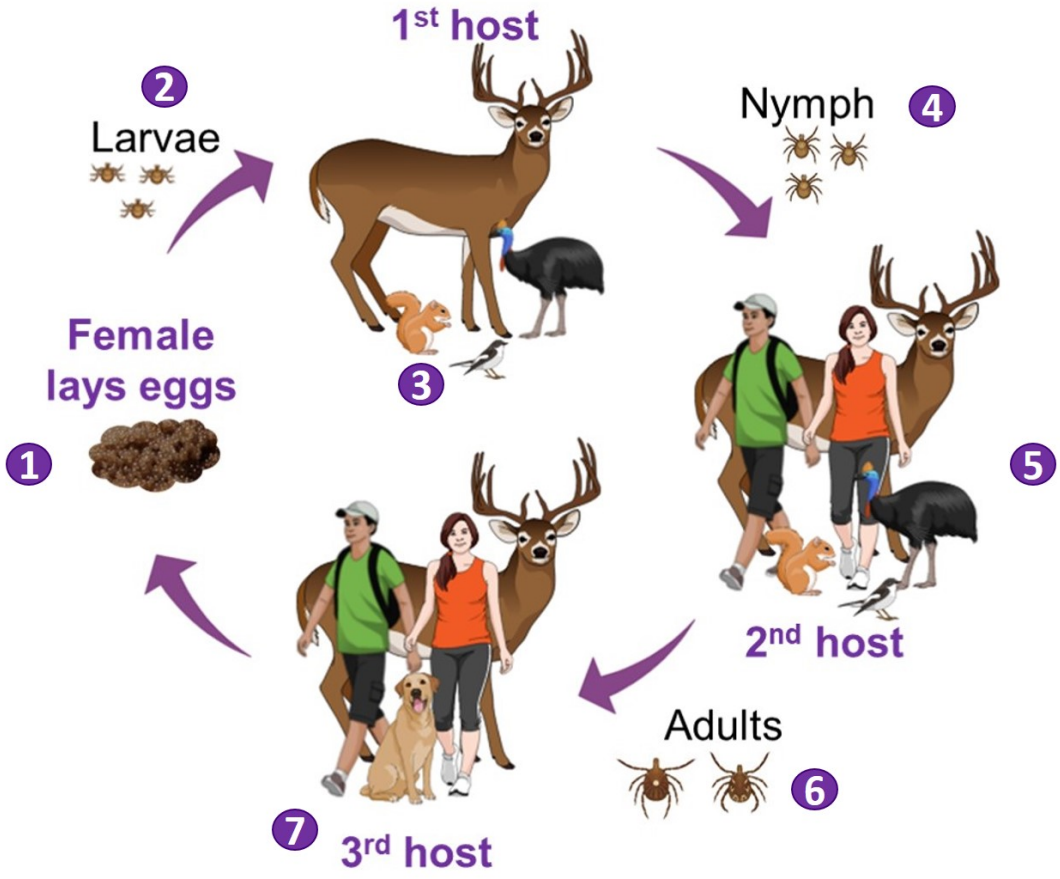

Figure 1.1. The three-host life cycle of *Amblyomma americanum*

This figure shows the three-host life cycle of *A. americanum*. In the first stage, adult females drop off the third host to lay eggs after feeding, ❶ usually in the fall. Next, eggs hatch into six-legged larvae ❷ and overwinter in the larval stage. In the spring, the larvae attach to the first host, usually a small animal❸. Later in the summer, engorged larvae leave the first host, molt into nymphs❹,

and usually overwinter in this stage. During the following spring, the nymphs seek out and attach to the second larger host❺. The nymphs feed on the second host and drop off later in the summer to molt into adults ❻ and overwinter. The next spring, adults seek out and attach to a third host the following spring. The third host is usually a larger animal or human❼. The adults feed and mate on the third host during the summer. Females drop off the host to continue the cycle. Females may reattach and feed multiple times. The three hosts are not necessarily different species, and humans may serve as first, second, or third hosts.

Recent reports on the distribution of *A. americanum* show a rapid expansion of the species' habitat (Figure 1.2A) (28). Predictive models show the potential for distribution of *A. americanum* to the upper Midwest and to California based on climate suitability (29). These distribution patterns are important for assessing the risk of disease associated to this tick species (Figure 1.2B) (29). Tick distribution largely depends on the ticks' ability to tolerate and adapt to harsh environments such as high temperatures and low relative humidity. Therefore, it is important to elucidate the physiological mechanisms that allow ticks to overcome stressful environmental conditions during the off-host period. Understanding the osmoregulatory and thermoregulaory mechanisms may offer new strategies for tick management, which could aid in mitigating tick distribution and tick-borne illnesses.

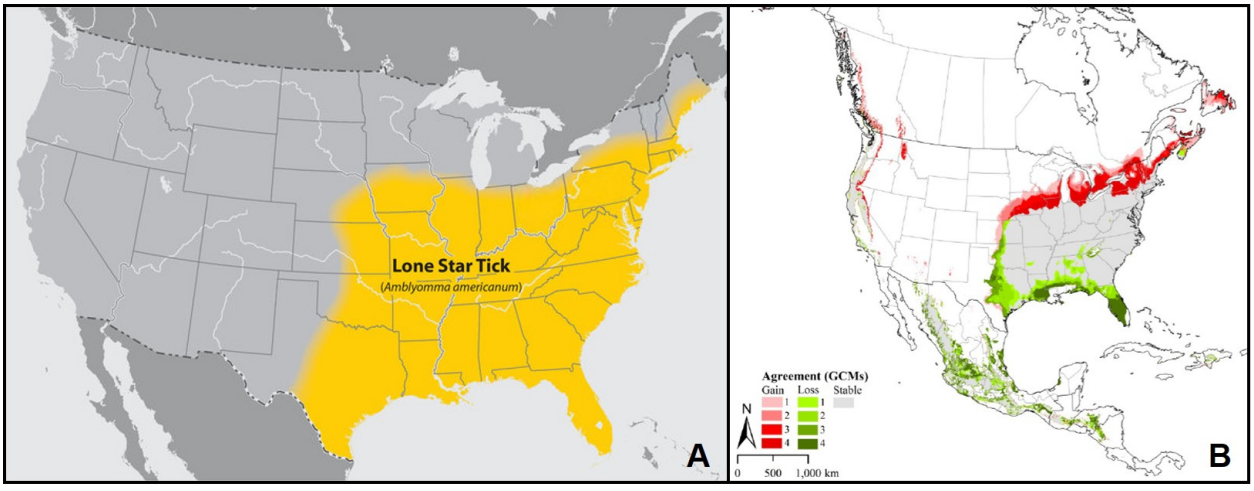

Figure 1.2. Current distribution of *Amblyomma americanum*
Distribution of *Amblyomma americanum* in the United States (CDC., 2019) **(A)**. The lone star tick is primarily distributed in the Midwest, South, and Northeast regions and predicted distribution in North America (Raghavan et al., 2019) **(B)**. Red represents areas where the gain of distribution is expected, while green represents predicted loss of distribution.

Osmoregulation

Water is the basis of life, comprising to 65 to 90% of all living organisms. All living organisms require a proper water balance for cellular functions, meaning they must maintain the ideal concentration of ions or electrolytes within the cell. At the organismal level, gaining water requires the uptake of dietary water while water is also constantly lost through the integument by evaporation and through the excretory system by metabolic waste.

Arthropods, including insect and arachnids, are especially susceptible to dehydration due to their small size and large surface-to-volume ratio compared to other animals. However, their osmoregulatory mechanisms, including a cuticle covered with a wax, allow a large number of species to withstand the most arid environments. For example, the fruit fly (*Drosophila melanogaster*) can maintain a constant hemolymph osmolarity even when subjected to a desiccation that reduces its hemolymph volume to less than 25% (30). Ticks' osmoregulatory processes are more complex, as ticks rely heavily on their ability to absorb water from the environment without nutritious diet during their long off-host periods.

Water homeostasis in the ixodid tick

Hard ticks imbibe a large amount of blood during a single blood meal. While they spend a relatively short time on the host for feeding, they survive extended off-host periods throughout the lifecycle. Hard ticks, including *A. americanum*, spend over 98% of their lifetime off-host (31). Therefore, tick survival is largely dependent on environmental conditions, which include fluctuating temperatures and relative humidity (RH) (32). Tick survival─and, thus, tick success─largely depends on their ability to maintain water balance during periods of low RH (31, 32). Evaporative loss of water through the integument, which is a physiological challenge for the small animal, is generally well prevented by the integument and its waxy sclerotized cuticle. Imminent water losses naturally occur through hindgut excretion, salivation (33), and dermal secretion (which is further explored in this study).

Ticks are known to be able to uptake water from water vapor. Water vapor uptake directly depends on Critical Equilibrium Activity (CEA), which is the minimum RH at which ticks can passively uptake water and maintain the water balance in the body (34-36). In most ixodid ticks the CEA is close to 90% RH (36); in *A. americanum*, water vapor uptake is between 85% and 93% RH and accounts for up to 77% of the water uptaken (37) in an experimental condition. Water

vapor uptake was first reported in *A. variegatum* with the mouthparts as the main site of this uptake (38). It was demonstrated that the salivary glands secrete to the mouthparts a hygroscopic saliva that is high in chloride, potassium, and sodium ions (32, 39), which captures water molecules from water vapor for ingestion (32). Although the ability of ticks to maintain water homeostasis has been of great interests in the last several decades, better understanding of tick success in water regulation may provide the tools for tick control.

Water uptake

Water drinking

It has been demonstrated that certain species of ticks actively and voluntarily drink water to maintain water balance during off-host periods. Drinking-like behavior was observed in several *Rhipicephalus* species, *D. nuttalli, A. hebraeum,* and the larvae of *A. americanum* (40-42). However, several studies reported no water drinking behavior in a number of other species, such as *Ixodes ricinus, D. marginatus,* and *D. reticulatus* (43-45). Across these reports, we find that metastrate ticks generally drink directly from water, whereas prostrate ticks tend to lack the water-drinking behavior. That being said, a previous study in our laboratory demonstrated that the prostrate tick *I. scapularis* can approach and touch water droplets and occasionally insert its hypostome (mouthparts) into and drink water. This behavior was observed in 5% of ticks tested (46). Other studies reported an increased weight gain in ticks after being in physical contact with water droplets, which was believed to occur *not* by direct ingestion through the mouthparts (47, 48). These studies suggest that further observations across varied conditions, such as the geographically different strains of ticks and their varied environmental conditions, are necessary to reach a firm conclusion. Based on a study tracing fluorescence dye in water, the water drunk through ticks' mouthparts is uptaken through the internal organs (e.g., type I acini of salivary glands and midgut) and transported into the hemolymph (46).

Water uptake mechanisms across different ticks will likely offer a new target system that could be utilized for development of tick control measures. We also consider the direct uptake of water by ticks as a potential route for delivering tick-specific toxic reagents. We must expand the current knowledge, which is limited in the aspects of the significance of liquid water drinking in tick biology and the mechanisms involved in this process that can be targeted for improved tick control.

Water excretion physiology

Excretory mechanisms in ixodid ticks are similar to those observed in insects, though they differ significantly in terms of the neural and hormonal components involved in the process (5). In hard ticks, the salivary glands, Malpighian tubules, and hindgut are the main osmoregulatory organs (Figure 1.3.C). The excretory organs, hindgut, and Malpighian tubules are believed to be controlled by the brain of the tick (synganglion) (Figure 1.3.A) (49), while the mechanisms controlling the salivary glands are not yet fully understood (50). In this chapter, we will give an overview of what is known about the control and regulation of excretion in ixodid ticks.

The salivary glands of ixodid ticks are the largest pair of glands in the tick body and they are responsible for maintaining water balance in both off-host and on-host ticks (5, 9). There are three types of acini (alveoli) in the female tick (Type I, Type II, and Type III) and four types in males (I-IV). Type I acini are attached directly to the anterior part (or proximal region) of the salivary duct. They are agranular and they have been associated primarily with off-host osmoregulation through the absorption of water after hygroscopic saliva is secreted during dehydration (5, 9, 34, 46, 50, 51). Type II (Figure 1.3.D) and Type III acini (Figure 1.3.E) are granular and more complex in structure, exhibiting drastic anatomical changes during feeding (5, 9, 51). Type II and Type III acini have been associated primarily with water and ion excretion during the feeding periods in addition to the production and secretion of bioactive molecules into their host (5, 50, 52-54).

It has been reported that salivary gland secretion is regulated through neuronal and hormonal signaling (55). The main regulator of the excretion and the influx of fluid into the lumens of Type II and Type III acini is dopamine, which acts as a paracrine/autocrine hormone (50, 55, 56). Two different dopamine receptors have been identified as the downstream physiological targets in Type III acini: the D1 receptor for water-solute influx and the InvD1L for pumping/gating (57, 58). Experiments utilizing heterologous receptor expression suggested that D1 is mainly coupled to cAMP, while InvD1L is coupled to Ca^{2+} elevation in the downstream signaling pathway (55, 57, 58). A number of neuropeptidergic projections reaching to the salivary glands as the upstream signal in the control of salivary glands have been described as SIFamide, Elevenin, and MIP (myoinhibitory peptide) (49). The locations of the neuropeptidergic varicosities and the receptors indicated their modulatory functions for control of secretion and for the feedback control of autocrine dopamine in the salivary glands. Overall, the salivary glands which play a

vital role in osmoregulatory processes in addition to contributing to vector competence as tick saliva is an important factor in pathogen transmission (54, 59, 60), are tightly controlled by neural and hormonal components for the activities.

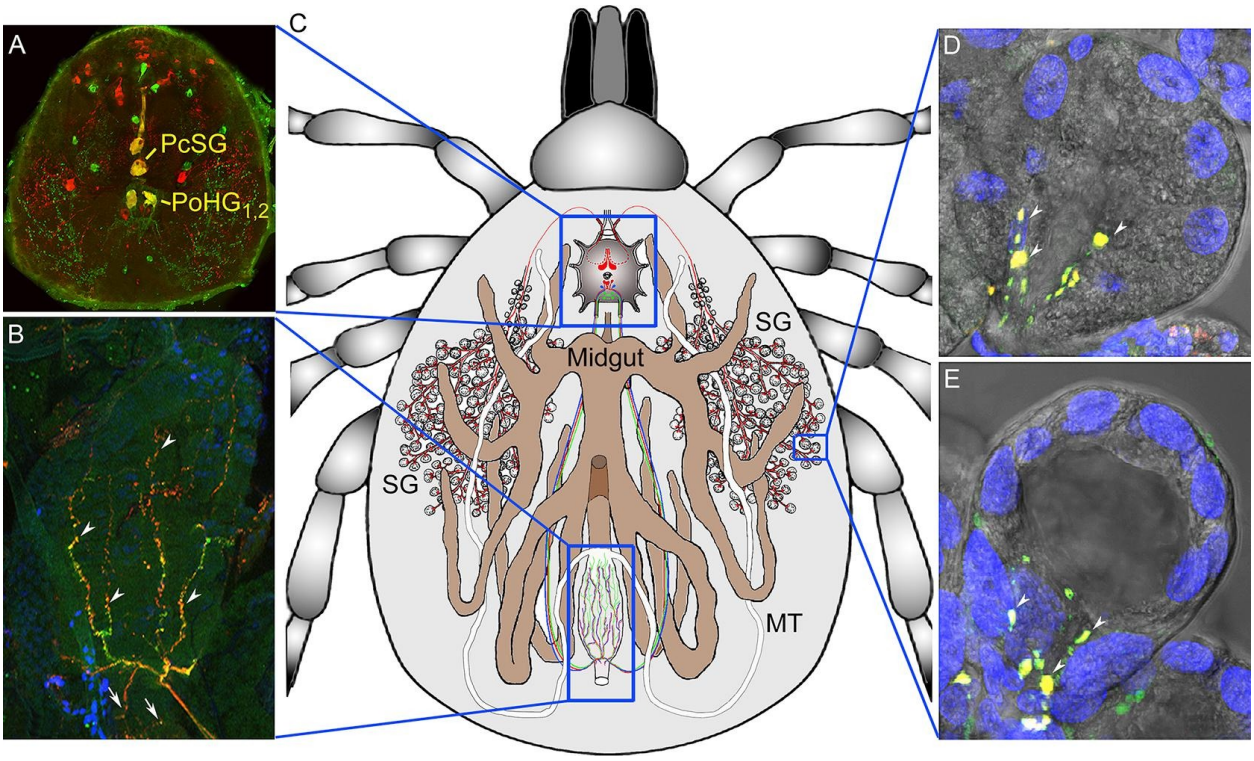

Figure 1.3. Main osmoregulatory organs in hard ticks (Kim et al., 2019).
Anatomy of hard ticks, showing the major internal organs. **A)** depicts the synganglion with cells showing positive immunoreactivity to antibodies against SIFamide (green) and MIP (red), where yellow indicates colocalization of both. PcSG is the protocerebral salivary gland neuron, while PoHG is the post-oesophageal hindgut neurons. **B)** shows the hindgut with projections immunoreactive to the same antibodies**. C)** depicts the internal distribution of organs. **D)** depicts the Type II acinus and **E)** the Type III acinus with axon terminals showing positive immunoreactivity to antibodies. Blue represents DAPI staining of nuclei. MT is Malphigian tubule and SG is salivary gland. This figure is reproduced and modified from Šimo et al. (2012, 2013) and Šimo and Park (2014).

Hindgut and Malpighian tubules

Malpighian tubules and the hindgut are located at the terminal end of the alimentary canal and primarily serve the function of metabolic waste excretion (5, 9). A pair of Malpighian tubules long tubes with dead ends arises at the midgut-hindgut junction. The hindgut is in direct contact

with the anus and is wrapped by longitudinal and cross muscles. A number of neural projections innervate the hindgut, which are neurons immunoreactive to SIFamide, MIP, and allatostain A. The neuropeptide SIFamide stimulates hindgut motility, which was antagonized by MIP in *Ixodes scapularis* (49).

It has been reported that K^+ is the major solute in the excretion being defecated through the hindgut, while Na^+ is the major solute in the saliva during feeding periods (52). In insects, the Malpighian tubules' primary role is the elimination of nitrogenous waste in the form of uric acid by the formation of hyperosmotic urine through the transport of K^+ from the hemolymph into the tubular lumen that is energized by V-ATPase and the transport of Na^+ out of the cell through Na/K-ATPase (5). Ticks' Malpighian tubules are also known to be the primary site for the formation of nitrogenous waste in the form of guanine-rich excreta (61-63). The mechanism controlling the Malpighian tubules in the tick has not been yet studied, while the Malpighian tubule in insects have been intensively studied for their hormonal components and their mechanisms of V-ATPase mediated fluid transport in the formation and secretion of the primary urine (62, 64)

In other blood feeding insects, like the kissing bug, potassium urate is formed in the Malpighian tubules and further urine concentration is achieved in the rectal sac through the reabsorption of water before excretion (5, 65, 66). In ixodid ticks, hindgut excretion is primarily for the removal of potassium-rich excreta. Guanine has been suggested as the main nitrogenous component of excretion, in addition to hematin and undigested blood. However, little is known about the molecular components regulating hindgut activity (5). Although natural water losses occur through the Malpighian tubules and hindgut through the excretion of waste, other imminent water losses occur through the cuticle, which amounts to a significant amount of water loss.

Dermal excretion through the cuticle

The phenomenon of dermal excretion through the cuticle in Ixodid ticks has long been observed, although the osmoregulatory implications of these physiology have not yet been investigated. Dermal excretion accounts for a significant amount of water lost in the tick's body, at least 2% of its body weight within a couple of seconds (67), and it is known to occur through the numerous dermal glands located on the epidermal cell layer of the ticks. Four different types of dermal glands and pore structures have been observed in ticks though their functions are yet controversial (5) and appear to vary in the structure depending on the tick species. This

characterization is based exclusively on the anatomy of the pore structures being non-setal and associated with no innervation. The dermal glands are classified into four structural categories: *sensilla auriformia, scnsilla sagittiformia, sensilla hastiformia*, and *sensilla latcniformia*, with the latter being absent in adult ticks for an unknown reason.

S*ensilla auriformia* in *R. appendiculatus* has been described as a sensory organ propioreceptor (68), while other studies defined it as a dermal gland (69). *Sensilla hastiformia*, also known as the Type I gland or small gland, is found in the alloscutum and scutum of both Metastriate and Prostriate ticks in all stages, though it appears to be underdeveloped in the non-fed stage (70). *Scnsilla sagittiformia*, also known as the Type II gland or large wax gland, is exclusive to Metastriate ticks and has been found in the alloscutum, edge of scutum, and anal plates of fed *Rhipicephalus* (67). Type II glands are known to produce secretions in ticks in the engorging stage but also in questing (non-fed) ticks (68, 70). The primary role of Type I and Type II glands is assumed to secrete defensive compound against predators and pathogens. The chemical components of the secretion includes squalene and other unidentified toxins which can be externalized through canal (pore) openings on the cuticle surface (71, 72), which is suggested to be acting as allomones against predatory ants. However, functional studies of the components of dermal secretions have not been successful in showing clear biological activities despite the predictions of the hypothetical functions (73). The dermal excretion has been reported to be triggered through mechanical stimulation (5, 70) and the mechanically induced secretion provided by heat tolerance in *R. sanguineus* ticks (74).

The microbiome and ticks

Arthropod disease vectors can carry a variety of disease-causing microorganisms, although the pathogen prevalence among arthropods largely varies depending on the vector and the pathogen; they all harbor a community of non-pathogenic symbiotic bacteria (75). The symbiotic relationships of arthropod-microorganism can be either parasitic (in which only one benefits), commensal (in which both are neutral), or mutualistic (in which both benefit). An obligate mutualistic symbiotic relationship has been extensively reported in arthropods, which is likely the consequence of a long evolutionary association between the animal host and the bacteria. Often in this association, both organisms are physiologically dependent on each another. When these bacterial cells commonly reside within host cells, they are referred to as “endosymbionts.” This

niche adaptation is normally seen in the midgut, hindgut, Malpighian tubules, and gonads of different blood feeding arthropods such as the body louse, the bed bug, mosquitoes, the tsetse fly, and ticks (76, 77).

It has been reported that over 90% of the microbiome is comprised by endosymbionts from the genus *Coxiella, Francisella*, and *Rickettsia* in ticks (78). These maternally transmitted endosymbionts have reduced genomes, compared to the bacterial pathogenic species from the same genera. The reduced symbiont genome implies that the long mutualistic association retained only the genes essential for the survival within the host cell. On the other hand, based on the ancestral relatedness between endosymbionts and pathogens in the genetic components (79), the boundary between these two forms may be easily modified to respond to the environment, which is determined by the hosts and their physiology (80). The endosymbiotic communities are reported to be abundant in blood-feeding arthropods, possibly due to the specialization of their food source, suggesting that their main function in the arthropod is to provide nutrients like vitamins to the host, which can impact their overall fitness (81).

Co-evolution of tick and microbes

The origin of ticks, which was estimated through fossil findings, is proposed to be somewhere between 54-65 million years ago (82) Since then, ticks have had to overcome many environmental challenges in the process of keeping their lineages. This process have included acquiring bacteria and/or bacterial origin genes through horizontal gene transfer (83). In addition, ticks have adapted to feeding exclusively on blood, which is not an ideal balanced nutrient source. It has been suggested that symbiots aid in vitamin production in *A. americanum* through the synthesis of biotin, folate, and other vitamins from the B complex (84). This has also been suggested for other obligate hematophagous arthropods like bed bugs and tsetse flies (76), which are exclusively hematophagous across both males and females.

Current studies of the arthropod vector microbiome

Many studies have focused on characterizing the bacterial community of arthropod vectors of diseases to better understand their role in vector competence. New technologies allowing large

scales of 16S sequence survey have provided innovative new concepts in vector biology and in control of vectors.

It has been reported in the case of mosquitoes that a major fraction of gut microbial community are environmentally acquired bacteria (85) and that these communities are important for the mosquitoes immune system development and completion of life cycle (86). Another important function of these microbial communities is **vector competence**, which is referred to as the ability of arthropods to acquire, maintain, and transmit microbial agents. Initial studies with mosquitoes showed that antibiotic treatment of *An. gambie* and *Ae. aegypti* mosquitoes affect the gut microbiota composition and increase the infection rates of *P. falciparum* in *An. gambie* and the DENV-2 titers in *Ae. aegypti* (87).

In the Lyme disease vector (*Ixodes scapularis*), it was demonstrated that composition of the gut microbiome influences *Borrelia burgdorferi* colonization. The following are proposed mechanisms by which bacterial communities impact vector competence:

1) Induction of host immune response: It was first observed in *Drosophila melanogaster* that PGRPs (peptide-glycan receptor proteins) present in the gut serve as surveillance receptors, which eventually lead to the production of AMP (antimicrobial peptides) and ROS (reactive oxygen species), with the intention to decrease microorganisms in the gut to prevent bacterial proliferation and thus maintain gut homeostasis (88). It has been shown that the absence of gut bacteria reduces AMP and ROS expression. In *Ixodes scapularis*, a dityrosine network (DTN) dependent of dual oxidases (Duox) influences pathogen survival in the midgut. Thus, it was shown that an impaired DNT in Doux knockdown or in specific peroxidase knockdown ticks results in reduced levels of *B. burgdorferi* persistence within ticks (89).

2) Direct competition: Some bacteria produce antiviral and/or anti-*Plasmodium* activity in *An. gambie*. This was shown when gut community members such as *Enterobacter* and *Chromobacterium* were isolated and cultured with arboviruses or *Plasmodium*, which showed an inhibition of the growth of pathogens (90), evidence of potential competition.

3) Barrier: Cririmoti et al. (2011) proposed that the microbiota also serves as potential barrier which can prevent the interaction of pathogenic bacteria with the peritrophic membrane (PM) and further prevent contact with midgut epithelium within the mosquito vector (90). In *I. scapularis*, the presence of *Anaplasma phagocytophilum* induces the expression of an antifreeze

glycoprotein, which alters bacterial biofilm formation, thus compromising the integrity of the PM and therefore facilitating pathogen invasion through the PM (91).

4) Promote infection: Although bacterial antagonistic infection effects are known, there are also some bacterial members which have been associated with increased infection, like the case of *Serratia* species in the gut of *Ae. aegypti*, which was shown to increase replication of Dengue virus (DENV-2).

Furthermore, recent studies with mosquitoes have focused on the potential of these bacterial communities in management and disease control (14, 92, 93). With the study of these microbial communities, it was shown that many of these communities are persistent and associated with the arthropod's environment. It was demonstrated that the mosquito larvae gut microbiome overlaps with their aquatic environments and that the adult gut microbiome also overlaps with those found in the larval stage (85). This is an important finding as it suggests that the gut microbiome acquired by the vector in the immature stages can be maintained during molting to the adult stage, functioning as the vector, which may permit the application of paratransgenic approaches for reducing the vectorial capacities of arthropod vectors.

Paratransgenesis in the arthropod disease vector

With the development of Integrated Pest Management (IPM), arthropod control has geared away from excessive use of pesticides into a more environmentally friendly control strategy. An innovative concept called paratransgenesis (94, 95) aims to reduce vectorial capacity of arthropod vectors by using genetically modified symbiont (or endosymbiont). Another modern technology, direct genetic modification of mosquitoes, has also arisen as a promising strategy for mosquito control and gene drive for lowering the vectorial capacity of the vector. The latter strategy has been rapidly moving forward recently with new biotechnologies, including CRISPR/Cas9 for vector control. Two main strategies have been conducted, namely the use of refractory (resistant to infection) mosquitoes to replace vector populations and the release of mosquitoes carrying a lethal gene to reduce mosquito populations (96, 97). However, these strategies have the disadvantage of potentially affecting mosquito fitness, and finding an effective drive mechanism and developing a stable mosquito germ-line may be challenging (92, 98, 99). Most of all, the negative public perception of using genetically modified eukaryotes in the field has hampered wide application of this strategy, though there has been partial success.

Use of genetic modification of endosymbiotic bacteria, which is more publicly accepted than generating transgenic mosquito due to the modification of bacteria instead of eukaryotic vector itself, has been proposed as a potential strategy for mosquito and vector-borne disease control (95). For this paratransgenic approach, the main requirement is to characterize microorganisms, which can successfully colonize the vector without affecting the overall fitness. Thus, endosymbiotic bacteria with the cytoplasmic incompatibility for invading the population are an ideal target due to their close evolutionary association with the vector.

Symbionts from the Chagas disease vector (*Rhodnius prolixus)* have been transformed using *R. rhodnii* and used as vehicles for the introduction of foreign genes into the disease vector (94). These transformed symbionts have been successfully introduced in the Chagas vector and recovered after molts. In the sleeping sickness (human African trypanosomiasis), vector (*Glossina spp*, or tsetse flies) similar genetic transformation approach has been applied using the modified *Sodalis* symbiont to repopulate the vector where they will express trypanocidal products with the potential to block parasite development (100-102). This is a promising strategy for the introduction of genes which could prevent pathogen colonization in the arthropod vector.

The concept of paratransgenesis may also be applicable in ticks. Previous studies on the microbiome of *A. americanum* have focused on characterization of intracellular endosymbionts (103-106). In addition, extracellular bacteria in the gut lumen may influence colonization of pathogens (75, 80, 107, 108) and overall vector competence of ticks as shown in mosquitoes. Characterizing the microbiome of non-intracellular symbionts in the tick might provide insight as to the tick's microbial habitats. This could aid in understanding the role of these communities in tick vector competence. Interestingly, most culture-independent microbiome studies on the tick gut reported the bacterial communities that should be easily culturable, such as *Bacillus*, *Pseudomonas*, and *Enterobacteriaceae* like *Escherichia*, *Shigella*, and *Proteus* (75, 109, 110), suggesting the microbiomes that can be potentially usable for the paratransgenic strategy.

Implications of water drinking behavior in tick microbiome

The role of non-pathogenic microorganisms, predominately gut microbiome rather than endosymbionts, found in ticks is still not well understood, although they were believed to play a role in vector competence (75). As has been discussed previously in this chapter, part of the tick microbiome is acquired through interaction with its environment; however, the role of voluntary

ingestion of microorganisms through drinking has not been investigated. Frequent ingestions of environmental water could serve as a route for ticks to acquire microorganisms. Thus, the impacts of their role in shaping tick microbiome need to be further explored. Using this natural phenomenon, water drinking behaviors can be exploited to deliver inoculated water with bacteria that could cause a microbial dysbiosis, leading to reduced fitness or vectorial capacity of the tick.

Dissertation structure and objectives

In this dissertation I have discussed and evaluated the water conservation biology of *A. americanum* and its implications for tick survival and management (Figure 1.4). An osmoregulatory process, the water drinking behavior, was further explored as a delivery route for toxic agents. Loss of large amounts of fluid through the dermal secretion was explored to understand the regulatory components and the signaling pathway, which can leverage the development of new strategies in tick control. I have addressed the water drinking behavior and its implication in shaping the tick microbiota in addition to its role in the acquisition of environmental bacteria.

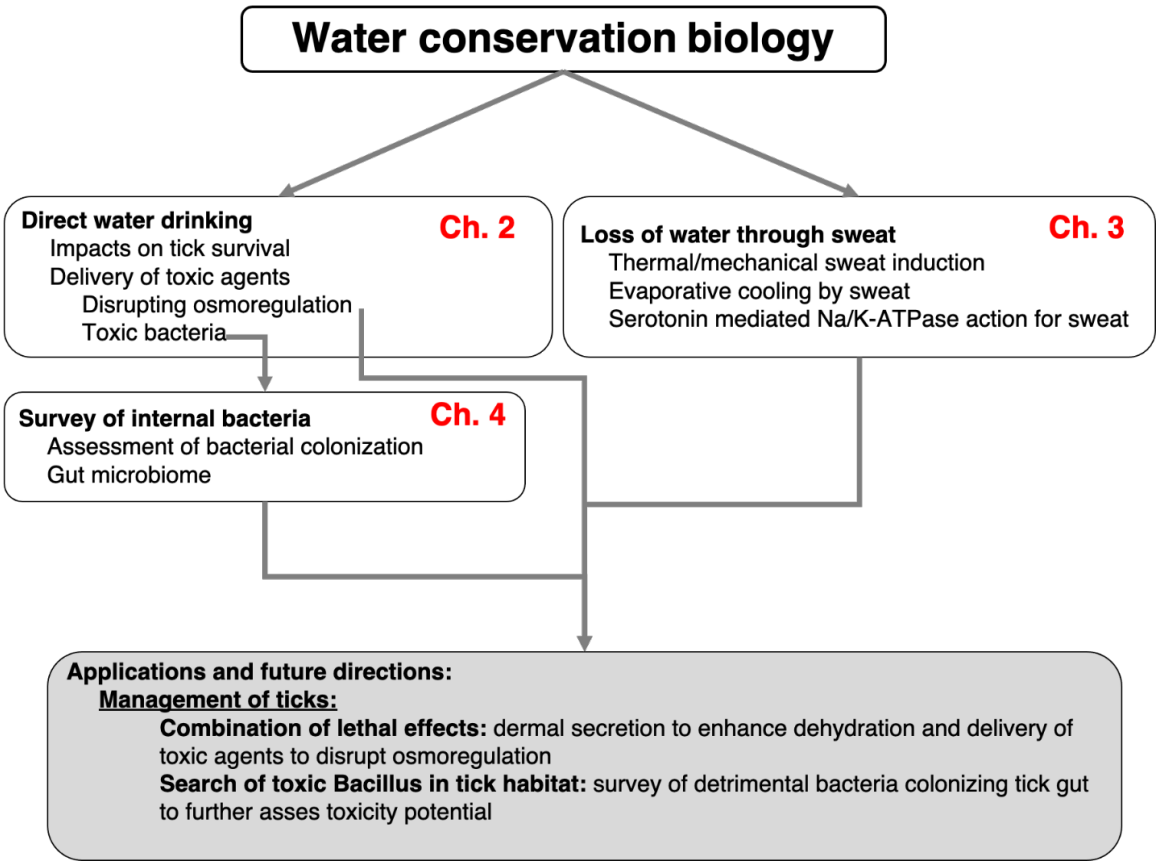

Figure 1.4. Flowchart diagram of the dissertation structure

In Chapter 2, I tested the hypothesis that *A. americanum* can actively drink water and that such a behavior can serve as a route for the delivery of toxic agents. I found that *A. americanum* can voluntarily drink nutrient-free water, which greatly increases their survival (111). In this study, I demonstrate that direct water drinking is the main contributing factor for extended survival, while water vapor alone has a moderate level of effect on tick survival. I was able to determine that water uptake occurs through the tick's mouthparts and that it can be later traced in the salivary glands and the midgut. In addition, by exploiting the water drinking behavior, I was able to deliver a toxic combination of inorganic compounds as an osmoregulation disruptor and toxic microorganisms to kill the tick. The study conducted in this chapter was further expanded across the studies in other chapters. Chapter 3 shows importance of another osmoregulatory physiology in dermal secretion, while Chapter 4 investigates the tick microbiome at the tick individual level that supports the tick in acquiring environmental bacteria.

In Chapter 3, I explored the implication of heat as a trigger for dermal secretion and its potential effects in thermoregulation and osmoregulation. In addition, I investigated the dermal gland and the mechanisms of secretion. I found that the temperature threshold for dermal secretion was >40º C. This observation led me to hypothesize that the dermal secretion could serve as a function of thermoregulation by evaporative cooling. I found that the secretion provided faster cooling rates. Thus, we called this mechanism "sweating" as an analogy to higher animals. Further anatomical studies revealed epidermal cell clusters in *A. americanum*, which is similar to the case of dermal glands described in *R. sanguineus* and *R. appendiculatus* (69, 112). A biogenic amine serotonin injection induced the dermal secretion and the serotonin-induced secretion was blocked by an injection of Ouabain (a Na^+/K^+ -ATPase blocker), suggesting neural or hormonal serotonin as the signal for activation of the Na^+/K^+ -ATPase in the dermal gland for fluid transport. Further study identified 5-HT1A as the only serotonin receptor (among four candidates) found in RNAseq of epidermal-enriched carcass of unfed *A. americanum* adult females. Application of this knowledge in tick control is suggested as the synergistic combination of osmoregulation disruptor and induction of dermal secretion.

In Chapter 4, I focused on the characterization of the microbial community of *A. americanum* identifying non-pathogenic microbes by culturable and culture-independent approaches. This idea stemmed from the phenomenon of ticks drinking water and acquiring environmental microorganisms. In theory, these culturable bacterial isolates would then become available for future studies on manipulation of the gut bacterial community and its effect on the tick vector competence. In addition, the ability of the tick to acquire environmental bacteria through water drinking provides an opportunity to control the tick by using the potential tick pathogenic bacteria as described in Chapter 2.

Our data showed that the midgut microbiome of *A. americanum* is very poor, with low abundance and with the majority of the 16S sequence being the endosymbionts *Coxiella* and *Rickettsia* (average 97% of 16S). The culture-dependent approach showed that the majority of the bacterial isolates were representative of soil- and plant-associated bacteria. This is not surprising, as *A. americanum* has been shown to actively ingest liquid water from the environment (as discussed in Chapter 2) and it is likely that these bacteria were ingested along with water (75). The soil and plant origin of the midgut bacteria was also suggested for *I. ricinus* (75, 113). This observation suggested a future direction for this work in the form of a survey of specific toxic

Bacillus strains, as it is the most abundant taxa found in field ticks in addition to the known toxicity of some species to many arthropods including ticks (114). I recommend further studies including larger scale surveys of bacteria that can colonize the tick alimentary cannel and play a role in pathogen colonization and in tick survival.

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Chapter 2 - Liquid water intake of the lone star tick, *Amblyomma americanum:* Implications for tick survival and management

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Abstract

Ixodid ticks are ectoparasites that feed exclusively on blood as their source of nutrients. Although ticks spend most of their life off the host, until now it has been assumed that the blood and the water vapor are the only sources of water to maintain water balance and prevent desiccation. Here we report for the first time that adult lone star ticks, *Amblyomma americanum*, also actively drink nutrient-free water, which greatly increases their survival. The volume of ingested water is greater in females than males (0.55 ± 0.06 vs 0.44 ± 0.07 µl) and most likely due to differences in tick size. Water uptake occurs through mouthparts and it can be later observed in the salivary glands and the midgut. We also exploited this behavior by adding a variety of inorganic compounds and microorganisms to water. Addition of inorganic salts to drinking water such as KH_2PO_4+NaCl+KNO_3 resulted in 100% tick mortality within 3 days. As a proof of concept for using the water drinking as a delivery route of toxic reagents for ticks, we also show that adding *Pseudomonas aeruginosa* to drinking water quickly leads to tick death. This tick behavior can be exploited to target important physiological systems, which would make ticks vulnerable to dehydration and microbial dysbiosis.

Key words: ticks, liquid water, drinking, survival, management, salts, microorganism

Introduction

Ticks are obligate blood feeding ectoparasites and one of the most important arthropod groups that vector pathogens to people and other animals worldwide (1-5). Hard ticks have to survive extended periods off the host (~ 90% of their lifetime) throughout their development.

Therefore, tick survival is dependent on their ability to maintain water balance, as they have to overcome fluctuating environmental conditions, primarily temperature and relative humidity (RH) (6, 7). Ticks maintain water balance by water vapor uptake. Water vapor uptake directly depends on the Critical Equilibrium Activity (CEA), which is the minimum RH required for water uptake and maintaining the water balance in the body (8-10). In most ixodid ticks the CEA is close to 90% RH (10); in *Amblyomma americanum* water vapor uptake is between 85% and 93% RH and accounts for up to 77% of the water taken in (11). Water vapor uptake was first reported in *Amblyomma variegatum* with the mouthparts as the main site of this uptake (12). It was demonstrated that the salivary glands secrete to the mouthparts a hygroscopic solution high in chlorine, potassium, and sodium (6, 13) which absorbs water vapor that is then ingested by the tick (6).

Off host ticks could also actively drink water to maintain water balance, whereas several studies reported no water drinking behavior in *Ixodes ricinus, Dermacentor marginatus,* and *D. reticulatus* (14-16). Our recent study demonstrated that *I. scapularis* can approach and touch water droplets and occasionally insert the hypostome and drink water (5% of ticks tested) (17). Drinking-like behavior was also observed for several *Rhipicephalus* species*, Dermacentor nuttalli, Amblyomma hebraeum,* and larvae of *A. americanum* (18-20). However, the significance of liquid water drinking in tick biology and fitness and its potential for management have not been investigated.

In this study, we aimed to determine 1) whether adults *A. americanum* ingest water; 2) the volume of water taken in and its distribution within the tick; 3) the significance of water drinking to tick survival; and 4) the potential for exploitation of water drinking behavior for tick management.

Materials and Methods

Ticks

Non-fed, 2-3 months old adults of *A. americanum* were obtained from the Oklahoma State University tick rearing facility and kept at 4°C and >95% RH to minimize tick activity until used for experiments. Before each experiment, ticks were preconditioned for 24 hrs at 28 ± 3°C and 30 ± 5% RH in individual 50 x 9 mm Tight-Fit Lid Dishes (Falcon® Brand Products, New York, USA).

Water drinking behavior

Laboratory-reared *A. americanum*

After preconditioning, ticks (20 males, 20 females) were placed individually into sterile 50 x 9 mm Tight-Fit Petri dishes and provided with a 5.0 µl droplet of deionized sterile water and kept at 40% RH and 26 ºC (laboratory conditions during experiments). Ticks were observed for 1 hour. Ticks that stayed in contact with droplet after 1 hour were left in the same dish until the next day. Ticks that did not drink during the first hour were transferred to a new dish without water, kept at 28ºC ± 3ºC, and provided with a 5.0 µl water droplet every 24 hrs until they were observed to drink water.

Field collected ticks

Questing adult ticks were collected from northeastern Kansas (Konza Prairie Biological Research Station) and southeastern Kansas (around Pittsburg) by cloth dragging. A total of 88 adult ticks were collected; 56 *A. americanum*, 30 *Dermacentor variabilis,* and 2 *A. maculatum*. Upon collection, ticks were kept in a cooler with ice (3-5ºC) and over 95% RH until arrival to the laboratory. Water drinking behavior was assessed as described above for laboratory-reared ticks.

Ticks survival with and without access to liquid water

Preconditioned laboratory-reared *A. americanum* adults were divided into 3 equal groups and placed individually in cardboard containers (volume: 236 ml, 8.5 x 4.8 cm) (Rigid paper Corp.) modified with a sterile petri dish base on the bottom (100 x 15 mm Fisherbrand) and a screen nylon mesh on the lid of the cup. Three different treatments were used after preconditioning: Group 1 (access to water) was provided with a 5.0 µl droplet of deionized sterile water daily. Group 2 (without access to water) was also provided daily with a 5.0 µl water droplet to maintain the same level of humidity as in the Group 1, but the droplet was not accessible to the ticks; water droplet was protected by a metal nut (1 x 2 cm) covered by a screen nylon mesh. Group 3 (no water) was provided with no water. Bioassays were conducted separately for males and females (n = 10 for each) and 3 replicates for each group were performed for all treatments. All experiments were conducted at 28 ± 3 °C and 30 ± 5% RH (in same experimental area). Room conditions were set to 30 % RH and 28 °C. During the experiments, conditions were recorded every 12 hours using a digital thermos-hygrometer (part 37950-03) (Cole-Parmer, IL, USA). Ticks were kept in same

conditions (28 ± 3 °C and 30 ± 5% RH) between treatments and mortality was recorded every 24 hrs for 30 days (until 100% mortality in Groups 2 and 3).

Quantification of water intake

In order to quantify the amount of drinking, two different approaches were used: a) bacteria mixed in the water to trace the amount of bacteria (and water) ingested, and b) capillary feeding showing the visually quantifiable volume of water. Ticks were preconditioned as described above.

1) Droplet drinking with bacteria

We used bacteria as a quantifiable tracer for the estimation of the liquid ingested amount. Deionized sterile water was inoculated with Gram-negative (*Escherichia coli*) or Gram-positive (*Staphylococcus epidermidis*) bacteria. Initial estimation of inoculum size was standardized by optical density at 600 nm using a BioMate^{TM} 3 series spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and confirmed by culturing on tryptic soy agar at 37˚C for 24 hours. Optic density at 600 nm was only used to measure turbidity (cell density) as a reference point to reach an approximation of 1,000 to 9,000 cells/µl. A 5.0 µl droplet of bacteria-inoculated water was provided to each preconditioned tick. The mean concentration of inoculum offered to each tick was 7.6 ± 0.7 x 10^3 CFU (colony forming units)/µl (*E. coli*) and 4.8 ± 0.7 x 10^3 CFU/µl (*S. epidermidis*). Ticks that drank from the inoculum droplet for 1 hour were processed (18 males and 20 females from each bacterial group). In order to minimize the loss of bacteria during the process, we harvested the bacteria immediately after the time allowed for the 1 hr drinking. Ticks were surface sterilized with 0.5% sodium hypochlorite, 70% ethanol, and washed with sterile water. Individual ticks were then cut in small pieces and homogenized in phosphate buffer saline. The homogenate was spread plated on tryptic soy agar and incubated at 37˚C for 24 hrs. We ensured that there were no culturable bacteria in the laboratory reared ticks by plating processed ticks on tryptic soy agar in an initial pilot test with 40 ticks. The volume of water ingested by ticks was determined by using the number of CFU obtained from the ticks and the bacterial inoculum size per µl. *Escherichia coli* and *S. epidermidis* were confirmed by colony and cell morphology and a rapid Gram staining test.

2) Capillary drinking

Preconditioned ticks (20 males and 20 females) were immobilized on dental wax and placed inside a Tight-fit lid 50 x 9 mm petri dish (5 ticks per dish). The tick hypostome was inserted into a

polyimide capillary tube (454 μm diameter x 2 cm length). Deionized sterile water was pipetted into capillary tubes and dishes were sealed during the assays. The decrease in water volume was measured (1mm = 0.1464 μl) every 15 minutes for 1 hr. Final measurements were recorded and an evaporation control (capillary tube without a tick) was subtracted from measurements to estimate the amount of water intake by ticks.

Internal distribution of ingested water (droplet and capillary feeding)

Ticks were preconditioned before experiments as described above. One mM rhodamine 123 (Rh123, Sigma-Aldrich, St. Louis, MO, USA) was used as a fluorescent dye to determine the distribution of water within tick internal organs after droplet and capillary feeding.

Droplet and capillary feeding were conducted as described above with a few modifications. To minimize the loss of fluorescence, dishes with ticks were kept under dim light during bioassays and drinking time was reduced to 30 min (capillary feeding) and to 15 min (droplet feeding). After the exposure, ticks (4 males and 4 females for capillary feeding and 2 males and 3 females from droplet feeding) were washed with sterile water and immobilized on dental wax for dissection in phosphate buffer saline. The dorsal integument of ticks was removed with a surgical scalpel and images of internal organs were captured using a camera (DFC400) attached to a stereo microscope (M205FA; Leica, Heerbrugg, Switzerland) with the GFP filter set (excitation BP470/40, dichromatic mirror 500, and emission filter 480LP) to visualize water by observing fluorescence in the internal tissues.

Exploitation of water drinking behavior for tick management

Non-fed males and females of *A. americanum* were obtained from the Oklahoma State University Tick rearing facility and kept at 4°C and 95% R.H. For all assays, ticks were preconditioned as described above.

Inorganic compounds: Different combinations and concentrations of inorganic compounds were tested to determine the optimal treatment to effect tick mortality (Table 2.1 and S1). Based on the function of the salivary glands, we focused primarily on salts with potassium, sodium, and chlorine. Ticks were individually provided with a 5.0 μl droplet of a tested compound in sterile deionized water every 24 or 48 hrs. Control ticks were kept at the same conditions and were

provided daily with a 5.0 µl droplet of deionized sterile water. Bioassays were conducted from 7 to 19 days and tick mortality was monitored daily.

Microorganisms: To assess tick survival after microbial ingestion, different bacterial species and strains in various dosages (Table 2.2 and S2) were provided to ticks in a 5.0 µl water droplet. Control ticks were kept in the same conditions and were provided daily with a 5.0 µl droplet of deionized sterile water. Tick mortality was monitored every 24 hrs. For assays with *Pseudomonas aeruginosa*, exposure to the inoculum was limited to 1 hr and a one-time application. For other assays, the exposure time varied depending on the treatment (Table 2.3). After the microbial exposure, ticks were transferred to new dishes and given a 5.0 µl droplet of deionized sterile water on daily basis. Dead ticks from different treatments and a subset of control live ticks were processed to determine the presence of the microorganisms in the body. Ticks were surface sterilized using 0.5% sodium hypochlorite, 70% ethanol, and washed with sterile water. Individual ticks were cut in small pieces by sterile scissors and homogenized in phosphate buffer saline. Homogenates were spread plated on tryptic soy agar and incubated at 26°C for 72 hrs.

LD_{50} for P. aeruginosa. To determine LD_{50} in ticks, 3 different bacterial concentrations were used: 10^4 CFU/µl, 10^3 CFU/µl, and 10^2 CFU/µl. Ticks were divided into 3 groups of 15 and preconditioned as described above. Ticks were immobilized on dental wax inside a Tight-fit lid 50 x 9 mm petri dish (5 ticks per dish and 1 water evaporation control). A polyimide capillary tube (454 µm diameter x 2 cm length) was inserted over the tick hypostome and filled with a bacterial suspension in sterile water (n=10 for each treatment) or sterile water (n=5) as control. The decrease in water volume was measured (1mm = 0.1619 µl) every 15 minutes for 1 hr. Final measurements were recorded and evaporation control (a tube with no tick) was subtracted from this measurement to determine the amount of liquid intake. After 1.0 hr exposure, ticks were removed from the wax, placed individually in sterile petri dishes, and given a 5.0 µl droplet of deionized sterile water immediately after treatment and then every 24 hrs during the assay. Ticks were kept in 28 ± 3°C at 30 ± 5% RH and mortality was recorded daily. All dead ticks were processed to determine the presence of *P. aeruginosa* as described above.

Statistical analysis

Kaplan-Meier survival curves (21) were generated using the data from surviving and dead individuals over time to assess median survival times of each treatment. Statistical significance

between survival curves of treatments was determined using multiple statistical comparison approaches. Using p=0.05 as cutoff for significance, we conducted Mantel-Cox, Genhan-Breslow-Wilcoxon and Chi Square tests using GraphPad Prism version 7.04 for Windows (GraphPad Software, California USA, www.graphpad.com).

Results

Water drinking behavior

All laboratory-reared ticks (n=40) preconditioned at 30 ± 5% RH and 28 ± 3 ^{0}C for 24 hrs displayed water drinking behavior when a drop of water was offered over the course of 3 days (5 µl daily). Few ticks were not attracted to drink water on day 1; however, eventually drank on day 2 or 3. Two types of behavior were observed: 1) slowly approaching the water (5 to 20 min) and placing the hypostome into the water droplet or 2) immediately reaching the water droplet and in less than a minute inserting the hypostome into the water. In both cases, slow movements of chelicera and pulsatile movements on the base of the hypostome were observed, indicating water drinking. Ticks often exhibited squatting behavior on the water droplet and had the entire ventral surface of the body submerged in the water.

The same behavior was observed in ticks collected in the field (Figure 2.1a). Questing adults of three tick species of unknown age collected in northeast and southeast Kansas, engaged in water drinking upon arrival to the laboratory: *Amblyomma americanum* [30 out of 56 (24/36 females and 6/20 males)], *D. variabilis* [20 out of 30 (14/20 females, 6/10 males)], and *A. maculatum* (2/2 females). Ticks that did not exhibit drinking behavior within the first 24-72 hrs after collection, died possibly from desiccation.

Tick survival with and without access to liquid water

The significance of direct liquid water drinking and water uptake through vapor was clearly shown in *A. americanum* survival. Ticks with free daily access to a water droplet had no mortality during the entire 30 day bioassay period. In contrast, all ticks with no water droplets died within 12 days (Figure 2.1b). Ticks with no direct access to the 5µl water droplet (covered with the screen mesh for blocking the direct access), survived significantly longer than ticks without any liquid water; however, they started dying on day 6 and 100% mortality was reached in 25 days (Figure 2.1b). In this treatment, ticks quickly moved to and stayed on top of the mesh covering the water

droplet (as close as possible to the droplet) presumably to take advantage of a higher humidity emanating from the droplet. Males and females had a similar pattern of survival; therefore, we combined these data (Figure 2.1b). Tick survival was significantly different among the three treatments; water droplets vs. no water ($p<0.0001$), water vapor vs. no water ($p=0.0099$), and water vapor vs. water droplets ($p<0.0001$).

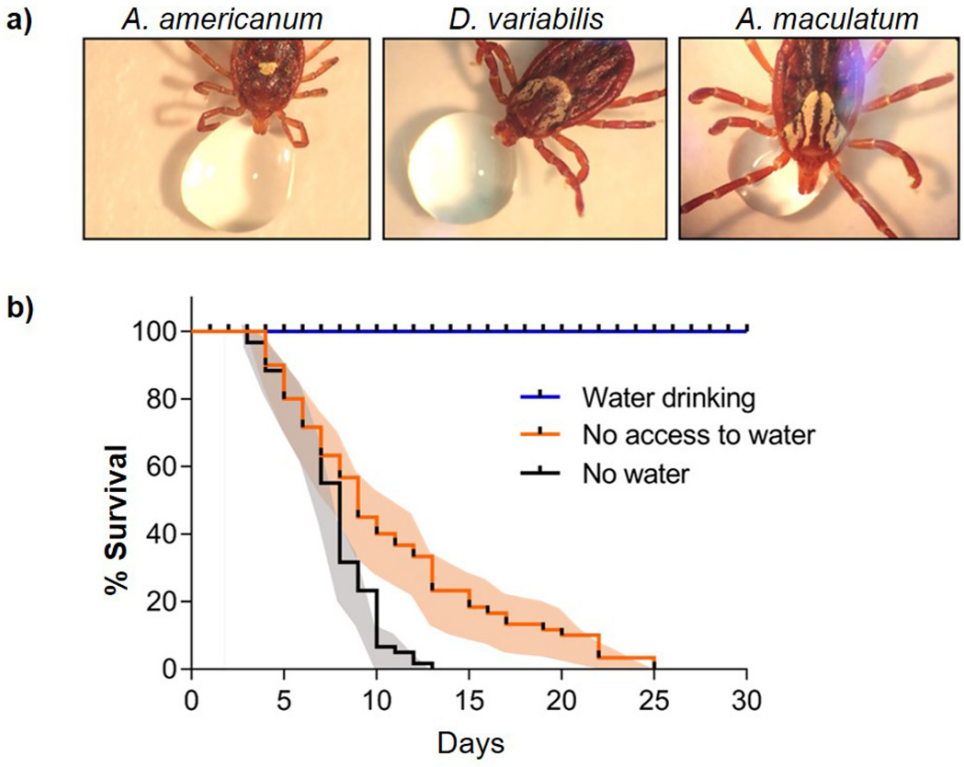

Figure 2.1. Water drinking and its impact in tick survival

Water drinking behavior exhibited by adult field collected female ticks (a); Impact of water drinking on male and female *Amblyomma americanum* survival (n=60/treatment) (b). Water drinking (direct contact), No access to water (no contact, water covered by screen mesh), No water: no droplet provided. Percent survival shown in Kaplan-Meier survival curves. Color shaded areas represent SEM for each data point.

Quantification of water intake

Using bacteria in a water droplet as quantifiable tracer, we were able to estimate by bacterial cell counts that males (n=20) ingested 0.20 ± 0.05 µl (*Staphylococcus epidermidis*) and 0.22 ± 0.03 µl (*E. coli*) of water within a period of 60 min. In females (n=18) water intake was 0.33 ± 0.07 µl (*S. epidermidis*) and 0.32 ± 0.05 µl (*E. coli*) (Table 2.1). Capillary feeding showed

that intake within 60 min was 0.44 ± 0.07 µl (n=20) and 0.55 ± 0.06 µl (n=20) in males and females, respectively (Table 2.1).

Internal distribution of ingested water (droplet and capillary feeding)

After 30 min of drinking from a capillary tube, we detected fluorescence in the type I acini of the salivary glands and in the midgut, in both males and females (n=4 each) (Figure 2.2e,g). After drinking from a fluorescent water droplet, all ticks showed fluorescence in type I acini of the salivary glands, while only 50% (1 of 2) and 75% (2 of 3) males and females, respectively, showed fluorescence also in the midgut (Figure 2.2d,f). This suggests that the uptake of water occurs through both, the salivary glands and the midgut as proposed for *I. scapularis* (17), while the uptake through the salivary glands is the primary mechanism in the voluntary feeding of the water droplet.

Table 2.1. Water intake of *Amblyomma americanum*

Droplet feeding				Capillary feeding	
Staphylococcus epidermidis (4.8 ± 0.7 x 10^3 CFU/µl)		***Escherichia coli*** (7.6 ± 0.7 x 10^3 CFU/µl)			
Males (n=20)	**Females (n=18)**	**Males (n=18)**	**Females (n=20)**	**Males (n = 20)**	**Females (n = 20)**
0.20 ± 0.05*	0.33 ± 0.07	0.22 ± 0.03	0.32 ± 0.05	0.44 ± 0.07	0.55 ± 0.06

*Mean µl ingested ± standard error of mean

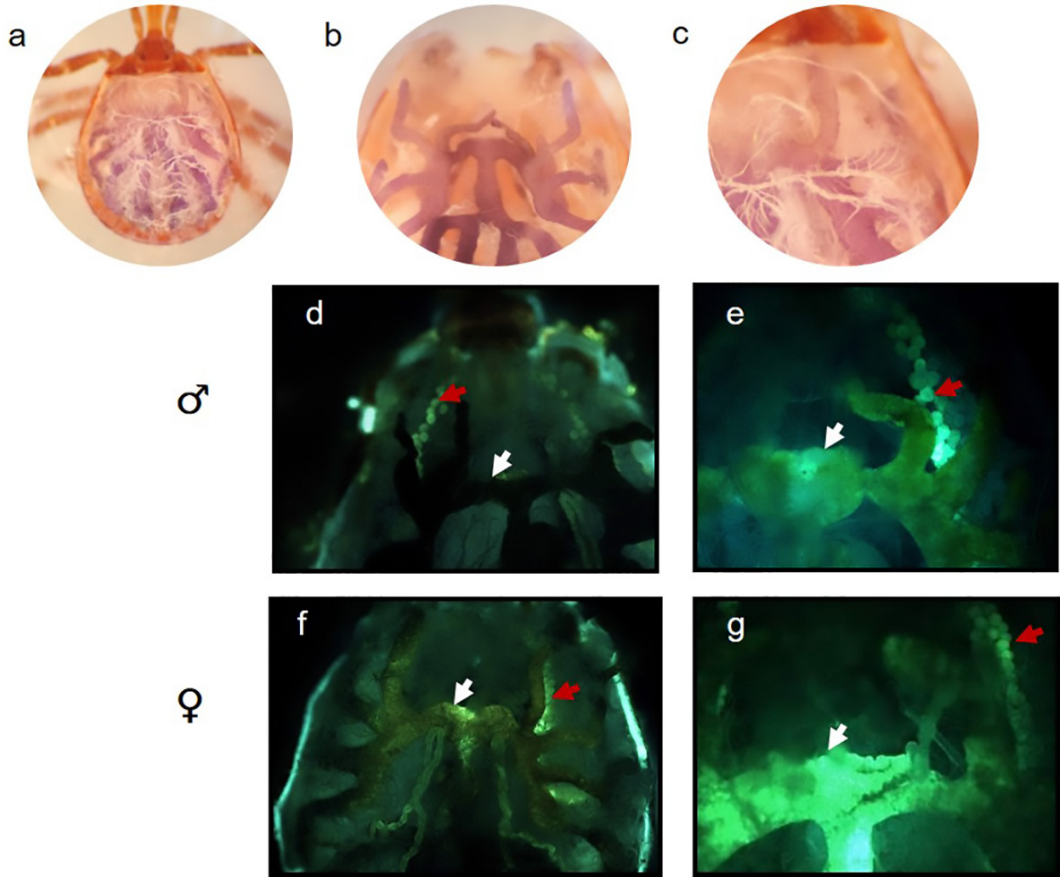

Figure 2.2. Water absorption organs in lone star ticks

From *a)* to *c)*: images of internal morphology of the adult ticks representing the location of the fluorescence below. *d)* Male after droplet feeding with Rh123 and *e)* after capillary feeding with Rh123. *f)* Female after droplet feeding with Rh123 and *g)* after capillary feeding with Rh123. White and red arrows point to fluorescence in the midgut and the salivary glands, respectively.

Exploitation of water drinking behavior for tick management

Toxicity of ingested ions

The tick osmoregulatory system must have limitations for transport of certain ions. Osmoregulation occurs through ion transport resulting in an electro-osmotic gradient across the cell layers. Therefore, we aimed to identify inorganic compounds that disrupt tick osmoregulation and water balance and lead to high tick mortality within 7 days (Table 2.2 and S1).

Table 2.2. Summary of inorganic compounds tested and their impact on *Amblyomma americanum* survival after water droplet ingestion

			Males		Females		Combined	
Treatment	**Final concentration mM (compound ratio)**	**Length of bioassay (days)**	***n***	**% survival (days)**	***n***	**% survival (days)**	***n***	**% survival (days)**
***Fert.20.10.20**	2.6 (KH_2PO_4)/4.7 (KNO_3)	7	5	100 (7)	5	100	10	100
***Fert.20.10.20 +NaCl**	2.6/4.7/5	7	5	100 (7)	5	100	10	100
***KH_2PO_4+NaCl**	2/250	12	10	70 (12)	10	30	20	50
KH_2PO_4 monobasic	500	17	10	0 (6)	10	0 (17)	20	0 (17)
Na_2HPO_4 dibasic	500	17	10	0 (7)	10	0 (9)	20	0 (9)
NaCl	500	19	10	0 (8)	10	0 (9)	20	0 (9)
KH_2PO_4 monobasic	100	10	10	40 (10)	10	30	20	35
Na_2HPO_4 dibasic	100	10	10	25(10)	10	25	20	25

			Males		Females		Combined	
Treatment	**Final concentration mM (compound ratio)**	**Length of bioassay (days)**	***n***	**% survival (days)**	***n***	**% survival (days)**	***n***	**% survival (days)**
KH_2PO_4+NaCl	125/62.5	7	5	0 (3)	5	0 (4)	10	0 (4)
$(NH_4)_2PO_4$ +NaCl	50/5	7	10	0 (6)	10	0 (7)	20	0 (7)
KH_2PO_4+NaCl+KNO_3	120/20/20	7	10	0 (2)	10	0 (3)	20	0 (3)
KH_2PO_4+NaCl+KNO_3	60/10/10	7	10	0 (2)	10	0 (3)	20	0 (3)
KH_2PO_4+NaCl+KNO_3	50/5/5	7	10	0 (5)	10	0 (6)	20	0 (6)
[1]KH_2PO_4+NaCl+KNO_3	240/40/40	7	8	0 (3)	8	0 (4)	16	0 (4)
[1]KH_2PO_4+NaCl+KNO_3	120/20/20	7	8	0 (2)	8	0 (3)	16	0 (3)
[1]KH_2PO_4+NaCl+KNO_3	60/5/5	7	10	0 (7)	10	0 (5)	20	0 (7)
[1]KH_2PO_4+NaCl+KNO_3	50/5/5	7	10	0 (5)	10	0 (6)	20	0 (6)

*Recommended concentration from Peter's fertilizer 20.10.20

[1] Every other day treatments

Solutions containing potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), and potassium nitrate (KNO_3) resulted in tick mortality in less than 7 days. For the most efficient treatment (60 mM KH_2PO_4/10 mM NaCl /10 mM KNO_3), 100% mortality was achieved by day 3 (Figure 2.3a). The lowest concentration to achieve 100% tick mortality in less than 7 days was 50 mM KH_2PO_4/5 mM NaCl/5 mM KNO_3 with a median survival at day 4 and 100% mortality at day 6 (Figure 2.3a). A two-fold increase in the ion concentration (120/20/20 mM) did not increase the speed of tick mortality, and the resulting survival curves were not statistically different (p=0.398) (Figure 2.3a). A decrease in treatment application frequency (every other day) using a 2-fold increase in concentration was not significantly different from that of daily applications (p= 0.499 and mortality day 3) (Figure 2.3b). Likewise, a 4-fold increase in ion concentration at the same application frequency did not increase mortality (Figure 2.3b).

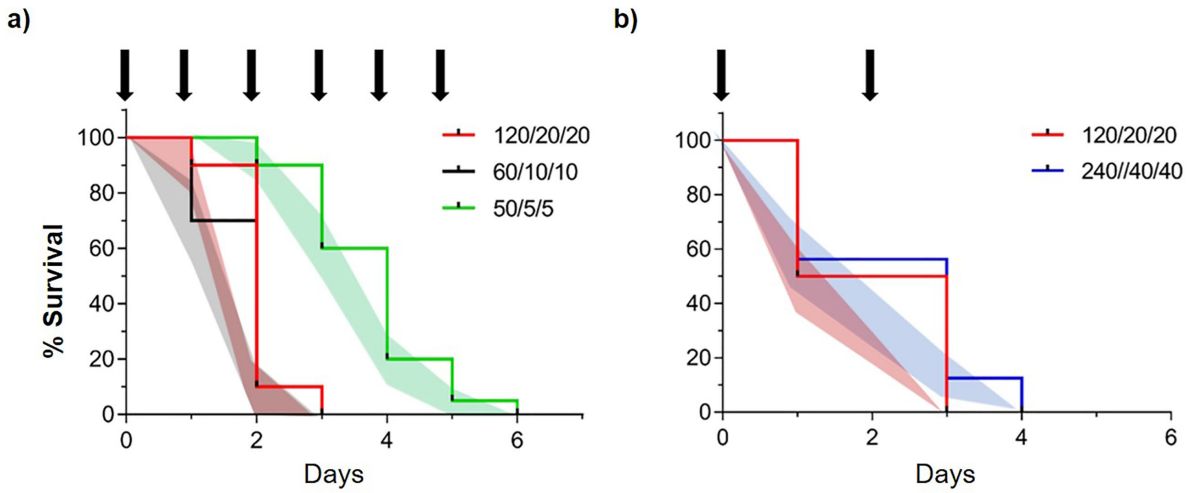

Figure 2.3. Kaplan-Meier survival curves after lone star ticks water droplet drinking with inorganic compounds.

All survival curves show treatments with KH_2PO_4 + NaCl + KNO_3 at different concentrations/ratios and different application frequency. a) Survival of *A. americanum* with concentrations in mM: 120/20/20; 60/10/10; and 50/5/5. Two replicates per treatment male and female ticks (n=20). b) Survival of *A. americanum* at 2-fold and 4-fold increase in compound concentration at a lower application frequency. Two replicates per treatment of male and female ticks (n=16). All control ticks (offered water droplet) in each treatment exhibited 100% survival

after day 15 (data not shown). Black arrows point to treatment days. Color shaded areas represent SEM for each data point.

Toxicity of ingested microorganisms

Water delivery of various bacteria, fungi, and their toxins was also tested in a search for agents lethal to ticks. Among bacteria, only *Pseudomonas aeruginosa* caused 100% mortality in less than 7 days post-treatment (Table 2.3). Presence of this bacterium was confirmed in dead ticks after surface sterilization, processing, and plating of individual ticks on trypticase soy agar. Although we observed some tick mortality (day 10 to 15 post-treatment) after treatments with *Bacillus thuringiensis,* entomopathogenic fungi, and *Bt* toxins, high tick mortality was not achieved (Tables 2.3,and S2).

Table 2.3. Summary of microorganisms tested and their impact on *Amblyomma americanum* survival after water droplet ingestion

Microorganisms					
Treatment	**Concentration (CFU/µl)**	**Days of exposure**	**Length of bioassay**	***n* (♂,♀)**	**% survival**
Pseudomonas aeruginosa	1.1×10^4	1	7	15 (0,15)	0
	1.8×10^3	1	7	17 (7,10)	53
	1.8×10^2	1	7	16 (9,7)	81
Bacillus thuringiensis					
***ser* kurstaki**	~1.5×10^4	3(♂), 7(♀)	15	18 (8,10)	38.8
***ser* israelensis**	1.5×10^4	3(♂), 7(♀)	15	18 (8,10)	55.5
***ser* morrisoni**	1.0×10^4	3(♂), 7(♀)	15	18 (8,10)	61.1
***ser* kurstaki**	1.8×10^4	7	7	10 (0,10)	70

*bioassay with nymphs

Based on the positive results with *P. aeruginosa*, we aimed to determine the LD_{50} for adult ticks using capillary and droplet drinking. We were able to achieve 100% mortality at day 3 post-treatment with 1.1 x 10^4 CFU/µl of *P. aeruginosa* in female ticks by droplet drinking with a median survival of 2 days. We were not able to reach 100% mortality with lower bacterial concentrations (Table 2.3, Figure 2.4a). In the capillary feeding approach, the mortality was lower than that in droplet feeding with the maximum mortality 90% in 7 days (Figure 2.4b). *Pseudomonas aeruginosa* was recovered from all dead ticks in this bioassay and bacterial concentrations in these ticks was very high (Table S3), while no bacteria were recovered from the control group of ticks.

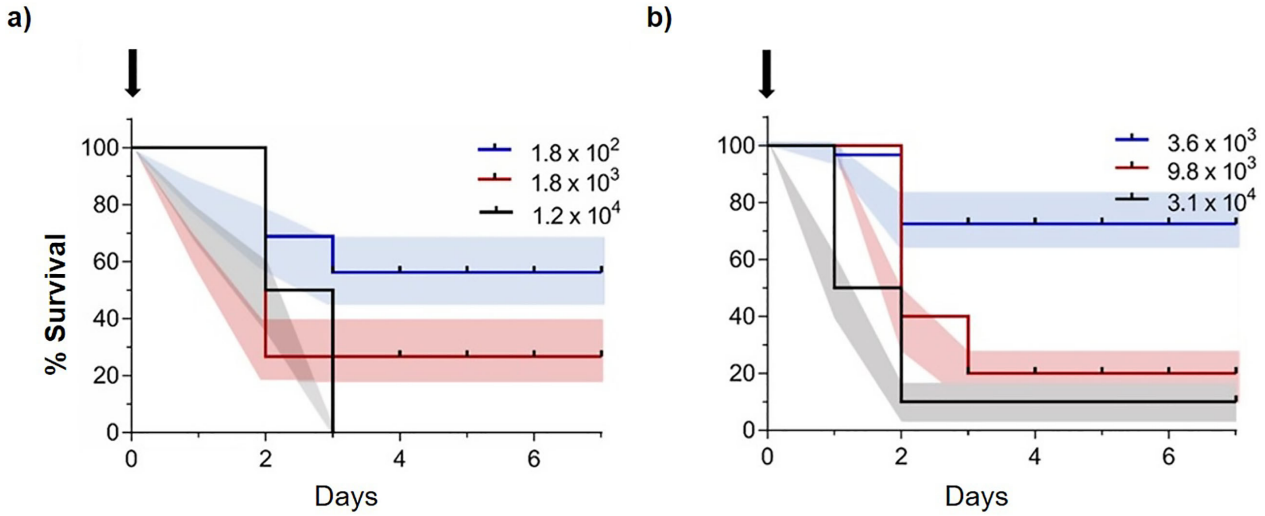

Figure 2.4. LD50 estimation with Kaplan-Meier survival curves after droplet and capillary drinking of water with *Pseudomonas aeruginosa*

a) Survival of *A. americanum* after droplet feeding. Two replicates per treatment in treatments 1.8 x 10^3 and 1.8 x 10^2 (n=17 and n=16 respectively). Treatment 1.2 x 10^4 was conducted only with females, 3 replicates (n=15); b) Survival of *A. americanum* after capillary feeding. One replicate per treatment, 20 individuals per group (10 males 10 females). Labels represent the inoculum size in CFU per microliter. Black arrows point to the treatment day. Color shaded areas represent the SEM for each data point.

Discussion

It has been established for a long time that ticks feed exclusively on host blood as the only source of nutrients. Since ticks spent most of their life off the host, it is critical for them to maintain water balance to prevent desiccation and death (6-9). Ticks use passive water vapor absorption and

active water vapor uptake (6, 8, 10, 16). In addition, there are several rather dated and sporadic observations that some ticks may actively uptake liquid water (18, 20). For *A. americanum*, Needham and Teel (1986) reported that larvae were seen gathering around and inserting their mouthparts in water droplets. Nonetheless, nothing is known about water ingestion and its significance in tick fitness and survival nor it was explored for potential tick management.

In our study, we describe for the first time adult lone star ticks ingesting liquid water. Active water uptake is shown by our observation of pulsatile movements in the base of hypostome with the mouthparts in the water and the recovery of bacteria and the fluorescent dye mixed in the water from the tick midgut and the salivary glands. More importantly, we show a significant difference in survival between ticks that did and did not have physical contact with water droplets; with no mortality for 30 days (up to 4 months, not shown) and 100% mortality by day 25 (median survival day 9), respectively. As expected, ticks without daily supply of water droplets died even faster with 100% mortality within 12 days. This clearly demonstrates the significance of direct contact with liquid water and water drinking in tick survival. Considering the critical importance of maintaining water balance to prevent desiccation, we propose that the ability of lone star ticks to drink liquid water is likely one of the main factors behind the great success and continuous expansion of this species in the United States (22). This is supported by modeling studies showing that vapor pressure is one of the most important determinants of tick suitable habitat (23) and continued expansion of the lone star tick (24). Nevertheless, additional studies assessing tick survival considering macro-, meso- and microclimatic levels of different habitats are needed to further asses the importance of tick water drinking and its spatial and temporal impacts.

The volume of water ingested differed between males and females and this is likely due to differences in the body size. The water volume measured in droplet feeding using bacteria, as a quantifiable tracer, was lower than that with the capillary feeding and this is likely because of the underestimation of water volume due to digestion of bacteria in the midgut and some loss during bacterial recovery and culturing. These bacteria were recovered from the internal soft tissues and likely derived from the midgut and the salivary glands, as these are the sites for water absorption (17).

Although other studies reported an increased weight gain of ticks after being in physical contact with water droplets, this was believed to occur mainly due to water vapor absorption through the cuticle (25, 26). Clearly, in our study we demonstrate that water vapor alone is not the

main contributing factor for extended survival rates. Previously, Londt and Whitehead (19) reported in a study from the South Africa that larvae of *A. hebraeum, Rhipicephalus appendiculatus, R. evertsi, R. decoloratus* were observed to imbibe water through the mouthparts from a moist filter paper and these ticks survived longer than those without wet filter paper. However, these authors could not distinguish between water vapor and water drinking. In our previous study on *I. scapularis* (17), we observed water in the type I acini of the salivary glands and the midgut after capillary feeding. However, water droplet drinking behavior was rare with only 5% (2/43) ticks ingesting water this way. Studies that examined other tick species including *Ixodes ricinus, Dermacentor marginatus,* and *D. reticulatus* reported no liquid water drinking (14-16). Interestingly, in addition, to *A. americanum* (laboratory-reared and from the field), we observed water drinking behavior in wild *A. maculatum* and *D. variabilis*. However; these results are preliminary and the significance of this behavior in tick biology and fitness remains to be examined.

We also aimed to exploit the water drinking behavior for *A. americanum* management. From the 48 inorganic compound combinations tested, daily applications of water with KH_2PO_4 (60mM) + NaCl (10mM) + KNO_3 (10mM) resulted in 100% mortality of ticks in three days. We also tested two-fold and 4-fold concentration of this solution combined with decrease application frequency (every other day); however, tick mortality was not significantly different from that of the original treatment. We suggest that this is an indication of a threshold of salt concentration at which certain combinations of ions disrupt tick osmoregulation and lead to irreversible dehydration and death. During dry periods, ticks prevent dehydration by reducing water excretion (27) and by secretion of hyperosmolar saliva rich in Na+, K+ and Cl- capturing water vapor from the air (28). Interestingly, in our study only assays with phosphates resulted in high tick mortality. It is likely that phosphate salts cause imbalance in ions and water regulation. In vertebrates, inorganic phosphate homeostasis is maintained by two families of phosphate transporters transporting Pi into the cells based on the electrochemical gradient provided by Na+/K+ ATPase (29). The mechanism for disruption of tick osmoregulation by phosphate salts could be due to a direct effect on the electrochemical gradient or due to disruption of anionic transporters of the excretory system; however, this remains to be further investigated. While this approach needs to be tested in the field, it is conceivable that for example, spraying water with a mix of KH_2PO_4,

NaCl, and KNO_3 in small droplets from a regular garden sprayer on a grass lawn, provides an effective control of lone star ticks.

In addition to the inorganic salts, we also tested different microorganisms including bacteria, fungi, and their toxins in search of agents that are lethal to ticks. After only one-time exposure, *P. aeruginosa* caused 100% mortality in ticks within three days post-treatment. Although some tick mortality was observed with other microorganisms tested, none of those led to high tick mortality within 7 days. While *P. aeruginosa* is a general opportunistic pathogen and therefore unsuitable for a biological control of ticks, these data show great potential for using this approach with other bacterial species and strains or their toxins.

Conclusion

Overall, our results demonstrate that water drinking behavior is common in *A. americanum* and possibly several other ixodid ticks, and has a significant impact on tick survival. In addition, water drinking can be used as a mean to deliver toxic compounds and microorganisms for tick management. We propose the exploitation of the tick water drinking behavior to target important physiological systems, which would make ticks vulnerable to dehydration and microbial dysbiosis.

Table S1. Inorganic compounds tested and their impact on *Amblyomma americanum* survival after water droplet ingestion.

Treatment	Final concentration mM (compound ratio)	Length of bioassay (days)	Males		Females		Combined	
			n	% survival (days)	*n*	% survival (days)	*n*	% survival (days)
NaCl	100	10	10	100 (10)	10	100	20	100
KH_2PO_4 + NaCl	500 (1:1)	7	8	0 (3)	8	0 (5)	16	0 (5)
KH_2PO_4 + NaCl	250 (1:1)	7	5	0 (4)	5	0 (3)	10	0 (4)
KH_2PO_4 + NaCl	125 (1:1)	7	5	0 (6)	5	0 (4)	10	0 (6)
KH_2PO_4 + NaCl	25 (1:1)	7	5	100 (7)	5	100	10	100
KH_2PO_4 + NaCl	20 (1:1)	7	5	60 (7)	5	80	10	70
KH_2PO_4 + NaCl	125/62.5	7	5	0 (3)	5	0 (4)	10	0 (4)
KH_2PO_4 + NaCl	125/30.12	7	5	0 (6)	5	0 (4)	10	0 (6)
KH_2PO_4 + NaCl	125/15	7	5	0 (4)	5	0 (3)	10	0 (4)
KH_2PO_4 + NaCl	62.5/15	7	5	0 (6)	5	0 (7)	10	0 (7)
ATP	10	19	10	50 (19)	10	90	20	70
Dopamine	10	19	10	80 (19)	10	50	20	65
Caffeine	10	19	9	0 (17)	10	20	19	10
ATP + NaCl	10/500	19	10	0 (7)	10	0 (9)	20	0 (9)
Dopamine + NaCl	10/500	19	10	0 (6)	10	0 (6)	20	0 (6)
Caffeine + NaCl	10/500	19	10	0 (4)	10	0 (7)	20	0 (7)
KH_2PO_4 + NaCl	60/10	7	5	0 (3)	5	20	10	10
KH_2PO_4 + NaCl	60/5	7	5	20 (7)	5	0 (4)	10	10
KH_2PO_4 + NaCl + KNO_3	60/10/5	7	5	20 (7)	5	0 (2)	10	10
NH_4N0_3 + NaCl	50/5	7	10	100 (7)	10	100	20	100
[i]KH_2PO_4 + NaCl + KNO_3	60/5/10	7	5	0 (5)	5	20	10	10
[i]KH_2PO_4 + NaCl + KNO_3	30/5/10	7	5	80 (7)	5	20	10	50
[i]KH_2PO_4 + NaCl + KNO_3	30/5/5	7	5	40 (7)	5	80	10	60
[i]NH_4N0_3	20	7	5	100 (7)	5	80	10	90
[i]$(NH_4)_2PO_4$	20	7	5	100 (7)	5	80	10	90
[i]NH_4N0_3 + NaCl	20/10	7	5	40 (7)	5	100	10	70
[i]NH_4N0_3 + NaCl	20/5	7	5	60 (7)	5	100	10	80
[i]$(NH_4)_2PO_4$ +NaCl	30/5	7	5	40 (7)	5	60	10	50
[i]$(NH_4)_2PO_4$ +NaCl	20/10	7	5	60 (7)	5	40	10	60
[i]$(NH_4)_2PO_4$ +NaCl	20/5	7	5	80 (7)	5	80	10	80

[i] every other day treatments

Table S2. Microorganisms and their impact on *Amblyomma americanum* survival after water droplet ingestion

Treatment	Concentration (CFU/µl)	Length of bioassay	*n* (♂,♀)	% survival
Microorganisms				
Bacillus thuringiensis				
Cry 4B	7.6×10^4	10	10 (5,5)	100
Cry 11A	3.7×10^4	10	10 (5,5)	100
Beauveria bassiana				
high concentration	unknown	10	20 (10,10)	100
low concentration	unknown	10	9 (9,0)	88.9
***high concentration**	unknown	7	10 (10,0)	70
***low concentration**	unknown	7	10 (10,0)	90
Isaria sinclairii				
high concentration	unknown	7	5 (5,0)	100
Toxins				
Protein toxin A	0.2 mg/ml	10	16 (8,8)	100
Protein toxin B	0.6 mg/ml	10	16 (8,8)	100

*bioassays with nymphs

Table S3. Mortality of *A. americanum* after ingestion of *Pseudomonas aeruginosa* through capillary feeding

Inoculum CFU/µL	Ingested cells ± SEM	[+]Mortality (%)	*P. aeruginosa* confirmation (%)*
$3.1x10^4$	$1.5 \pm 0.2 \times 10^4$	90	100
$9.8x10^3$	$3.2 \pm 0.4 \times 10^3$	80	100
$3.6x10^3$	$1.3 \pm 0.2 \times 10^3$	30	100

[+]Mortality (%) for each inoculum was calculated from 20 individuals tested (10 males and 10 females)

*Percentage calculated from the total dead individuals.

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Chapter 3 - Dermal secretion physiology in Metastriata ticks; thermoregulation in the lone star tick *Amblyomma americanum*

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Abstract

Ticks are blood feeding ectoparasites that transmit a wide range of pathogens. The lone star tick, *Amblyomma americanum*, is one of the most widely distributed ticks in the Midwest and Eastern United States. Lone star ticks, like most three-host ixodid ticks, can survive in harsh environments for extended periods without consuming a blood meal. Physiological mechanisms that allow them to survive during hot and dry season include thermal tolerance and water homeostasis. Large quantity of dermal fluid secretions induced by mechanical stimulation of tick legs has been described in metastriate ticks including *Amblyomma*. We hypothesize that a function of tick dermal secretion is similar to the sweating in large homeothermal animals. In this study, we found that a contact with a heat probe at 45ºC can trigger dermal secretion. We demonstrated that dermal secretion plays a role in evaporative cooling when ticks are exposed to high temperature. We observed that direct contact to a heat probe for 5 seconds at ~52ºC caused an exhaustive dermal secretion with ~4% loss of body weight and resulted in the lethality in 24-hour, indicating that the secretion is associated with significant costs of water loss. We identified type II dermal glands having paired two cells forming large glandular structures. The secretion is triggered by an injection of serotonin and the serotonin-mediated secretion was suppressed by a pretreatment of Ouabain, a Na/K-ATPase blocker, implying that the secretion is controlled by serotonin and the downstream Na/K-ATPase.

Key words: Dermal glands, evaporative cooling, serotonin, Na/K-ATPase, *Ixodidae, Rhipicephalus*

Introduction

Ticks are blood feeding arthropods and the number one cause of vector-borne illness in the United States (1, 2). The Family *Ixodidae*, hard tick containing the major vector species, is mainly divided into two groups; Metastriata group including *Amblyomma, Rhipicephalus,* and *Dermacentor,* and Prostriata group including *Ixodes*. (2-4). The lone star tick, *Amblyomma americanum*, is a hard tick that belongs to the Metastriata group, which is widely distributed in the Midwest region of the United States (5). This tick vectors pathogens, including *Fransicella tularensis*, *Ehrlichia chaffeens*is, and *E. ewingii*. Each are causative agents of Ehrlichiosis and a number of viruses (6-8). This tick can also cause southern tick associated rash illness (STARI) and red meat allergy, which is caused by a specific glycan (alpha-gal or galactose-α-1,3-galactose) present in their salivary glands (9, 10).

Multi-host hard ticks spend a relatively small part of their lifetime on the host for feeding. Over 90% of their lifetime is spent off-host, mostly in vegetated ground. Tick survival and success largely depends on their ability to maintain water balance during off-host periods in high temperature and low relative humidity (RH) (11, 12). Water absorption physiology in hard tick during the off-host periods has been reviewed previously (13-15). Ticks capture water molecules in environmental vapor by using a hygroscopic saliva (16) that is rich in chloride, potassium, and sodium (11, 17). Water absorption then takes place through the type I salivary gland acini (18). In addition, active drinking directly from water drops has also been observed (13, 18, 19). The physiology for obtaining water is to recuperate from the natural imminent water losses that occur through evaporation and excretion (3, 20-22).

Ticks have thick sclerotized cuticle covered with wax (13) preventing evaporative water loss. However, an important route of water loss through the integument may be secretion through dermal glands which accounts for 2.3–2.5% of the body weight in the case of *R. sanguineus* (23) and 4% in *A. americanum* (in this study). In metastriate ticks, the dermal secretion occurs through a large number of a subset of dermal glands opened to dorsal and ventral surface, which has been demonstrated to be triggered through mechanical stimulation (3, 24). It has been proposed that the primary function of this secretion is to defend against predators and pathogens as it contains squalene and other unknown toxic compounds (25-27). Other reports also show that the gland secretion contains compounds with pheromonal activity for aggregation, such as: *o*-nitrophenol and methy-salicylate in the fed male *A. variegatum* (28) and in *A. haebreum* (29). In addition, it

has been also reported that dermal excretion through mechanical stimulation provides heat tolerance in *R. sanguineus* (30), opening possibilities of multiple functions of the dermal secretion, although the analytical studies for different types of secretion is yet lacking. We hypothesize that the tick dermal secretion, large volume of fluid containing small portion of the active compounds, functions for evaporative cooling like the case of sweating in large animals.

In this study, we propose that dermal secretion allows ticks to cope with a sudden exposure to hot temperature by using evaporative cooling of the body. We show here heat-probe induced dermal secretions and rapid evaporative cooling, supporting the hypothesis. The dermal secretion physiology is further investigated by identifying the molecular components, serotonin and Na/K-ATPase, involved in the control of the dermal secretion.

Materials and Methods

Ticks

Unfed 2 to 3-month old female adult *A. americanum, D. variabilis, R. sanguineus*, and *I. scapularis* were obtained from the Oklahoma State University tick rearing facility. They were kept at room temperature and >95% RH until used for experiments. Ticks were kept at 28 °C and >90% RH prior to experiments.

Induction of dermal secretion by heat probe

Ticks were immobilized on a flat surface with double-sided sticky tape (3M Scotch permanent mounting tape, MN, USA). The dorsal surface of the tick was exposed to heat by a direct contact with a heat probe made of 28-gauge nichrome 80 wire for 1–5 sec., depending on the experiments specified. The temperature of the wire was controlled by a current controller (Stoelting Co, IL, USA) and the temperature was monitored by a thermal camera (SeekTermal, CompactXR).

In order to measure the threshold temperature for secretion response in *A. americanum*, we treated the ticks with different temperatures for 1 sec. with the temperature increasing from 30 to 70 ºC by 5 ºC interval. Eight ticks were tested at each temperature to avoid acclimation or sensitization of ticks to prior exposure to the heat probe. Another modified method was used for measuring the heat sensitivity in the test including other Metastriata ticks (*A. americanum, R. sanguineus and D. variabilis).* In this test, individual ticks were exposed to the probe repeatedly

with sequential increments of the probe temperature. In each trial, the probe temperature was sequentially increased by ~3 to 4 °C (*n*=10 for *R. sanguineus, n*=12 for *A. americanum*, and *n*=12 for *D. variabilis*). If the dermal secretion was not observed before 5 seconds of contact at the given temperature, the probe was detached from the tick and the next higher temperature was set for another contact that was followed by ~ 1 min. interval. A control group was treated with the probe at room temperature (RT) to ensure the response was not due to mechanical simulation by contact from the probe. During this operation, the thermal image was recorded and analyzed by SeekThermal application software (V2.1.9.1) and the image processing software (V2.6.1.12).

To assess the impact of dermal secretion on the body cooling rate, a set of 5 ticks per group were used; a group with no sweat and with sweat. Both were treated with the probe at the temperature between 40-42 °C. Ticks reported as; no sweat means they did not exhibit the secretion after 5 seconds of the heat probe contact. Thermal images were analyzed as 20 frames per second using ImageJ 1.53a (31). The cooling rate was assessed by the average pixel values of 3 different regions of interest (ROI) surrounding the probe contact point on the tick surface. The average pixel value of the ROI was used for accurate estimation of temperature. The rate of lowering the temperature from 40 °C in each tick were fitted to an ExpDec1 curve (OriginPro 2020b, Fig 3b) and values obtained for each rate of decay, $A_1/t1$, were used in a Student's T-test for the analysis (Fig 3b). ExpDec1 regression provided the fits with R values in the range of 0.97 to 0.99. The regression and the data analyses were conducted in OriginPro 2020b (9.7.5.184).

To assess the amount of weight loss after exhaustive dermal secretion, the ticks kept in high RH (>95%) for one day were weighted before and after the contact with heat probe at 55–58°C for 5 sec. The ticks after the treatments were placed on the high RH for recovery and monitored for survivorship.

Pharmacology of dermal secretion

To identify the neural or hormonal components involved in dermal excretion, we injected a series of biogenic amines, neuropeptides, and secondary signaling messengers. The chemicals used for biogenic amines were: Octopamine ((±)-Octopamine hydrochloride, Sigma, Cas#00250), Norepinephrine ((±)-Norepinephrine (+)-bitartrate salt, Sigma, Cas#3414-63-9), Dopamine (Dopamine hydrochloride, Sigma, Cas#H8502), Serotonin (5-hydroxytriptamine hydrochloride, Sigma Cas#H9523), for neuropeptides were: SIFamide (AYRKPPFNGSIFamide, (32, 33)), MIP-

1 (Mioinhibitory peptide-1, ASDWNRLSGMWamide, (32, 33)), Proctolin (RYLPT, ELV-1 (Elevenin, LDCRKYPFYYRCRGISA, (34)), for secondary signaling messengers were: Dibutyryl cAMP (Dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt, Santa Cruz Biotechnology, Cas# 16980-89-5), Forskolin (Sigma, Cas # F3917), Dibutyryl cGMP (Santa Cruz Biotechnology, Cas# 51116-00-8) SNAP (N-(acetyloxy)-3-nitrosothiovaline, Cayman chemicals Cas#67776-06-1).

The compounds dissolved in water were injected through the base of the second coxal segment from lateral side using a Nanoject III nano-injector (Drummond Scientific Company, PA, USA) (Table 1). All injections were made for 10 nL unless it is specified. The strong secretion inducer, serotonin, was further tested for obtaining the full dose-responses.

We tested the role of Na^+/K^+-ATPase in the dermal secretion by a pretreatment of the tick with Oubaian, a Na^+/K^+-ATPase inhibitor. Ticks were injected with 10 nl of 100 µM Oubaian 30 min before injection of serotonin (10 nl of 1mM). In rare cases (3 out of 20), the ticks that showed dermal secretion in Ouabain injection were excluded in data analysis because they were considered as the response to mechanical stimulation by insertion of the needle, which also occurred in water control.

Localization of dermal secretion and the anatomy

Naïve *A.mericanum* females were used to visualize the structure of the dermal gland on the dorsal and ventral dermal integuments. Immobilized ticks were placed on a double-sided sticky tape and treated by a heat contact to the legs. Dorsal dermal secretion was observed under fluorescent light with CFP filter set (excitation BP436/7, dichromatic mirror 455, and emission filter 470LP). The majority of dermal secretions displayed fluorescence drops with the CFP filter set. For internal view of the dermal glands, naïve ticks were injected with a local anesthetic, 20 µl of 740 mM of Procaine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), to prevent the dermal secretion triggered in the processes of dissection, which empties the gland contents. Ten minutes after procaine injection, the dorsal integument of ticks was removed with a surgical scalpel to visualize the internal glands. External and internal images were captured using a camera (DFC400) attached to a stereo microscope (M205FA; Leica, Heerbrugg, Switzerland) with the CFP filter set.

For confocal imaging of the glands, cellular structures, and molecular components, dorsally opened tissues were washed with PBST fixed in 4% paraformaldehyde for 3 hours. The tissues were then incubated with 5% normal goat serum (Jackson ImmunoResearch), containing the target antibodies overnight at room temperature. Immunohistochemistry (IHC) was performed using beta-tubulin mouse antibody (GenScript, Piscataway, NJ, USA) at a final concentration of 0.5 mg/ml, mouse monoclonal antibody (a5) raised against chicken Na/K-ATPase (Developmental Study Hybridoma Bank, University of Iowa) at 4.4 µg/ml. To localize Na/K-ATPase, we used a procedure already established by our laboratory (35). Following primary antibody incubation, tissues were washed with PBST and subsequently incubated overnight at room temperature with the secondary antibody, goat-anti-mouse IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). In addition, goat polyclonal antibody against horseradish peroxidase (HRP) conjugated with Cyanine Cy™3 (8 µg/ml, Jackson Immunoresearch, West Grove, PA. USA), containing 5% NGS, was used. The HRP-antibody has been used for characterization of the tissues having neural properties in insect (36), due to its immunoreactivity against an N-linked oligosaccharide epitope expressed on neuronal glycoproteins in insects (37). After the secondary antibody incubations, tissues were washed with PBST, incubated in 300nM 4′,6′-diamino-2-phenylindole (DAPI, Sigma) or 2.5 µg/ml Hoechst 33342 (Invitrogen, Carlsbad, CA, US) and 40 fold dilution of Phalloidin conjugated with Alexa Fluor™ 555 (used for actin staining), (Molecular Probes, Eugene, OR, USA) or 5 µg/ml CellMask™ (Invitrogen, Carlsbad, CA, US) for 10 minutes, washed for 30 minutes and then mounted in glycerol. Images were captured with a confocal microscope (Zeiss LSM 700).

In microtome sections to visualize the cuticle integument and epidermal layers, ticks were cut into 2–3 pieces directly alive or after snap-freezing using liquid Nitrogen. Ticks were fixed for 3 hours at room temperature in non-alcoholic Bouin's fixative and washed with PBS containing 0.5% Triton X-100 (PBST). Samples were dehydrated with series of increasing ethanol solution (50 to 95%) and an additional cuticle plasticization step was conducted by placing samples in n-Butanol (3 hours incubation at room temperature with rotation). Following dehydration, samples were placed in 100% chloroform for overnight at 60 °C and transferred to paraffin for up to 96 hrs. Tissue sections were made by using a Leica microtome at 8 to 10 µm thickness and placed on a slide with 0.5% gelatin. Samples were dried in an incubator at 40 °C overnight. Deparaffinization

was conducted using xylene, tissues were rehydrated with decreasing series of ethanol solutions (95 to 50%) and washed with PBST.

For visualization of dermal layers under bright field, staining with methylene blue (10 seconds) was conducted, using solution II from the Hema 3^{TM} staining kit (Protocol^{TM}, Fisher Scientific, Waltham, MA, USA). Slides were visualized using a Nikon Eclipse E800 compound microscope.

Results

Tick dermal excretion is induced by a contact to the heat probe

The dermal secretion observed for the dorsal side was induced by a dorsal contact of the thermal probe (Figure 3.1) at 35 °C in 12.5% individuals, and 100% individuals responded at 45 ºC (Figure 3.2a) in *A. americanum*. When the treatments were made by sequential increases in the probe temperature on the same individual, where rapid acclimation to prior exposure to the heat probe could occur, the temperature required for induction of dermal secretion was 42 ºC in *A. americanum*, Other tick species also showed secretion responses with moderate levels of shifts in the sensitivities: *R. sanguineus* (45 °C) and *D. variabilis* (50 °C) where total percent response was 90 and 65% respectively (Figure 3.2B). No dermal secretion was observed in *Ixodes scapularis*.

When *A. americanum secretion* was induced by the heat-probe at 55-58 °C for up to 5 seconds, the exhaustive dermal secretion resulted in significant loss of the weight, 4.1 % loss from the average 6.1 mg to 5.8 mg (n=40). These group of the ticks were all dead in a day kept in 95% RH after the treatment. The temperature in an edge of a typical tick habitat in Kansas in a sunny summer day with 33 ºC ambient temperature was found to be in a range of ~55-65 °C on the surface of soil and ~37-50 °C on the surface of grasses (Figure 3.2C).

In an investigation of the effect of dermal secretion on evaporative cooling, the temperature change monitored on the dorsal surface showed significantly higher cooling rate in the ticks with dermal secretions than in those without secretion (Figure 3A). When the cooling rate was regressed with an exponential decay formula, the slopes of decay were significantly different; -1.7 compared to that of no secretion -1.2 (Figure 3.3B). The evaporative cooling in the ticks with the secretion resulted in 1.2 degrees cooler at the 10 second after the contact with heat probe (Figure 3.3A).

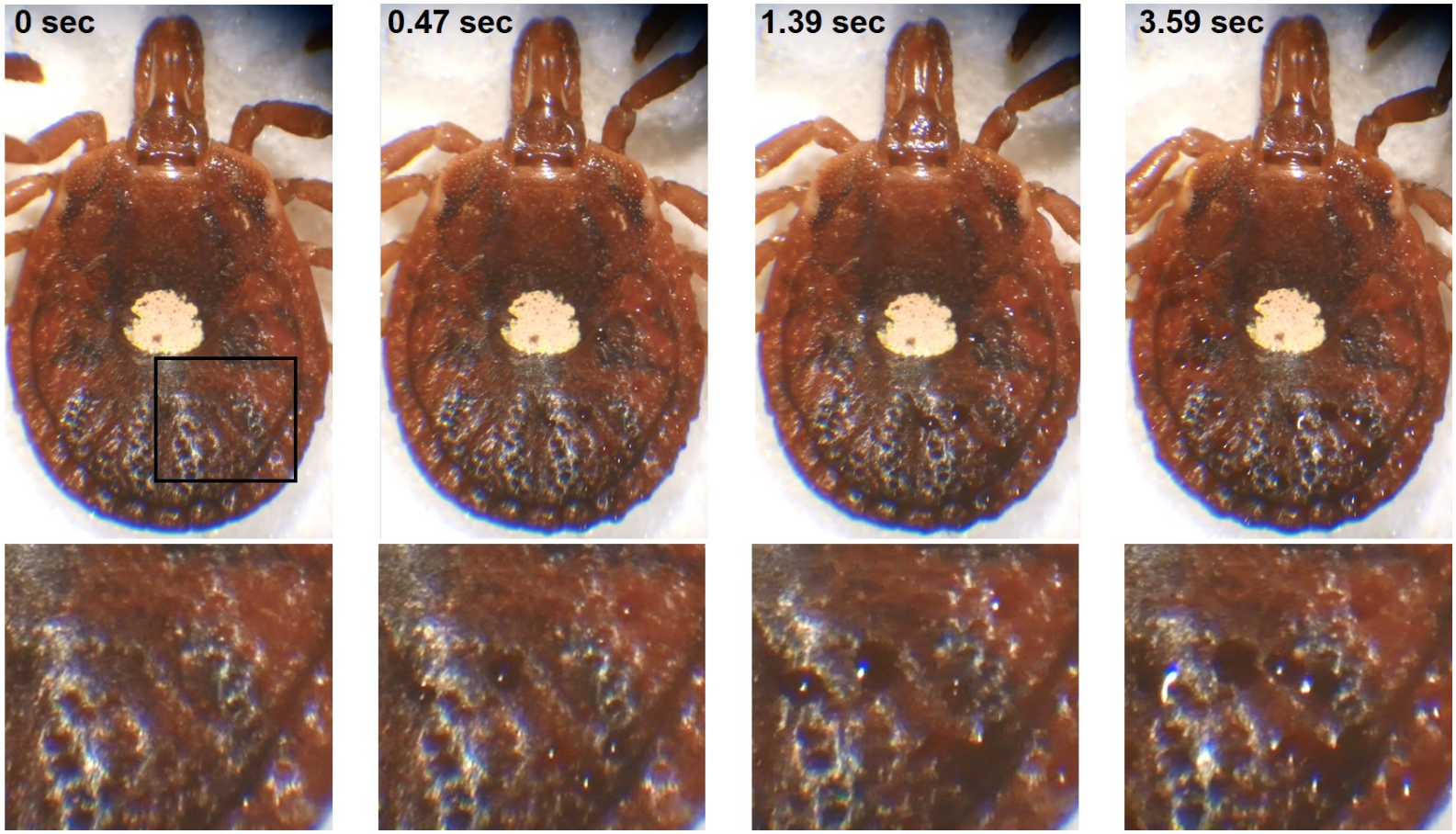

Figure 3.1. Dermal secretion upon heat probe contact

Time 0 represents the moment the heated probe contacted the legs of the ticks. Top images show the full dorsal view of the tick over the course of 4 seconds after touching the dorsum with the heated probe. Lower images show the magnification of the insertion in the time 0 for each time point.

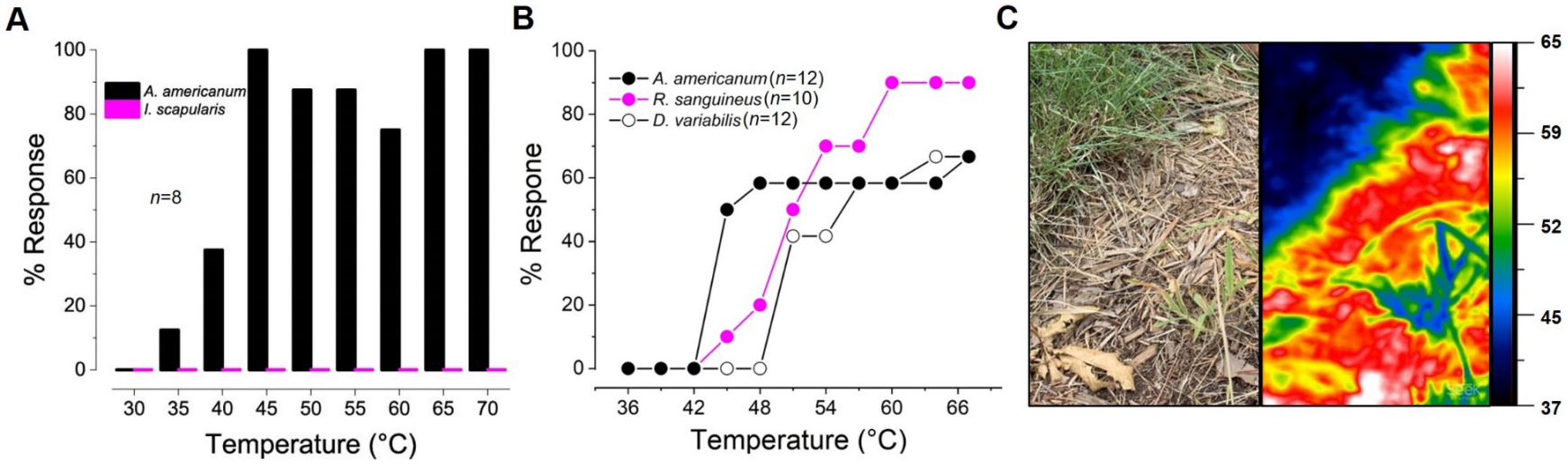

Figure 3.2. Dermal secretion in response to heat probe contacts.

A) The response by percentage for dermal secretion at different temperature in female *Amblyomma americanum* (n=8 ticks/temperature). **B)** Dermal secretion responses to a heat probe contact tested in a same tick with sequentially increased temperature of the heat probe. **C)** Thermal image with regular bright field image for a typical tick habitat showing the temperature range of 38 to 65ºC. The image was captured on a sunny summer day with 33 ºC ambient temperature.

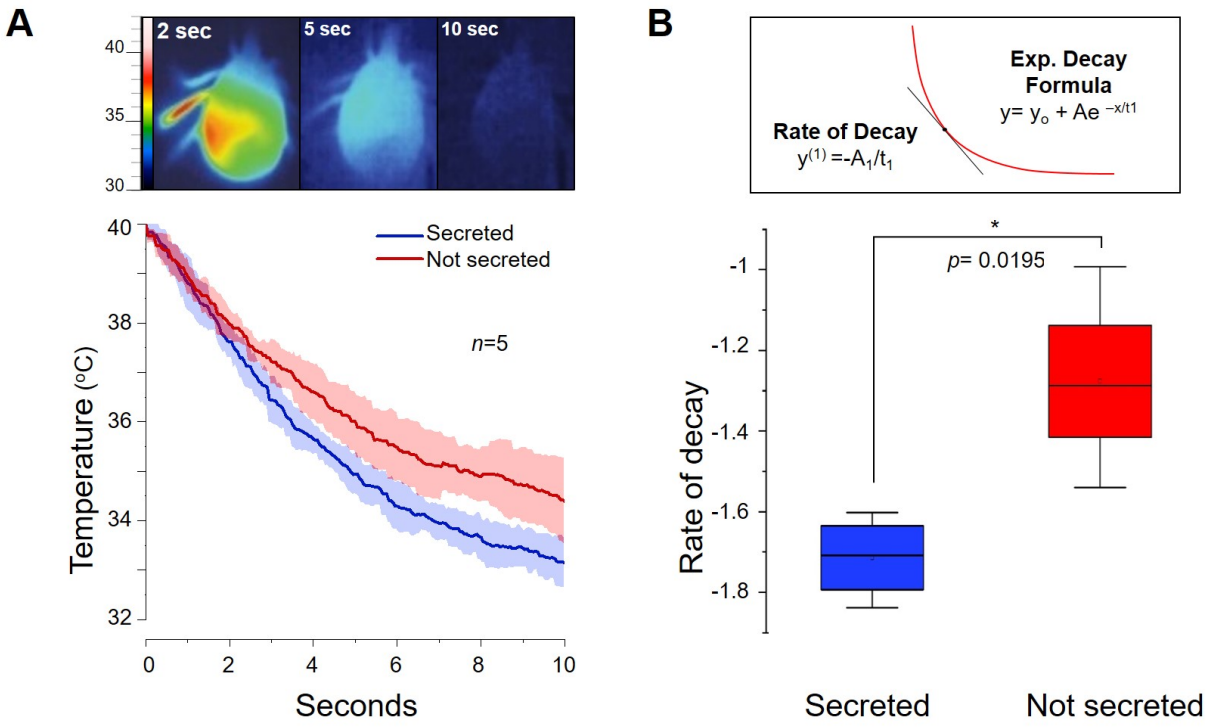

Figure 3.3. Cooling rate after the contact with heat probe with and without dermal secretion.

A) Cooling rate measured for 10 seconds after contact with heat probe in *Amblyomma americanum*. The blue line represents the ticks with dermal secretion and the red line is for the ticks without secretion (n=5 ticks/treatment). **B)** The regression formula and lines for the rate of decay that was used for the statistics by student T-test.

Dermal secretion is induced by serotonin and inhibited by Ouabain

In injection of different biogenic amines, neuropeptides and secondary signaling molecules, we found that a biogenic amine serotonin triggered an immediate dermal secretion at 1mM (Table 1). This response was observed in 75% of *A. americanum* (Figure 3.4). In other Metastriata ticks, we observed that 75% individuals responded in *R. sanguineous*, 50% in *D. variabilis*, whereas no response was observed in a Prostriata *Ixodes scapularis* (Figure 3.4A). Dose-response of serotonin showed that the dose required for the secretion is higher than 100 μM in 10 nL injection and reached to the maximum response to a plateau at 1 mM with 75% responders, which was also found in an increased concentration to 10 mM (Figure 3.4B).

Table 3.1. Compounds injected into ticks for dermal secretion responses. Further details of the compound information are in the materials and methods.

Compound	Concentration	Total ticks tested	Response %
Biogenic amines			
Octopamine	10 mM	10	0%
Norepinephrine	10 mM	10	0%
Dopamine	10 mM	10	10%
Serotonin	1 mM	20	75%
Neuropeptides			
SIFamide	10 mM	10	0%
MIP-1	1 mM	10	0%
Proctolin	10 mM	5	0%
ELV-1	10 mM	10	10%
Secondary signaling messengers			
Dibutyryl cAMP	10 mM	5	0%
Forskolin	10 mM	10	10%
Dibutyryl cGMP	10 mM	20	10%
SNAP	5 mM	10	20%

We expanded the study to investigate the downstream machinery for fluid transport. A pre-treatment of Ouabain, a Na/K-ATPase inhibitor, by an injection of 10 nL 100 μM at the -30 min. of the serotonin injection, blocked the secretion response to the serotonin injection. Ouabain significantly lower the response to serotonin than that in control (water injection) (p=0.0134 in a chi-square test) (Figure 3.4C).

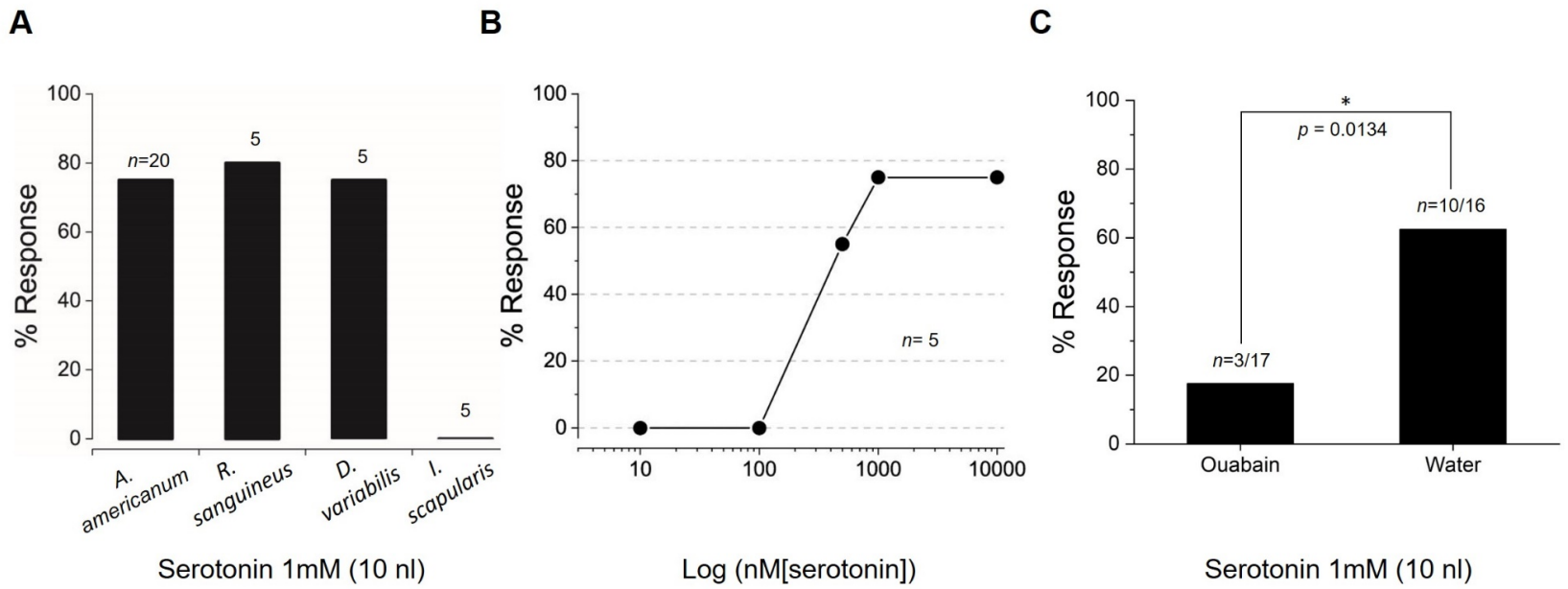

Figure 3.4. Dermal secretion induced by serotonin injection

A) The dermal secretion responses by percentage after serotonin injection (10nl of 1mM serotonin) in metastriata ticks, *Amblyomma americanum, Rhipicephalus sanguineus, Dermacentor variabilis,* and *Ixodes scapularis*. **B)** Dose-response to serotonin injection in *A. americanum* (10nl injections). N=5 for each dose. **C)** Effect of ouabain pre-treatment on the serotonin-mediated dermal secretion. Ouabain pre-treatment was made with an injection of 10nl of 100 μM at 30 minutes before the serotonin treatment. Water injections were used for the control. The statistics were Chi-square test.

An interesting observation worth to be mentioned in this set of experiment was bilateral asymmetric responses (Supplementary Table S1). In one batch of ticks, when ticks were injected on the left side, majority of the ticks responded with the secretion on the right side and vice versa. However, in another batch of ticks, the secretion responses were observed on the same side of injections; right side injections resulted in the right side response and vice versa. (Table S1).

Dermal secretion and type II dermal glands were visible with the aid of blue fluorescent light

After the induction of dermal secretion by a heat-probe to the legs, we found that the drops of dermal secretions, ~ 2nL from each dermal pore, which were dried immediately on the surface of the integument, had blue fluorescence under CFP filter (Figure 3.5A-D) in a fluorescence stereoscope. Dissections and visualization of the dermal glands were performed after we blocked the secretory response by an injection of local anesthetic Procaine hydrochloride to naïve ticks at 10 minutes prior dissection (n=13) to preserve the intact gland structure during the dissection procedure, which induces the secretion and collapses the gland. The Procaine injected individuals were tested by a heat-probe before dissection to ensure lack of secretion as the anesthetic response. Only the individuals with a negative secretory response (9/13) were used for dissection and visualization for the internal structure. The glands under the fluorescent light with CFP filter set showed blue fluorescent glandular structures in both dermal and ventral layers (Figure 3.5E-G). The blue fluorescent glands were rich in the festoon region, while a number of the glands were also observed in the region surrounding the base of coxa and also in the regions near the clusters of dorso-ventral muscles.

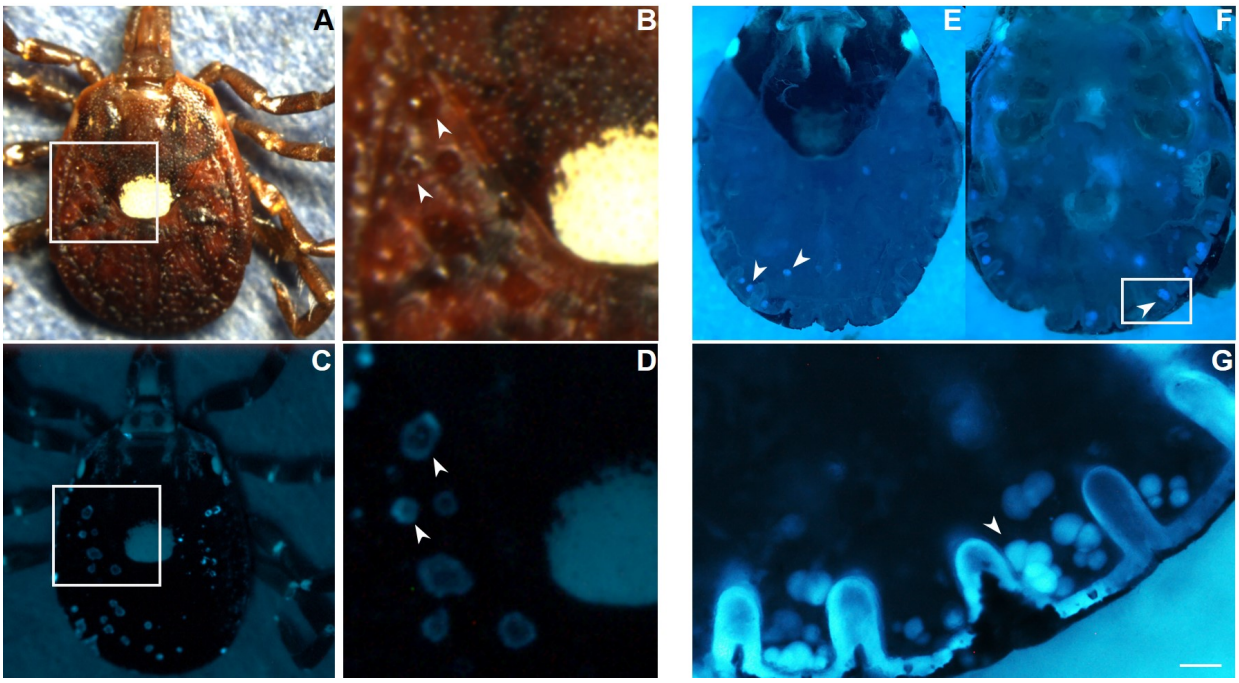

Figure 3.5. Localization of dermal secretion and putative type II gland

A) Dorsal view of *Amblyomma americanum* female after dermal secretion stimulated by heat exposure on the legs. The white square highlights a subset of secretion spots. **B)** Inset image of "A" where white arrowheads point specific secretion drops. **C)** Image of "A" under CFP light. The white square shows the same region of image "A." **D)** Inset image depicted on white square of image "C." **E)** Internal view of dorsal integument under CFP light and **F)** Internal view of ventral cuticle integument. White arrowheads point to the putative dermal glands type II. **G)** Internal view of ventral cuticle where white arrow heads point to cluster of type II glands located on the ticks festoons. Scale bar shown at 100 µm.

Confocal images of dorsal and ventral integuments showed the same patterns of localization for the type II gland structures as were observed in the CFP filter set; the type II glands mainly in the tick festoons and also in the area surrounding the coxal regions and near the dorso-ventral muscles, while the glands lost the intactness of the round shapes during the sample processing. A pair of large nuclei associated with balloon-like thin membrane structure was observed on the internal surface of epidermal cell layer. All the staining reagents that we employed, Na/K-ATPase, cell membrane (CellMask^{TM}), beta-tubulin, and HRP (neural marker in insects), helped the visualization of the type II dermal glands. The anti-HRP antibody and CellMask^{TM} showed the robust membrane staining (Figure 3.6A-F). Anti-beta-tubulin antibodies stained subcellular part of the glands, but with high variability among different glands (Figure 3.6B-F).

The entire membrane for small number of glands were stained for beta-tubulin (Figure 3.6C, E, and F), but mostly having only small spots on the membrane (Figure 3.6B), whereas all type I glands were positive for the beta-tubulin (Figure 3.6D). Often, we observed clustered subcellular region of basal part of the glands that was positive for β-tubulin (Figure 3.6E and F). Anti-HRP stained the clusters of the cells in the type I glands (Figure 3.6D and F) and the membrane of type II glands. Anti-Na/K-ATPase antibody stained small spots on the membrane of type II glands (Figure 3.6H and J).

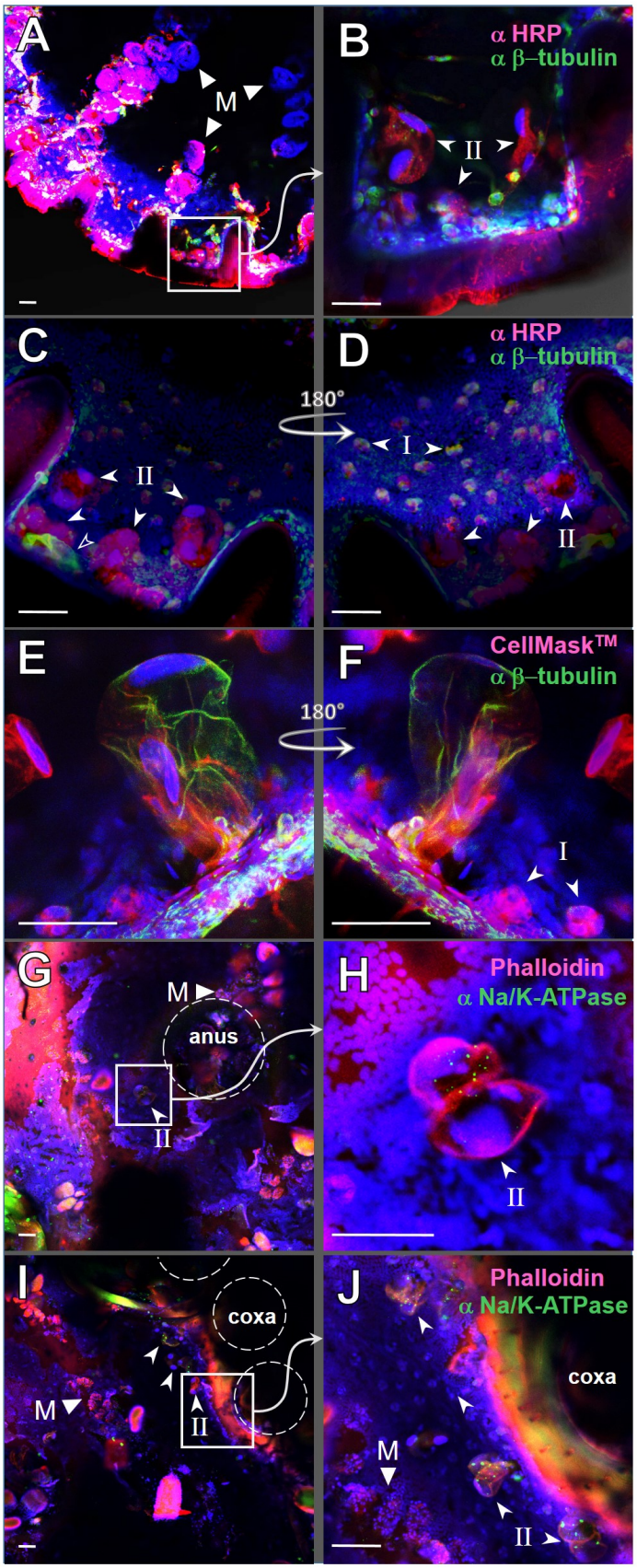

Figure 3.6. Confocal images of the dermal layer showing type II and type I glands.

A) Ventral layer of unfed tick. The white triangles points to the clusters of round shapes are for the dorso-ventral muscle (M). The inset containing red (anti-HRP) is a festoon showing typical type II glands magnified in B. **B)** Inset of A showing the type II dermal glands indicated by white arrowheads. **C)** A confocal image with a Z-stack for 9 layers of a total of 27.54 µm. The white arrowheads point to type II dermal glands, empty arrowhead points the type II gland stained for β-tubulin. **D)** Lower layers of the region shown in C, a Z-stack for 12 layers of a total of 36.72 µm. This image shows both type I type II glands. The front and back views were made by different degrees of transparency in the Z-stack algorism. **E)** A paired type II gland with the nuclei staining in each. The image is the Z-stack of 14 layers of total 37.38 µm. **F)** The backside view of E made by different degrees of transparency in the Z-stack. Note that β-tubulin immunoreactivity is clustered in the basal region of the gland. Type I glands are marked with the arrowheads. **G)** Type II glands in the central/anus region of the ventral dermal layer, where white square shows type II glands and white triangle the dorso-ventral muscles (M). **H)** Inset of G showing the type II dermal glands. Note spotted granular green staining of Na/K-ATPase. **I)** Type II glands in the lateral/coxa region of the

ventral dermal integument, where white arrowheads are for type II glands and white triangle the dorso-ventral muscles (M) **J)** Inset of I showing the type II dermal glands with white arrowheads and spotted granular staining of Na/K-ATPase. White triangle is for the dorso-ventral muscles (M). **A-D**: Blue; nuclei staining with Hoeschst, Green; β-tubulin staining and Red; Horseradish Peroxidase staining (Cy^{TM}3). **E** and **F**: nuclei staining with Hoeschst, Green; β-tubulin and Red; plasma membrane stained with CellMask^{TM}. **G-J:** nuclei staining with Hoeschst, Green; Na/K-ATPase and Red; Phalloidin used for actin staining. All scale bars shown represent 50 µm.

In microtome sections of the dermal layers, two types of dermal glands were obviously categorized: narrow duct with a cluster of small cells for type I (Figure 3.7 A-C) and the large dermal glands connected to the wide dermal pores for type II (Figure 3.7D-F). The location and the shape of the type II glands are well correlated with the structures observed in fluorescence stereoscope and confocal images. Each wide dermal pore is mostly connected to a paired two glandular structures, which was shown in the confocal microscopy with two large nuclei.

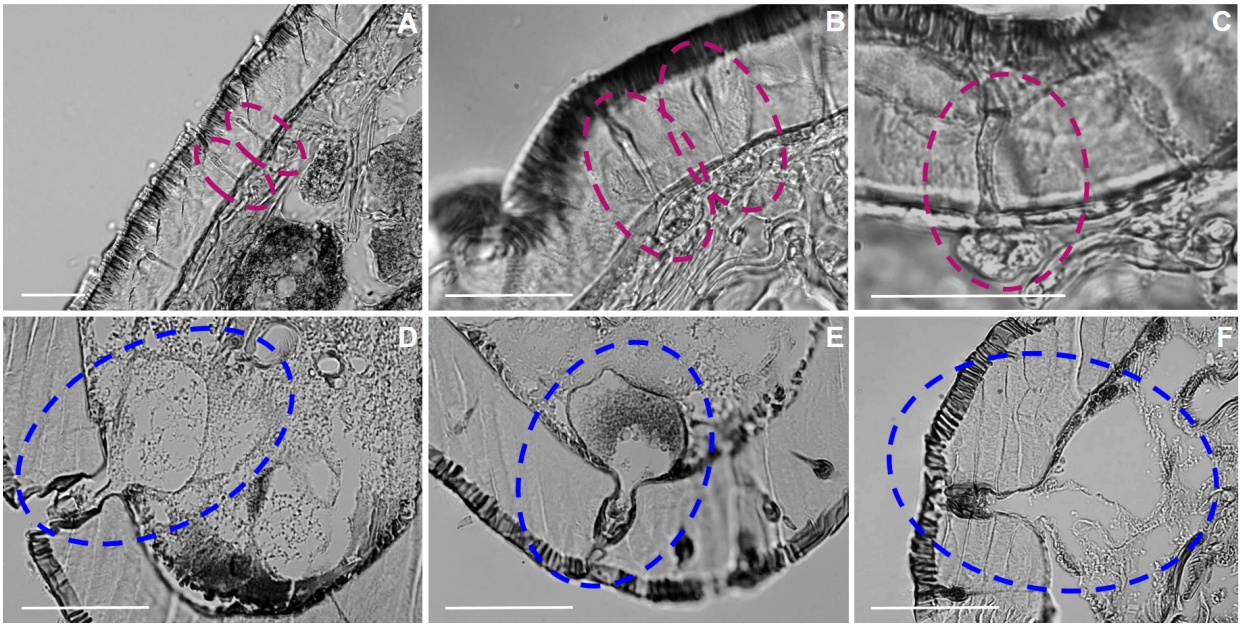

Figure 3.7. Putative dermal gland type I and II shown in association with duct on the cuticle layer in 10 µm sections.

Magenta circle shows the pore for putative dermal gland type I, blue circles show pore of the putative dermal gland type II. **A)** Unfed tick integument showing type I dermal glands of an unfed female tick. **B and C)** Close-up image of type I glands characterized by thin pore and small gland size/cell clusters**. D–F**) Type II glands shown in different locations of alloscutum of unfed ticks.

Discussion

We demonstrated that tick dermal secretion is triggered by contact with a high temperature substrate in this study. Evaporation of the dermal secretion significantly helped the rapid cooling of the body. These data support that a function of tick dermal secretion is to provide the evaporative cooling of the body in hot environments, which can be up to 65 °C shown in a typical tick habitat in a summer day in Kansas (Figure 3.2C).

Evaporative cooling in small invertebrate is an unexpected observation; while, endothermic/homeothermic large animals are well known for dermal secretion, sweating, for evaporative cooling in thermoregulation through apocrine and eccrine glands (38). Small arthropods, having high surface to volume ratio, are vulnerable to evaporative loss of water. The integument covered with a wax layer in arthropods is efficiently preventing evaporative loss of water. The cost of dermal secretion is shown by the death of the individual having exhaustive dermal secretion by a contact of 55–58 °C heat probe for 5 seconds. Excessive loss of fluid (4.1 % of body weight) is likely leading to death although the accurate cause of the death in this case needs to be further investigated. Despite of the costs of water loss, tick dermal secretion is likely important for tick survival with the tight control of the secretion. Indeed, previous studies have shown that the ticks with dermal secretion can survive better when they are exposed to high temperature. This finding was reported in *R. sanguineus* where dermal secretion provided heat tolerance after ticks were mechanically stimulated through leg pinching (30). In this study, the tick survival after 1 hr. heat shock in naïve ticks was 27% at 52°C and 18% at 54°C, whereas ticks that experienced dermal secretion by pinching the legs were much more tolerable in the same condition, *i.e.*, 93% survival at 52°C and 89% at 54°C. The authors suggest that heat tolerance may be the result of internal changes in the dermal glands that occur after secretion. The physiology behind this tolerance needs to be investigated further.

The secretion is proposed to contain defensive compounds against predators and pathogens. Such toxic compounds, like antimicrobials and squalene, can be externalized through pore openings on the cuticle surface (25, 26) and may act as allomones against predatory ants like the case of squalene (23). An earlier study identified *o*-nitrophenol and methyl salicylate in the type II glands of fed male ticks of *A. variegatum* and *A. hebraeum*, which are thought to have aggregation pheromonal activities (28, 29). In this study, we add an additional function of the dermal secretion for thermoregulation by evaporative cooling.

Two types of dermal glands and pores have been described in *R. sanguineus* (39), namely type I and type II. Type I glands are also known as small gland (40) and type II are known as large wax gland or type A gland (40-42). Dermal secretion has been reported to occur presumably through type II dermal glands connected to *sensilla sagittiformia* types of pore, which are exclusive to metastriate ticks (43) and absent in prostriate ticks (41, 44), although previous reports have not been able to directly link this type of secretion with the specific type II glands. A number of our observations support that the type II gland we described is the gland for the dermal secretion, *i.e.*, the number, size, and location of the glands are equivalent to the external pore that produces secretions. The blue fluorescence we observed in the secretion is also localized to the type II glands.

Gland morphology consisted of paired two cells each having thin membrane forming a balloon-like sac, which is similar to the previous description for *R. sanguineus* (28). Immunohistochemistry of the type II glands suggests the absence of neuronal projections connecting the dermal gland, based on the lack of axon-like projections in HRP-immunoreactivity. Interestingly, the HRP immunoreactivity, which presumes staining neuronal associated glycans in arthropods, also appears to be present in subcellular structures of the gland itself. Noticeably, however, the anatomically categorized dermal gland type II appear to be having subcategories based on the blue fluorescence of the contents and immunoreactivities in the dermal gland sac for Na/K-ATPase. The dermal secretion often contained non-fluorescent oily product externally (Figure 5d), and the type II glands in the internal structure often was not fluorescent in the CFP filter set. Na/K-ATPase and beta-tubulin immunoreactivities were also vary for different type II glands (Figure 3.6B, C, H, and J). It is not clear whether the variation is caused by the intrinsic, environmental, developmental variations at this time.

While the multifunctional dermal secretion is a novel biological innovation, it costs a large amount of water loss as is shown by the lethality caused by exhaustive secretion. Therefore, the dermal secretion is likely a process tightly controlled by neural/hormonal mechanisms. In ticks, the water homeostasis is a tightly controlled physiology. The excretions occurring through the saliva and hindgut are controlled by neural and hormonal controllers (21, 45). An immediate response of dermal secretion upon the injection of serotonin (sub-second) favors serotonin as a direct hormonal/neural factor for activating the dermal glands. Blocking the serotonin-mediated dermal secretion by Ouabain suggests that the main downstream transporter is Na/K-ATPase in

the dermal glands. This is supported by our IHC results showing granular spots of Na/K-ATPase staining in both type I and type II glands (Figure 3.6H and J), So far, the roles of Na/K-ATPase in ticks have been described for the secretory activities in the salivary gland type 2 and 3 acini and resorptive activity in the salivary gland type I. A previous study for RNAi of Na/K-ATPase in a feeding stage tick found that the phenotype was associated with incomplete feeding and limited cuticle expansion during feeding (46). An involvement of Na/K-ATPase in the dermal secretion suggests another function of Na/K-ATPase in different physiological processes.

In conclusion, tick dermal secretion provides evaporative cooling when the ticks encounter the contact with hot substrates in microhabitats. This could be a factor contributing to their extended survival rates and habitat expansions to geographical areas with hot temperature. There are two main molecular controllers of the dermal secretion pathway: serotonin and Na/K-ATPase. The dermal secretion involved in osmoregulatory physiology and thermoregulation may offer a vulnerable tick physiology that can be targeted in development of tick control measures. Current study brought in an interesting aspect of this exocrine gland that is responsible for the massive amount of dermal secretion in a short time.

Supplementary material

Table S1. Bilateral asymmetric responses observed after the injections of 10 nl of 1mM serotonin on left or right side of the second coxal region.

Trial 1	**Injection position**	**Left**			**Right**		
	Responder/total n	9/9			3/4		
	Response position	**Left**	**Both**	**Right**	**Left**	**Both**	**Right**
		1/9	1/9	7/9	3/4	0/4	0/4
Trial 2	**Injection position**	**Left**			**Right**		
	Responder/total n	5/5			4/8		
	Response position	**Left**	**Both**	**Right**	**Left**	**Both**	**Right**
		3/5	2/5	0/5	0/8	0/8	4/8

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Chapter 4 - The bacterial community of the lone star tick (*Amblyomma americanum*)

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Abstract

The lone star tick (*Amblyomma americanum*), an important vector of a wide range of human and animal pathogens, is very common throughout the East and Midwest of the United States. Ticks are known to carry non-pathogenic bacteria that may play a role in their vector competence for pathogens. Several previous studies reported the commensal bacteria in a tick midgut as abundant and diverse using High throughput sequencing (HTS) technologies. In contrast, in our preliminary survey of the field collected adult lone star ticks, we found the number of culturable/viable bacteria very low. We aimed to address the differences in bacterial community by a parallel culture-dependent and a culture-independent approach applied to individual ticks. We analyzed 94 adult *A. americanum* females collected in eastern Kansas, and found that 60.8% of ticks had no culturable bacteria and the remaining ticks carried only 67.7± 42.8 colony forming units (CFUs)/tick representing 26 genera. HTS of the 16S rRNA gene resulted in a total of 32 operational taxonomic units (OTUs) with the dominant endosymbiotic genera *Coxiella* and *Rickettsia* (>95%). Remaining OTUs with very low abundance were typical soil bacterial taxa indicating their environmental origin. No correlation was found between the CFU abundance and the relative abundance from the culture-independent approach. This suggests that many culturable taxa detected by HTS but not detected by culture-dependent method, were possibly not viable or were not in their culturable state. Overall, our HTS results show that the midgut bacterial community of *A. americanum* is very poor without a core microbiome and the majority of bacteria are endosymbiotic.

Key words: microbiome, bacterial diversity, midgut, culturing, high throughput sequencing

Introduction

Hard ticks (Ixodidae) are among the most important arthropod vectors of human and animal pathogens in the United States and worldwide (1-3). The lone star tick (*Amblyomma americanum*) is common in the Midwest and Eastern USA (4) and an important vector of *Fransicella tularensis, Ehrlichia chaffeensis, E. ewingii,* and heartland virus (5-7). This tick is also known to cause red meat allergy due to alpha-gal in their salivary glands (8, 9). In addition to pathogens, lone star ticks carry a commensal and symbiotic bacterial community (10) which may play a role in the vector competence for pathogens (11, 12) although this role remains to be elucidated (12). Most studies on the microbiome of *A. americanum* focused on intracellular endosymbionts (13-16) although extracellular bacteria in the gut lumen may influence colonization of pathogens (10, 12, 17) and overall vector competence of ticks (10, 11). Using culture-independent approaches, earlier studies have shown great microbial diversity in the lone star tick, reporting several hundreds of operational taxonomic units (OTUs) with high alpha diversity and at least 99 bacterial families and over 100 genera (18-20).

Interestingly, most culture-independent microbiome studies on the tick gut report bacterial communities that should be easily culturable, such as *Bacillus*, *Pseudomonas, Escherichia*, *Shigella,* and *Proteus* (10, 21, 22) and therefore should be coupled with a culturing approach to determine the abundance of viable bacterial taxa. These culturable bacterial isolates might then become available for future studies on manipulation of the gut bacterial community and its effect on the tick vector competence. In this study, we aimed to survey the microbiome of *A. americanum* using the parallel culture-dependent and culture-independent approach.

Methods

Field sites, tick collection and sample preparation

Adult females of *A. americanum* (n=120) were collected from northeastern Kansas (Konza Prairie Biological Research Station 39°06'23.4"N 96°36'11.4"W and 39°06'16.6"N 96°35'43.7"W) and southeastern Kansas (Pittsburg Wilderness Park 37°27'09.8"N 94°42'41.0"W) by flagging. Ticks were placed in a cooler with high humidity (>90% RH) and transported to the laboratory. Ticks were surface sterilized upon arrival using 0.5% sodium hypochlorite (5 minutes), 70% ethanol (3 minutes), and washed 3 times with sterile water. Mouthparts and anus of ticks were sealed with a glue (SuperGlue, Pacer Technology, Inc. ID, USA) to prevent access of chemicals

to the gut lumen during sterilization. Then, individual ticks were immobilized on sterile wax surface and aseptically dissected in phosphate buffered saline (PBS; MP Biomedicals, LLC, CA, USA) to remove soft tissues (midgut, salivary glands, and ovaries). Tissues from each individual tick were homogenized in PBS in a total volume of 200µl and divided into two equal parts. One half (100 µl) was immediately used for culturing and the other half was stored at -80 ˚C for DNA extraction and culture-independent analysis. This homogenates were analyzed individually and could be traced back to each individual tick.

Culture-dependent method

Tissue homogenates (100µl) were serially diluted in PBS spread plated on trypticase soy agar (TSA) (BD, Sparks, MD, USA), and incubated at 25 and 37˚C for 72 hrs in aerobic and microaerophilic (CampyPakPlus™ GasPak™ system jars, BD, BBL™ Franklin Lakes, NJ, USA) conditions. Colony forming units (CFU) per tick of each distinct colony morphology were counted and calculated in CFU per tick. Morphologically distinct colonies were sub-cultured on TSA for characterization and identification. Rapid tests for catalase activity using hydrogen peroxide and Gram test using potassium hydroxide were conducted. DNA extraction was performed with the ZymoBiomics DNA Miniprep kit (Zymo Research, CA. USA). The 16S rRNA gene was amplified using universal bacterial primers for the V1-V4 regions (8F and 806R) (23) (Figure 4.1) and sequenced by the Sanger method. Sequences were edited, aligned, and phylogenetically analyzed in MEGA-X (Molecular Evolutionary Genetics Analysis version 10.1.6). Alignment was generated using the multiple sequence comparison by Log-expectation (MUSCLE) and the UPGMA clustering methods. Tree was generated by maximum likelihood (ML) method with 500 bootstrapping. Taxonomic affiliation of each sequence was determined using Basic Local Alignment Search Tool (BLASTn) at the NCBI GenBank database (24) and verified through the Ribosomal Database Project (RDP) classifier, using the sequence matching tool (25, 26).

Culture-independent approach

A total of 94 samples from the original 120 individual tick sampleswere randomly selected for sequencing the individual samples. The total DNA from individual homogenates was extracted with the ZymoBiomics® DNA Miniprep kit (Zymo Research, CA. USA) following the manufacturer protocol. As control we used water used for sample preparation containing DNA

from *E. faecalis* V583, extracted using the same DNA extraction protocol. A second positive control was used, which contained the DNA of a laboratory-reared tick as reference. The DNA was quantified using a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) and a fluorometer (PicoGreen, Invitrogen, MA. USA). In order to assess the quantity of 16S rDNA, serial dilutions of known amount of tick DNA were used for quantitative PCR (qPCR) standardization curves using the single copy gene of the tick *V-ATPase subunit C*. We used similar DNA template amounts (between 5-20 ng across samples) for both genes in qPCR. The qPCR reaction was prepared using the 2x Luna® Universal qPCR Master mix following the manufacturer instructions. A single copy gene from *A. americanum* (*V-ATPase* subunit *C*) was used as a reference for known amount of tick cell number (Primers: 894F: 5'-CCC TGA GGC TTT TTG TTG AG-3'and 1043R: 5' CCT GGG CAA TGC TTG TGT-3'). For quantification of the 16S rRNA gene, the V4 region amplification with universal eubacterial primers: 515F: 5'-GTG YCA GCM GCC GCG GTA A-3' and 806R: 5'-GGA CTA CNV GGG TWT CTA AT-3'(modified from Caporaso et al., 2012) (27) (Figure 4.1) was used. Delta Ct values were calculated by the difference in qPCR Ct values of the 16S rDNA and the tick V-ATPase subunit C.

Library preparation and sequencing of the V3 and V4 region of the 16S rRNA gene (341F and 806R)(Figure 4.1) was performed at the Genome Sequencing Core of the University of Kansas. Libraries were generated using unique dual indexing (UDI) and prepared using the Nextera XT indices kit. Sequencing was conducted using the MiSeq Next Generation Sequencer. Raw sequence reads were analyzed using the software package Mothur (version 1.39.5, (28)). Paired-end sequences for 300 nt were joined, and sequence reads with low quality ($q < 25$), ambiguous base and ambiguous length (< 100 and > 450 bp) were removed. All sequences other than that of *E. faecalis* from positive control sample were also filtered out. High quality sequences were aligned with SSU rRNA SILVA reference alignment (29) using Needleman-Wunsch global alignment algorithm (30). Chimeric sequences were checked using UCHIME (31) and removed. Non-*E. faecalis* sequences from the positive control sample were also removed. Sequence reads were then clustered into OTUs using the average neighbor algorithm with the 97% sequence similarity criterion. For each OTU, taxonomy was assigned using the naïve Bayesian classifier algorithm (25). Low abundance and erroneous OTUs (abundance ≤ 0.005 % of total abundance) were filtered out as described previously (32). Further, to lower the bias due to variation in sequence numbers across the samples, the OTU table was normalized by subsampling to equal

sequence numbers (15,613) per sample. Rarefaction curves show that full richness of a community has been sampled showing good sequencing depth (supplementary material: Figure S1). OTUs with the same taxonomic identification were grouped into same genera for further analysis at the genus level and taxa with relative abundance <0.005% were grouped under the "others" category.

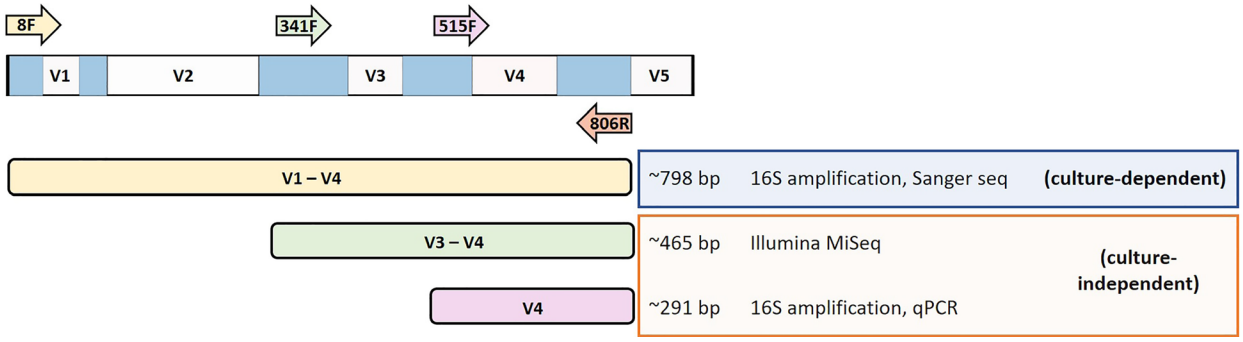

Figure 4.1. Schematic representation of primers in the hypervariable regions. (V1-V5) of the 16S rRNA gene for Sanger sequencing, high throughput sequencing, and quantitative PCR.

Statistical analysis

Species richness, species diversity index (Shannon diversity index) were calculated using vegan package in R statistical platform (version 3.5.3). Abundance and diversity figures representing the genus and phylum level were generated in GraphPad Prism version 8.4.1 for Windows (GraphPad Software, California USA). *In silico* removal of likely endosymbionts was conducted and OTU abundance was normalized accordingly**.** Initially, Pearson *r* correlation was used to calculate the correlation between CFU abundance and bacterial abundance by the culture-independent method and to statistically compare agonistic patterns among specific OTUs. Then we conducted Spearman *r* correlation testing (non-parametric) for accurate representation of non-normally distributed data. Statistical analyzes and plots were generated using GraphPad Prims version 8.4.1.

OTU downstream analysis

Phylogenetic analysis using the sequences obtained by 97% sequence identity criterion and an additional analysis and at 99% sequence identity were conducted to construct phylogenetic trees

in the search of endosymbiont genotypes and unclassified taxa. Reference sequences were obtained from the Genbank database at NCBI and phylogenetic trees were generated in MEGA-X: Molecular Evolutionary Genetics Analysis version 10.1.6 (33). Alignments were generated using the multiple sequence comparison by Log-expectation (MUSCLE) and the UPGMA clustering methods. Trees were generated by maximum likelihood (ML) method with 500 bootstrapping (supplementary material: Figure S2), which was also supported by neighbor joining (NJ), and unweighted pair group method with arithmetic mean (UPGMA).

Results

Culture-dependent method:

We detected culturable bacteria from only 39.2% of ticks with abundance of 67.7 ± 42.8 CFU/tick (Figure 4.2a). Sequencing of the 16S rRNA gene (~800 bp) revealed 3 bacterial phyla: Actinobacteria (54.2 %), Firmicutes (33.9 %), and Proteobacteria (11.9 %) (Figures 4.2b and S1) with a heavy bias toward Gram-positive (88.1%) and catalase positive (92.1%) taxa. A total of 45 species from 23 genera (Figures 4.2c and S1) were identified. The most prevalent genera were *Micrococcus* (26.6%), *Staphyloccocus* (13.3%), and *Bacillus* (11.6%) with the highest abundance of *Bacillus* (54.9%) and *Pseudomonas* (26.9%).

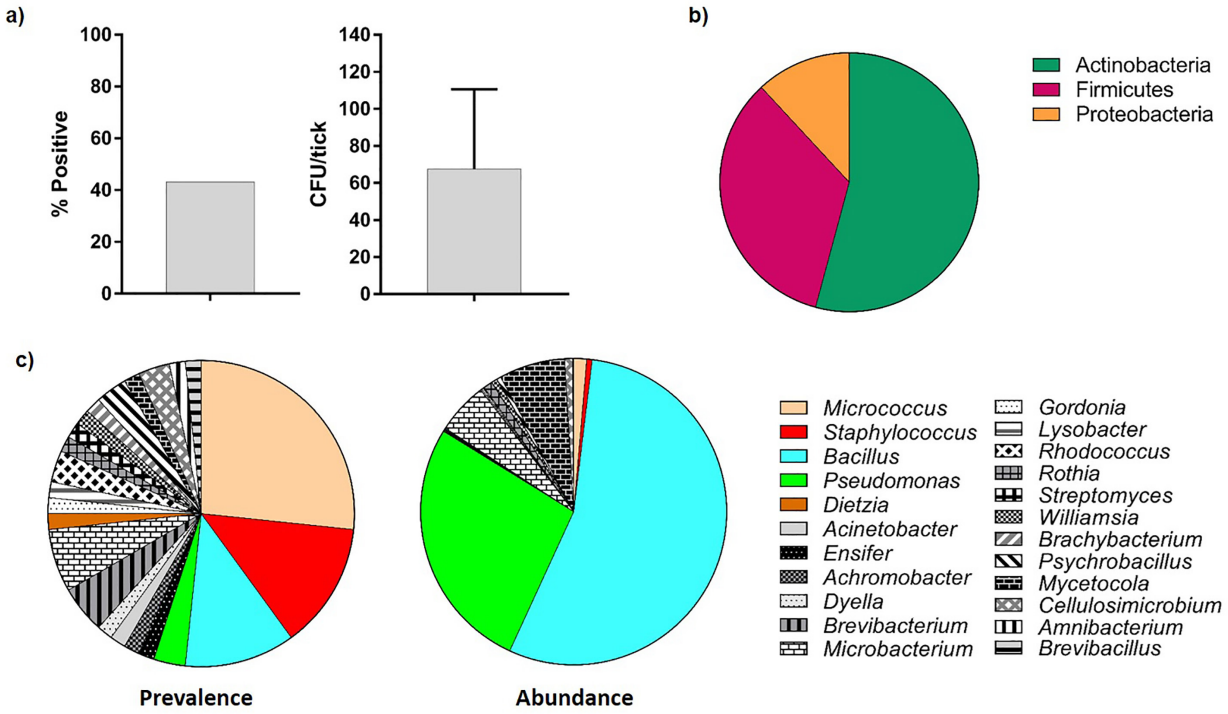

Figure 4.2. cultured from *Ambylomma americanum*.

a) Prevalence (left) and abundance (right) in colony-forming units (CFU)/tick; b) phylum abundance: Actinobacteria (54.2 %), Firmicutes (33.9 %), and Proteobacteria (11.9 %); c) prevalence (in %) and abundance (in %) of bacterial genera (colored genera represent taxa also detected by culture-independent approach; black and white genera represent taxa identiied by culture-dependent approach only).

Culture-independent method:

DNA extracts from individual 94 ticks, resulted in a total of 236 OTUs using average neighbor algorithm with 97% sequence similarity criterion. After removal of taxa with very low relative abundance (<0.005%) and grouping OTUs by the genus level, we obtained a total of 32 OTUs. Dominant genera were endosymbionts (*Coxiella* sp. and *Rickettsia* sp., 97.8% ± 0.4 of the reads per tick) (Figure 4.3a). We performed *in silico* removal of the endosymbionts to better visualize the abundance of the extracellular bacteria. Our analysis revealed that the most abundant taxa (excluding endosymbionts) were those of typical soil and plant associated bacteria including *Pseudomonas, Bradyrhizobium, Micrococcus, Methylobacterium, Herbaspirillum, Acinetobacter* and others (Figure 4.3b). In an attempt to determine if, high 16S rDNA abundance correlates with high CFU counts; we first measured the 16S rDNA abundance (measured by quantitative PCR) of each individual tick. This analysis included all the 16S rDNA present within each individual. We

observed that variation of 16S rDNA was very high (six orders of magnitude for the largest difference). The average of 16S rDNA copy number in each tick was 2.82 ($Log_{10}[2^{-\Delta CT}]$), presenting ~630x more 16S copy number compared to the tick single copy gene *V-ATPase subunit C*. The major bacterial species, *Coxiella,* is known to have one copy of 16S, allowing direct conversion of 16S copy number to bacterial number, although bacterial 16S copy number varies depending on the species. Therefore, we conclude that there are approximately ~630 times more bacteria than the tick cell numbers with large variations among individual ticks. No correlation was found between the CFU abundance in the culture and the 16S rRNA qPCR abundance (r = 0.045; p = 0.66) (Figure 4.4a).

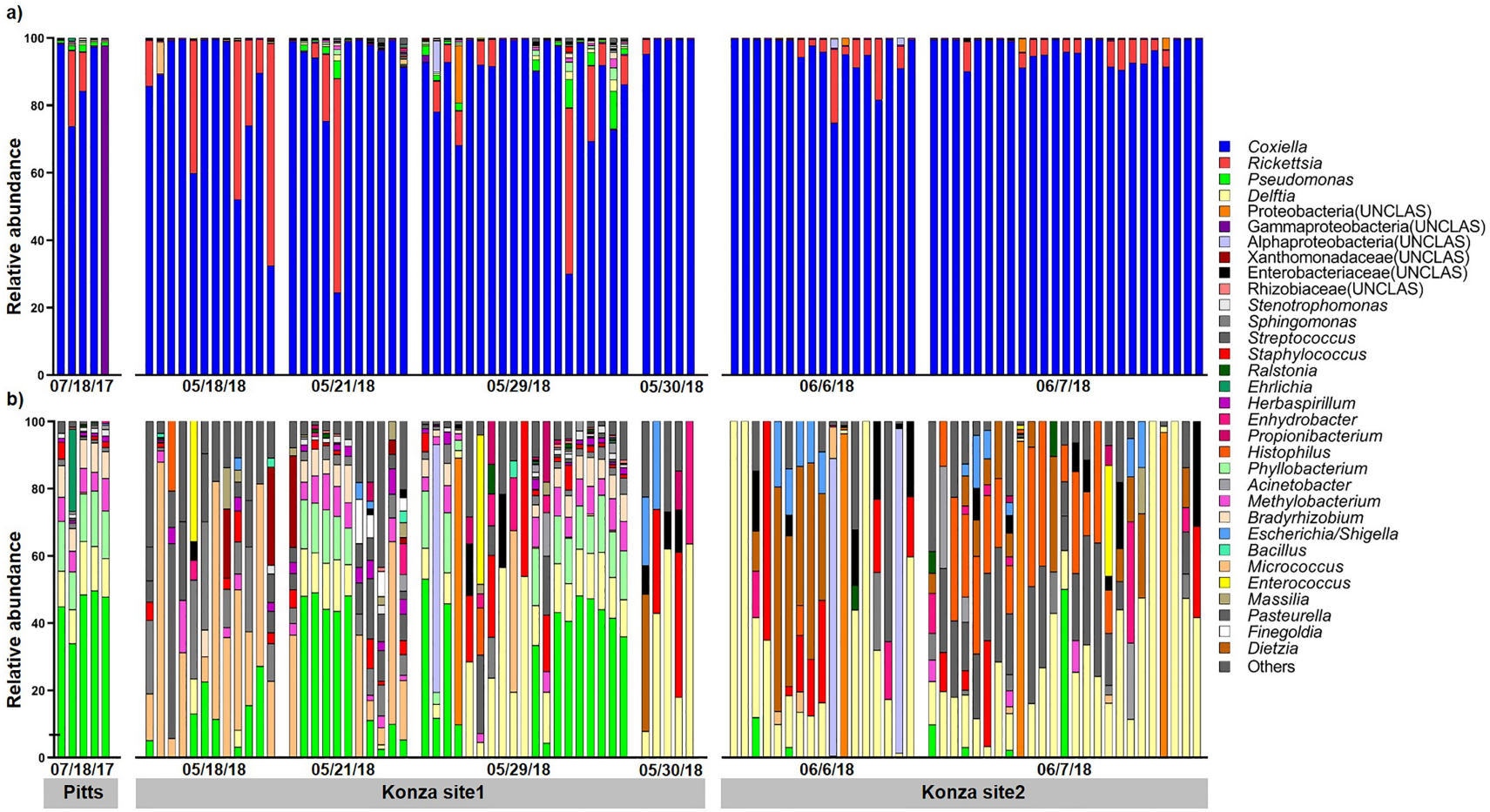

Figure 4.3. Relative abundance of bacterial genera obtained by the culture-independent method

a) Relative abundance by genera of individual ticks; b) relative abundance by genera after removal of endosymbionts (Coxiella, Rickettsia and Gammaproteobacteria). UNCLAS represent unclassified bacterial families or phyla

In addition, no correlation was found between CFU abundance and relative abundance of non-endosymbiotic taxa and taxa identified by both approaches (culture-dependent and independent methods); (r= 0.07; p=0.48 and r=0.09; p=0.38, respectively) (Figure 4.4b). This suggests that high amounts of 16S rDNA from endosymbionts masked other OTUs in most cases. Several ticks with high CFU counts had low relative abundance of culturable taxa from sequencing (Figure 4.4c). For example, the tick 82 (white arrow in Figure 4.4c) with the high CFU abundance of *Pseudomonas* sp. did not have any detectable *Pseudomonas* sp. reads. Likewise, high abundance of *Pseudomonas, Bradyrhizobium, Methylobacterium, Streptococcus* and *Phyllobacterium* in the tick 46 (black arrow in Figure 4.4c) was not matched in CFUs from the culturing approach.

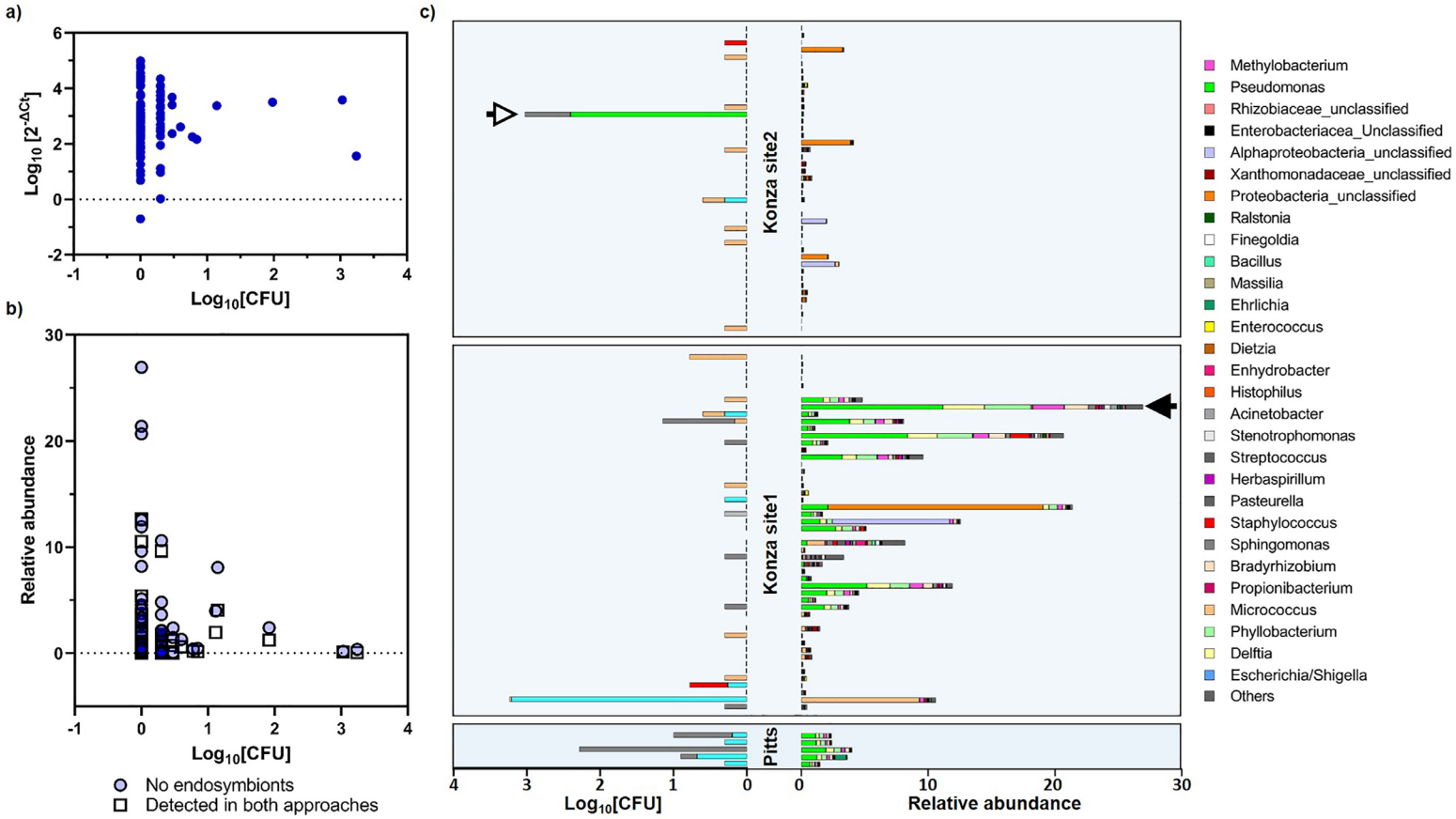

Figure 4.4. The 16S rRNA gene abundance by qPCR and its correlation to CFU abundance.

The 16S rRNA gene abundance by qPCR and its correlation to CFU abundance. a) Scatterplot showing no correlation (p = 0.66) (between16S rDNA abundance from qPCR and CFU abundance). Mean 16S copies per individual tick was 2.82 (log10[2−ΔCT]). B) Scatterplot for correlation; circles represent total abundance of taxa excluding Coxiella, Rickettsia, and Gammaproteobacteria (UNCLAS)] (p = 0.48); squares represent added abundance of taxa that were detected by culturing and identified in a culture-independent approach (p = 0.38). c) CFU abundance diversity in individual ticks contrasted with the relative abundance of bacterial taxa from sequencing after exclusion of Coxiella, Rickettsia, and Gammaproteobacteria. Empty and black arrows point to tick samples 82 and 46 where no correlation between CFU abundance and HTS is observed.

Phylogenetic trees showed that unclassified (UNCLAS) Gammaproteobacteria clustered with endosymbionts of *Amblyomma* sp. We also detected two *Coxiella* genotypes (Genotype Aa1 and Aa2, with 98.6 and 99.3% identity, respectively to *Coxiella* endosymbiont (AY939824.1) (Figure 4.5). *Coxiella* Aa1 was dominant (100% frequency) and abundant (88.3%) while the *Coxiella* Aa2 was less frequent (89%) and with very low abundance (0.1%) (Figure 4.5).

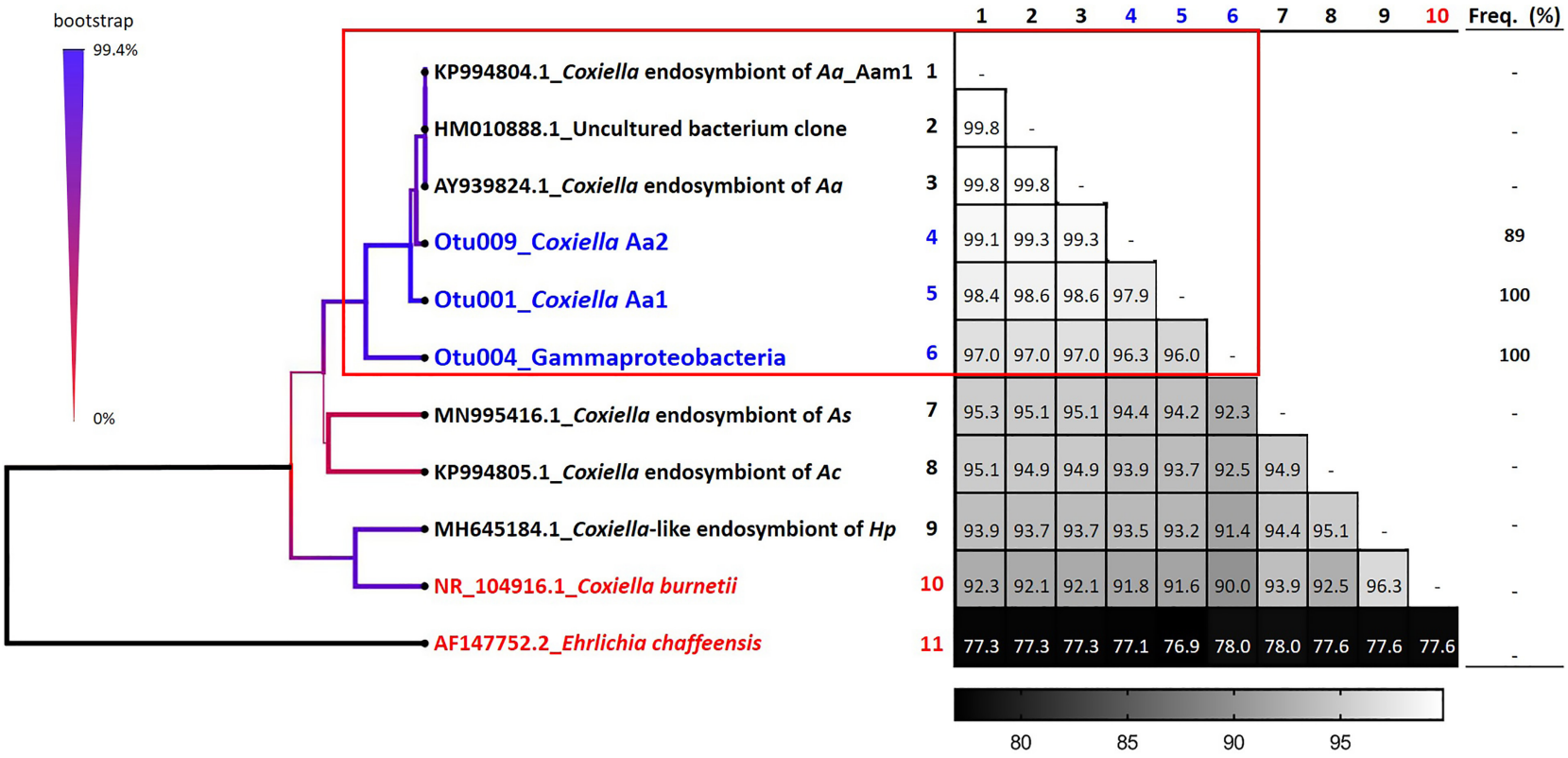

Figure 4.5. Phylogenetic tree showing the relationship among *Coxiella* endosymbionts, Gammaproteobacteria UNCLAS, and *Coxiella burnetii* of *Amblyomma sp*.

OTUs obtained in this study (blue), references from data sets of known endosymbionts (black), and pathogens (red) of the *A.americanum*. UPGMA phylogram with bootstrap test (500 replicates) depict bootstrap values as a weighted line and in blue and red color scale (values > 70 are shown in blue and purple). Matrix table shows % identity of each taxon against the other. Frequency (%) shows the frequency at which the taxa were found in the tick samples

We also found distribution patterns of bacterial taxa implying antagonistic and agonistic relationships among certain bacteria. A closely related taxonomic group, *Delfia, Phyllobacterium, Methylobacterium* and *Bradyrhizobium* (OTUs 8,9,11 and 12) were found in the same individual ticks, while *Micrococcus* sp. (OTU10) and *Streptococcus* (OTU31) were also found together, but in different individual ticks (Figure 4.6). This speculated antagonistic distribution pattern was found in 47 ticks (53% of total). These two groups appeared to be in a mutually exclusive manner in each individual tick through manual search. However, only the agonistic taxa (OTUs 8,9,11 and 12) were found to be statistically correlated to each other (Pearson's correlation coefficients r=0.95-0.98) and no significant correlation was observed between OTU10 and OTU31 (r=0.34, p=0.052).

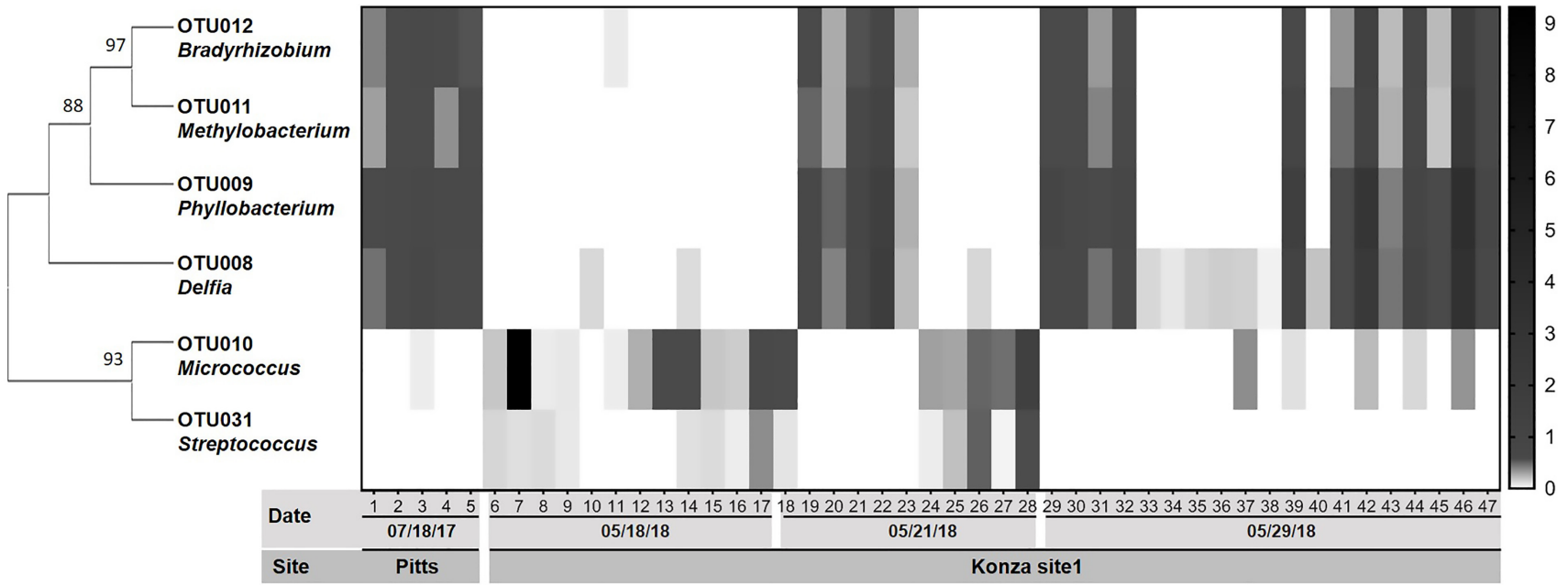

Figure 4.6. Heat map and phylogeny of taxa with potential agonist and antagonistic relationships in *Amblyomma americanum* (n = 47)

Absence in white and presence in gray to black. Phylogeny is based on the maximum likelihood clustering with 500 bootstraps.

Discussion

Data from our culture-dependent approach clearly show that the midgut microbiome of *A. americanum* is very poor with low abundance and no core bacterial community when compared to that of other blood-feeding arthropods (34). This is in agreement with recent studies on the gut microbiome of other tick species including *I. scapularis* (35) and *I. ricinus* (36). The majority of the bacterial taxa were representative of soil and plant associated bacteria. This is not surprising since *A. americanum* has been shown to actively ingest liquid water from the environment to recuperate the imminent water losses occurred through excretion mechanisms (37-39) and it this therefore likely that these bacteria were ingested along with water (10). The soil and plant origin of the midgut bacteria was also suggested for *I.ricinus* (10, 40). It is however intriguing that the majority of isolates were Gram-positive and catalase-positive indicating that these attributes likely play a role in bacterial resisting tick epithelial immunity responses including action of dual oxidases maintaining tick bacterial homeostasis (41). Nevertheless, even for these taxa, the abundance was very low and in more than half of the tick samples no culturable bacteria were found.

The culture-independent approach revealed that the majority of the tick microbiome is composed of known endosymbiotic bacteria with *Coxiella* sp. as the dominant taxon followed by *Rickettsia* sp. Other bacterial community members were low in abundance and also dominated by bacteria typical for the soil and plant environment, as previously suggested (42), and this corroborates the results from the culturing approach. Overall, our findings contrast previous reports of an abundant bacterial community in ticks reporting several hundreds of OTUs (13, 19, 20) using similar culture-independent methods. It is possible that the rich and abundant bacterial community detected in other culture-independent studies in ticks was a result of contamination by bacteria from tick surface as recently suggested by Binetruy et al. (36). In that study, it is shown that surface sterilization methods significantly impact internal bacterial community composition. We have used a thorough surface sterilization protocol with sodium hypochlorite and ethanol as the most effective method of sterilization (36) and this very likely avoided any major contamination from the tick surface.

Standardizing optimal conditions for the culture and isolation of all culturable bacteria are challenging (43), and although our culture-dependent approach is limited to culturable aerobic and microaerophilic bacteria on a broad spectrum nutrient agar, we believe it provides the sufficient

evidence to show limited abundance of extracellular bacteria in the tick midgut. Many culturable taxa that were also commonly identified in the culture-independent approach were Gram-negative such as *Pseudomonas, Delftia,* and unidentified *Proteobacteria*. The culturable taxa captured in only 16S sequencing, but not in culture of the same homogenates, is likely a result of amplification of DNA from lysed cells or DNA of cells in viable but not culturable state (44). Clearly, the lack of correlation between results of culture- and culture-independent (Miseq and qPCR) approach indicates the limits of both approaches. Our culture-independent approach results revealed that over 95% of bacteria represented endosymbionts, which is in accordance with other tick microbiome studies (13-16). In our study, we used a 0.005% cutoff for OTU downstream analysis, which is very low for microbial community studies and allowed us to show bacterial taxa with very low abundance some of which were also isolated by culturing. Overall, it is very likely that many bacteria, especially those with low abundance, were masked by the dominant endosymbionts *Coxiella* and *Rickettsia*, which could explain why some of the isolates identified using the culture-dependent approach were not captured in the culture-dependent approach.

Phylogenetic analysis revealed two different *Coxiella* genotypes and unidentified Gamma-proteobacterium (OTU 004) all of which were closely related to known *Coxiella* endosymbiont of *A. americanum.* Since all three were detected in high frequency, it greatly reduces the likelihood of artifacts including sequencing errors. Nonetheless, more in depth analysis including sequencing of the entire 16S rRNA gene is needed (45) to uncover the phylogeny of these symbionts.

We also detected potential agonist and antagonist relationships among specific bacterial genera by manual search. Specifically, *Micrococcus* sp. and *Streptococcus* sp. were absent when taxa *Delfia, Phyllobacterium, Methylobacterium,* and *Bradyrhizobium* were present, and *vice versa*. The statistical analysis revealed strong correlation among the agonist taxa; however, no antagonistic effect was shown with our statistical analysis. The antagonist phenomena is common among bacteria in other animals (46), and it could also have biological significance for the vector competence of *A. americanum* for pathogens such *Francisella tularensis* and *Anaplasma* sp. as proposed for the midgut bacteria of *I. scapularis* and *Borrelia burgdorferi* (47).

Shannon's diversity index and species richness varied across samples (0.269 ± 0.03, and 9.04 ± 0.44; respectively); however, the overall diversity and species richness was significantly lower in comparison to that in other studies on *Ixodes scapularis* (22) where the overall Shannon index was between 1.0 and 2.5 and the overall species richness between 10.0 and 20.0. This

variation can be attributed to the difference in tick species, tick distribution and potentially the microhabitat, which could influence tick microbial community (40, 48, 49).

In conclusion, the microbiome of *A. americanum* is dominated by endosymbionts and these are likely more diverse than believed previously. The midgut bacterial community of this tick species is poor without a core microbiome. Nevertheless, there are several culturable bacterial taxa that could be used for further experimental studies addressing: 1) whether these are transient or capable of midgut colonization, 2) how midgut epithelial immunity maintains such a low bacterial abundance, and 3) the role these bacteria play in the vector competence of *A. americanum* for pathogens.

Supplementary material

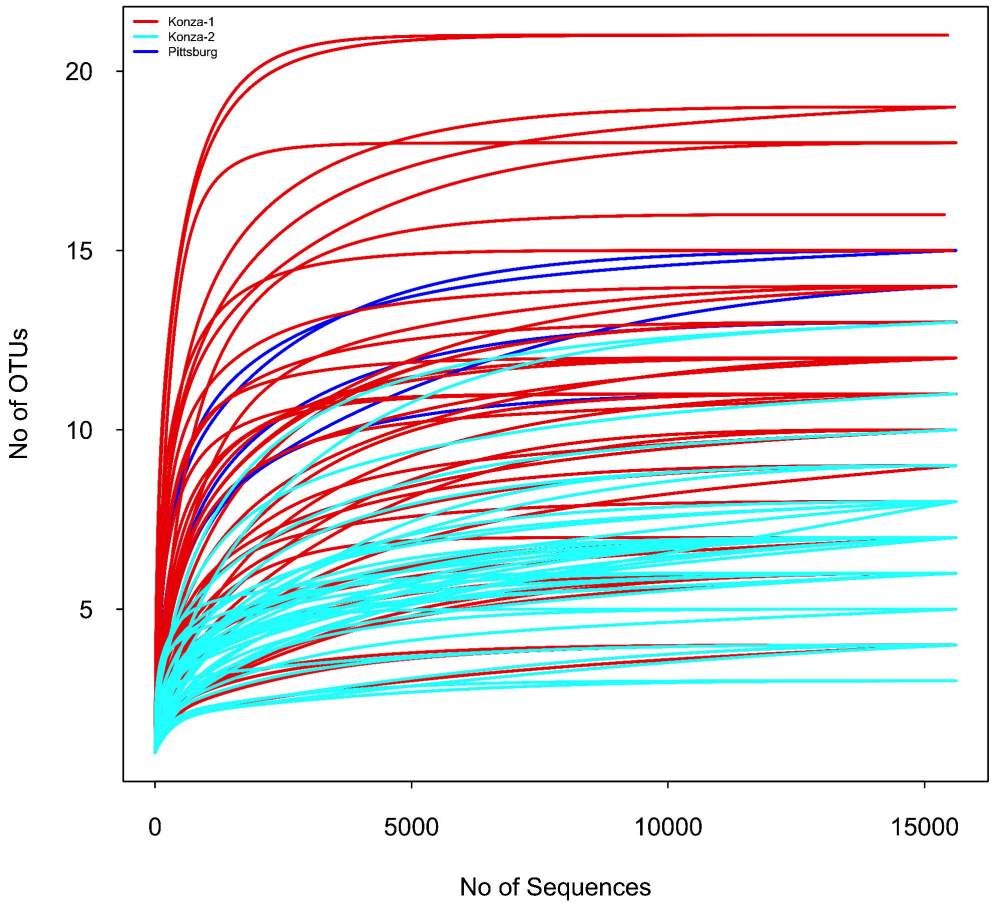

Figure S1. Rarefaction curves of individual tick samples.
The OTU table was rareied to equal sequence numbers (15,613) per sample. Curves are color coded by location: red: Konza-1; cyan: Konza-2; blue: Pittsburg.

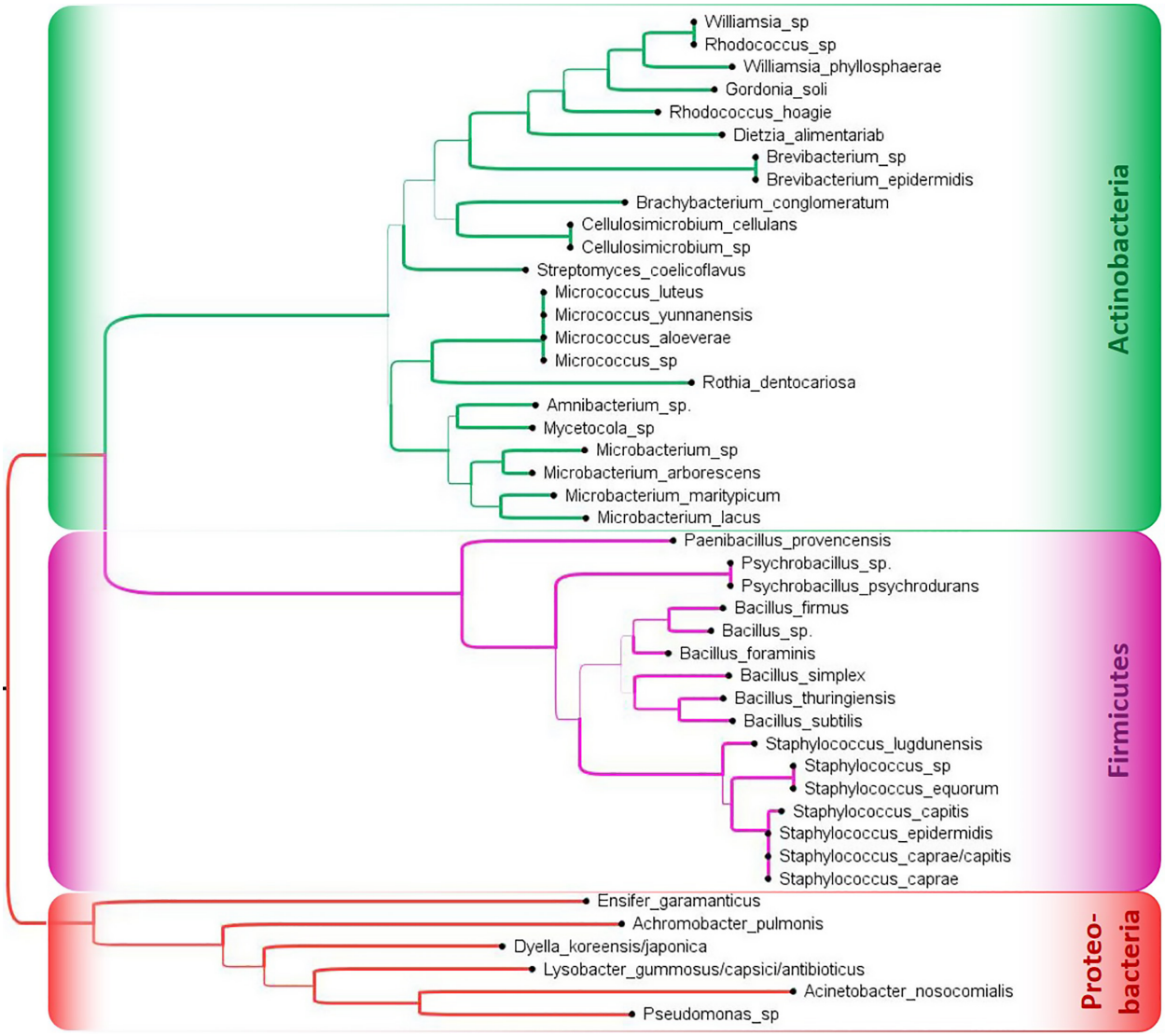

Figure S2. Maximum likelihood tree of the 16S rDNA of bacterial isolates from *Amblyomma americanum*.

The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (500 replicates). Initial trees for the heuristic search were obtained automatically by applying neighbor joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with a superior log likelihood value. This analysis involved 45 nucleotide sequences. There were a total of 785 positions in the inal dataset. Colors of the tree branches indicate the phyla: Actinobacteria (green); Firmicutes (magenta); Proteobacteria (red).

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Chapter 5 - Research summary and future directions

In recent years, the research in ticks has significantly increased due to the growing numbers of cases of tick-borne diseases, although the knowledge we currently have is yet limited in comparison to other blood feeding arthropods, like mosquitoes. Control of ticks in the field is heavily relying upon the use of conventional insecticides and acaricides. Although the method of application and the formulation of toxic chemicals in tick control have been improved, new classes of chemicals for controlling ticks are urgently in need, as ticks have developed acaricide resistance (1, 2). The safety and the environmental issues became more important in the development of tick control measures. Rational design of the tools and methods for tick control can be made on the bases of understanding tick biology.

My dissertation work with the lone star tick contributes to the knowledge of tick physiology, aids in the understanding of tick survival strategies, and proposes to direct alternative management strategies. Ticks directly drinking environmental water, as an important physiology in water homeostasis, has been exploited to control ticks by delivering the osmoregulatory disruptors in chapter 2. The research has expanded to the importance of osmoregulatory and thermoregulatory physiology in dermal gland secretions in ixodid ticks in chapter 3. Finally, in chapter 4, I have explored the microbiomes of tick, which is relevant to the ticks directly acquiring environmental microorganisms through water drinking. Within these chapters, I explored a new area of tick physiology, which contributes to the development of the measures for tick control.

Chapter 2 demonstrated that water drinking behavior is common in *Amblyomma americanum* and other ixodid ticks, having a significant impact on tick survival. This observation led us to test the possibilities of delivering toxic compounds and microorganisms through the water for tick management purposes. In conclusion of this study, we have propose that the tick water drinking behavior is a part of important osmoregulatory physiology and can be utilized as a delivery route of tick-specific toxic reagents, which would contribute the knowledge on development of the tools in tick control.

It is critical for ticks to maintain water balance to avoid desiccation and death (3-6) as previous studies suggested that some ticks may actively uptake liquid water (7, 8). In this chapter, we showed a significant difference in survival between ticks that ingested water in comparison to

those that did not. This clearly demonstrates the significance of direct contact with liquid water and water drinking in tick survival. Other studies reported water vapor uptake and an increased weight gain of ticks (9, 10), suggesting water vapor uptake is a major way for tick rehydration. However, our results show that water vapor alone is not the main factor contributing for extended survival rates. We propose that the ability of lone star ticks to drink liquid water is likely a contributing factor for the success and continuous expansion of this species in the United States (11). Studies modeling the geographical distributions of tick have shown that vapor pressure is one of the most important determinants of tick suitable habitat (12) and continued habitat expansion of the lone star tick (13) also support our claim.

We found that feeding the ticks a mixture of salt solution, containing phosphates at the reasonably low concentrations, resulted in 100% mortality of ticks in three days. We suggest that spraying water with this solution in small droplets on a grass lawn, could provide an effective tick control. Specific formula for efficiently delivering the inorganic ions would be further developed for a field application strategy. In addition to the inorganic salts, we found that the ticks fed on inoculated water containing *P. aeruginosa* caused 100% tick mortality. While *P. aeruginosa* is an opportunistic human pathogen and therefore not suitable for a biological control of ticks, these data provide the proof of the concept for using this approach with other bacterial species and strains or their toxins. The delivery of toxic agents through drinking water is a promising proposal for management and adds new avenues for tick research. The ingestion of bacteria, which can be later traced to the tick midgut provides opportunity for elucidating tick vector competence if certain bacteria can successfully colonize the tick gut.

In chapter 3, I have explored the impact of dermal secretion in thermoregulation and dehydration. Our results suggest that dermal secretion provides evaporative cooling, which could be occurring in nature in *A. americanum*'s hot microhabitats. This could be a factor contributing to their extended survival rates and habitat expansions to geographical areas with hot temperature. Furthermore, we report that there are two main molecular controllers of the dermal secretion pathway; serotonin and Na/K-ATPase. The dermal secretion involved in osmoregulatory physiology and thermoregulation may offer a vulnerable tick physiology that can be targeted in development of tick control measures.

Dermal secretion-mediated evaporative cooling is an unexpected observation in such a small heterothermal animal. The data in this chapter support that a function of tick dermal

secretion is likely to provide the evaporative cooling of the body in hot environments. This phenomenon in the tick explains such large volume of fluid secretion (4% of the body weight) in a fraction of seconds. In the case of a male human adult the rates of perspiration is 3 liters per hour, on average (14), which amounts to 4.2% of the body weight of an average adult being lost during an hour of exercise under heat. Although this extrapolation might not seem comparable, it provides a general perspective in regards to the intensity of the secretory response observed in ticks.

Tick dermal secretion has been reported to occur through type II dermal glands (15-17) also known as: large wax gland, type A glands or *sensilla sagittiformia*, which are exclusively in metastriate ticks (18), but absent in prostriate ticks (16, 19). In our study we showed that other metastriata ticks are also exhibiting secretion triggered by heat, while *Ixodes scapularis* does not. Unfortunately, previous reports have not been able to directly link this type of secretion with the specific type II glands. In this study, the blue fluorescence in the secretion coincided with the internal type II dermal glands having the blue fluorescence. Further anatomical studies are required for understanding this exocrine gland responsible for the massive amount, ~ 2nL in each dermal pore, of secretion in a short time.

Furthermore, I found that serotonin is one of the controllers of this response, which likely functions as a hormonal factor directly activating large number of dermal glands. An immediate response of dermal secretion upon the injection of serotonin (sub-second) favors serotonin as a direct hormonal factor for activating the dermal glands, although possible involvement of neural controls could not be excluded.

Blocking the serotonin-mediated dermal secretion by ouabain, a well-known Na/K-ATPase inhibitor, suggests that the main downstream transporter is Na/K-ATPase in the dermal glands. So far, the roles of Na/K-ATPase in tick have been described for the secretory activities in the salivary gland type 2 and 3 acini and resorptive activity in the salivary gland type 1 (20). An involvement of Na/K-ATPase in the dermal secretion suggests another function of Na/K-ATPase in different physiological processes.

In chapter 4, I have explored the internal microbiome of ticks, which is likely influenced by drinking environmental water. Results showed that the microbiome of *A. americanum* is predominantly comprised by endosymbionts and these are likely more diverse than believed previously. Our data show that the midgut bacterial community of this tick species is low in diversity and abundance. Nevertheless, there are several culturable bacterial taxa, which we were

able to isolate and characterize, from bacterial genera known for containing typical soil members. Whether these bacteria are successfully colonizing the tick gut or whether they are transient, we propose this finding leads to further experimental studies addressing: 1) how midgut epithelial immunity maintains such a low bacterial abundance, 2) the role these bacteria play in the vector competence of *A. americanum* for pathogens and 3) the use of these bacteria for a paratransgenic approach.

Recent studies on the Lyme disease vector *I. scapularis* (21) and *I. ricinus* (22) showed that the gut microbiome is also low in the diversity and the abundance in comparison to other blood feeding arthropods. I found that the majority of the bacterial taxa in the internal organ of *A. americanum*, were representative of soil and plant associated bacteria. This is not surprising since *A. americanum* has been shown to actively ingest liquid water from the environment (23), as discussed in chapter 2, and it is therefore likely that these bacteria were ingested along with water (24). Midgut bacteria from soil and plant origin was also suggested for *I. ricinus* (24, 25) . In addition, the bias towards Gram-positive and catalase-positive bacteria indicates that these attributes likely play a role in survival of the bacteria in tick epithelial immune responses including action of dual oxidases maintaining tick bacterial homeostasis (26)

The results in the study chapter 4 suggest that it is necessary to conduct a culture-dependent approach for the identification of extracellular bacterial taxa with biological function within the tick gut. Although standardizing optimal conditions for the culture and isolation of all culturable bacteria are challenging (27), our data show that there are culturable taxa not captured by high throughput 16S sequencing, but isolated through culturing methods from the same homogenates. This is likely a result of the sequencing approach not being able to capture less abundant bacteria due to the high abundance of the endosymbiotic bacteria masking the rare OTUs. Thus, culturing methods are imperative for the identification of viable extracellular bacteria with potential roles in tick vector competence or with colonization potential.

In this work, we also observed agonist and antagonist relationship patterns among specific bacterial genera identified from the culture-independent approach. This observation provides the foundation for potential usage of bacterial antagonism to control vectorial capacity of tick. Bacteria ingested by ticks through the environmental water, might be capable of colonizing the tick gut in the long-term. The isolation and identification of such bacteria could aid in the understanding of

the role of these bacterial communities in the tick or may be exploited for a paratransgenic approach directed towards tick management.

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